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# Multi-class/residue method for determination of veterinary drug residues, mycotoxins and pesticide in urine using LC-MS/MS technique

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## Abstract

**Background** Veterinary drugs are widely used in animals to prevent diseases and are a complex set of drugs with very different chemical properties. Multiclass and multi-residue methods for simultaneous detection of residues from veterinary drugs and contaminants in urine are very rare or non-existent. Therefore, the aim of this study was to develop and validate a sensitive and reliable quantitative LC-MS/MS method for simultaneous determination of a wide range of veterinary drug and pesticide residues and mycotoxins in bovine urine. This involved 42 veterinary drug residues (4 thyrostats, 6 anabolic hormones, 2 lactones, 10 beta agonists, 15 antibiotics, 5 sulphonamides), 28 pesticides and 2 mycotoxins. Stable isotopically labelled internal standards were used to facilitate effective quantification of the analytes. Analysis was performed in both positive and negative ionization modes with multiple reaction monitoring transitions over a period of 12 min.

**Results** The parameters validated included linearity, limit of detection (LOD), limit of quantification (LOQ), detection capability (CC $\beta$ ), decision limit (CC $\alpha$ ), stability, accuracy and precision. The process followed guidelines of the regulation 2021/808/EC. The calibration curves were linear with coefficient of correlation (R<sup>2</sup>) from 0.991 to 0.999. The LODs were from 0.01 to 2.71  $\mu\text{g/L}$ , while the LOQs were from 0.05 to 7.52  $\mu\text{g/L}$ . The CC $\alpha$  and CC $\beta$  were in range 0.05–12.11  $\mu\text{g/L}$  and 0.08–15.16  $\mu\text{g/L}$ . In addition, the average recoveries of the spiked urine samples were from 71.0 to 117.0% and coefficient of variation (CV) < 21.38% (intraday and interday).

**Conclusion** A new isotopic LC-MS/MS method has been developed, validated and applied for identification and quantification of 72 residues of veterinary drugs and pesticides and other contaminants such as mycotoxins in bovine urine. The most appropriated sample preparation procedures involved sodium acetate buffer, enzymatic hydrolysis using  $\beta$ -glucuronidase and cleanup solid phase extraction with OASIS SPE cartridges. The parameters were satisfactorily validated fulfilling requirements under Regulation 2021/808/EC. Consequently, the method could be

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used in routine analysis of bovine urine samples for simultaneous detection of veterinary drug and pesticide residues as well as contaminants such as mycotoxins.

**Keywords** Veterinary drugs, Mycotoxins, Pesticides, Urine, LC-MS/MS, Method development

## Introduction

Veterinary drugs are widely used in livestock production to prevent or treat diseases. While in some countries certain veterinary drugs such as thyreostats, anabolic hormones and  $\beta$ -agonists may be used as a growth-promoting agents [1]. This is generally prohibited in other regions such as the European Union. The residues of veterinary drugs in animal tissues/matrices resulting from incorrect use and/or nonobservance of withdrawal period can affect humans [2]. To ensure food safety and safeguard human health, the monitoring of residues in animal products and live animals is very important [3–4]. The measures for monitoring of these residues in live animals and animal products are prescribed in Regulation (EU) 2017/625 [5]. An advantage of analyzing urine or blood from live animals for presence of veterinary drug residues is the possibility to retest animals in case of a suspect result. Veterinary drugs are metabolized by animals, and some of the drugs remain in the animal body, while others enter the environment through excreta. Also, generally the drug concentration in urine tends to be higher than in the muscle or other tissues [6].

Animals are also often simultaneously exposed to other hazards such as mycotoxins pesticides or heavy metals, and since these may also end up in food consumption, multi-residue and multi-toxin exposure studies are therefore very relevant to public health. Appropriate analytical methods are therefore required [7]. The determination of the multiple substances in biological matrices such as urine and blood from live animals could be reliable and useful in exposure assessment (short- and long-term) and may predict future effects on human health [8].

The establishment of sensitive analytical methods to detect residues and contaminants in food from animal origin as well as live animals is important for safety and public health. Currently, more sensitive methods for the determination of residues and contaminants in food of animal origin have been developed, but there are few multi-residue and multiclass analytical methods for simultaneous detection of residues of veterinary drugs and other hazards such as pesticides and mycotoxins. Zhan et al., (2013) [9], developed an LC–MS/MS method for screening for multi-class veterinary drug residues and other contaminants in muscle while Hajrulai-Musliu et al., (2021), reported an LC–MS/MS method for multiple residues and contaminants in bovine meat [10]. Danezis et al., (2016) [11], developed a HILIC chromatography-MS/MS method for detection of pesticides, plant hormones, veterinary drugs and mycotoxins in various food

matrices while Gómez-Pérez et al., (2015) [12], published a method for analysis of pesticide and veterinary drug residues in baby food by liquid chromatography coupled to Orbitrap high resolution mass spectrometry, as well as Wei et al., (2015) [13], who developed multi-residue screening method for analysis of veterinary drugs, their metabolites and pesticides in meat by LC-MS/MS. Xie et al., (2015), developed LC-MS/MS methods for analysis of veterinary drugs, pesticides and mycotoxins in dairy products [14].

Multiclass and multi-residue methods for simultaneous detection of residues from veterinary drugs and contaminants in urine are very rare or non-existent. A range of methods for residues of veterinary drugs and contaminants have been published from different authors although these involve single class or groups [15–22]. Stanley and Foo (2006) published a multiresidue method for simultaneous screening of more than 250 veterinary drugs alone in equine urine [23]. The multiclass/residue methods present several advantages to testing laboratories and monitoring programs [10, 24].

The objective of this study was to develop and validate a sensitive and reliable quantitative LC-MS/MS method for simultaneous determination of a wide range of veterinary drug and pesticide residues and mycotoxins in bovine urine. This involved 72 analyses including 42 veterinary drug residues (4 thyreostats, 6 anabolic hormones, 2 lactones, 10 beta agonists, 15 antibiotics, 5 sulphonamides), 28 pesticides and 2 mycotoxins. Analysis was performed in both positive and negative ionization modes with multiple reaction monitoring transitions over a period of 12 min.

Six extraction protocols for effective and rugged multiresidue extraction of 72 compounds from urine were investigated. Four of the protocols followed solid phase extraction (two with and two without enzymatic hydrolysis), while the other two protocol involved usage of liquid-liquid extraction (also with and without enzymatic hydrolysis). The parameters validated included: limit of detection (LOD), limit of quantification (LOQ), decision limit ( $CC\alpha$ ), detection capability ( $CC\beta$ ), linearity, accuracy and precision. The criteria prescribed in Regulation 2021/808/EC were followed [25].

## Materials and methods

### Chemicals and reagents

Methanol, acetonitrile and water (LC-MS/MS grade), ethyl acetate, 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.),  $\beta$ -glucuronidase aryl sulfatase from *Helix pomatia* and trichloroacetic acid ( $\geq 99.5\%$ ), Discovery® DSC-MCAX cartridges (300 mg/6 ml) were from Merck (Darmstadt, Germany) and Oasis HLB cartridges (500 mg/6ml) were from Waters (Milford, MA, USA).

### Standards and isotopically-labelled internal standards

**The analytical standards and purity levels included** Thyreostats: thiouracil (100%), propylthiouracil (99.6%), methylthiouracil ( $\geq 98.0\%$ ) and tapazole (99.7%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Anabolic steroids: 19 nortestosterone (99.8%), clostebol (99.1%), boldenone (99.1%), methyltestosterone (99.5%) and testosterone (100.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while stanozolol (99.8%) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany); Lactones of resorcylic acid: taleranol (99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA), while zeranol (99.9%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany);  $\beta$ -agonists: clenbuterol HCl (99.1%), isoxsuprine HCl (100%), salbutamol (99.4%), zilpaterol HCl (96.0%), ractopamine HCl (95.5%) and terbutaline hemisulfate salt (100.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while brombuterol (98.0%), mabuterol HCl (98.0%), cimbuterol (98.0%) and clenpenterol HCl (98.0%) were obtained from Witega (Berlin, Germany); Antimicrobials: Amoxicillin (99.6%), ampicillin (99.8%), benzylpenicillin (99.3%), cloxacillin (98.7%), oxacillin (98.4%), lincomycin (100.3%), tylosin (87.9%), trimethoprim (99.5%), tetracycline (96.8%) and cephapirin (98.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the rest of antimicrobials: ceftiofur (98.01%), cephalixin (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); Pesticides: 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%), dicholphos (99.8%), chlorpyrifos (99.8%), boscalid (99.5%), fentoate (98.8%), fenthion (98.5%), fenvalerate (99.4%), monocrotophos (99.8%), malaaxon (99.0%), methamidophos (98.1%), metacrifos (96.1%), amitraz (99.8%), omethoate (98.4%), vamidothion ( $\geq 98.0\%$ ), phosmet (99.8%), heptenophos (98.7%), bifenthrin (99.0%), methomyl (99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Mycotoxins: Ochratoxin A ( $\geq 98.0\%$ ) and zearalenone (99.0%)

were obtained from Trilogy Analytical Laboratory, Inc. (Washington, USA).

The isotopic labelled internal standards used were: anabolic steroid: 19–17  $\beta$  Nortestosteron-d3 was obtained from the RIVM, Netherlands; lactones of resorcylic acid:  $\beta$ -zearalenol-d4 ( $\geq 98.0\%$ ) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada);  $\beta$  agonists: Clenbuterol-d6 HCl (98.0%), brombuterol-d9 HCl (98.0%), mabuterol-d9 HCl (98.0%), clenpenterol-d5 HCl (98.0%) and cimbuterol-d9 (98.0%) obtained from Witega (Berlin, Germany), isoxsuprine-d5 hemifumarate ( $\geq 98.0\%$ ) and ractopamine-d6 HCl ( $\geq 98.0\%$ ) from the European reference laboratory (EURL) at RIKILT, The Netherlands, salbutamol (albuterol)-d9 ( $\geq 98.0\%$ ) from Dr. Ehrenstorfer GmbH (Augsburg, Germany), zilpaterol-d7 ( $\geq 98.0\%$ ) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada), while terbutaline-d9 acetate hemihydrate (99.3%) was obtained from Sigma-Aldrich (St. Louis, MO, USA); antimicrobials: flunixin-d3 (100.0%), penicillin G-d7 N-ethylpiperidinium (98.1%) salt and pesticides: atrazine-d5 (99.7%), chlorpyrifos-d10 (100%) and carbofuran-d3 (99.3%) were obtained from Sigma-Aldrich (St. Louis, MO, USA) [10, 26].

### Preparation of stock standard solutions

To prepare the stock standards solutions, 5 to 10 mg of each standard and internal standard was weighed and transferred to a 10 ml volumetric flask which contained methanol. The concentration of individual stock solutions was in range from 0.5 mg/ml to 1.0 mg/ml. The solutions were stored at  $-20\text{ }^{\circ}\text{C}$ .

### Preparation of working standard solutions

A total of five mixed working solutions were prepared in methanol from the standards stock solutions. According to the values for minimum method performance requirements (MMPRs) the standards and internal standards were divided in groups [27]. The standards from analytes with the same values for MMPRs (Table 1) were placed in the same group, while standards from analytes without MMPSs and MRL were placed in another group, because they had similar sensitivity levels. Group 1 (mixed working solution 1) consisted of: thiouracil, methylthiouracil, propylthiouracil and tapazole; group 2 (mixed working solution 2) had: methyltestosterone, 19 nortestosterone, stanozolol, isoxsuprine, ractopamine, salbutamol, zilpaterol, terbutaline; group 3 (mixed working solution 3) consisted of: clenbuterol, brombuterol, mabuterol, clenpenterol and cimbuterol; group 4 (mixed working solution 4) contained: boldenone, zeranol and taleranol and the rest of the analyses were placed in group 5 (mixed working solution 5). The initial concentration of all five mixed working solutions which were prepared from individual standard stock solutions was 10  $\mu\text{g/ml}$ , but this

**Table 1** MMPR values for target analytes included in the study

| Analytes                     | MMPR (µg/L) |
|------------------------------|-------------|
| Thiouracil (TU)              | 10          |
| Methylthiouracil (MTU)       | 10          |
| Propylthiouracil (PTU)       | 10          |
| Tapazole (TAP)               | 10          |
| Testosterone (TEST)          | /           |
| Methyltestosterone (MEST)    | 0.5         |
| Boldenone (BOLD)             | 1           |
| 19 Nortestosterone (19 NO)   | 0.5         |
| Stanozolol (STZL)            | 0.5         |
| Clostebol (CLBL)             | /           |
| Zeranol (ZENL)               | 1           |
| Taleranol (TANL)             | 1           |
| Clenbuterol (CLEN)           | 0.1         |
| Brombuterol (BROM)           | 0.1         |
| Mabuterol (MABT)             | 0.1         |
| Clenpenterol (CLEP)          | 0.1         |
| Isoxuprin (ISOX)             | 0.5         |
| Cimbuterol (CIMB)            | 0.1         |
| Ractopamine (RACTO)          | 0.5         |
| Salbutamol (SALB)            | 0.5         |
| Zilpaterol (ZILP)            | 0.5         |
| Terbutaline (TERB)           | 0.5         |
| Amoxicillin (AMOX)           | /           |
| Ampicillin (AMPO)            | /           |
| Benzylpenicillin (BNPC)      | /           |
| Lincomycin (LINK)            | /           |
| Tylosin (TYLS)               | /           |
| Trimethoprim (TRIP)          | /           |
| Cephapirin (CEPR)            | /           |
| Tetracycline (TETC)          | /           |
| Cloxacillin (CLCN)           | /           |
| Oxacillin (OXIN)             | /           |
| Cefalexin (CEFA)             | /           |
| Ceftiofur (CEFT)             | /           |
| Enrofloxacin (ENRO)          | /           |
| Ciprofloxacin (CIPR)         | /           |
| Oxytetracycline (OXTT)       | /           |
| Sulfachloropyridazine (SUPZ) | /           |
| Sulfadiazine (SUDI)          | /           |
| Sulfadimethoxine (SUDM)      | /           |
| Sulfadimidine (SULD)         | /           |
| Sulfamethoxazole (SULM)      | /           |
| Carbofuran (CRL)             | /           |
| Carbaryl (CRB)               | /           |
| Parathion (PTN)              | /           |
| Malathion (MTN)              | /           |
| Diazinon (DNN)               | /           |
| Dimethoate (DIM)             | /           |
| Atrazine (ATRZ)              | /           |
| Permethrine (PEMT)           | /           |
| Cypermethrine (CIRM)         | /           |
| Deltamethrine (DELM)         | /           |
| Coumaphos (COU)              | /           |

**Table 1** (continued)

| Analytes             | MMPR (µg/L) |
|----------------------|-------------|
| Dichlorophos (DIRP)  | /           |
| Chlorpyrifos (CHRS)  | /           |
| Fenvalerate (FERT)   | /           |
| Boscalid (BOS)       | /           |
| Fenthoate (FETE)     | /           |
| Fenthione (FEON)     | /           |
| Monocrotophos (MOCR) | /           |
| Malaoxon (MAON)      | /           |
| Methamidophos MEDF   | /           |
| Metacrifos (MECF)    | /           |
| Amitraz (AMRZ)       | /           |
| Omethoate (OMAT)     | /           |
| Vamidothion (VAON)   | /           |
| Phosmet (FOST)       | /           |
| Heptenophos (HEPH)   | /           |
| Bifenthrin (BFNT)    | /           |
| Methomyl (MEML)      | /           |
| Zearalenone (ZEAN)   | /           |
| Ochratoxin A (OTAA)  | /           |

**Table 2** Sample preparation steps

| Sample preparation steps | Initial sample preparation | Spiking | Addition of buffer | Enzymatic hydrolyze | Centrifugation | LLE | SPE | Evaporation | Reconstitution | LC-MS/MS analysis |
|--------------------------|----------------------------|---------|--------------------|---------------------|----------------|-----|-----|-------------|----------------|-------------------|
| Protocol no              |                            |         |                    |                     |                |     |     |             |                |                   |
| 1                        | +                          | +       | +                  | -                   | +              | +   | -   | +           | +              | +                 |
| 2                        | +                          | +       | +                  | +                   | +              | +   | -   | +           | +              | +                 |
| 3                        | +                          | +       | +                  | -                   | +              | -   | +   | +           | +              | +                 |
| 4                        | +                          | +       | +                  | +                   | +              | -   | +   | +           | +              | +                 |
| 5                        | +                          | +       | +                  | -                   | +              | -   | +   | +           | +              | +                 |
| 6                        | +                          | +       | +                  | +                   | +              | -   | +   | +           | +              | +                 |

\*SPE with OASIS HLB cartridges; \*\*SPE with DSC-MCAX cartridges

was diluted further while preparing the calibration curve, depending on the required concentrations. From the appropriate mixed working solutions from 10 µg/ml were prepared the next mixed working solutions: for group 1: 1.0 µg/ml (prepared from mixed working solution 1), for group 2: 1.0 µg/ml and 0.1 µg/ml (prepared from mixed working solution 2), for group 3: 1.0 µg/ml and 0.01 µg/ml (prepared from mixed working solution 3), for group 4: 1.0 µg/ml and 0.1 µg/ml (prepared from mixed working solution 4) and for group 5: 1.0 µg/ml (prepared from mixed working solution 5); (the solutions were prepared in the volumetric flask from 10 ml and dissolved in methanol). The mixed working solutions were used for preparation of matrix match calibration curve and spiking of the urine samples. Matrix match calibration curves were prepared in blank bovine urine samples (Table 2).

The mixed working solutions from internal standards were prepared in methanol from the internal standards stock solutions. The internal standards were also divided

in two groups, the first one (with concentration of 5 µg/l in the samples, after spiking) consisted of: 19–17β Nortestosteron-d3, isoxsuprine-d5, ractopamine-d6, salbutamol-d9, zilpaterol-d7, terbutaline-d9, Clenbuterol-d6, brombuterol-d9, mabuterol-d9, clenpenterol-d5, cimbuterol-d9 and β-zearalenol-d4. The concentration of the mixed internal standard working solution was 10 µg/ml, from which additionally, for spiking of the samples and preparation of matrix match calibration curve, was prepared mixed internal standard working solution with concentration from 1000 ng/ml. The second group (with concentration of 50 µg/l in the samples, after spiking) consisted of: flunixin-d3, penicillin G-d7, atrazine-d5, chlorpyrifos-d10 and carbofuran-d3. The concentration of this mixed internal standard working solution for spiking of the samples and preparation of matrix match calibration curve was 10 µg/ml.

Matrix-matched calibration prepared in blank bovine urine and internal standard were utilized for



quantification to compensate the matrix effects that influence analytical response. Due to aspects of availability, cost, and convenience the use of internal standards was feasible only for  $\beta$ -agonists, 7 antibiotics, 3 pesticides, 1 anabolic steroid and 1 mycotoxin (Table 3).

### Sample preparation

Six extraction protocols during the optimization of the extraction method were investigated for extraction of the 72 compounds from urine. In two extraction protocols liquid-liquid extraction (LLE) was applied, while solid phase extraction (SPE) was applied to the remaining four protocols. This characterization was as follows: LLE without enzymatic hydrolysis (protocol 1); LLE with enzymatic hydrolysis (protocol 2); SPE using OASIS HLB cartridges without enzymatic hydrolysis (protocol 3); SPE using OASIS HLB cartridges with enzymatic hydrolysis (protocol 4); SPE using DSC-MCAX cartridges without enzymatic hydrolysis (protocol 5) and lastly SPE using DSC-MCAX cartridges with enzymatic hydrolysis (protocol 6). Enzymatic hydrolysis was performed with  $\beta$ -glucuronidase aryl sulfatase (*Helix pomatia*). Sample preparation steps for all protocols are given below. The initial preparation of the samples prior to extraction, as well as the spiking of urine samples with standards and internal standards are the same for all protocols.

**All protocols** In the first step, 30 ml urine sample was centrifuged 5 min, at 2000 rpm and room temperature. This step was used to remove proteins from the matrix. After centrifugation, 5 ml of urine sample was fortified with the standards and internal standards. Prior to extraction the samples were left to stand for 10 min. Next, the samples were prepared with different protocols, as follows.

**Protocol 1** 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 to the samples, and the samples were shaken for 1 min on a vortex. After this step samples were centrifuged 5 min, at 2000 rpm, at room temperature followed by LLE. 10 ml methanol:acetonitrile:acetic acid (49:49:2, v/v/v) was added and the samples were shaken for 1 min on a vortex and centrifuged again for 5 min, at 2000 rpm and room temperature and the supernatant was transferred to new test tubes. The LLE was repeated with 10 ml of ethyl acetate: hexane (40:60, v/v). The samples were shaken for 1 min on a vortex and centrifuged 5 min, at 2000 rpm and room temperature. The supernatant was collected, added to the first supernatant and the mixture 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), filtered through a 0.45  $\mu$ m membrane filter into 2 ml autosampler

vials and analysed on the LC-MS/MS after separation on a reverse phase column.

**Protocol 2** 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 to the samples, and the samples were shaken for 1 min on a vortex. Further, 20  $\mu$ l of  $\beta$ -glucuronidase aryl sulfatase was added and the samples were incubated for 17 h at 37 °C. After cooling to room temperature, the samples were centrifuged for 5 min, at 2000 rpm and room temperature, followed by LLE. The LLE and the next steps for sample preparation were the same as in protocol 1.

**Protocol 3** 5 ml of 0.2 M sodium acetate buffer (pH=5) and 5 ml 0.02 M phosphate buffer (PBS, pH=7.2) were added and the samples were shaken for 1 min on a vortex before spinning of a centrifuge for 5 min at 2000 rpm and room temperature. This was followed SPE extraction. Oasis HLB cartridges were activated and conditioned with 5 ml of methanol and 5 ml of water. The 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 first with 4 ml of methanol:acetonitrile:ammonium hydroxide (48.5:48.5:3, v/v/v) and then with 4 ml of methanol: dichloromethane (1.5:8.5, v/v). The eluent was evaporated to dryness at 35 °C under nitrogen, the residues were reconstituted with 1 ml of the mobile phase (Mobile phase A: Mobile phase B, 95:5, v/v, B) pressed through 0.45  $\mu$ m membrane filter into 2 ml autosampler vials prior to LC-MS/MS analysis after separation on a C-18 column.

**Protocol 4** 5 ml of 0.2 M sodium acetate buffer (pH=5) and 5 ml 0.02 M Phosphate buffer (PBS, pH=7.2) were added and the samples were shaken for 1 min on a vortex before spinning of a centrifuge for 5 min at 2000 rpm and room temperature. In the next step,  $\beta$ -glucuronidase aryl sulfatase (20  $\mu$ l) was added and the samples incubated for 17 h at 37 °C followed after cooling, by centrifugation for 5 min at 2000 rpm and room temperature. Further, the same approach was applied as well as in protocol 3.

**Protocol 5** In this protocol, DSC-MCAX cartridges instead of Oasis HLB cartridges were used and the rest of the conditions remaining the same as in the protocol 3.

**Protocol 6** In this protocol, Oasis HLB cartridges were replaced by DSC-MCAX cartridges and the rest of the conditions remaining the same as in the protocol 6.

The sample preparation steps for all protocols are given in the Table 4.

**Table 3** MRM parameters for the target analytes

| Standard                       | Polarity | Precursor ion (m/z) | Product ion (m/z) | Collision energy | Cone voltage | Retention time |
|--------------------------------|----------|---------------------|-------------------|------------------|--------------|----------------|
| Thiouracil (TU)                | +        | 128.80              | 112.0<br>69.86    | 20<br>18         | 30           | 4.90           |
| Methylthiouracil (MTU)         | +        | 142.83              | 125.90<br>83.85   | 18<br>22         | 30           | 2.35           |
| Propylthiouracil (PTU)         | +        | 170.88              | 154.30<br>111.91  | 20<br>24         | 32           | 3.91           |
| Tapazole (TAP)                 | +        | 114.82              | 110.15<br>87.83   | 16<br>16         | 36           | 1.23           |
| Testosterone (TEST)            | +        | 289.16              | 108.99<br>96.95   | 24<br>28         | 36           | 7.70           |
| Methyltestosterone (MEST)      | +        | 303.22              | 96.96<br>109.0    | 28<br>24         | 36           | 7.79           |
| Boldenone (BOLD)               | +        | 287.16              | 121.03<br>135.02  | 24<br>16         | 34           | 7.50           |
| 19 Nortestosterone (19 NO)     | +        | 275.14              | 80.56<br>109.0    | 34<br>26         | 38           | 7.32           |
| Stanozolol (STZL)              | +        | 329.22              | 80.95<br>95.00    | 46<br>46         | 64           | 7.90           |
| Clostebol (CLBL)               | +        | 323.16              | 130.98<br>142.96  | 26<br>26         | 40           | 7.98           |
| Zeranol (ZENL)                 | -        | 321.03              | 90.87<br>40.90    | 40<br>40         | 74           | 6.83           |
| Taleranol (TANL)               | -        | 321.03              | 90.87<br>40.90    | 34<br>40         | 74           | 7.48           |
| Clenbuterol (CLEN)             | +        | 276.97              | 202.95<br>131.87  | 16<br>30         | 22           | 4.89           |
| Brombuterol (BROM)             | +        | 366.90              | 292.84<br>211.42  | 20<br>34         | 26           | 5.41           |
| Mabuterol (MABT)               | +        | 310.95              | 236.99<br>216.96  | 18<br>26         | 24           | 5.26           |
| Clenpenterol (CLEP)            | +        | 291.00              | 202.92<br>131.89  | 16<br>30         | 28           | 5.62           |
| Isoxuprin (ISOX)               | +        | 302.04              | 106.96<br>164.01  | 30<br>16         | 26           | 6.29           |
| Cimbuterol (CIMB)              | +        | 234.03              | 159.98<br>142.94  | 16<br>28         | 22           | 3.01           |
| Ractopamine (RACT)             | +        | 302.04              | 164.01<br>106.96  | 16<br>28         | 24           | 4.29           |
| Salbutamol (SALB)              | +        | 240.03              | 147.96<br>165.98  | 20<br>14         | 22           | 2.81           |
| Zilpaterol (ZILP)              | +        | 262.03              | 202.05<br>185.01  | 22<br>24         | 22           | 3.93           |
| Terbutaline hemisulfate (TERB) | +        | 226.00              | 152.00<br>106.97  | 14<br>30         | 26           | 2.73           |
| Amoxicillin (AMOX)             | +        | 367.07              | 159.96<br>90.89   | 16<br>40         | 28           | 6.43           |
| Ampicillin (AMP)               | +        | 349.97              | 105.95<br>159.94  | 20<br>14         | 34           | 4.97           |
| Benzylpenicillin (BNPC)        | +        | 334.99              | 90.96<br>80.94    | 42<br>52         | 44           | 7.39           |
| Lincomycin (LINK)              | +        | 407.06              | 126.02<br>41.75   | 34<br>72         | 22           | 3.89           |
| Tylosin (TYLS)                 | +        | 916.3               | 173.99<br>100.88  | 46<br>52         | 74           | 6.86           |
| Trimethoprim (TRIP)            | +        | 290.97              | 122.94<br>229.94  | 28<br>24         | 26           | 3.81           |

**Table 3** (continued)

| Standard                     | Polarity | Precursor ion (m/z) | Product ion (m/z) | Collision energy | Cone voltage | Retention time |
|------------------------------|----------|---------------------|-------------------|------------------|--------------|----------------|
| Cephapirin (CEPR)            | +        | 423.93              | 291.93<br>151.89  | 14<br>28         | 42           | 6.08           |
| Tetracycline (TETC)          | +        | 445.03              | 410.01<br>153.90  | 20<br>34         | 40           | 4.18           |
| Cloxacillin (CLCN)           | +        | 435.94              | 159.97<br>276.96  | 18<br>14         | 26           | 6.94           |
| Oxacillin (OXIN)             | +        | 402.05              | 159.96<br>243.03  | 10<br>12         | 24           | 6.90           |
| Cefalexin (CEFA)             | +        | 347.97              | 157.86<br>173.93  | 8<br>14          | 30           | 2.75           |
| Ceftiofur (CEFT)             | +        | 523.96              | 241.00<br>125.17  | 16<br>58         | 34           | 9.40           |
| Enrofloxacin (ENRO)          | +        | 360.05              | 245.09<br>72.02   | 30<br>36         | 36           | 4.65           |
| Ciprofloxacin (CIPR)         | +        | 332.01              | 245.05<br>230.94  | 40<br>28         | 38           | 4.49           |
| Oxytetracycline (OXTT)       | +        | 462.01              | 426.02<br>200.93  | 38<br>30         | 36           | 4.37           |
| Sulfachloropyridazine (SUPZ) | +        | 284.90              | 155.93<br>91.93   | 16<br>34         | 28           | 3.93           |
| Sulfadiazine (SUDI)          | +        | 250.97              | 91.93<br>155.93   | 30<br>14         | 28           | 2.49           |
| Sulfadimethoxine (SUDM)      | +        | 310.97              | 155.93<br>91.93   | 20<br>32         | 36           | 5.41           |
| Sulfadimidine (SULD)         | +        | 278.95              | 185.93<br>91.93   | 18<br>36         | 34           | 3.57           |
| Sulfamethoxazole (SULM)      | +        | 253.91              | 92.00<br>155.94   | 30<br>16         | 28           | 4.07           |
| Carbofuran (CRL)             | +        | 222.1               | 165.0<br>123.0    | 12<br>22         | 32           | 6.39           |
| Carbaryl (CRB)               | +        | 202.0               | 145.05<br>127.0   | 10<br>32         | 26           | 5.93           |
| Parathion (PTN)              | +        | 292.0               | 210.0<br>180.0    | 12<br>26         | 30           | 6.86           |
| Malathion (MTN)              | +        | 331.1               | 98.93<br>127.0    | 14<br>26         | 30           | 3.85           |
| Diazinon (DNN)               | +        | 304.97              | 168.94<br>153.00  | 24<br>24         | 44           | 8.18           |
| Dimethoate (DIM)             | +        | 229.90              | 198.83<br>124.84  | 10<br>20         | 30           | 4.77           |
| Atrazine (ATRZ)              | +        | 216.0               | 174.22<br>104.14  | 15<br>30         | 32           | 6.98           |
| Permethrin (PEMT)            | +        | 390.97              | 355.02<br>182.92  | 6<br>12          | 34           | 6.35           |
| Cypermethrin (CIRM)          | +        | 433.0               | 192.80<br>90.93   | 20<br>12         | 28           | 8.07           |
| Deltamethrin (DELM)          | +        | 229.84              | 198.83<br>124.85  | 30<br>14         | 30           | 4.50           |
| Coumaphos (COU)              | +        | 362.90              | 226.86 306.86     | 26<br>18         | 52           | 7.75           |
| Dichlorophos (DIRP)          | +        | 220.78              | 108.89<br>78.83   | 20<br>30         | 44           | 6.13           |
| Chlorpyrifos (CHRS)          | +        | 351.78              | 96.79<br>199.77   | 32<br>16         | 38           | 7.47           |
| Fenvalerate (FERT)           | +        | 419.97              | 166.89<br>124.88  | 14<br>42         | 38           | 7.17           |



**Table 3** (continued)

| Standard                                 | Polarity | Precursor ion (m/z) | Product ion (m/z) | Collision energy | Cone voltage | Retention time |
|--|----------|---------------------|-------------------|------------------|--------------|----------------|
| Boscalid (BOS)                           | +        | 342.94              | 306.94<br>139.85  | 20<br>20         | 56           | 7.46           |
| Fenthoate (FETE)                         | +        | 320.86              | 162.87<br>246.84  | 12<br>12         | 28           | 7.31           |
| Fenthion (FEON)                          | +        | 278.82              | 168.87<br>104.86  | 18<br>28         | 38           | 7.98           |
| Monocrotophos (MOCR)                     | +        | 223.16              | 192.87<br>97.83   | 8<br>12          | 30           | 3.65           |
| Malaoxon (MAON)                          | +        | 314.94              | 126.84<br>98.80   | 1426             | 38           | 6.79           |
| Methamidophos (MEDF)                     | +        | 141.78              | 93.80<br>46.82    | 14<br>24         | 38           | 1.69           |
| Methacrifos (MECF)                       | +        | 240.93              | 208.83<br>124.83  | 8<br>20          | 32           | 8.59           |
| Amitraz (AMRZ)                           | +        | 294.05              | 162.96<br>121.91  | 14<br>32         | 30           | 7.40           |
| Omethoate (OMAT)                         | +        | 213.84              | 182.82<br>154.84  | 12<br>18         | 32           | 2.18           |
| Vamidothion (VAON)                       | +        | 287.78              | 145.92<br>117.87  | 14<br>24         | 30           | 4.89           |
| Phosmet (FOST)                           | +        | 320.86              | 246.84<br>162.87  | 14<br>58         | 32           | 7.20           |
| Heptenophos (HEPH)                       | +        | 250.78              | 126.83<br>89.04   | 16<br>34         | 42           | 7.10           |
| Bifenthrin (BFNT)                        | +        | 440.03              | 180.96<br>165.87  | 22<br>42         | 24           | 9.20           |
| Methomyl (MEML)                          | +        | 162.84              | 87.88 105.90      | 8<br>10          | 30           | 3.29           |
| Zearalenone (ZEAN)                       | -        | 316.97              | 130.87 174.91     | 30<br>26         | 62           | 6.85           |
| Ochratoxin A (OTAA)                      | +        | 404.03              | 238.92<br>101.80  | 30<br>10         | 46           | 7.62           |
| 19–17 $\beta$ Nortestosterone D3 (19ND3) | +        | 278.10              | 108.95            | 26               | 46           | 7.26           |
| Clenbuterol-d6 (CLENL6)                  | +        | 283.03              | 203.56            | 16               | 22           | 4.89           |
| Brombuterol-d9 (BROMD9)                  | +        | 375.93              | 293.87            | 18               | 24           | 5.45           |
| Mabuterol-d9 (MABTD9)                    | +        | 320.07              | 237.94            | 18               | 24           | 5.50           |
| Clenpenterol-d5 (CLEPD5)                 | +        | 296.00              | 203.10            | 16               | 24           | 5.71           |
| Isosuxprin-d5 hemifumarate (ISOXD5)      | +        | 308.15              | 120.95            | 16               | 26           | 4.37           |
| Cimbuterol-d9 (CIMBD9)                   | +        | 243.07              | 160.96            | 16               | 20           | 3.25           |
| Ractopamine-d6 (RACTD6)                  | +        | 308.10              | 168.05            | 16               | 24           | 4.33           |
| Salbutamol-d9 (SALBD9)                   | +        | 249.08              | 148.59            | 20               | 24           | 2.81           |
| Zilpaterol-d7 (ZILPD7)                   | +        | 269.08              | 185.15            | 24               | 22           | 3.93           |
| Terbutalin-d9 (TERBD9)                   | +        | 235.07              | 152.83            | 16               | 34           | 5.52           |
| Flunixin-3 (FLUXD3)                      | +        | 300.03              | 263.98            | 36               | 28           | 7.74           |
| Penicillin G-d7 (PENGD7)                 | +        | 374.03              | 159.94            | 16               | 32           | 6.39           |
| $\beta$ -zearalenol-d4 (ZEAND4)          | -        | 323.03              | 160.02            | 30               | 68           | 7.20           |
| Carbofuran-d3 (CARBD3)                   | +        | 224.97              | 164.91            | 12               | 22           | 6.46           |
| Atrazine-d5 (ATRZD5)                     | +        | 220.98              | 100.90            | 24               | 34           | 7.02           |
| Chlorpyrifos-d10 (CHRD10)                | +        | 361.82              | 98.74             | 30               | 26           | 6.90           |

**LC-MS/MS analysis**

The LC-MS/MS (Waters ACQUITY™ Ultra Performance LC coupled to Waters ACQUITY™ TOQ, Milford, MA, USA) used for identification and quantification of the

analytes was equipped with a binary pump, vacuum degasser, thermostated autosampler, thermostated column manager. A Kinetex C18 LC column (50×2.1 mm, 2.6  $\mu$ m, Phenomenex, Torrance, CA, USA) was used for

**Table 4** Recovery in bovine urine samples for each protocol

| Analytes                   | Added concentration (µg/ L) | LLE* without EH** (%) | LLE with EH | SPE*** with Oasis HLB cartridges without EH (%) | SPE with Oasis HLB cartridges (%) with EH | SPE with DSC-MCAX cartridges (%) without EH | SPE with DSC-MCAX cartridges (%) with EH |
|----------------------------|-----------------------------|-----------------------|-------------|---|---|---|--|
| Thiouracil (TU)            | 5                           | nd****                | nd          | 88.6  | 93.0                                      | 65.8  | 70.4                                     |
|                            | 10                          |                       |             | 92.4  | 101.1                                     | 62.4  | 75.2                                     |
|                            | 15                          |                       |             | 94.1  | 101.5                                     | 55.7  | 77.8                                     |
| Methylthiouracil (MTU)     | 5                           | nd                    | nd          | 80.3  | 95.6                                      | 72.4  | 79.8                                     |
|                            | 10                          |                       |             | 85.3  | 94.6                                      | 77.4  | 80.1                                     |
|                            | 15                          |                       |             | 93.5  | 96.1                                      | 75.6  | 84.3                                     |
| Propylthiouracil (PTU)     | 5                           | nd                    | nd          | 70.2  | 77.0                                      | 67.8  | 71.4                                     |
|                            | 10                          |                       |             | 73.1  | 84.5                                      | 66.4  | 74.2                                     |
|                            | 15                          |                       |             | 77.5  | 89.9                                      | 60.2  | 77.4                                     |
| Tapazole (TAP)             | 5                           | nd                    | nd          | 73.5  | 86.4                                      | 74.7  | 81.4                                     |
|                            | 10                          |                       |             | 71.5  | 84.8                                      | 72.3  | 77.8                                     |
|                            | 15                          |                       |             | 79.9  | 93.9                                      | 78.5  | 79.3                                     |
| Testosterone (TEST)        | 7.5                         | 65.6                  | 69.6        | 74.2  | 87.2                                      | 69.7  | 80.4                                     |
|                            | 15                          | 60.2                  | 65.4        | 73.5  | 88.2                                      | 74.3  | 76.3                                     |
|                            | 22.5                        | 61.3                  | 64.3        | 70.0  | 90.5                                      | 72.1  | 71.2                                     |
| Methyltestosterone (MEST)  | 0.25                        | 48.9                  | 58.9        | 65.2  | 86.0                                      | 60.2  | 67.8                                     |
|                            | 0.50                        | 45.3                  | 59.0        | 60.1  | 91.0                                      | 61.3  | 71.2                                     |
|                            | 0.75                        | 50.2                  | 52.4        | 67.7  | 94.8                                      | 64.7  | 65.1                                     |
| Boldenone (BOLD)           | 0.5                         | 51.3                  | 55.4        | 63.4  | 84.8                                      | 54.6  | 64.5                                     |
|                            | 1.0                         | 54.8                  | 59.7        | 71.2  | 104.2                                     | 52.2  | 60.2                                     |
|                            | 1.5                         | 60.2                  | 61.6        | 60.1  | 91.6                                      | 58.3  | 67.4                                     |
| 19 Nortestosterone (19 NO) | 0.25                        | 44.6                  | 60.1        | 55.4  | 84.4                                      | 65.4  | 70.4                                     |
|                            | 0.5                         | 40.2                  | 62.2        | 61.2  | 94.2                                      | 60.7  | 66.3                                     |
|                            | 0.75                        | 51.3                  | 57.7        | 63.1  | 95.2                                      | 59.2  | 67.1                                     |
| Stanozolol (STZL)          | 0.25                        | 65.6                  | 69.6        | 70.0  | 88.8                                      | 71.3  | 75.6                                     |
|                            | 0.5                         | 60.2                  | 66.7        | 59.4  | 91.4                                      | 69.2  | 71.3                                     |
|                            | 0.75                        | 63.8                  | 70.2        | 63.1  | 94.8                                      | 61.3  | 69.4                                     |
| Clostebol (CLBL)           | 7.5                         | 39.4                  | 52.5        | 71.2  | 89.6                                      | 57.6  | 65.6                                     |
|                            | 15                          | 35.5                  | 54.8        | 62.4  | 93.6                                      | 54.3  | 69.7                                     |
|                            | 22.5                        | 41.3                  | 50.3        | 60.1  | 93.0                                      | 61.8  | 66.3                                     |
| Zeranol (ZENL)             | 0.5                         | 49.7                  | 60.3        | 54.1  | 79.2                                      | 67.3  | 71.3                                     |
|                            | 1.0                         | 42.5                  | 61.6        | 55.2  | 81.6                                      | 70.5  | 72.5                                     |
|                            | 1.5                         | 44.5                  | 54.8        | 61.1  | 89.9                                      | 62.4  | 79.2                                     |
| Talaranol (TANL)           | 0.5                         | 55.2                  | 64.1        | 58.4  | 87.2                                      | 61.6  | 78.2                                     |
|                            | 1.0                         | 51.1                  | 63.2        | 67.8  | 90.8                                      | 67.3  | 69.1                                     |
|                            | 1.5                         | 57.8                  | 58.8        | 71.3  | 81.9                                      | 70.2  | 66.7                                     |
| Clenbuterol (CLEN)         | 0.05                        | 41.6                  | 64.3        | 49.4  | 74.0                                      | 56.5  | 70.3                                     |
|                            | 0.1                         | 43.1                  | 56.4        | 54.1  | 79.0                                      | 59.2  | 71.2                                     |
|                            | 0.15                        | 47.8                  | 60.0        | 61.2  | 88.0                                      | 55.4  | 65.6                                     |
| Brombuterol (BROM)         | 0.05                        | 51.4                  | 61.3        | 64.3  | 76.0                                      | 66.3  | 69.4                                     |
|                            | 0.1                         | 50.8                  | 65.4        | 69.1  | 86.0                                      | 67.1  | 77.6                                     |
|                            | 0.15                        | 57.2                  | 63.2        | 58.3  | 78.0                                      | 75.4  | 80.2                                     |
| Mabuterol (MABT)           | 0.05                        | 35.4                  | 47.8        | 51.3  | 84.0                                      | 57.4  | 60.2                                     |
|                            | 0.1                         | 32.1                  | 45.2        | 49.1  | 71.0                                      | 59.2  | 59.9                                     |
|                            | 0.15                        | 40.2                  | 43.4        | 53.5  | 75.3                                      | 51.5  | 54.3                                     |
| Clenpenterol (CLEP)        | 0.05                        | 46.8                  | 54.7        | 61.6  | 96.0                                      | 66.3  | 75.6                                     |
|                            | 0.1                         | 49.2                  | 53.2        | 54.1  | 113.0                                     | 69.2  | 70.2                                     |
|                            | 0.15                        | 50.1                  | 64.5        | 61.3  | 91.3                                      | 71.8  | 80.5                                     |
| Isoxuprin (ISOX)           | 0.25                        | 51.2                  | 60.8        | 71.4  | 90.8                                      | 54.6  | 66.3                                     |
|                            | 0.50                        | 47.8                  | 58.9        | 75.3  | 102.8                                     | 50.2  | 57.8                                     |
|                            | 0.75                        | 55.7                  | 53.4        | 81.2  | 98.4                                      | 59.8  | 70.2                                     |
| Cimbuterol (CIMB)          | 0.05                        | 40.8                  | 60.2        | 54.5  | 74.0                                      | 59.6  | 62.4                                     |
|                            | 0.1                         | 45.6                  | 55.7        | 61.3  | 117.0                                     | 64.3  | 75.4                                     |
|                            | 0.15                        | 44.7                  | 61.3        | 64.1  | 88.0                                      | 71.8  | 72.1                                     |

**Table 4** (continued)

| Analytes                | Added concentration (µg/L) | LLE* without EH** (%) | LLE with EH | SPE*** with Oasis HLB cartridges without EH (%) | SPE with Oasis HLB cartridges (%) with EH | SPE with DSC-MCAX cartridges (%) without EH | SPE with DSC-MCAX cartridges (%) with EH |
|-------------------------|----------------------------|-----------------------|-------------|---|---|---|--|
| Ractopamine (RACT)      | 0.25                       | 42.4                  | 60.8        | 46.1  | 95.2                                      | 63.2  | 77.4                                     |
|                         | 0.50                       | 45.0                  | 63.2        | 49.7  | 91.4                                      | 66.4  | 70.5                                     |
|                         | 0.75                       | 51.2                  | 59.5        | 59.4  | 93.0                                      | 71.3  | 73.2                                     |
| Salbutamol (SALB)       | 0.25                       | 61.5                  | 69.7        | 69.7  | 78.8                                      | 74.6  | 83.1                                     |
|                         | 0.50                       | 64.2                  | 66.4        | 66.3  | 97.4                                      | 70.2  | 75.6                                     |
|                         | 0.75                       | 59.4                  | 67.8        | 60.1  | 85.9                                      | 79.4  | 88.1                                     |
| Zilpaterol (ZILP)       | 0.25                       | 39.4                  | 51.2        | 66.3  | 85.2                                      | 55.4  | 63.8                                     |
|                         | 0.50                       | 33.5                  | 43.1        | 59.7  | 103.4                                     | 59.2  | 70.2                                     |
|                         | 0.75                       | 37.8                  | 58.7        | 63.1  | 102.1                                     | 53.1  | 61.5                                     |
| Terbutaline (Terb)      | 0.25                       | 45.4                  | 59.8        | 47.3  | 77.6                                      | 65.4  | 70.2                                     |
|                         | 0.50                       | 55.2                  | 59.9        | 51.3  | 103.8                                     | 60.2  | 75.4                                     |
|                         | 0.75                       | 53.4                  | 62.3        | 55.1  | 88.8                                      | 59.8  | 77.9                                     |
| Amoxicillin (AMOX)      | 7.5                        | 62.5                  | 70.0        | 64.8  | 96.0                                      | 63.5  | 78.2                                     |
|                         | 15                         | 63.8                  | 65.4        | 63.0  | 94.0                                      | 66.8  | 81.3                                     |
|                         | 22.5                       | 57.6                  | 61.8        | 59.3  | 85.8                                      | 71.3  | 71.5                                     |
| Ampicillin (AMP)        | 7.5                        | 44.6                  | 54.8        | 74.2  | 92.0                                      | 69.4  | 81.4                                     |
|                         | 15                         | 50.2                  | 55.9        | 73.8  | 98.2                                      | 65.6  | 75.6                                     |
|                         | 22.5                       | 45.3                  | 49.3        | 85.4  | 94.7                                      | 60.2  | 78.3                                     |
| Benzylpenicillin (BNPC) | 7.5                        | 39.0                  | 57.0        | 84.3  | 101.3                                     | 64.6  | 74.5                                     |
|                         | 15                         | 41.5                  | 54.2        | 81.5  | 101.3                                     | 69.2  | 78.8                                     |
|                         | 22.5                       | 46.0                  | 61.3        | 75.2  | 83.4                                      | 60.4  | 77.3                                     |
| Lincomycin (LINK)       | 7.5                        | 36.5                  | 51.8        | 78.2  | 89.5                                      | 75.5  | 81.3                                     |
|                         | 15                         | 42.8                  | 52.3        | 85.3  | 82.6                                      | 74.1  | 77.4                                     |
|                         | 22.5                       | 44.6                  | 54.6        | 74.6  | 92.1                                      | 79.2  | 78.2                                     |
| Tylosin (TYLS)          | 7.5                        | 55.8                  | 62.4        | 82.2  | 90.7                                      | 61.2  | 75.5                                     |
|                         | 15                         | 52.1                  | 60.2        | 87.5  | 88.0                                      | 67.4  | 79.8                                     |
|                         | 22.5                       | 59.3                  | 67.8        | 91.3  | 92.6                                      | 70.2  | 74.6                                     |
| Trimethoprim (TRIP)     | 7.5                        | 44.6                  | 59.2        | 84.1  | 94.4                                      | 59.8  | 75.6                                     |
|                         | 15                         | 47.5                  | 54.7        | 91.2  | 93.3                                      | 55.3  | 77.8                                     |
|                         | 22.5                       | 54.3                  | 60.2        | 87.3  | 83.1                                      | 56.2  | 71.4                                     |
| Cephapirin (CEPR)       | 7.5                        | 55.4                  | 63.4        | 74.1  | 92.0                                      | 66.3  | 77.8                                     |
|                         | 15                         | 55.3                  | 65.8        | 81.3  | 90.4                                      | 65.2  | 74.5                                     |
|                         | 22.5                       | 61.4                  | 60.2        | 83.2  | 96.5                                      | 70.4  | 71.3                                     |
| Tetracycline (TETC)     | 7.5                        | 50.2                  | 61.3        | 84.5  | 86.8                                      | 64.5  | 73.4                                     |
|                         | 15                         | 51.0                  | 59.8        | 79.4  | 91.3                                      | 60.2  | 75.2                                     |
|                         | 22.5                       | 47.6                  | 59.3        | 83.8  | 89.6                                      | 70.1  | 80.4                                     |
| Cloxacillin (CLCN)      | 7.5                        | 33.6                  | 49.8        | 75.2  | 92.4                                      | 74.3  | 79.4                                     |
|                         | 15                         | 39.8                  | 52.3        | 81.3  | 86.7                                      | 71.8  | 72.3                                     |
|                         | 22.5                       | 34.2                  | 47.8        | 86.8  | 83.5                                      | 88.2  | 89.5                                     |
| Oxacillin (OXIN)        | 7.5                        | 39.0                  | 55.6        | 77.8  | 88.4                                      | 66.3  | 74.2                                     |
|                         | 15                         | 43.5                  | 60.2        | 71.2  | 92.1                                      | 60.2  | 77.1                                     |
|                         | 22.5                       | 41.0                  | 51.3        | 83.4  | 102.7                                     | 69.7  | 74.3                                     |
| Cefalexin (CEFA)        | 7.5                        | 36.5                  | 50.2        | 80.2  | 101.3                                     | 60.3  | 62.4                                     |
|                         | 15                         | 41.3                  | 44.6        | 75.3  | 108.8                                     | 61.5  | 66.4                                     |
|                         | 22.5                       | 47.8                  | 47.2        | 78.8  | 97.3                                      | 69.2  | 67.1                                     |
| Ceftiofur (CEFT)        | 7.5                        | 45.2                  | 55.6        | 69.5  | 81.3                                      | 54.3  | 71.3                                     |
|                         | 15                         | 41.3                  | 59.3        | 65.4  | 92.1                                      | 59.2  | 75.6                                     |
|                         | 22.5                       | 46.2                  | 51.8        | 71.4  | 81.8                                      | 60.1  | 77.9                                     |
| Enrofloxacin (ENRO)     | 7.5                        | 55.8                  | 56.8        | 90.7  | 89.4                                      | 67.8  | 80.4                                     |
|                         | 15                         | 62.3                  | 59.4        | 84.4  | 88.9                                      | 72.3  | 79.2                                     |
|                         | 22.5                       | 61.5                  | 64.5        | 85.2  | 85.3                                      | 75.6  | 81.3                                     |
| Ciprofloxacin (CIPR)    | 7.5                        | 60.0                  | 65.2        | 81.3  | 93.4                                      | 64.6  | 74.6                                     |
|                         | 15                         | 64.8                  | 64.3        | 88.7  | 94.2                                      | 68.2  | 77.2                                     |
|                         | 22.5                       | 58.2                  | 61.8        | 90.4  | 82.7                                      | 70.2  | 70.5                                     |

**Table 4** (continued)

| Analytes                     | Added concentration (µg/L) | LLE* without EH** (%) | LLE with EH | SPE*** with Oasis HLB cartridges without EH (%) | SPE with Oasis HLB cartridges (%) with EH | SPE with DSC-MCAX cartridges (%) without EH | SPE with DSC-MCAX cartridges (%) with EH |
|------------------------------|----------------------------|-----------------------|-------------|---|---|---|--|
| Oxytetracycline (OXTT)       | 7.5                        | 53.5                  | 63.2        | 75.5  | 80.2                                      | 71.3  | 75.6                                     |
|                              | 15                         | 59.6                  | 61.7        | 72.1  | 88.4                                      | 77.6  | 77.8                                     |
|                              | 22.5                       | 48.2                  | 66.3        | 78.3  | 81.8                                      | 70.2  | 73.2                                     |
| Sulfachloropyridazine (SUPZ) | 7.5                        | 55.4                  | 65.7        | 88.2  | 84.2                                      | 71.3  | 79.8                                     |
|                              | 15                         | 56.2                  | 69.2        | 84.6  | 84.0                                      | 77.6  | 80.7                                     |
|                              | 22.5                       | 60.2                  | 63.8        | 91.3  | 86.2                                      | 69.2  | 74.3                                     |
| Sulfadiazine (SUDI)          | 7.5                        | 47.8                  | 60.4        | 88.4  | 90.7                                      | 66.5  | 69.2                                     |
|                              | 15                         | 44.3                  | 65.2        | 81.0  | 90.0                                      | 60.2  | 70.1                                     |
|                              | 22.5                       | 51.2                  | 63.5        | 82.7  | 103.5                                     | 61.3  | 75.6                                     |
| Sulfadimethoxine (SUDM)      | 7.5                        | 50.2                  | 59.2        | 92.4  | 82.6                                      | 71.3  | 77.6                                     |
|                              | 15                         | 53.8                  | 57.6        | 91.7  | 85.3                                      | 66.2  | 74.3                                     |
|                              | 22.5                       | 55.6                  | 61.3        | 85.2  | 82.7                                      | 69.7  | 71.2                                     |
| Sulfadimidine (SULD)         | 7.5                        | 47.5                  | 55.4        | 97.6  | 98.7                                      | 55.8  | 61.3                                     |
|                              | 15                         | 43.2                  | 52.3        | 91.3  | 90.2                                      | 59.2  | 62.4                                     |
|                              | 22.5                       | 51.7                  | 57.9        | 95.5  | 83.5                                      | 57.3  | 66.6                                     |
| Sulfamethoxazole (SULM)      | 7.5                        | 55.2                  | 60.2        | 87.4  | 98.7                                      | 64.5  | 65.4                                     |
|                              | 15                         | 59.6                  | 64.5        | 80.2  | 93.3                                      | 69.1  | 73.5                                     |
|                              | 22.5                       | 50.1                  | 62.0        | 83.5  | 104.1                                     | 60.2  | 77.2                                     |
| Carbofuran (CRL)             | 7.5                        | 60.1                  | 65.6        | 86.3  | 90.6                                      | 74.3  | 81.5                                     |
|                              | 15                         | 54.3                  | 60.2        | 81.2  | 88.4                                      | 70.8  | 79.8                                     |
|                              | 22.5                       | 55.2                  | 63.2        | 91.5  | 93.3                                      | 78.5  | 79.4                                     |
| Carbaryl (CRB)               | 7.5                        | 47.8                  | 65.7        | 75.5  | 94.6                                      | 60.2  | 65.6                                     |
|                              | 15                         | 52.6                  | 66.4        | 71.3  | 96.0                                      | 57.6  | 70.9                                     |
|                              | 22.5                       | 55.3                  | 60.2        | 72.1  | 90.2                                      | 53.8  | 73.5                                     |
| Parathion (PTN)              | 7.5                        | 35.8                  | 50.2        | 69.6  | 89.3                                      | 66.2  | 80.4                                     |
|                              | 15                         | 39.2                  | 53.5        | 74.5  | 92.0                                      | 70.4  | 75.2                                     |
|                              | 22.5                       | 37.4                  | 51.4        | 71.8  | 87.6                                      | 74.2  | 76.3                                     |
| Malathion (MTN)              | 7.5                        | 37.7                  | 55.6        | 66.5  | 101.3                                     | 60.4  | 70.3                                     |
|                              | 15                         | 36.2                  | 39.8        | 69.4  | 98.2                                      | 60.2  | 65.6                                     |
|                              | 22.5                       | 43.5                  | 47.5        | 60.2  | 107.1                                     | 67.4  | 68.4                                     |
| Diazinon (DNN)               | 7.5                        | 44.6                  | 55.4        | 82.4  | 88.0                                      | 66.3  | 71.4                                     |
|                              | 15                         | 51.8                  | 56.3        | 87.5  | 85.3                                      | 61.2  | 72.5                                     |
|                              | 22.5                       | 50.2                  | 49.7        | 79.8  | 85.7                                      | 63.4  | 79.4                                     |
| Dimethoate (DIM)             | 7.5                        | 46.3                  | 60.3        | 88.6  | 92.1                                      | 59.8  | 70.2                                     |
|                              | 15                         | 47.5                  | 64.2        | 82.3  | 92.0                                      | 54.3  | 71.8                                     |
|                              | 22.5                       | 44.2                  | 57.4        | 84.4  | 94.2                                      | 57.5  | 66.4                                     |
| Atrazine (ATRZ)              | 7.5                        | 51.6                  | 60.3        | 75.5  | 96.0                                      | 64.6  | 74.5                                     |
|                              | 15                         | 57.8                  | 66.4        | 71.3  | 91.3                                      | 67.8  | 78.2                                     |
|                              | 22.5                       | 53.6                  | 67.5        | 82.8  | 89.3                                      | 69.5  | 81.2                                     |
| Permethrin (PEMT)            | 7.5                        | 50.4                  | 61.3        | 80.5  | 96.0                                      | 65.6  | 69.5                                     |
|                              | 15                         | 51.3                  | 55.4        | 81.3  | 94.0                                      | 61.3  | 69.1                                     |
|                              | 22.5                       | 47.6                  | 60.2        | 87.8  | 89.8                                      | 67.2  | 68.4                                     |
| Cypermethrin (CIRM)          | 7.5                        | 44.6                  | 67.4        | 80.2  | 85.3                                      | 55.6  | 71.4                                     |
|                              | 15                         | 45.2                  | 61.3        | 80.8  | 95.4                                      | 60.2  | 75.2                                     |
|                              | 22.5                       | 54.1                  | 61.0        | 75.4  | 87.6                                      | 63.8  | 71.3                                     |
| Deltamethrin (DELM)          | 7.5                        | 61.5                  | 69.8        | 77.1  | 90.7                                      | 62.1  | 63.1                                     |
|                              | 15                         | 57.4                  | 71.3        | 72.5  | 89.3                                      | 66.4  | 67.4                                     |
|                              | 22.5                       | 55.3                  | 70.0        | 80.6  | 87.6                                      | 60.8  | 69.2                                     |
| Coumaphos (COU)              | 7.5                        | 47.8                  | 66.3        | 82.4  | 92.0                                      | 55.4  | 66.4                                     |
|                              | 15                         | 52.7                  | 61.2        | 87.5  | 86.3                                      | 59.2  | 69.1                                     |
|                              | 22.5                       | 54.6                  | 60.4        | 92.4  | 83.1                                      | 61.5  | 65.6                                     |
| Dichlorvos (DIRP)            | 7.5                        | 51.5                  | 67.4        | 78.4  | 96.2                                      | 66.4  | 71.5                                     |
|                              | 15                         | 54.2                  | 59.2        | 71.3  | 87.3                                      | 61.3  | 75.4                                     |
|                              | 22.5                       | 57.8                  | 57.1        | 67.5  | 88.1                                      | 63.8  | 72.3                                     |

**Table 4** (continued)

| Analytes             | Added concentration (µg/L) | LLE* without EH** (%) | LLE with EH | SPE*** with Oasis HLB cartridges without EH (%) | SPE with Oasis HLB cartridges (%) with EH | SPE with DSC-MCAX cartridges (%) without EH | SPE with DSC-MCAX cartridges (%) with EH |
|----------------------|----------------------------|-----------------------|-------------|---|---|---|--|
| Chlorpyrifos (CHRS)  | 7.5                        | 46.6                  | 55.2        | 67.8  | 84.1                                      | 59.8  | 71.4                                     |
|                      | 15                         | 45.2                  | 50.7        | 74.3  | 86.3                                      | 54.6  | 72.7                                     |
|                      | 22.5                       | 40.2                  | 51.6        | 81.4  | 89.8                                      | 57.3  | 79.2                                     |
| Fenvalerate (FERT)   | 7.5                        | 43.2                  | 50.4        | 84.6  | 90.6                                      | 60.2  | 75.4                                     |
|                      | 15                         | 43.6                  | 54.3        | 80.2  | 87.7                                      | 64.5  | 70.2                                     |
|                      | 22.5                       | 49.5                  | 57.8        | 87.4  | 92.4                                      | 66.1  | 71.3                                     |
| Boscalid (BOS)       | 7.5                        | 51.5                  | 60.4        | 90.4  | 93.3                                      | 78.2  | 80.1                                     |
|                      | 15                         | 54.6                  | 66.4        | 86.6  | 92.0                                      | 70.4  | 85.5                                     |
|                      | 22.5                       | 52.2                  | 62.8        | 87.1  | 81.2                                      | 73.3  | 74.3                                     |
| Phenthoate (FETE)    | 7.5                        | 49.5                  | 55.7        | 77.1  | 85.3                                      | 65.1  | 69.3                                     |
|                      | 15                         | 43.5                  | 55.9        | 70.2  | 97.3                                      | 60.8  | 77.5                                     |
|                      | 22.5                       | 52.5                  | 50.3        | 79.4  | 103.1                                     | 67.2  | 72.1                                     |
| Fenthion (FEON)      | 7.5                        | 39.5                  | 51.7        | 84.5  | 92.0                                      | 74.5  | 78.4                                     |
|                      | 15                         | 43.5                  | 54.3        | 77.8  | 92.1                                      | 70.2  | 72.3                                     |
|                      | 22.5                       | 47.2                  | 60.0        | 81.3  | 86.2                                      | 72.3  | 79.5                                     |
| Monocrotophos (MOCR) | 7.5                        | 53.5                  | 64.6        | 87.5  | 94.6                                      | 66.3  | 69.7                                     |
|                      | 15                         | 59.8                  | 64.0        | 84.4  | 84.0                                      | 64.2  | 64.6                                     |
|                      | 22.5                       | 55.5                  | 59.8        | 91.7  | 83.1                                      | 67.1  | 69.2                                     |
| Malaaxon (MAON)      | 7.5                        | 55.0                  | 61.0        | 85.6  | 97.3                                      | 54.7  | 71.4                                     |
|                      | 15                         | 50.1                  | 61.0        | 81.7  | 90.67                                     | 59.6  | 74.4                                     |
|                      | 22.5                       | 54.3                  | 64.6        | 91.3  | 94.67                                     | 61.3  | 69.7                                     |
| Methamidophos (MEDF) | 7.5                        | 49.5                  | 51.3        | 80.2  | 102.6                                     | 55.7  | 65.6                                     |
|                      | 15                         | 47.6                  | 49.2        | 84.4  | 90.1                                      | 59.2  | 69.5                                     |
|                      | 22.5                       | 42.1                  | 47.8        | 80.8  | 88.4                                      | 57.4  | 64.1                                     |
| Metacrifos (MECF)    | 7.5                        | 60.2                  | 69.2        | 87.6  | 101.3                                     | 64.7  | 74.5                                     |
|                      | 15                         | 58.5                  | 64.7        | 78.2  | 92.0                                      | 69.2  | 71.5                                     |
|                      | 22.5                       | 66.4                  | 67.1        | 80.3  | 88.4                                      | 71.3  | 77.8                                     |
| Amitraz (AMRZ)       | 7.5                        | 39.8                  | 48.5        | 84.6  | 93.3                                      | 54.3  | 70.4                                     |
|                      | 15                         | 43.5                  | 47.4        | 87.8  | 94.0                                      | 59.6  | 74.4                                     |
|                      | 22.5                       | 47.8                  | 51.2        | 93.5  | 85.3                                      | 61.4  | 79.2                                     |
| Omethoate (OMAT)     | 7.5                        | 44.1                  | 51.3        | 82.2  | 92.0                                      | 70.4  | 80.5                                     |
|                      | 15                         | 47.8                  | 55.6        | 87.4  | 97.3                                      | 64.6  | 74.6                                     |
|                      | 22.5                       | 41.3                  | 60.3        | 88.6  | 99.6                                      | 67.3  | 79.8                                     |
| Vamidothion (VAON)   | 7.5                        | 56.6                  | 60.2        | 74.6  | 85.3                                      | 59.6  | 64.6                                     |
|                      | 15                         | 60.3                  | 51.3        | 74.8  | 92.1                                      | 64.8  | 73.2                                     |
|                      | 22.5                       | 54.2                  | 57.2        | 81.4  | 84.0                                      | 69.7  | 70.4                                     |
| Phosmet (FOST)       | 7.5                        | 55.7                  | 60.4        | 84.6  | 96.0                                      | 70.5  | 71.8                                     |
|                      | 15                         | 59.6                  | 60.8        | 80.7  | 89.3                                      | 64.7  | 69.6                                     |
|                      | 22.5                       | 49.8                  | 63.5        | 85.3  | 93.3                                      | 66.8  | 74.2                                     |
| Heptenophos (HEPH)   | 7.5                        | 50.2                  | 57.8        | 69.7  | 93.3                                      | 58.8  | 66.6                                     |
|                      | 15                         | 57.6                  | 58.2        | 65.4  | 94.0                                      | 56.4  | 69.2                                     |
|                      | 22.5                       | 61.4                  | 67.4        | 74.7  | 86.2                                      | 63.8  | 64.6                                     |
| Bifenthrin (BFNT)    | 7.5                        | 40.5                  | 51.2        | 80.7  | 85.3                                      | 66.4  | 81.5                                     |
|                      | 15                         | 46.2                  | 53.2        | 85.4  | 88.7                                      | 70.5  | 79.3                                     |
|                      | 22.5                       | 47.8                  | 57.4        | 79.9  | 87.5                                      | 74.5  | 78.2                                     |
| Methomyl (MEML)      | 7.5                        | 50.6                  | 63.2        | 84.8  | 104.0                                     | 70.2  | 80.5                                     |
|                      | 15                         | 53.2                  | 66.1        | 90.2  | 99.3                                      | 75.5  | 79.5                                     |
|                      | 22.5                       | 57.8                  | 66.2        | 82.4  | 85.78                                     | 71.3  | 72.8                                     |
| Zearalenone (ZEAN)   | 7.5                        | 53.5                  | 60.8        | 92.5  | 89.3                                      | 77.8  | 81.2                                     |
|                      | 15                         | 54.2                  | 67.5        | 95.7  | 84.0                                      | 72.5  | 74.6                                     |
|                      | 22.5                       | 47.8                  | 62.1        | 99.4  | 92.4                                      | 70.4  | 79.7                                     |
| Ochratoxin A (OTAA)  | 7.5                        | 44.6                  | 53.8        | 88.5  | 98.7                                      | 66.5  | 77.8                                     |
|                      | 15                         | 49.2                  | 55.1        | 91.3  | 86.0                                      | 61.2  | 76.4                                     |
|                      | 22.5                       | 49.0                  | 61.3        | 80.8  | 88.4                                      | 69.3  | 72.4                                     |

\* LLE – liquid-liquid extraction, \*\* EH - enzymatic hydrolyze, \*\*\* SPE – solid-phase extraction, \*\*\*\* nd - not detected

chromatographic separation. MassLynx version 4.1 software (Waters, Milford, MA, USA) was used for instrument control, data acquisition and processing of results.

The LC conditions were as follow: mobile phase flow rate: 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 mM ammonium acetate, 0.01% formic acid and 0.01% trichloroacetic acid; mobile phase B contains: methanol with 0.1% formic acid, oven temperature: 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. Both positive and negative electrospray ionization were used along with multiple reaction monitoring (MRM) [10, 26].

### Validation study

Validation of the method was performed according to the European Commission Regulation 2021/808 [25]. The parameters evaluated were: linearity, limit of detection (LOD), limit of quantification (LOQ), decision limit ( $CC\alpha$ ), detection capability ( $CC\beta$ ), accuracy determined by estimating trueness (recovery), precision (repeatability and reproducibility) and stability.

Matrix-matched calibration curves were prepared to determine the linearity. The blank urine samples were fortified at six concentration levels (for group 1: 2.0; 10.0; 25.0; 50.0 75.0 and 100 µg/ L; for group 2: 0.25; 1.0; 2.5; 5.0; 7.5 and 10.0 µg/ L; for group 3: 0.05; 1.0; 2.5; 5.0; 7.5 and 10.0 µg/ L; for group 4: 0.5; 1.0; 2.5; 5.0; 7.5 and 10 µg/ L; for group 5: 5.0; 10.0; 25.0; 50.0; 75.0 and 100.0 µg/ L). The calibration curves were prepared every day with each series of analyzed samples, each concentration level performed injected in triplicate. The coefficient of correlation ( $R^2$ ) was calculated by least squares linear regression analysis.

Decision limit ( $CC\alpha$ ). The  $CC\alpha$  means the concentration of target analyte at and above which it can be concluded with an error probability of  $\alpha$  ( $\alpha=1\%$ ) that a sample is non-compliant and the value  $1 - \alpha$  means statistical certainty in percentage that the permitted limit has been exceeded.  $CC\alpha$  was calculated according to 2021/808/EC [25].

Detection capability ( $CC\beta$ ). The  $CC\beta$  means the smallest concentration of the target analyte that may be detected or quantified in a sample with an error probability of  $\beta$  ( $\beta=5\%$ ).  $CC\beta$  was calculated according to 2021/808/EC [25].

The LOD and LOQ were determined as the lowest spiked concentration evaluated that gave a signal to noise (S/N) ratio of 3:1 for LOD and 10:1 for LOQ [28–30].

The intraday (single day,  $n=6$ ) and interday (three consecutive days,  $n=18$ ) recovery values were calculated at three spiking levels ( $\times 0.5/ \times 1$ , and  $\times 1.5$  the MMPR for substances with MMPR; while for substances without MMPRs and maximum residue limits (MRLs), the samples were fortified according to the sensitivity of the method) (Tables 5 and 6, respectively). Repeatability and reproducibility, in terms of the CV (coefficient of variation for repeatability ( $CV_r$ ) and coefficient of variation for reproducibility ( $CV_R$ )) of precision were estimated with spiking of the urine samples with standards and internal standards at the same concentration levels as well as recovery. The spiking levels are given in Tables 5 and 6. For reproducibility, the procedure was performed for three consecutive days by three analysts using different batches of blank material, solvents and reagents on different days.

Stability tests are performed for stock standard solutions, working standard solutions and standards in final matrix. Stock standard solutions were tested at -20 °C within 1 year, once per month. Working standard solutions were tested at -20 °C every week. Stability in the final extract was tested at -20 °C within 1 week, once per day, at different concentrations.

## Results and discussion

### MS/MS optimization

The MS/MS conditions including collision energy, cone voltage and dwell time (0.025 s), as well as selection of appropriate diagnostic ions are summarized in Table 7. This follows direct infusion of standard and internal standard working solutions (1.0 µg/ml).

### Optimization of the sample preparation

The recovery test results for the different sample preparation protocols are summarized in Table 8. As can be seen, the thyrostats were not detected with the LLE protocols (first and second protocols). The first protocol results (32.1–66.4%) and those from the second protocol (39.8–71.3%) were not satisfactory according to 2021/808/EC [25]. In protocols 3, 5 and 6 most recoveries were not in range described in 2021/808/EC, namely 50–120% for fortification concentration  $\leq 1$  µg/kg, 70–120% for  $>1$  to 10 µg/kg and 80–120% for  $\geq 10$  µg/kg [25]. The best recoveries were obtained using SPE extraction protocols including enzymatic hydrolysis (protocol 4). The recoveries ranged were from 71.0% for mabuterol spiked at the concentration 0.1 µg/L to 117.0% for cimbuterol spiked at the concentration 0.1 µg/L. The protocol 4 was therefore selected for further validation.

**Table 5** Linearity of the method

| Analytes                     | Calibration range ( $\mu\text{g/L}$ ) | R <sup>2</sup> |
|------------------------------|---------------------------------------|----------------|
| Thiouracil (TU)              | 2.0-100.0                             | 0.997          |
| Methylthiouracil (MTU)       | 2.0-100.0                             | 0.991          |
| Propylthiouracil (PTU)       | 2.0-100.0                             | 0.997          |
| Tapazole (TAP)               | 2.0-100.0                             | 0.994          |
| Testosterone (TEST)          | 5.0-100.0                             | 0.992          |
| Methyltestosterone (MEST)    | 0.25-10.0                             | 0.993          |
| Boldenone (BOLD)             | 0.5-10.0                              | 0.995          |
| 19 Nortestosterone (19 NO)   | 0.25-10.0                             | 0.994          |
| Stanozolol (STZL)            | 0.25-10.0                             | 0.994          |
| Clostebol (CLBL)             | 5.0-100.0                             | 0.993          |
| Zeranol (ZENL)               | 0.5-10.0                              | 0.992          |
| Taleranol (TANL)             | 0.5-10.0                              | 0.992          |
| Clenbuterol (CLEN)           | 0.05-10.0                             | 0.993          |
| Brombuterol (BROM)           | 0.05-10.0                             | 0.993          |
| Mabuterol (MABT)             | 0.05-10.0                             | 0.992          |
| Clenpenterol (CLEP)          | 0.05-10.0                             | 0.994          |
| Isoxuprin (ISOX)             | 0.25-10.0                             | 0.994          |
| Cimbuterol (CIMB)            | 0.05-10.0                             | 0.995          |
| Ractopamine (RACT)           | 0.25-10.0                             | 0.992          |
| Salbutamol (SALB)            | 0.25-10.0                             | 0.992          |
| Zilpaterol HCl (ZILP)        | 0.25-10.0                             | 0.993          |
| Terbutaline (TERB)           | 0.25-10.0                             | 0.994          |
| Amoxicillin (AMOX)           | 5.0-100.0                             | 0.995          |
| Ampicillin (AMP)             | 5.0-100.0                             | 0.992          |
| Benzylpenicillin (BNPC)      | 5.0-100.0                             | 0.998          |
| Lincomycin (LINK)            | 5.0-100.0                             | 0.994          |
| Tylosin (TYLS)               | 5.0-100.0                             | 0.994          |
| Trimethoprim (TRIP)          | 5.0-100.0                             | 0.992          |
| Cephapirin (CEPR)            | 5.0-100.0                             | 0.993          |
| Tetracycline (TETC)          | 5.0-100.0                             | 0.993          |
| Cloxacillin (CLCN)           | 5.0-100.0                             | 0.996          |
| Oxacillin (OXIN)             | 5.0-100.0                             | 0.997          |
| Cefalexin (CEFA)             | 5.0-100.0                             | 0.997          |
| Ceftiofur (CEFT)             | 5.0-100.0                             | 0.996          |
| Enrofloxacin (ENRO)          | 5.0-100.0                             | 0.998          |
| Ciprofloxacin (CIPR)         | 5.0-100.0                             | 0.992          |
| Oxytetracycline (OXTT)       | 5.0-100.0                             | 0.995          |
| Sulfachloropyridazine (SUPZ) | 5.0-100.0                             | 0.998          |
| Sulfadiazine (SUDI)          | 5.0-100.0                             | 0.997          |
| Sulfadimethoxine (SUDM)      | 5.0-100.0                             | 0.998          |
| Sulfadimidine (SULD)         | 5.0-100.0                             | 0.999          |
| Sulfamethoxazole (SULM)      | 5.0-100.0                             | 0.995          |
| Carbofuran (CRL)             | 5.0-100.0                             | 0.998          |
| Carbaryl (CRB)               | 5.0-100.0                             | 0.994          |
| Parathion (PTN)              | 5.0-100.0                             | 0.994          |
| Malathion (MTN)              | 5.0-100.0                             | 0.994          |
| Diazinon (DNN)               | 5.0-100.0                             | 0.994          |
| Dimethoate (DIM)             | 5.0-100.0                             | 0.993          |
| Atrazine (ATRZ)              | 5.0-100.0                             | 0.997          |
| Permethrin (PEMT)            | 5.0-100.0                             | 0.993          |
| Cypermethrin (CIRM)          | 5.0-100.0                             | 0.997          |
| Deltamethrin (DELM)          | 5.0-100.0                             | 0.997          |
| Coumaphos (COU)              | 5.0-100.0                             | 0.994          |



**Table 5** (continued)

| Analytes             | Calibration range ( $\mu\text{g/L}$ ) | $R^2$ |
|----------------------|---------------------------------------|-------|
| Dichlorophos (DIRP)  | 5.0-100.0                             | 0.994 |
| Chloropyrifos (CHRS) | 5.0-100.0                             | 0.993 |
| Fenvalerate (FERT)   | 5.0-100.0                             | 0.991 |
| Boscalid (BOS)       | 5.0-100.0                             | 0.997 |
| Fenthoate (FETE)     | 5.0-100.0                             | 0.996 |
| Fenthion (FEON)      | 5.0-100.0                             | 0.992 |
| Monocrotophos (MOCR) | 5.0-100.0                             | 0.997 |
| Malaoxon (MAON)      | 5.0-100.0                             | 0.998 |
| Methamidophos (MEDF) | 5.0-100.0                             | 0.995 |
| Metacrifos (MECF)    | 5.0-100.0                             | 0.993 |
| Amitraz (AMRZ)       | 5.0-100.0                             | 0.994 |
| Omethoate (OMAT)     | 5.0-100.0                             | 0.995 |
| Vamidothione (VAON)  | 5.0-100.0                             | 0.995 |
| Phosmet (FOST)       | 5.0-100.0                             | 0.999 |
| Heptenophos (HEPH)   | 5.0-100.0                             | 0.998 |
| Bifenthrin (BFNT)    | 5.0-100.0                             | 0.998 |
| Methomyl (MEML)      | 5.0-100.0                             | 0.996 |
| Zearalenone (ZEAN)   | 5.0-100.0                             | 0.998 |
| Ochratoxin A (OTAA)  | 5.0-100.0                             | 0.999 |

## Method validation

### Linearity

Very good linearity was attained with coefficient of correlation ( $R^2$ ) from 0.991 for methylthiouracil and fenvalerate to 0.999 for sulfadimidine, phosmet and ochratoxin A. The range of calibration curve and  $R^2$  values for all compounds are presented in Table 2.

### LOD, LOQ, $CC\alpha$ , $CC\beta$

The results for LOD, LOQ,  $CC\alpha$  and  $CC\beta$  are shown in the Table 3. The LODs were from 0.01  $\mu\text{g/L}$  for cimbuterol to 2.71  $\mu\text{g/L}$  for ampicillin, while the LOQs were from 0.05  $\mu\text{g/L}$  for cimbuterol to 7.52  $\mu\text{g/L}$  for oxacillin. The  $CC\alpha$  values ranged from 0.05  $\mu\text{g/L}$  for cimbuterol to 12.11  $\mu\text{g/L}$  for cephalixin, while  $CC\beta$  values ranged from 0.08  $\mu\text{g/L}$  for clenpenterol to 15.16  $\mu\text{g/L}$  for cephalixin.

### Recovery, repeatability and reproducibility

Recovery, repeatability (intraday) and reproducibility (interday) were used for evaluation of the accuracy of the method. The intraday recovery range was from 71.0% for mabuterol spiked at 0.1  $\mu\text{g/l}$  to 117% for cimbuterol at 0.1  $\mu\text{g/L}$ , while the interday recovery range was from 76.2% to 19 nortestosterone spiked at 0.25  $\mu\text{g/L}$  to 109.3% for coumaphos spiked at 7.5  $\mu\text{g/L}$ . The CV for repeatability ranged from 1.69% for tetracycline to 16.76% for sulfadiazine, while the CV for reproducibility was from 5.65% for tetracycline to 21.38% for methomyl. The results are summarized in Tables 5 and 6. The chromatograms of the analytes in urine samples spiked with standards at concentration level two from Table 5 are shown in Fig. 1 (a, b, c).

### Stability

Stock standards of the anabolic hormones, lactones, beta agonists, thyreostats, mycotoxins and most antibiotics (except amoxicillin, ampicillin, benzylpenicillin, cloxacillin and oxacillin) were stable for 6 months at  $-20^\circ\text{C}$ , while pesticide standards were stable for 1 year, and amoxicillin, ampicillin, benzylpenicillin, cloxacillin as well as oxacillin were stable for 3 months. Working standard solutions were stable for 1 month when kept at  $-20^\circ\text{C}$ . The standards in the final extract were stable 3 days.

### Discussion

The testing of forbidden substances in media such as urine is an important task of regulatory authorities and laboratories. In the European Union, the use of antimicrobials, thyreostats, anabolic hormones and  $\beta$ -agonist drugs for fattening slaughter animals has been banned since 1981 under Council Directive 81/602/EEC [31]. For protection of consumer health against unwanted residues and in compliance with Directive 96/23, each EU country must monitor these substances in samples of animal origin. One precursor and two product ions were selected for each analyte as recommended elsewhere [32] and most were analyzed in positive ionization mode, except zeranol, taleranol, zearalenone and  $\beta$ -zearalenol-d4 analyzed in the negative ionization mode. The most abundant product ion was used for quantification, while the second product ion was used for confirmation.

Urine is widely used to monitor the illegal use of growth-promoting agents and veterinary drugs with a good number of these substances showing high clearance rates in urine [33–35]. While preparation of urine

**Table 6** Decision limit, detection capability, limit of detection, limit of quantification of the method and used internal standards

| Analytes                     | CC $\alpha$ * ( $\mu\text{g/L}$ ) | CC $\beta$ ** ( $\mu\text{g/L}$ ) | LOD*** ( $\mu\text{g/L}$ ) | LOQ**** ( $\mu\text{g/L}$ ) | Internal standards              |
|------------------------------|-----------------------------------|-----------------------------------|----------------------------|-----------------------------|---------------------------------|
| Thiouracil (TU)              | 5.17                              | 8.46                              | 2.03                       | 5.78                        | /                               |
| Methylthiouracil (MTU)       | 2.26                              | 4.88                              | 1.04                       | 3.56                        | /                               |
| Propylthiouracil (PTU)       | 2.23                              | 5.22                              | 1.48                       | 4.75                        | /                               |
| Tapazole (TAP)               | 5.03                              | 8.42                              | 2.22                       | 5.60                        | /                               |
| Testosterone (TEST)          | 4.87                              | 6.36                              | 1.88                       | 5.45                        | /                               |
| Methyltestosterone (MEST)    | 0.27                              | 0.48                              | 0.12                       | 0.40                        | /                               |
| Boldenone (BOLD)             | 0.69                              | 0.95                              | 0.31                       | 0.95                        | /                               |
| 19 Nortestosterone (19 NO)   | 0.25                              | 0.48                              | 0.15                       | 0.47                        | 19–17 $\beta$ Nortestosteron-d3 |
| Stanozolol (STZL)            | 0.34                              | 0.50                              | 0.14                       | 0.38                        | /                               |
| Clostebol (CLBL)             | 4.36                              | 8.02                              | 1.56                       | 4.88                        | /                               |
| Zeranol (ZENL)               | 0.62                              | 0.93                              | 0.26                       | 0.89                        | /                               |
| Taleranol (TANL)             | 0.58                              | 0.87                              | 0.19                       | 0.94                        | /                               |
| Clenbuterol (CLEN)           | 0.06                              | 0.09                              | 0.02                       | 0.08                        | Clenbuterol-d6                  |
| Brombuterol (BROM)           | 0.07                              | 0.09                              | 0.03                       | 0.08                        | Brombuterol-d9                  |
| Mabuterol (MABT)             | 0.08                              | 0.10                              | 0.03                       | 0.09                        | Mabuterol-d9                    |
| Clenpenterol (CLEP)          | 0.06                              | 0.08                              | 0.03                       | 0.09                        | Clenpenterol-d5                 |
| Isoxuprin (ISOX)             | 0.28                              | 0.38                              | 0.17                       | 0.32                        | Isoxuprine-d5                   |
| Cimbuterol (CIMB)            | 0.05                              | 0.10                              | 0.01                       | 0.05                        | Cimbuterol-d9                   |
| Ractopamine (RACTO)          | 0.38                              | 0.49                              | 0.16                       | 0.49                        | Ractopamine-d6                  |
| Salbutamol (SALB)            | 0.26                              | 0.41                              | 0.17                       | 0.48                        | Salbutamol-d9                   |
| Zilpaterol (ZILP)            | 0.36                              | 0.49                              | 0.14                       | 0.40                        | Zilpaterol-d7                   |
| Terbutaline (TERB)           | 0.22                              | 0.43                              | 0.11                       | 0.42                        | Terbutaline-d9                  |
| Amoxicillin (AMOX)           | 9.25                              | 12.18                             | 2.26                       | 7.03                        | Penicillin G-d7                 |
| Ampicillin (AMPO)            | 8.14                              | 11.25                             | 2.71                       | 7.12                        | Penicillin G-d7                 |
| Benzylpenicillin (BNPC)      | 10.12                             | 12.18                             | 2.05                       | 6.94                        | Penicillin G-d7                 |
| Lincomycin (LINK)            | 7.15                              | 9.18                              | 2.16                       | 6.85                        | /                               |
| Tylosin (TYLS)               | 9.11                              | 13.54                             | 1.36                       | 6.41                        | /                               |
| Trimethoprim (TRIP)          | 8.82                              | 12.46                             | 1.78                       | 7.15                        | /                               |
| Cephapirin (CEPR)            | 9.12                              | 11.35                             | 2.54                       | 7.46                        | /                               |
| Tetracycline (TETC)          | 7.00                              | 9.12                              | 2.01                       | 6.54                        | /                               |
| Cloxacillin (CLCN)           | 10.54                             | 12.83                             | 2.22                       | 7.10                        | Penicillin G-d7                 |
| Oxacillin (OXIN)             | 8.15                              | 10.41                             | 1.51                       | 7.52                        | Penicillin G-d7                 |
| Cefalexin (CEFA)             | 12.11                             | 15.16                             | 2.09                       | 6.57                        | /                               |
| Ceftiofur (CEFT)             | 7.35                              | 10.12                             | 1.58                       | 6.96                        | /                               |
| Enrofloxacin (ENRO)          | 9.28                              | 12.75                             | 2.04                       | 7.17                        | Flunixin-d3                     |
| Ciprofloxacin (CIPR)         | 8.64                              | 12.00                             | 0.88                       | 4.02                        | Flunixin-d3                     |
| Oxytetracycline (OXTT)       | 6.28                              | 8.17                              | 2.11                       | 6.78                        | /                               |
| Sulfachloropyridazine (SUPZ) | 8.25                              | 10.22                             | 1.56                       | 7.02                        | /                               |
| Sulfadiazine (SUDI)          | 7.17                              | 9.25                              | 1.14                       | 5.95                        | /                               |
| Sulfadimethoxine (SUDM)      | 7.38                              | 8.92                              | 2.46                       | 7.26                        | /                               |
| Sulfadimidine (SULD)         | 11.35                             | 14.80                             | 2.11                       | 6.58                        | /                               |
| Sulfamethoxazole (SULM)      | 9.18                              | 12.35                             | 1.57                       | 5.92                        | /                               |
| Carbofuran (CRL)             | 6.11                              | 9.28                              | 2.54                       | 7.35                        | Carbofuran-d3                   |
| Carbaryl (CRB)               | 8.25                              | 12.41                             | 1.88                       | 6.51                        | /                               |
| Parathion (PTN)              | 7.15                              | 9.12                              | 0.95                       | 4.12                        | /                               |
| Malathion (MTN)              | 7.81                              | 10.14                             | 1.36                       | 5.06                        | /                               |
| Diazinon (DNN)               | 9.52                              | 11.88                             | 1.95                       | 6.71                        | /                               |
| Dimethoate (DIM)             | 8.35                              | 9.22                              | 2.07                       | 6.52                        | /                               |
| Atrazine (ATRZ)              | 11.80                             | 13.59                             | 2.38                       | 7.28                        | Atrazine-d5                     |
| Permethrine (PEMT)           | 9.87                              | 12.35                             | 2.17                       | 7.11                        | /                               |
| Cypermethrine (CIRM)         | 9.38                              | 12.13                             | 1.41                       | 6.36                        | /                               |
| Deltamethrine (DELM)         | 11.85                             | 14.45                             | 0.85                       | 2.92                        | /                               |
| Coumaphos (COU)              | 10.35                             | 13.12                             | 2.68                       | 7.51                        | /                               |

**Table 6** (continued)

| Analytes             | CC $\alpha$ * ( $\mu\text{g/L}$ ) | CC $\beta$ ** ( $\mu\text{g/L}$ ) | LOD*** ( $\mu\text{g/L}$ ) | LOQ**** ( $\mu\text{g/L}$ ) | Internal standards     |
|----------------------|-----------------------------------|-----------------------------------|----------------------------|-----------------------------|------------------------|
| Dichlorophos (DIRP)  | 8.81                              | 11.35                             | 1.55                       | 7.25                        | /                      |
| Chlorpyrifos (CHRS)  | 7.38                              | 9.86                              | 1.47                       | 5.48                        | Chlorpyrifos-d10       |
| Fenvalerate (FERT)   | 9.12                              | 10.92                             | 2.42                       | 7.50                        | /                      |
| Boscalid (BOS)       | 6.54                              | 8.25                              | 1.78                       | 6.28                        | /                      |
| Fenthoate (FETE)     | 8.78                              | 11.38                             | 1.41                       | 5.11                        | /                      |
| Fenthione (FEON)     | 7.05                              | 11.06                             | 2.12                       | 6.25                        | /                      |
| Monocrotophos (MOCR) | 8.12                              | 11.48                             | 2.30                       | 7.08                        | /                      |
| Malaoxon (MAON)      | 7.48                              | 9.86                              | 2.67                       | 7.35                        | /                      |
| Methamidophos MEDF   | 10.15                             | 14.00                             | 1.45                       | 5.00                        | /                      |
| Metacrifos (MECF)    | 8.15                              | 11.48                             | 2.00                       | 6.28                        | /                      |
| Amitraz (AMRZ)       | 9.01                              | 10.56                             | 1.42                       | 6.02                        | /                      |
| Omethoate (OMAT)     | 8.45                              | 11.48                             | 1.88                       | 6.89                        | /                      |
| Vamidothion (VAON)   | 9.12                              | 12.90                             | 1.56                       | 7.25                        | /                      |
| Phosmet (FOST)       | 9.41                              | 11.52                             | 1.01                       | 4.11                        | /                      |
| Heptenophos (HEPH)   | 7.08                              | 11.06                             | 1.47                       | 7.51                        | /                      |
| Bifenthrin (BFNT)    | 7.35                              | 9.42                              | 2.05                       | 7.35                        | /                      |
| Methomyl (MEML)      | 10.14                             | 13.78                             | 1.92                       | 5.91                        | /                      |
| Zearalenone (ZEAN)   | 6.35                              | 10.08                             | 0.98                       | 4.38                        | $\beta$ -zearalenol-d4 |
| Ochratoxin A (OTAA)  | 8.12                              | 11.35                             | 1.14                       | 4.08                        | /                      |

\*CC $\alpha$  - decision limit, \*\*CC $\beta$  - detection capability, \*\*\*LOD - limit of detection, \*\*\*\*LOQ - limit of quantification

can generally be easier than for other matrices, sample preparation for analysis of mixed hazards is a challenge to many laboratories thus requiring rigorous extraction and cleanup [10]. The simplest methods for detection of pesticides in urine are direct injection of urine samples or dilute-and-shoot procedures although urinary salts or macromolecules decrease instrument sensitivity, clog the injection syringe or the ESI probe [19, 36]. To avoid such effects solid phase extraction and liquid-liquid extraction procedures are applied for residues of veterinary drugs and contaminants [36–38].

Six extraction protocols were investigated. In the first and second protocols involving LLE, thyreostats were not detected due to poor recovery. The results were comparable with previous studies by Kellman et al. (2009) and Eeckhaut et al. (2009) who concluded that while the LLE is simpler and easier than SPE, matrix (urine) interferences lead to low extraction efficiency hence the need for SPE [39, 40]. Gómez-Pérez et al. (2015), used Florisil cartridges for determination of pesticide and veterinary drug residues, Kaufman et al. (2008), used Oasis HLB cartridge for detection of different classes of veterinary drugs, while Kaklamanos et al. (2009), used Oasis HLB and Amino Supelclean NH<sub>2</sub> cartridges for analysis of anabolic steroids [12, 38, 41].

Furthermore, Ho et al. (2006), used C8-SCX mixed-mode cartridge for analysis of anabolic steroids, corticosteroids and acidic drugs, while Leon et al. (2012), used Oasis HLB cartridges in a method for 87 analytes in different families of banned or unauthorized substances [42, 43]. In this study SPE extraction involved Oasis HLB and

DSC-MCAX cartridges in procedures 3, 4, 5 and 6. Better recoveries can be attained when Oasis HLB cartridges are used along with enzymatic hydrolysis. Kinsella et al. (2009), reported that steroids in urine can be present in free, glucuronic acid and sulphate forms [44]. Also, phenolic  $\beta$ -agonists contain conjugated esters, especially in the form of glucuronides and sulphates. Enzymatic hydrolysis (with  $\beta$ -glucuronidase aryl sulfatase) deconjugate steroid glucuronides and sulfates and this can improve recovery [44, 45]. Overall, the validation parameters demonstrate suitability of the method to analyse a mixture of residues/contaminants such as veterinary drugs, pesticides and mycotoxins in bovine urine, and in agreement with criteria described in 2021/808/EC [25] and is applicable to field /real sample analysis.

#### Real sample analysis

A total of 83 bovine urine samples were collected from local farms and tested using the developed and validated method during 2021–2022. In five samples of urine, methyl- and propylthiouracil were found. No other veterinary drug residues and contaminants were found. Methylthiouracil (veterinary drug) residue amount was found between 1.08 and 1.31  $\mu\text{g/L}$  in 5 samples and propylthiouracil (veterinary drug) amount was found to be 1.67–2.63  $\mu\text{g/L}$  in 4 samples. In Fig. 2 is given chromatogram of bovine urine with methylthiouracil, while in Fig. 3 is given chromatogram of bovine urine with propylthiouracil. The presence of thiouracil and its derivatives in urine samples is most likely due to feeding animals diet containing cruciferous plants. The proposed method,

**Table 7** Intraday recovery and repeatability of the method

| Analytes                  | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 6) | Standard deviation (µg/L) | Recovery (%) | Repeat-ability (CvR, %) |
|---------------------------|----------------------------|---|---------------------------|--------------|-------------------------|
| Thiouracil (TU)           | 5                          | 4.65  | 0.25                      | 93.0         | 5.38                    |
|                           | 10                         | 10.11   | 0.75                      | 101.1        | 7.42                    |
|                           | 15                         | 15.22   | 1.12                      | 101.5        | 7.77                    |
| Methylthiouracil (MTU)    | 5                          | 4.78  | 0.31                      | 95.6         | 6.49                    |
|                           | 10                         | 9.46  | 0.62                      | 94.6         | 6.55                    |
|                           | 15                         | 14.42   | 1.27                      | 96.1         | 8.81                    |
| Propylthiouracil (PTU)    | 5                          | 3.85  | 0.12                      | 77.0         | 3.12                    |
|                           | 10                         | 8.45  | 0.62                      | 84.5         | 7.34                    |
|                           | 15                         | 13.48   | 1.01                      | 89.9         | 7.49                    |
| Tapazole (TAP)            | 5                          | 4.32  | 0.28                      | 86.4         | 6.48                    |
|                           | 10                         | 8.48  | 0.39                      | 84.8         | 4.60                    |
|                           | 15                         | 14.08   | 0.78                      | 93.9         | 5.54                    |
| Testosterone (TEST)       | 7.5                        | 4.36  | 0.38                      | 87.20        | 8.72                    |
|                           | 15                         | 8.82  | 0.72                      | 88.20        | 8.16                    |
|                           | 22.5                       | 13.58   | 1.05                      | 90.53        | 7.73                    |
| Methyltestosterone (MEST) | 0.25                       | 0.21  | 0.014                     | 86.0         | 6.67                    |
|                           | 0.50                       | 0.46  | 0.041                     | 91.0         | 8.91                    |
|                           | 0.75                       | 0.70  | 0.052                     | 94.8         | 7.43                    |
| Boldenone (BOLD)          | 0.5                        | 0.42  | 0.034                     | 84.8         | 8.10                    |
|                           | 1.0                        | 1.04  | 0.079                     | 104.2        | 7.60                    |
|                           | 1.5                        | 1.37  | 0.171                     | 91.6         | 12.48                   |
| 19 Nortestosteron (19 NO) | 0.25                       | 0.21  | 0.011                     | 84.4         | 5.24                    |
|                           | 0.5                        | 0.47  | 0.032                     | 94.2         | 6.81                    |
|                           | 0.75                       | 0.71  | 0.048                     | 95.2         | 6.76                    |
| Stanozolol (STZL)         | 0.25                       | 0.22  | 0.017                     | 88.8         | 7.28                    |
|                           | 0.5                        | 0.46  | 0.051                     | 91.4         | 11.09                   |
|                           | 0.75                       | 0.71  | 0.074                     | 94.8         | 10.42                   |
| Clostebol (CLBL)          | 7.5                        | 4.48  | 0.25                      | 89.6         | 5.58                    |
|                           | 15                         | 9.36  | 1.15                      | 93.6         | 12.29                   |
|                           | 22.5                       | 13.95   | 1.66                      | 93.0         | 11.90                   |
| Zeranol (ZENL)            | 0.5                        | 0.41  | 0.056                     | 81.6         | 13.66                   |
|                           | 1.0                        | 0.88  | 0.111                     | 88.7         | 12.61                   |
|                           | 1.5                        | 1.27  | 0.068                     | 84.8         | 5.35                    |
| Taleranol (TANL)          | 0.5                        | 0.45  | 0.038                     | 90.8         | 8.44                    |
|                           | 1.0                        | 0.92  | 0.084                     | 92.4         | 9.13                    |
|                           | 1.5                        | 1.43  | 0.092                     | 95.3         | 6.43                    |
| Clenbuterol (CLEN)        | 0.05                       | 0.04  | 0.003                     | 74.0         | 6.50                    |
|                           | 0.1                        | 0.08  | 0.004                     | 79.0         | 5.25                    |
|                           | 0.15                       | 0.13  | 0.009                     | 88.0         | 6.92                    |
| Brombuterol (BROM)        | 0.05                       | 0.04  | 0.005                     | 76.0         | 11.75                   |
|                           | 0.1                        | 0.09  | 0.008                     | 86.0         | 8.67                    |
|                           | 0.15                       | 0.12  | 0.014                     | 78.0         | 11.67                   |
| Mabuterol (MABT)          | 0.05                       | 0.04  | 0.004                     | 84.0         | 8.75                    |
|                           | 0.1                        | 0.07  | 0.007                     | 67.0         | 9.71                    |
|                           | 0.15                       | 0.11  | 0.012                     | 75.3         | 10.91                   |
| Clenpenterol (CLEP)       | 0.05                       | 0.05  | 0.005                     | 96.0         | 9.60                    |
|                           | 0.1                        | 0.11  | 0.009                     | 113.0        | 7.82                    |
|                           | 0.15                       | 0.14  | 0.010                     | 91.3         | 7.30                    |
| Isoxuprin (ISOX)          | 0.25                       | 0.22  | 0.027                     | 90.8         | 12.27                   |
|                           | 0.50                       | 0.51  | 0.054                     | 102.8        | 10.59                   |
|                           | 0.75                       | 0.74  | 0.079                     | 98.4         | 10.68                   |
| Cimbuterol (CIMB)         | 0.05                       | 0.04  | 0.005                     | 74.0         | 11.22                   |
|                           | 0.1                        | 0.12  | 0.008                     | 117.0        | 6.42                    |
|                           | 0.15                       | 0.13  | 0.014                     | 88.0         | 10.77                   |
| Ractopamine (RACT)        | 0.25                       | 0.24  | 0.021                     | 95.2         | 8.75                    |
|                           | 0.50                       | 0.46  | 0.052                     | 91.4         | 11.30                   |
|                           | 0.75                       | 0.70  | 0.071                     | 93.0         | 10.14                   |

**Table 7** (continued)

| Analytes                    | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 6) | Standard deviation (µg/L) | Recovery (%) | Repeat-ability (Cvr, %) |
|-----------------------------|----------------------------|---|---------------------------|--------------|-------------------------|
| Salbutamol (SALB)           | 0.25                       | 0.20  | 0.022                     | 78.8         | 11.00                   |
|                             | 0.50                       | 0.49  | 0.057                     | 97.4         | 11.63                   |
|                             | 0.75                       | 0.64  | 0.062                     | 85.9         | 9.69                    |
| Zilpaterol HCl (ZILP)       | 0.25                       | 0.21  | 0.029                     | 85.2         | 13.81                   |
|                             | 0.50                       | 0.52  | 0.050                     | 103.4        | 9.62                    |
|                             | 0.75                       | 0.77  | 0.065                     | 102.1        | 8.44                    |
| Terbutaline (TERB)          | 0.25                       | 0.19  | 0.017                     | 77.6         | 8.95                    |
|                             | 0.50                       | 0.52  | 0.036                     | 103.8        | 6.92                    |
|                             | 0.75                       | 0.67  | 0.078                     | 88.8         | 11.64                   |
| Amoxicillin (AMOX)          | 7.5                        | 7.2   | 0.31                      | 96.0         | 4.31                    |
|                             | 15                         | 14.1  | 1.42                      | 94.0         | 10.07                   |
|                             | 22.5                       | 19.3  | 1.74                      | 85.8         | 9.02                    |
| Ampicillin (AMP)            | 7.5                        | 6.9   | 0.78                      | 92.0         | 11.30                   |
|                             | 15                         | 14.7  | 1.13                      | 98.2         | 7.69                    |
|                             | 22.5                       | 21.3  | 1.45                      | 94.7         | 6.81                    |
| Benzylpenicillin (BNPC)     | 7.5                        | 7.6   | 0.28                      | 101.3        | 3.68                    |
|                             | 15                         | 15.2  | 1.01                      | 101.3        | 6.64                    |
|                             | 22.5                       | 18.7  | 0.75                      | 83.4         | 4.01                    |
| Lincomycin (LINK)           | 7.5                        | 6.7   | 0.29                      | 89.5         | 4.33                    |
|                             | 15                         | 12.4  | 1.43                      | 82.6         | 11.53                   |
|                             | 22.5                       | 20.7  | 1.95                      | 92.1         | 9.40                    |
| Tylosin (TYLS)              | 7.5                        | 6.8   | 0.48                      | 90.7         | 7.06                    |
|                             | 15                         | 13.2  | 1.32                      | 88.0         | 10.0                    |
|                             | 22.5                       | 20.9  | 1.14                      | 92.6         | 8.84                    |
| Trimethoprim (TRIP)         | 7.5                        | 7.1   | 0.65                      | 94.4         | 9.15                    |
|                             | 15                         | 14.0  | 1.10                      | 93.3         | 7.85                    |
|                             | 22.5                       | 18.7  | 2.05                      | 83.1         | 10.96                   |
| Cephapirin (CEPR)           | 7.5                        | 6.9   | 0.38                      | 92.0         | 5.51                    |
|                             | 15                         | 13.5  | 0.75                      | 90.4         | 5.56                    |
|                             | 22.5                       | 21.8  | 1.94                      | 96.5         | 8.51                    |
| Tetracycline (TETC)         | 7.5                        | 6.5   | 0.11                      | 86.8         | 1.69                    |
|                             | 15                         | 13.7  | 0.72                      | 91.3         | 5.26                    |
|                             | 22.5                       | 20.1  | 0.96                      | 89.6         | 4.78                    |
| Cloxacillin (CLCN)          | 7.5                        | 6.9   | 0.25                      | 92.4         | 3.62                    |
|                             | 15                         | 13.0  | 1.10                      | 86.7         | 8.46                    |
|                             | 22.5                       | 18.8  | 1.15                      | 83.5         | 6.12                    |
| Oxacillin (OXIN)            | 7.5                        | 6.6   | 0.36                      | 88.4         | 5.45                    |
|                             | 15                         | 13.8  | 1.22                      | 92.1         | 8.84                    |
|                             | 22.5                       | 23.0  | 2.14                      | 102.7        | 9.30                    |
| Cefalexin (CEFA)            | 7.5                        | 7.6   | 0.78                      | 101.3        | 10.26                   |
|                             | 15                         | 16.3  | 1.02                      | 108.8        | 6.26                    |
|                             | 22.5                       | 21.9  | 1.73                      | 97.3         | 7.90                    |
| Ceftiofur (CEFT)            | 7.5                        | 6.1   | 0.46                      | 81.3         | 7.54                    |
|                             | 15                         | 13.8  | 1.18                      | 92.1         | 8.55                    |
|                             | 22.5                       | 18.4  | 2.13                      | 81.8         | 11.58                   |
| Enrofloxacin (ENRO)         | 7.5                        | 6.7   | 0.94                      | 89.4         | 14.02                   |
|                             | 15                         | 13.8  | 1.45                      | 88.9         | 10.50                   |
|                             | 22.5                       | 19.2  | 1.74                      | 85.3         | 9.06                    |
| Ciprofloxacin (CIPR)        | 7.5                        | 7.0   | 0.85                      | 93.4         | 12.14                   |
|                             | 15                         | 14.1  | 1.74                      | 94.2         | 12.34                   |
|                             | 22.5                       | 18.6  | 2.04                      | 82.7         | 10.97                   |
| Oxytetracycline (OXTT)      | 7.5                        | 6.0   | 0.64                      | 80.2         | 10.67                   |
|                             | 15                         | 13.2  | 1.11                      | 88.4         | 8.41                    |
|                             | 22.5                       | 18.4  | 1.45                      | 81.8         | 7.88                    |
| Sulfachlorpyridazine (SUPZ) | 7.5                        | 6.3   | 0.95                      | 84.2         | 15.07                   |
|                             | 15                         | 13.7  | 1.12                      | 84.0         | 8.17                    |
|                             | 22.5                       | 19.4  | 1.17                      | 86.2         | 6.03                    |

**Table 7** (continued)

| Analytes                | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 6) | Standard deviation (µg/L) | Recovery (%) | Repeat-ability (CvR, %) |
|-------------------------|----------------------------|---|---------------------------|--------------|-------------------------|
| Sulfadiazine (SUDI)     | 7.5                        | 6.8   | 1.14                      | 90.7         | 16.76                   |
|                         | 15                         | 13.5  | 1.75                      | 90.0         | 12.96                   |
|                         | 22.5                       | 21.3  | 1.41                      | 103.5        | 6.62                    |
| Sulfadimethoxine (SUDM) | 7.5                        | 6.2   | 0.61                      | 82.6         | 9.84                    |
|                         | 15                         | 12.8  | 0.78                      | 85.3         | 6.09                    |
|                         | 22.5                       | 18.6  | 1.31                      | 82.7         | 7.04                    |
| Sulfadimidine (SULD)    | 7.5                        | 6.9   | 0.65                      | 98.7         | 9.42                    |
|                         | 15                         | 13.5  | 1.17                      | 90.2         | 8.67                    |
|                         | 22.5                       | 18.8  | 1.48                      | 83.5         | 7.87                    |
| Sulfamethoxazole (SULM) | 7.5                        | 7.4   | 1.22                      | 98.7         | 16.48                   |
|                         | 15                         | 14.0  | 1.78                      | 93.3         | 12.71                   |
|                         | 22.5                       | 23.4  | 1.21                      | 104.1        | 5.17                    |
| Carbofuran (CRL)        | 7.5                        | 6.8   | 0.35                      | 90.6         | 5.15                    |
|                         | 15                         | 13.2  | 0.61                      | 88.4         | 4.62                    |
|                         | 22.5                       | 21.0  | 1.11                      | 93.3         | 5.29                    |
| Carbaryl (CRB)          | 7.5                        | 7.1   | 0.78                      | 94.6         | 10.98                   |
|                         | 15                         | 14.4  | 1.02                      | 96.0         | 7.08                    |
|                         | 22.5                       | 20.3  | 1.12                      | 90.2         | 9.31                    |
| Parathion (PTN)         | 7.5                        | 6.7   | 0.78                      | 89.3         | 11.64                   |
|                         | 15                         | 13.8  | 1.11                      | 92.0         | 8.04                    |
|                         | 22.5                       | 19.7  | 1.23                      | 87.6         | 6.24                    |
| Malathion (MTN)         | 7.5                        | 7.6   | 0.64                      | 101.3        | 8.42                    |
|                         | 15                         | 14.7  | 0.92                      | 98.2         | 6.26                    |
|                         | 22.5                       | 24.1  | 1.47                      | 107.1        | 6.10                    |
| Diazinon (DNN)          | 7.5                        | 6.6   | 0.32                      | 88.0         | 4.85                    |
|                         | 15                         | 12.8  | 0.75                      | 85.3         | 5.86                    |
|                         | 22.5                       | 19.3  | 1.12                      | 85.7         | 5.80                    |
| Dimethoate (DIM)        | 7.5                        | 6.9   | 1.01                      | 92.1         | 14.63                   |
|                         | 15                         | 13.8  | 1.27                      | 92.0         | 9.20                    |
|                         | 22.5                       | 21.2  | 1.59                      | 94.2         | 7.50                    |
| Atrazine (ATRZ)         | 7.5                        | 7.2   | 0.54                      | 96.0         | 7.06                    |
|                         | 15                         | 13.7  | 0.75                      | 91.3         | 5.48                    |
|                         | 22.5                       | 20.1  | 1.13                      | 89.3         | 5.62                    |
| Permethrin (PEMT)       | 7.5                        | 7.2   | 1.02                      | 96.0         | 14.16                   |
|                         | 15                         | 14.1  | 1.41                      | 94.0         | 10.00                   |
|                         | 22.5                       | 20.2  | 1.51                      | 89.8         | 7.48                    |
| Cypermethrin (CIRM)     | 7.5                        | 6.4   | 0.78                      | 85.3         | 12.19                   |
|                         | 15                         | 14.3  | 0.95                      | 95.4         | 6.64                    |
|                         | 22.5                       | 19.7  | 1.43                      | 87.6         | 7.26                    |
| Deltamethrin (DELM)     | 7.5                        | 6.8   | 0.91                      | 90.7         | 13.38                   |
|                         | 15                         | 13.4  | 1.32                      | 89.3         | 9.85                    |
|                         | 22.5                       | 19.7  | 1.74                      | 87.6         | 8.83                    |
| Coumaphos (COU)         | 7.5                        | 6.9   | 0.56                      | 92.0         | 8.12                    |
|                         | 15                         | 12.9  | 0.92                      | 86.3         | 7.13                    |
|                         | 22.5                       | 18.7  | 1.36                      | 83.1         | 7.27                    |
| Dichlorvos (DIRP)       | 7.5                        | 7.2   | 0.78                      | 96.2         | 10.83                   |
|                         | 15                         | 13.1  | 1.45                      | 87.3         | 11.07                   |
|                         | 22.5                       | 19.8  | 1.92                      | 88.1         | 9.70                    |
| Chlorpyrifos (CHRS)     | 7.5                        | 6.3   | 0.28                      | 84.1         | 4.45                    |
|                         | 15                         | 12.9  | 1.43                      | 86.3         | 11.09                   |
|                         | 22.5                       | 20.2  | 1.21                      | 89.8         | 5.99                    |
| Fenvalerate (FERT)      | 7.5                        | 6.8   | 0.34                      | 90.6         | 5.00                    |
|                         | 15                         | 13.5  | 0.92                      | 87.7         | 6.81                    |
|                         | 22.5                       | 20.8  | 1.13                      | 92.4         | 5.43                    |
| Boscalid (BOS)          | 7.5                        | 7.0   | 0.46                      | 93.3         | 6.57                    |
|                         | 15                         | 13.8  | 1.11                      | 92.0         | 8.04                    |
|                         | 22.5                       | 18.4  | 1.42                      | 81.2         | 7.72                    |

**Table 7** (continued)

| Analytes             | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 6) | Standard deviation (µg/L) | Recovery (%) | Repeat-ability (CVr, %) |
|----------------------|----------------------------|---|---------------------------|--------------|-------------------------|
| Fenthoate (FETE)     | 7.5                        | 6.4   | 0.45                      | 85.3         | 7.03                    |
|                      | 15                         | 14.6  | 1.01                      | 97.3         | 6.92                    |
|                      | 22.5                       | 23.2  | 1.41                      | 103.1        | 6.08                    |
| Fenthion (FEON)      | 7.5                        | 6.9   | 0.27                      | 92.0         | 3.91                    |
|                      | 15                         | 13.8  | 0.88                      | 92.1         | 6.37                    |
|                      | 22.5                       | 19.4  | 1.02                      | 86.2         | 5.26                    |
| Monocrotophos (MOCR) | 7.5                        | 7.1   | 0.65                      | 94.6         | 9.15                    |
|                      | 15                         | 12.6  | 0.68                      | 84.0         | 5.40                    |
|                      | 22.5                       | 18.7  | 1.05                      | 83.1         | 5.61                    |
| Malaoxon (MAON)      | 7.5                        | 7.3   | 1.14                      | 97.3         | 15.62                   |
|                      | 15                         | 13.6  | 1.25                      | 90.67        | 9.19                    |
|                      | 22.5                       | 21.3  | 1.38                      | 94.67        | 6.48                    |
| Methamidophos (MEDF) | 7.5                        | 7.7   | 1.11                      | 102.6        | 14.41                   |
|                      | 15                         | 13.5  | 1.82                      | 90.1         | 13.48                   |
|                      | 22.5                       | 19.8  | 2.01                      | 88.4         | 10.15                   |
| Metacrifos (MECF)    | 7.5                        | 7.6   | 0.41                      | 101.3        | 5.39                    |
|                      | 15                         | 13.8  | 0.95                      | 92.0         | 6.88                    |
|                      | 22.5                       | 22.1  | 1.17                      | 88.4         | 5.29                    |
| Amitraz (AMRZ)       | 7.5                        | 7.0   | 1.12                      | 93.3         | 16.00                   |
|                      | 15                         | 14.1  | 1.41                      | 94.0         | 10.00                   |
|                      | 22.5                       | 19.2  | 1.52                      | 85.3         | 7.92                    |
| Omethoate (OMAT)     | 7.5                        | 6.9   | 0.28                      | 92.0         | 4.06                    |
|                      | 15                         | 14.6  | 0.71                      | 97.3         | 4.86                    |
|                      | 22.5                       | 22.4  | 1.13                      | 99.6         | 5.04                    |
| Vamidothion (VAON)   | 7.5                        | 6.4   | 0.95                      | 85.3         | 14.84                   |
|                      | 15                         | 13.8  | 1.14                      | 92.1         | 8.26                    |
|                      | 22.5                       | 18.9  | 1.38                      | 84.0         | 7.30                    |
| Phosmet (FOST)       | 7.5                        | 7.2   | 0.65                      | 96.0         | 9.02                    |
|                      | 15                         | 13.4  | 0.92                      | 89.3         | 6.87                    |
|                      | 22.5                       | 21.0  | 1.12                      | 93.3         | 5.33                    |
| Heptenophos (HEPH)   | 7.5                        | 7.0   | 0.65                      | 93.3         | 9.29                    |
|                      | 15                         | 14.1  | 0.95                      | 94.0         | 6.74                    |
|                      | 22.5                       | 19.4  | 1.52                      | 86.2         | 7.83                    |
| Bifenthrin (BFNT)    | 7.5                        | 6.4   | 0.54                      | 85.3         | 8.43                    |
|                      | 15                         | 13.3  | 0.68                      | 88.7         | 5.11                    |
|                      | 22.5                       | 19.7  | 0.92                      | 87.5         | 4.67                    |
| Methomyl (MEML)      | 7.5                        | 7.8   | 1.11                      | 104.0        | 14.23                   |
|                      | 15                         | 14.9  | 1.48                      | 99.3         | 9.93                    |
|                      | 22.5                       | 19.3  | 1.23                      | 85.78        | 6.37                    |
| Zearalenone (ZEAN)   | 7.5                        | 6.7   | 0.49                      | 89.3         | 7.31                    |
|                      | 15                         | 12.6  | 0.61                      | 84.0         | 4.84                    |
|                      | 22.5                       | 20.8  | 0.68                      | 92.4         | 3.27                    |
| Ochratoxin A (OTAA)  | 7.5                        | 7.4   | 0.92                      | 98.7         | 12.43                   |
|                      | 15                         | 12.9  | 1.45                      | 86.0         | 11.24                   |
|                      | 22.5                       | 19.9  | 1.52                      | 88.4         | 7.63                    |

consisting of enzymatic hydrolysis using  $\beta$ -glucuronidase and cleanup solid phase extraction with OASIS SPE cartridges, allowed analysis at low level concentrations without any matrix interference for all samples, indicating that the method was very effective for regulatory monitoring of bovine urine for 72 residues of veterinary drugs residues, pesticides and mycotoxins.

## Conclusion

A new isotopic LC-MS/MS method has been developed, validated and applied for identification and quantification of 72 residues of veterinary drugs and pesticides and other contaminants such as mycotoxins in bovine urine. The most appropriated sample preparation procedures involved sodium acetate buffer, enzymatic hydrolysis using  $\beta$ -glucuronidase and cleanup solid phase extraction with OASIS SPE cartridges. The parameters were satisfactorily validated fulfilling requirements under



**Table 8** Interday recovery and reproducibility of the method

| Analytes                  | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 18) | Standard deviation (µg/L) | Recovery (%) | Reproducibility (CV <sub>R</sub> %) |
|---------------------------|----------------------------|--|---------------------------|--------------|-------------------------------------|
| Thiouracil (TU)           | 5                          | 4.88   | 0.41                      | 97.6         | 8.32                                |
|                           | 10                         | 10.51  | 1.20                      | 105.1        | 11.40                               |
|                           | 15                         | 14.32  | 1.80                      | 95.5         | 12.54                               |
| Methylthiouracil (MTU)    | 5                          | 4.28   | 0.40                      | 85.6         | 9.36                                |
|                           | 10                         | 8.85   | 1.02                      | 88.5         | 11.55                               |
|                           | 15                         | 13.95  | 1.92                      | 93.0         | 13.84                               |
| Propylthiouracil (PTU)    | 5                          | 4.88   | 0.30                      | 97.6         | 6.11                                |
|                           | 10                         | 10.10  | 1.27                      | 101.0        | 12.54                               |
|                           | 15                         | 15.14  | 2.11                      | 100.9        | 13.95                               |
| Tapazole (TAP)            | 5                          | 4.23   | 0.39                      | 84.6         | 9.22                                |
|                           | 10                         | 9.78   | 0.73                      | 97.8         | 7.40                                |
|                           | 15                         | 13.66  | 1.56                      | 91.1         | 11.54                               |
| Testosterone (TEST)       | 7.5                        | 7.02   | 0.87                      | 93.6         | 12.41                               |
|                           | 15                         | 14.51  | 1.61                      | 96.7         | 11.08                               |
|                           | 22.5                       | 21.46  | 2.12                      | 95.4         | 9.87                                |
| Methyltestosterone (MEST) | 0.25                       | 0.22   | 0.031                     | 88.0         | 13.88                               |
|                           | 0.50                       | 0.42   | 0.057                     | 84.0         | 13.51                               |
|                           | 0.75                       | 0.77   | 0.089                     | 102.7        | 11.65                               |
| Boldenone (BOLD)          | 0.5                        | 0.45   | 0.056                     | 90.0         | 12.46                               |
|                           | 1.0                        | 1.09   | 0.142                     | 109.2        | 13.08                               |
|                           | 1.5                        | 1.20   | 0.176                     | 80.4         | 14.66                               |
| 19 Nortestosteron (19 NO) | 0.25                       | 0.19   | 0.016                     | 76.2         | 8.57                                |
|                           | 0.5                        | 0.53   | 0.065                     | 106.4        | 12.35                               |
|                           | 0.75                       | 0.65   | 0.066                     | 86.7         | 10.12                               |
| Stanozolol (STZL)         | 0.25                       | 0.24   | 0.028                     | 96.3         | 11.84                               |
|                           | 0.5                        | 0.52   | 0.075                     | 104.7        | 14.46                               |
|                           | 0.75                       | 0.73   | 0.101                     | 97.3         | 13.50                               |
| Clostebol (CLBL)          | 7.5                        | 7.10   | 0.87                      | 94.7         | 12.16                               |
|                           | 15                         | 14.95  | 2.30                      | 99.7         | 15.38                               |
|                           | 22.5                       | 18.65  | 2.96                      | 82.9         | 15.89                               |
| Zeranol (ZENL)            | 0.5                        | 0.47   | 0.074                     | 94.1         | 15.88                               |
|                           | 1.0                        | 0.80   | 0.122                     | 80.2         | 15.29                               |
|                           | 1.5                        | 1.36   | 0.121                     | 90.7         | 8.90                                |
| Taleranol (TANL)          | 0.5                        | 0.42   | 0.057                     | 84.5         | 13.65                               |
|                           | 1.0                        | 0.98   | 0.145                     | 98.4         | 14.80                               |
|                           | 1.5                        | 1.35   | 0.156                     | 90.2         | 11.54                               |
| Clenbuterol (CLEN)        | 0.05                       | 0.05   | 0.005                     | 94.0         | 11.54                               |
|                           | 0.1                        | 0.08   | 0.011                     | 84.2         | 12.82                               |
|                           | 0.15                       | 0.14   | 0.014                     | 92.7         | 10.14                               |
| Brombuterol (BROM)        | 0.05                       | 0.04   | 0.006                     | 82.0         | 14.90                               |
|                           | 0.1                        | 0.10   | 0.012                     | 95.0         | 12.56                               |
|                           | 0.15                       | 0.12   | 0.019                     | 82.7         | 15.58                               |
| Mabuterol (MABT)          | 0.05                       | 0.04   | 0.005                     | 84.2         | 12.35                               |
|                           | 0.1                        | 0.08   | 0.012                     | 81.0         | 14.46                               |
|                           | 0.15                       | 0.15   | 0.023                     | 97.3         | 15.80                               |
| Clenpenterol (CLEP)       | 0.05                       | 0.04   | 0.006                     | 84.0         | 14.47                               |
|                           | 0.1                        | 0.09   | 0.010                     | 86.0         | 11.10                               |
|                           | 0.15                       | 0.15   | 0.022                     | 101.3        | 14.22                               |
| Isoxuprin (ISOX)          | 0.25                       | 0.26   | 0.041                     | 104.4        | 15.84                               |
|                           | 0.50                       | 0.41   | 0.059                     | 82.2         | 14.46                               |
|                           | 0.75                       | 0.76   | 0.105                     | 101.2        | 13.85                               |
| Cimbuterol (CIMB)         | 0.05                       | 0.05   | 0.007                     | 92.0         | 15.11                               |
|                           | 0.1                        | 0.08   | 0.008                     | 84.0         | 9.25                                |
|                           | 0.15                       | 0.14   | 0.021                     | 90.7         | 15.56                               |

**Table 8** (continued)

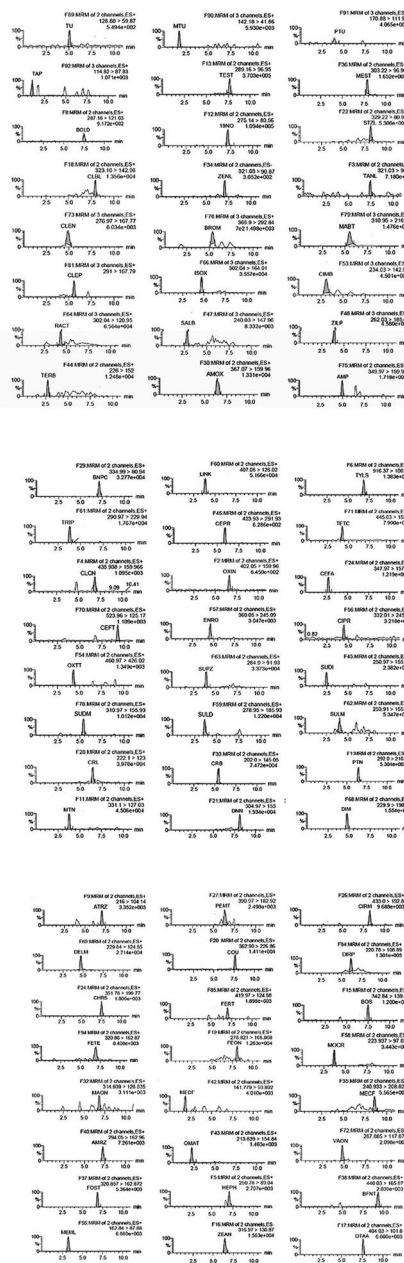
| Analytes                | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 18) | Standard deviation (µg/L) | Recovery (%) | Reproducibility (CV <sub>R</sub> %) |
|-------------------------|----------------------------|--|---------------------------|--------------|-------------------------------------|
| Ractopamine (RACT)      | 0.25                       | 0.25   | 0.028                     | 100.4        | 11.26                               |
|                         | 0.50                       | 0.44   | 0.067                     | 88.2         | 15.11                               |
|                         | 0.75                       | 0.72   | 0.106                     | 95.3         | 14.88                               |
| Salbutamol (SALB)       | 0.25                       | 0.22   | 0.036                     | 89.2         | 15.98                               |
|                         | 0.50                       | 0.47   | 0.067                     | 93.4         | 14.36                               |
|                         | 0.75                       | 0.62   | 0.079                     | 82.8         | 12.68                               |
| Zilpaterol HCl (ZILP)   | 0.25                       | 0.24   | 0.038                     | 95.2         | 15.95                               |
|                         | 0.50                       | 0.52   | 0.065                     | 104.8        | 12.38                               |
|                         | 0.75                       | 0.71   | 0.095                     | 95.2         | 13.29                               |
| Terbutaline (TERB)      | 0.25                       | 0.22   | 0.029                     | 88.4         | 13.26                               |
|                         | 0.50                       | 0.48   | 0.050                     | 95.6         | 10.52                               |
|                         | 0.75                       | 0.76   | 0.120                     | 101.5        | 15.78                               |
| Amoxicillin (AMOX)      | 7.5                        | 7.0  | 0.71                      | 93.3         | 10.15                               |
|                         | 15                         | 13.5   | 2.13                      | 90.2         | 15.74                               |
|                         | 22.5                       | 21.4   | 3.79                      | 95.4         | 17.70                               |
| Ampicillin (AMP)        | 7.5                        | 6.6  | 2.64                      | 88.4         | 17.40                               |
|                         | 15                         | 16.1   | 1.96                      | 107.3        | 12.24                               |
|                         | 22.5                       | 23.4   | 2.14                      | 104.2        | 9.15                                |
| Benzylpenicillin (BNPC) | 7.5                        | 7.0  | 0.43                      | 93.6         | 6.14                                |
|                         | 15                         | 13.2   | 1.35                      | 88.4         | 10.21                               |
|                         | 22.5                       | 19.4   | 1.82                      | 86.2         | 9.46                                |
| Lincomycin (LINK)       | 7.5                        | 7.5  | 0.64                      | 100.4        | 8.48                                |
|                         | 15                         | 13.4   | 2.20                      | 89.5         | 16.45                               |
|                         | 22.5                       | 18.9   | 2.82                      | 84.7         | 14.94                               |
| Tylosin (TYLS)          | 7.5                        | 7.6  | 0.93                      | 101.3        | 12.25                               |
|                         | 15                         | 14.8   | 2.43                      | 98.7         | 16.41                               |
|                         | 22.5                       | 19.4   | 2.68                      | 86.0         | 13.85                               |
| Trimethoprim (TRIP)     | 7.5                        | 7.4  | 1.10                      | 98.7         | 15.48                               |
|                         | 15                         | 15.7   | 1.96                      | 104.8        | 12.46                               |
|                         | 22.5                       | 22.1   | 3.30                      | 98.3         | 14.95                               |
| Cephapirin (CEPR)       | 7.5                        | 6.4  | 0.58                      | 85.6         | 9.12                                |
|                         | 15                         | 12.8   | 1.08                      | 85.2         | 8.46                                |
|                         | 22.5                       | 18.9   | 2.37                      | 84.4         | 12.52                               |
| Tetracycline (TETC)     | 7.5                        | 6.9  | 0.39                      | 92.2         | 5.65                                |
|                         | 15                         | 12.5   | 1.64                      | 83.7         | 13.10                               |
|                         | 22.5                       | 23.1   | 1.65                      | 102.8        | 7.14                                |
| Cloxacillin (CLCN)      | 7.5                        | 7.2  | 0.66                      | 96.0         | 9.15                                |
|                         | 15                         | 13.8   | 1.68                      | 92.0         | 12.20                               |
|                         | 22.5                       | 19.9   | 2.56                      | 88.4         | 12.84                               |
| Oxacillin (OXIN)        | 7.5                        | 7.1  | 0.53                      | 94.7         | 7.45                                |
|                         | 15                         | 14.5   | 1.90                      | 96.7         | 13.12                               |
|                         | 22.5                       | 21.0   | 2.57                      | 93.3         | 12.25                               |
| Cefalexin (CEFA)        | 7.5                        | 7.0  | 1.20                      | 93.3         | 17.11                               |
|                         | 15                         | 14.4   | 1.85                      | 96.0         | 12.84                               |
|                         | 22.5                       | 23.8   | 2.73                      | 105.8        | 11.46                               |
| Ceftiofur (CEFT)        | 7.5                        | 6.9  | 0.79                      | 92.0         | 11.41                               |
|                         | 15                         | 13.0   | 1.60                      | 86.7         | 12.25                               |
|                         | 22.5                       | 21.7   | 3.57                      | 96.4         | 16.41                               |
| Enrofloxacin (ENRO)     | 7.5                        | 6.1  | 1.18                      | 81.3         | 19.35                               |
|                         | 15                         | 12.5   | 2.19                      | 83.3         | 17.48                               |
|                         | 22.5                       | 22.3   | 3.01                      | 99.1         | 13.51                               |
| Ciprofloxacin (CIPR)    | 7.5                        | 7.4  | 1.49                      | 98.7         | 20.12                               |
|                         | 15                         | 14.9   | 2.59                      | 99.3         | 17.35                               |
|                         | 22.5                       | 22.8   | 3.77                      | 101.3        | 16.48                               |

**Table 8** (continued)

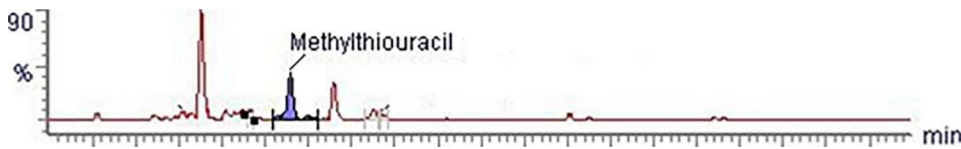
| Analytes                    | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 18) | Standard deviation (µg/L) | Recovery (%) | Reproducibility (CV <sub>R</sub> %) |
|-----------------------------|----------------------------|--|---------------------------|--------------|-------------------------------------|
| Oxytetracycline (OXTT)      | 7.5                        | 6.9  | 1.04                      | 92.0         | 15.12                               |
|                             | 15                         | 15.4   | 2.56                      | 102.7        | 10.14                               |
|                             | 22.5                       | 19.9   | 1.62                      | 88.4         | 13.18                               |
| Sulfachlorpyridazine (SUPZ) | 7.5                        | 7.7  | 1.65                      | 102.7        | 21.35                               |
|                             | 15                         | 16.3   | 2.48                      | 108.7        | 15.22                               |
|                             | 22.5                       | 20.8   | 1.94                      | 92.4         | 9.31                                |
| Sulfadiazine (SUDI)         | 7.5                        | 6.2  | 1.31                      | 82.7         | 21.13                               |
|                             | 15                         | 12.5   | 2.15                      | 83.3         | 17.11                               |
|                             | 22.5                       | 18.2   | 1.85                      | 80.9         | 10.15                               |
| Sulfadimethoxine (SUDM)     | 7.5                        | 7.4  | 0.99                      | 98.7         | 13.41                               |
|                             | 15                         | 13.8   | 1.26                      | 92.0         | 9.12                                |
|                             | 22.5                       | 22.0   | 3.05                      | 97.8         | 13.85                               |
| Sulfadimidine (SULD)        | 7.5                        | 6.6  | 0.89                      | 88.0         | 13.46                               |
|                             | 15                         | 14.7   | 1.70                      | 98.0         | 11.54                               |
|                             | 22.5                       | 21.0   | 2.57                      | 93.3         | 12.25                               |
| Sulfamethoxazole (SULM)     | 7.5                        | 8.1  | 1.69                      | 108.4        | 20.81                               |
|                             | 15                         | 15.9   | 2.33                      | 106.0        | 14.66                               |
|                             | 22.5                       | 22.5   | 2.52                      | 100.2        | 11.22                               |
| Carbofuran (CRL)            | 7.5                        | 7.1  | 0.59                      | 94.7         | 8.35                                |
|                             | 15                         | 14.6   | 1.63                      | 97.3         | 11.14                               |
|                             | 22.5                       | 19.4   | 1.72                      | 86.4         | 8.88                                |
| Carbaryl (CRB)              | 7.5                        | 7.1  | 1.17                      | 94.7         | 16.46                               |
|                             | 15                         | 13.0   | 2.55                      | 86.4         | 19.58                               |
|                             | 22.5                       | 18.9   | 2.33                      | 84.2         | 12.35                               |
| Parathion (PTN)             | 7.5                        | 7.4  | 1.27                      | 98.7         | 17.12                               |
|                             | 15                         | 13.8   | 1.80                      | 92.1         | 13.01                               |
|                             | 22.5                       | 22.1   | 2.02                      | 98.2         | 9.15                                |
| Malathion (MTN)             | 7.5                        | 6.4  | 0.78                      | 85.3         | 12.15                               |
|                             | 15                         | 13.2   | 1.22                      | 88.6         | 9.21                                |
|                             | 22.5                       | 20.1   | 2.04                      | 89.3         | 10.15                               |
| Diazinon (DNN)              | 7.5                        | 7.2  | 0.66                      | 96.1         | 9.12                                |
|                             | 15                         | 13.7   | 1.16                      | 91.4         | 8.46                                |
|                             | 22.5                       | 21.2   | 2.37                      | 94.0         | 11.2                                |
| Dimethoate (DIM)            | 7.5                        | 6.4  | 1.23                      | 85.3         | 19.23                               |
|                             | 15                         | 13.0   | 1.80                      | 86.7         | 13.81                               |
|                             | 22.5                       | 18.4   | 1.87                      | 81.8         | 10.15                               |
| Atrazine (ATRZ)             | 7.5                        | 7.4  | 1.00                      | 98.7         | 13.52                               |
|                             | 15                         | 13.7   | 1.39                      | 91.3         | 10.15                               |
|                             | 22.5                       | 21.3   | 1.81                      | 94.7         | 8.48                                |
| Permethrin (PEMT)           | 7.5                        | 7.0  | 1.49                      | 93.3         | 21.35                               |
|                             | 15                         | 13.5   | 1.86                      | 90.0         | 13.78                               |
|                             | 22.5                       | 20.8   | 2.61                      | 92.4         | 12.54                               |
| Cypermethrin (CIRM)         | 7.5                        | 6.9  | 1.06                      | 92.0         | 15.31                               |
|                             | 15                         | 14.7   | 1.54                      | 98.0         | 10.48                               |
|                             | 22.5                       | 18.2   | 2.07                      | 80.9         | 11.35                               |
| Deltamethrin (DELM)         | 7.5                        | 7.4  | 1.35                      | 98.7         | 18.22                               |
|                             | 15                         | 15.8   | 2.13                      | 105.3        | 13.48                               |
|                             | 22.5                       | 22.9   | 3.32                      | 101.8        | 14.51                               |
| Coumaphos (COU)             | 7.5                        | 8.2  | 1.25                      | 109.3        | 15.21                               |
|                             | 15                         | 14.6   | 1.98                      | 97.3         | 13.58                               |
|                             | 22.5                       | 23.4   | 3.07                      | 104.0        | 13.11                               |
| Dichlorvos (DIRP)           | 7.5                        | 6.9  | 1.26                      | 92.0         | 18.21                               |
|                             | 15                         | 14.6   | 2.53                      | 97.3         | 17.35                               |
|                             | 22.5                       | 21.8   | 2.95                      | 96.9         | 13.54                               |

**Table 8** (continued)

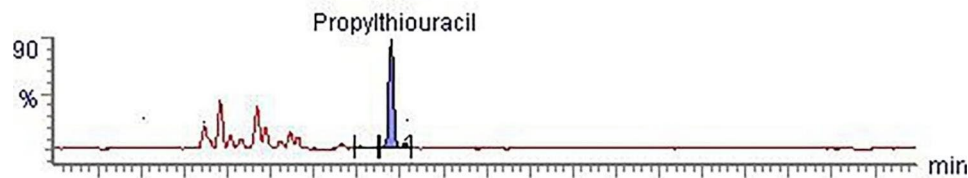
| Analytes             | Added concentration (µg/L) | Average concentration in samples (µg/L) (n = 18) | Standard deviation (µg/L) | Recovery (%) | Reproducibility (CV <sub>R</sub> %) |
|----------------------|----------------------------|--|---------------------------|--------------|-------------------------------------|
| Chlorpyrifos (CHRS)  | 7.5                        | 6.6  | 0.50                      | 88.0         | 7.54                                |
|                      | 15                         | 13.8   | 1.92                      | 92.0         | 13.88                               |
|                      | 22.5                       | 19.5   | 1.58                      | 86.7         | 8.11                                |
| Fenvalerate (FERT)   | 7.5                        | 7.1  | 0.65                      | 94.7         | 9.11                                |
|                      | 15                         | 15.8   | 1.82                      | 105.3        | 11.52                               |
|                      | 22.5                       | 22.0   | 1.79                      | 97.8         | 8.14                                |
| Boscalid (BOS)       | 7.5                        | 7.1  | 0.65                      | 94.7         | 9.15                                |
|                      | 15                         | 14.6   | 1.77                      | 97.3         | 12.11                               |
|                      | 22.5                       | 18.9   | 2.25                      | 84.0         | 11.90                               |
| Fenthoate (FETE)     | 7.5                        | 6.7  | 0.97                      | 89.3         | 14.42                               |
|                      | 15                         | 16.0   | 2.06                      | 106.7        | 12.86                               |
|                      | 22.5                       | 22.1   | 2.24                      | 98.2         | 10.14                               |
| Fenthion (FEON)      | 7.5                        | 7.1  | 0.50                      | 94.7         | 7.11                                |
|                      | 15                         | 13.9   | 1.29                      | 92.7         | 9.25                                |
|                      | 22.5                       | 18.5   | 1.89                      | 82.2         | 10.14                               |
| Monocrotophos (MOCR) | 7.5                        | 7.5  | 1.04                      | 100.0        | 13.84                               |
|                      | 15                         | 14.8   | 1.40                      | 98.7         | 9.48                                |
|                      | 22.5                       | 22.0   | 1.78                      | 97.8         | 8.11                                |
| Malaoxon (MAON)      | 7.5                        | 6.7  | 1.35                      | 89.3         | 20.11                               |
|                      | 15                         | 12.9   | 1.69                      | 86.0         | 13.11                               |
|                      | 22.5                       | 22.3   | 2.00                      | 99.1         | 8.95                                |
| Methamidophos (MEDF) | 7.5                        | 7.0  | 1.42                      | 93.3         | 20.22                               |
|                      | 15                         | 12.8   | 2.23                      | 85.3         | 17.46                               |
|                      | 22.5                       | 20.9   | 2.69                      | 92.9         | 12.88                               |
| Metacrifos (MECF)    | 7.5                        | 6.4  | 0.52                      | 85.3         | 8.12                                |
|                      | 15                         | 12.5   | 1.17                      | 83.3         | 9.36                                |
|                      | 22.5                       | 22.1   | 1.79                      | 98.2         | 8.10                                |
| Amitraz (AMRZ)       | 7.5                        | 7.0  | 1.49                      | 93.3         | 21.35                               |
|                      | 15                         | 14.1   | 1.86                      | 94.0         | 13.22                               |
|                      | 22.5                       | 18.3   | 1.80                      | 81.3         | 9.88                                |
| Omethoate (OMAT)     | 7.5                        | 6.2  | 0.50                      | 82.7         | 8.11                                |
|                      | 15                         | 13.1   | 0.99                      | 87.3         | 7.52                                |
|                      | 22.5                       | 19.9   | 2.26                      | 88.4         | 11.35                               |
| Vamidothion (VAON)   | 7.5                        | 7.4  | 1.23                      | 98.7         | 16.60                               |
|                      | 15                         | 14.6   | 1.75                      | 97.3         | 12.02                               |
|                      | 22.5                       | 22.1   | 2.32                      | 98.2         | 10.51                               |
| Phosmet (FOST)       | 7.5                        | 7.4  | 0.90                      | 98.7         | 12.20                               |
|                      | 15                         | 14.6   | 1.22                      | 97.4         | 8.36                                |
|                      | 22.5                       | 18.8   | 1.71                      | 83.6         | 9.11                                |
| Heptenophos (HEPH)   | 7.5                        | 6.6  | 0.83                      | 84.8         | 12.51                               |
|                      | 15                         | 13.2   | 1.21                      | 88.2         | 9.15                                |
|                      | 22.5                       | 21.7   | 2.95                      | 96.4         | 13.61                               |
| Bifenthrin (BFNT)    | 7.5                        | 6.9  | 0.97                      | 92.2         | 14.11                               |
|                      | 15                         | 12.8   | 1.17                      | 85.3         | 9.12                                |
|                      | 22.5                       | 23.4   | 1.71                      | 104.6        | 7.35                                |
| Methomyl (MEML)      | 7.5                        | 7.5  | 1.60                      | 100.2        | 21.38                               |
|                      | 15                         | 13.6   | 2.07                      | 91.0         | 15.22                               |
|                      | 22.5                       | 24.3   | 2.27                      | 108.1        | 9.35                                |
| Zearalenone (ZEAN)   | 7.5                        | 7.1  | 0.89                      | 94.6         | 12.58                               |
|                      | 15                         | 14.0   | 1.43                      | 93.4         | 10.22                               |
|                      | 22.5                       | 19.6   | 1.20                      | 87.1         | 6.11                                |
| Ochratoxin A (OTAA)  | 7.5                        | 6.7  | 1.24                      | 89.3         | 18.54                               |
|                      | 15                         | 13.6   | 2.00                      | 90.6         | 14.65                               |
|                      | 22.5                       | 18.7   | 2.10                      | 83.1         | 11.22                               |



**Fig. 1** **A** The chromatograms of spiked urine samples - concentration level is level 2 from Table 5 (TU (10 µg/L), MTU (10 µg/L), PTU (10 µg/L), TAP (10 µg/L), TEST (15 µg/L), MEST (0.5 µg/L), BOLD (1.0 µg/L), 19 NO (0.5 µg/L), STZL (0.5 µg/L), CLBL (15 µg/L), ZENL (1.0 µg/L), TANL (1.0 µg/L), CLEN (0.1 µg/L), BROM (0.1 µg/L), MABT (0.1 µg/L), CLEP (0.1 µg/L), ISOX (0.5 µg/L), CIMB (0.1 µg/L), RACT (0.5 µg/L), SALB (0.5 µg/L), ZILP (0.5 µg/L), TERB (0.5 µg/L), AMOX (15 µg/L), AMP (15 µg/L). **B** The chromatograms of spiked urine samples - concentration level is level 2 from Table 5 (BNPC, LINK, TYLS, TRIP, CEPR, TETC, CLCN, OXIN, CEFA, CEFT, ENRO, CIPR, OXTT, SUPZ, SUDI, SUDM, SULD, SULM, CRL, CRB, PTN, MTN, DNN, DIM – 15 µg/L for all). **C** The chromatograms of spiked urine samples - concentration level is level 2 from Table 5 (ATRZ, PEMT, CIRN, DELM, COU, DIPR, CHR, FERT, BOS, FETE, FEON, MOCR, MAON, MEDF, MECF, AMRZ, OMAT, VAON, FOST, HEPH, BFNT, MEML, ZEAN, OTAA – 15 µg/L for all)



**Fig. 2** Chromatogram – Methylthiouracil in bovine urine



**Fig. 3** Propylthiouracil in bovine urine

Regulation 2021/808/EC. Consequently, the method could be used in routine analysis of bovine urine samples for simultaneous detection of veterinary drug and pesticide residues as well as contaminants such as mycotoxins.

#### Abbreviations

|                 |  |
|-----------------|--|
| CC $\alpha$     | Decision limit                                 |
| CC $\beta$      | Detection capability                           |
| CV <sub>r</sub> | Coefficient of variation for repeatability     |
| CV <sub>R</sub> | Coefficient of variation for reproducibility   |
| EC              | European Commission                            |
| EURL            | European Union Reference Laboratory            |
| LC-MS/MS        | Liquid chromatography tandem mass spectrometry |
| LLE             | Liquid-liquid extraction                       |
| LOD             | Limit of detection                             |
| LOQ             | Limit of quantification                        |
| MMPRs           | Minimum method performance requirements        |
| MRM             | Multiple reaction monitoring                   |
| MRLs            | Maximum residue limit                          |
| PBS             | Phosphate buffer                               |
| R <sup>2</sup>  | Coefficient of correlation                     |
| S/N             | Signal to noise                                |
| SPE             | Solid phase extraction                         |
| USA             | United States of America                       |

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Not applicable.

#### Authors' contributions

Zehra Hajrulai-Musliu: participated in writing the manuscript, experimental design, methodology, data analysis, review and editing resources, project administration and supervision. Risto Uzunov participating in writing the manuscript, experimental design, methodology, sample preparation, analysis and data analysis. Elizabeta Dimitrieska-Stojkovikj and Biljana Stojanovska-Dimzoska participated in experimental design, methodology and data analysis. Stefan Jovanov and Aleksandra Angeleska carried out sampling, sample preparation, analysis and data analysis. Dea Musliu carried out real sample analysis and data analysis. Velimir Stojkovski participated in methodology, conceptualization and investigation. James Jacob Sasanya participated in project administration, funding acquisition, review and editing. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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#### Data Availability

All data and materials analyzed during the current study are included in the manuscript.

#### Declarations

#### Ethical approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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#### References

- Dasenaki EM, Thomaidis SN. Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography–tandem mass spectrometry. *Anal Chim Acta*. 2015;880:103–21. <https://doi.org/10.1016/j.aca.2015.04.013>.
- Canton L, Lanusse C, Moreno L. Rational pharmacotherapy in Infectious Diseases: issues related to drug residues in Edible Animal tissues. *Anim (Basel)*. 2021;11(10):2878. <https://doi.org/10.3390/ani11102878>. PMID: 34679899; PMCID: PMC8532868.
- Lin YP, Lee LL, Hung CY, Huang WJ, Lin SC. Determination of multiresidue analysis of  $\beta$ -agonists in muscle and viscera using liquid chromatograph/tandem mass spectrometry with quick, easy, cheap, effective, rugged, and safe methodologies. *J Food Drug Anal*. 2017;25:275–84. <https://doi.org/10.1016/j.jfda.2016.06.010>.
- Uzunov R, Hajrulai-Musliu Z, Dimitrieska Stojkovic E, Stojanovska-Dimzoska B, Sekulovski P, Stojkovski V. (2013) Use of ELISA for Preliminary Screening of 19 Nortestosterone Anabolic Steroid in Cattle Meat in Republic of Macedonia. *Kafkas Univ Vet Fak Derg* 19:173–177. <https://doi.org/10.9775/kvfd.2012.7527>.
- Regulation, EU) 2017/625 of the European Parliament and of the Council of 15. (March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation). *OJEU*, L95/1, 2017.
- Scarth J, Akre C, van Ginkel L, Le Bizec B, De Brabander H, Korth W, Points J, Teale P, Kay J. Presence and metabolism of endogenous androgenic-anabolic steroid hormones in meat producing animals: a review. *Food Addit Contam*. 2009;26:640–71. <https://doi.org/10.1080/02652030802627160>.
- Agriopoulou S, Stamatelopoulou E, Varzakas T. Advances in occurrence, importance, and mycotoxin control strategies: prevention and detoxification in foods. *Foods*. 2020;9:137. <https://doi.org/10.3390/foods9020137>.

8. Awuchi CG, Ondari EN, Ogbonna CU, Upadhyay AK, Baran K, Okpala COR, Korzeniowska M, Guine RPF. Mycotoxins affecting animals, foods, humans, and plants: types, occurrence, toxicities, action mechanisms, prevention, and detoxification Strategies-A revisit. *Foods*. 2021;10:1279. <https://doi.org/10.3390/foods10061279>.
9. Zhan J, Xu DM, Wang SJ, Sun J, Xu YJ, Ni ML, Yin JY, Chen J, Yu XJ, Huang ZQ. Comprehensive screening for multi-class veterinary drug residues and other contaminants in muscle using column-switching UPLC–MS/MS. *Food Addit Contam Part A*. 2013;30:1888–99. <https://doi.org/10.1080/19440049.2013.833670>.
10. Hajrulai-Musliu Z, Uzunov R, Jovanov S, Jankuloski D, Stojkovski V, Pendovski L, Sasanya JJ. A new LC–MS/MS method for multiple residues/contaminants in bovine meat. *BMC Chem*. 2021;15:62. <https://doi.org/10.1186/s13065-021-00788-5>.
11. Danezis GP, Anagnostopoulos CJ, Liapis K, Koupparis MA. Multi-residue analysis of pesticides, plant hormones, veterinary drugs and mycotoxins using HILIC chromatography—MS/MS in various food matrices. *Anal Chim Acta*. 2016;942:121–38. <https://doi.org/10.1016/j.aca.2016.09.011>.
12. Gómez-Pérez ML, Romero-González R, Vidal JLM, Frenich AG. Analysis of pesticide and veterinary drug residues in baby food by liquid chromatography coupled to Orbitrap high resolution mass spectrometry. *Talanta*. 2015;131:1–7. <https://doi.org/10.1016/j.talanta.2014.07.066>.
13. Wei H, Tao Y, Chen D, Xie S, Pan Y, Liu Z, Huang L, Yuan Z. Development and validation of a multi-residue screening method for veterinary drugs, their metabolites and pesticides in meat using liquid chromatography–tandem mass spectrometry. *Food Addit Contam Part A*. 2015;32:686–701. <https://doi.org/10.1080/19440049.2015.1008588>.
14. Xie J, Peng T, Zhu A, He J, Chang Q, Hu X, Chen H, Fan C, Jiang W, Chen M, Li J, Ding S, Jiang H. Multi-residue analysis of veterinary drugs, pesticides and mycotoxins in dairy products by liquid chromatography–tandem mass spectrometry using low-temperature cleanup and solid phase extraction. *J Chromatogr B*. 2015;1002:19–29. <https://doi.org/10.1016/j.jchromb.2015.08.005>.
15. Ahn J, Kim D, Kim H, Jahng KY. Quantitative determination of mycotoxins in urine by LC–MS/MS. *Food Addit Contam Part A*. 2010;27:1674–82. <https://doi.org/10.1080/19440049.2010.505201>.
16. Akre C, Mizuno M. A screening and determinative method for the analysis of natural and synthetic steroids, stilbenes and resorcylic acid lactones in bovine urine. *Drug Test Anal*. 2016;8:448–57. <https://doi.org/10.1002/dta.2012>.
17. Escrivá L, Oueslati S, Font G, Manyes L. Alternaria Mycotoxins in Food and feed: an overview. *J Food Qual*. 2017;2017:1569748. <https://doi.org/10.1155/2017/1569748>.
18. Uzunov R, Hajrulai-Musliu SV, Dimitrieska-Stojkovic E, Stojanovska-Dimzoska B, Sekulovski P, Jankuloski D. Development and validation of LC–MS/MS method for determination of ten beta agonists in bovine urine. *Kafkas Univ Vet Fak Derg*. 2019;25:55–60. <https://doi.org/10.9775/kvfd.2018.20324>.
19. Kaufmann A, Butcher P, Maden K, Widmer M. Ultra-performance liquid chromatography coupled to time of flight mass spectrometry (UPLC–TOF): a novel tool for multiresidue screening of veterinary drugs in urine. *Anal Chim Acta*. 2007;586:13–21. <https://doi.org/10.1016/j.aca.2006.10.026>.
20. Tuerk J, Reinders M, Dreyer D, Kiffmeyer T, Schmidt KG, Kuss HM. Analysis of antibiotics in urine and wipe samples from environmental and biological monitoring—comparison of HPLC with UV-, single MS- and tandem MS-detection. *J Chromatogr B*. 2006;831:72–80. <https://doi.org/10.1016/j.jchromb.2005.11.030>.
21. De la Huebra MJG, Bordin G, Rodriguez AR. A multiresidue method for the simultaneous determination of ten macrolide antibiotics in human urine based on gradient elution liquid chromatography coupled to coulometric detection (HPLC–ECD). *Anal Chim Acta*. 2004;517:53–63. <https://doi.org/10.1016/j.aca.2004.04.042>.
22. Shealy DB, Bonin MA, Wooten JV, Ashley DL, Needham LL, Bond AE. Application of an improved method for the analysis of pesticides and their metabolites in the urine of farmer applicators and their families. *Environ Int*. 1996;22:661–75. [https://doi.org/10.1016/S0160-4120\(96\)00058-X](https://doi.org/10.1016/S0160-4120(96)00058-X).
23. Stanley SMR, Foo HC. Screening for basic drugs in equine urine using direct-injection differential-gradient LC–LC coupled to hybrid tandem MS/MS. *J Chromatogr B*. 2006;836:1–14. <https://doi.org/10.1016/j.jchromb.2006.03.034>.
24. Biselli S, Schwab S, Meyer A, Hartig L. A multi-class, multi-analyte method for routine analysis of 84 veterinary drugs in chicken muscle using simple extraction and LC–MS/MS. *Food Addit Contam Part A*. 2013;30:921–39. <https://doi.org/10.1080/19440049.2013.777976>.
25. Commission Implementing Regulation (EU). 2021/808 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. *Official Journal of the European Union*. L 180/84. 21.5.2021.
26. Hajrulai-Musliu Z, Uzunov R, Jovanov S, Kerluku M, Jankuloski D, Stojkovski V, Pendovski L, Sasanya JJ. Determination of veterinary drug residues, mycotoxins, and pesticide residues in bovine milk by liquid chromatography electrospray ionisation–tandem mass spectrometry. *J Vet Res*. 2022;66:215–24. <https://doi.org/10.2478/jvetres-2022-0027>.
27. MMPR - EURL guidance. EURL guidance on minimum method performance requirements (MMPRs) for specific pharmacologically active substances in specific animal matrices. 2020.
28. Pozo OJ, Van Eenoo P, Deventer K, Delbeke TF. Development and validation of a qualitative screening method for the detection of exogenous anabolic steroids in urine by liquid chromatography–tandem mass spectrometry. *Anal Bioanal Chem*. 2007;389:1209–24. <https://doi.org/10.1007/s00216-007-1530-6>.
29. Saadati N, Abdullah MP, Zakaria Z, Sany SBT, Rezayi M, Hassonizadeh H. Limit of detection and limit of quantification development procedures for organochlorine pesticides analysis in water and sediment matrices. *Chem Cent Journal*. 2013;7:63. <https://doi.org/10.1186/1752-153X-7-63>.
30. Qu CH, Li HL, Zhang L, Xi CX, Wang GM, Li NB, Luo HQ. Simultaneous determination of cimaterol, salbutamol, terbutaline and ractopamine in feed by SPE coupled to UPLC. *Chromatographia*. 2011;73:243–9. <https://doi.org/10.1007/s10337-010-1873-6>.
31. Council Directive 81/602/EEC of 31. July 1981 concerning the prohibition of certain substances having a hormonal action and of any substances having a thyrostatic action (OJ L 222 07.08.1981, p. 32, ELI: <http://data.europa.eu/eli/dir/1981/602/oj>).
32. Guidance SANTE. 11312/2021 – Analytical quality control and method validation procedures for pesticide residues analysis in food and feed. SANTE; 2021.
33. Stolker AAM, Brinkman UATH. Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals - a review. *J Chromatogr A*. 2005;1067:15–53. <https://doi.org/10.1016/j.chroma.2005.02.037>.
34. Stolker AAM, Zuidema T, Nielen MWF. Residue analysis of veterinary drugs and growth-promoting agents. *Trends Anal Chem*. 2007;26:967–79. <https://doi.org/10.1016/j.trac.2007.09.008>.
35. Courttheyn D, Le Bizec B, Brambilla G, Cobbaert E, de Wiele MV, Verkammen J, De Wasch K. Recent developments in the use and abuse of growth promoters. *Anal Chim Acta*. 2002;473:71–82. [https://doi.org/10.1016/S0003-2670\(02\)00753-5](https://doi.org/10.1016/S0003-2670(02)00753-5).
36. Kaufmann A, Butcher P, Maden K, Walker S, Widmer M. Semi-targeted residue screening in complex matrices with liquid chromatography coupled to high resolution mass spectrometry: current possibilities and limitations. *Analyst*. 2011;136:1898–909. <https://doi.org/10.1039/C0AN00902D>.
37. Leung GNW, Chung EW, Ho ENM, Kwok WH, Leung DKK, Tang FPW, Wan TSM, Yu NH. High-throughput screening of corticosteroids and basic drugs in horse urine by liquid chromatography–tandem mass spectrometry. *J Chromatogr B*. 2005;825:47–56. <https://doi.org/10.1016/j.jchromb.2004.12.039>.
38. Kaufmann A, Butcher P, Maden K, Widmer M. Quantitative multiresidue method for about 100 veterinary drugs in different meat matrices by sub 2-microm particulate high-performance liquid chromatography coupled to time of flight mass spectrometry. *J Chromatogr A*. 2008;1194:66–79. <https://doi.org/10.1016/j.chroma.2008.03.089>.
39. Kellman M, Muenster H, Zomer P, Mol H. Full scan MS in comprehensive qualitative and quantitative residue analysis in food and feed matrices: how much resolving power is required? *J Am Soc Mass Spectrom*. 2009;20:1464–76. <https://doi.org/10.1016/j.jasms.2009.05.010>.
40. Eeckhaut AV, Lanckmans K, Sarre S, Smolders I, Michotte Y. Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects. *J Chromatogr B*. 2009;877:2198–207. <https://doi.org/10.1016/j.jchromb.2009.01.003>.
41. Kakkamanos G, Theodoridis G, Dabalís T. Determination of anabolic steroids in bovine urine by liquid chromatography–tandem mass spectrometry. *J Chromatogr B*. 2009;877:2330–6. <https://doi.org/10.1016/j.jchromb.2009.03.033>.
42. Ho ENM, Leung DKK, Wan TSM, Yu NH. Comprehensive screening of anabolic steroids, corticosteroids, and acidic drugs in horse urine by solid-phase extraction and liquid chromatography–mass spectrometry. *J Chromatogr A*. 2006;1120:38–53. <https://doi.org/10.1016/j.chroma.2006.03.089>.
43. León N, Roca M, Igualada C, Martins CPB, Pastor A, Yusa V. Wide-range screening of banned veterinary drugs in urine by ultra-high liquid



- chromatography coupled to high-resolution mass spectrometry. *J Chromatogr A*. 2012;1258:55–65. <https://doi.org/10.1016/j.chroma.2012.08.031>.
44. Kinsella B, O'Mahony J, Malone E, Moloney M, Cantwell H, Furey A, Danaher M. Current trend in sample preparation for growth promoter and veterinary drug residue analysis. *J Chromatogr A*. 2009;1216:7977–8015. <https://doi.org/10.1016/j.chroma.2009.09.005>.
45. Biancotto G, Stella R, Barrucci F, Lega F, Angeletti R. (2016) Urinary Concentrations of Steroids in Bulls under Anabolic Treatment by

Revalor-XS Implant. *J Anal Methods Chem Article* 2016: 8013175. <https://doi.org/10.1155/2016/8013175>.

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