Microchemical Journal 204 (2024) 110916

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Comprehensive steroid screening in bovine and porcine urine by GC-HRMS

Josha Jager, Marco Blokland, Rachelle Linders, Paul Zoontjes, Eric van Bennekom, Saskia Sterk, Esmer Jongedijk *

Wageningen Food Safety Research (WFSR), part of Wageningen University & Research, P.O. Box 230, 6700 AE Wageningen, the Netherlands

ARTICLE INFO

Keywords:
GC-Q-Orbitrap HRMS
Residue analysis
Food safety
Doping control
Risk-based monitoring
Method development

ABSTRACT

Steroids are a large and diverse group of molecules containing both natural hormones and synthetic derivatives. Steroid administration can have a growth-promoting effect in food-producing livestock. However, the exogenic use of steroids to promote growth has adverse health effects in livestock and consumers, and, consequently, is forbidden in the European Union (EU). Therefore, broad monitoring of steroids and other growth-promoting compounds in animal matrices is desirable. Commission Implementing Regulation (EU) 2021/808 was recently implemented to provide explicit room and quality guidance for analyzing food residues, including steroids and growth-promoting compounds, using the technique of high-resolution mass spectrometry (HRMS). This manuscript presents a method for broad steroid monitoring in animal urine by gas chromatography (GC) Q-Orbitrap HRMS, using a minimal cleanup of liquid–liquid extractions and a 96-well plate solid-phase extraction (SPE). An in-house library was built to detect 104 steroid and steroid-like molecules from subclasses of androgens, estrogens, progestogens, stilbenes, and resorcylic acid lactones (RALs) in the HRMS datafiles. The method was fully validated according to Regulation (EU) 2021/808 as a qualitative screening method for bovine and porcine urine, and used for analysis of bovine urine samples originating from previously performed animal studies and from the Dutch National Residue Control Plan. Analyses of samples from previous animal studies show performance of the developed method for monitoring synthetic steroid misuse, as all incurred samples were flagged non-compliant. To our knowledge, it is the first time a method was developed and validated with such a broad scope of growth promoters in cattle with GC-HRMS. Since the sample preparation is generic for multiple steroid classes, and the HRMS data acquisition is untargeted, the generated data can be used for further expansion of the scope with other steroids, for investigating unexpected or new steroids, and for retrospective (trend-)analysis, thus facilitating risk-based monitoring of steroids and other growth promoting compounds.

1. Introduction

Steroids are a diverse class of bioactive molecules that play an important role in mammals, regulating processes such as muscle growth [1,2]. Conversely, the importance of steroids for regulating muscle growth makes them popular agents for misuse. The misuse of steroids to promote muscle growth has adverse health effects. Therefore, administration of steroids in livestock for growth promotion is forbidden in the European Union (EU) [3]. For control purposes, steroid residues are being monitored in urine by food safety and anti-doping laboratories worldwide [4,5]. Steroid subclasses that are included in food safety monitoring programs are, among others, subclasses of androgens, estrogens, and progestogens (Fig. 1). Besides these, other classes of growth-promoting molecules are relevant for monitoring misuse, including stilbenes and resorcylic acid lactones (RALs).

Traditionally, official control laboratories mainly perform routine residue monitoring by triple quadrupole mass analyzers [6,7]. While sensitive and specific, these analyzers only focus on predefined substances [7]. Currently, food safety monitoring programs have to change in two plans as described in Commission Delegated Regulation (EU) 2022/1644: "risk-based plans and randomized surveillance plan focused on official controls on the use of pharmacologically active substances authorized as veterinary medicinal products or as feed additives and of prohibited or unauthorized pharmacologically active substances and residues" [4]. Regulation (EU) 2022/1644 means that broad (and ideally untargeted) detection can be considered since these techniques

* Corresponding author.
E-mail address: esmer.jongedijk@wur.nl (E. Jongedijk).

https://doi.org/10.1016/j.microc.2024.110916
Received 8 April 2024; Received in revised form 31 May 2024; Accepted 3 June 2024
Available online 4 June 2024

have no fixed scope, while triple-quad techniques have. High-resolution mass spectrometry (HRMS) methods using time of flight (TOF) or Orbitrap analyzers have demonstrated their potential for residue analysis and offer broad and untargeted detection [7–11].

The EU Reference Laboratories (EURLs) and National Reference Laboratories (NRLs) have implemented HRMS methods for various substances. Mainly liquid chromatography (LC) based HRMS methods are documented [12–17]. LC-based methods are especially challenging for steroid analysis due to the general non-polar structure and low proton affinities of steroids [18,19]. Preferably, a complimentary gas chromatography (GC) strategy, on derivatized steroids (Fig. 1), should be used. Only a limited number of studies have been performed for targeted screening of steroids on GC-HRMS, in doping and medical settings [5,20–23]. According to EU legislation for official food control laboratories, none of the yet-reported GC-HRMS methods have been validated. As until now only targeted GC–MS methods to a limited number of steroids have been validated according to official legislative criteria, *e.g.* [24,25]. With the updated 2021/808 EU regulation in mind, at the same time including a broad scope of steroids, we developed a GC-Q-Orbitrap HRMS method for the broad screening of steroidal substances. The method was fully validated for qualitative screening of synthetic steroidal substances in bovine and porcine urine according to Regulation (EU) 2021/808 [26]. The validated method was applied to bovine urine samples originating from previously performed animal studies and from the Dutch National Residue Control Plan (NRCP).

The aim of this paper is to present a validated method for broad steroid monitoring in animal urine by gas chromatography (GC) Q-Orbitrap HRMS. The results of this study can be used to facilitate expansion to other steroids, to investigate new or unexpected steroids, and for retrospective (trend-)analyses, all of which can contribute to risk-based monitoring of steroids.

2. Materials and methods

2.1. Chemicals

Methanol (MeOH), acetonitrile (ACN), Milli-Q water, and formic acid (FA) were purchased from Actu-All Chemicals B.V. (Oss, the Netherlands). Tert-butyl methyl ether (TBME) and n-pentane were purchased from Biosolve B.V. (Valkenswaard, the Netherlands). Acetic acid, disodium hydrogen phosphate, potassium dihydrogen phosphate, and *iso*-octane were obtained from Merck (Darmstadt, Germany), and β-glucuronidase from *Escherichia coli* K12, tris(hydroxymethyl)-aminomethane (TRIS), ammonium iodide, and DL-dithiothreitol were purchased from Sigma-Aldrich (Steinheim, Germany). N-methyl-N-trimethylsilyl trifluoroacetamide ('MSTFA++', MSTFA + 1 % TMCS) was

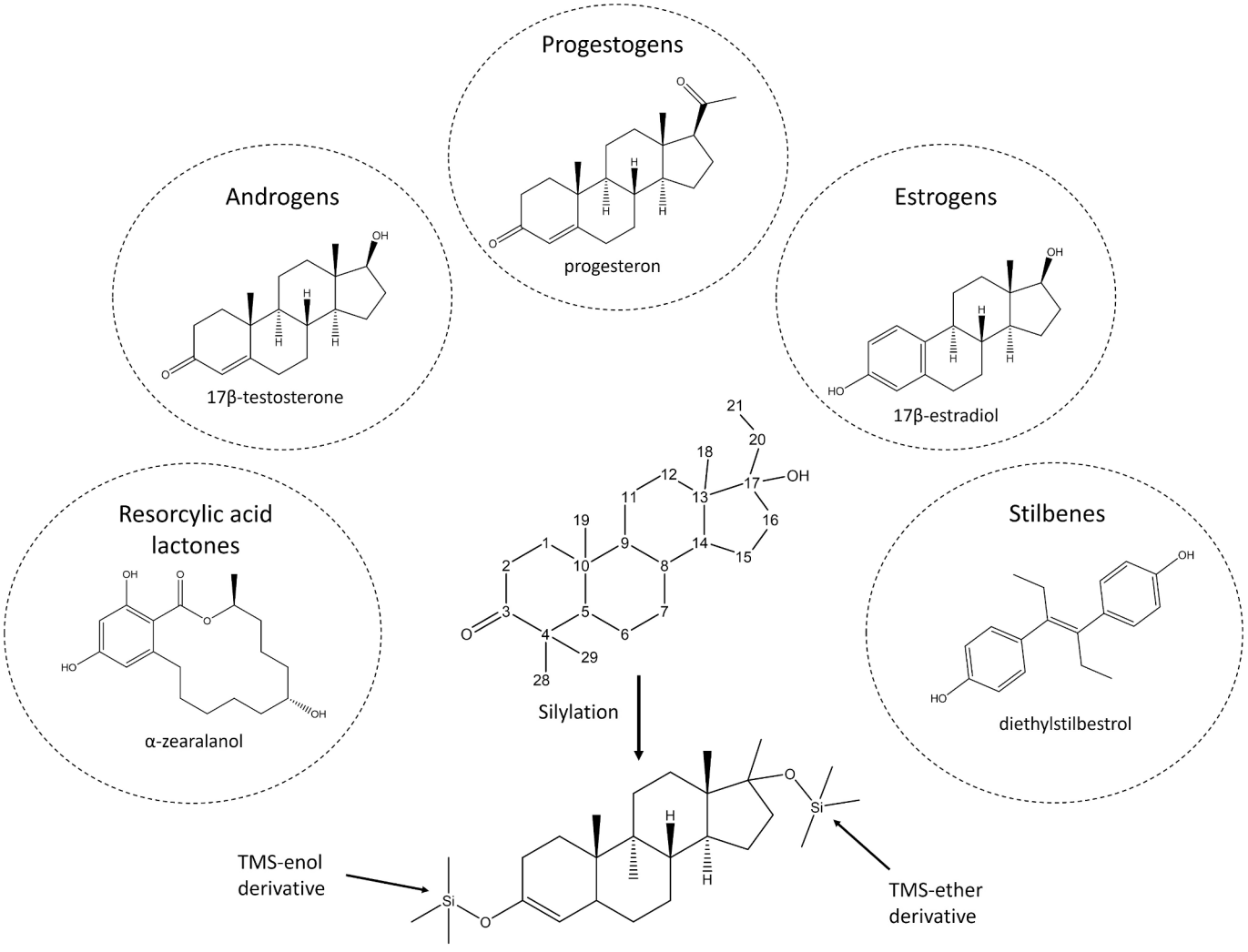

Fig. 1. Molecular structures of a selection of steroidal substances from resorcylic acid lactones (18 carbon atoms), androgens (19 carbon atoms), progestogens (21 carbon atoms), estrogens (18 carbon atoms), and stilbenes (synthetic estrogens). Silylation using trimethylsiloxysilicate (TMS) of enol and ether functions of steroidal molecules are shown.

obtained from Thermo Scientific (San Jose, CA, USA). Analytical standards of the steroidal substances were obtained from Steraloids, National Measurement Institute (NMI), Biosynth Carbosynth, Sigma, Dalton Research, Fisher Scientific, BDG Synthesis, BOC Science, Cayman chem., Cerilliant, Toronto Research Chemicals (TRC) and LGC Standards. A complete overview of these standards is shown in Table S1.

2.2. Sample preparation and derivatization

Bovine and porcine urine samples were homogenized and centrifuged, and 0.5 ml was taken into preparation. After adding internal standards at 3 μg/L, 0.5 mL of phosphate buffer (0.2 M, pH 7.0) was added, and the pH was set between 6.5 and 7.5. Urine samples were hydrolyzed for 16 h at 37 °C. For extraction purposes, 3 mL of TBME was added to the hydrolyzed samples. Samples were shaken and centrifuged at 2500 rpm for 10 min. The organic upper layer was transferred into a new glass tube, and the extraction was repeated once. Combined TBME layers were evaporated at 40 °C, and the residue was redissolved into 10 μL of MeOH, which was then ultrasonicated. After that, 1.0 mL of phosphate buffer (0.2 M, pH 7.0) was added for solid phase extraction (SPE) purposes. During SPE cleanup, an Oasis HLB 96-well plate (Waters, Milford, USA) was conditioned with 1.0 mL of MeOH, followed by 1.0 mL of Milli-Q water. Extracts were applied and washed with 0.2 ml of Milli-Q water. Columns were dried using a mild vacuum and eluted with 1.0 ml of ACN/water (90/10 v/v). Eluted samples were evaporated to dryness under a constant nitrogen flow and a temperature of 40 °C. The residue was redissolved into 2.5 ml TRIS buffer and extracted with 6 ml of n-pentane. Samples were shaken and centrifuged at 2500 rpm for 10 min. The organic upper layer was transferred into a new glass tube, and the extraction was repeated. Combined n-pentane layers were evaporated at 40 °C, and the residue was redissolved into 150 μL of MeOH, which was transferred to derivatization vials and evaporated at 55 °C under a gentle stream of nitrogen until dry. For TMS derivatization (Fig. 1), 25 μL of derivatization reagent MSTFA++ was added and left at 60 °C for 1 h. In order to limit potential background effects caused by the derivatization mixture, the remaining MSTFA++ was evaporated, and 40 μL of PCB-138 (0.1 mg/L) solution in *iso*-octane was added and transferred to glass vials with glass 50 μL inserts (Agilent Technologies) for analysis.

In each sample series, mixed standard solutions were included that contained natural (0.05 mg/L) and synthetic steroids (0.005, 0.02, 0.1 and 0.2 mg/L). These concentrations were based on the standard screening concentration (STC) in animal urine samples as described in method validation. Mixed standard solutions were transferred to derivatization vials and the organic solvent was evaporated at 55 °C under a gentle stream of nitrogen until dry. For TMS derivatization, 25 μL of derivatization reagent MSTFA++ was added and left at 60 °C for 1 h.

2.3. GC-Q-Orbitrap analysis

Gas chromatography was performed using a GC Trace 1300 equipped with a Thermo TriPlus™ RSH autosampler (Thermo Scientific, Waltham, USA) coupled with a Q-Orbitrap (Q Exactive™ GC, Thermo Scientific, Bremen, Germany). The injection port temperature was set to 250 °C. The injection volume was set to 2.0 μL under pulsed-splitless conditions and using a Thermo LinerGOLD liner (4 x 6.5 x 78.5 mm, Thermo Scientific, Waltham, USA). The analytes were separated using a DB-35MS capillary column (60 m, ID: 0.250 mm, film: 0.25 μm, Agilent Technologies, Santa Clara, USA) with helium carrier gas using a programmed temperature gradient. The oven temperature program started at 110 °C and was held for 2 min. Then, it was ramped to 230 °C at 30 °C/min and held for 1 min. This was followed by a second ramp to 325 °C at 5 °C/min and held stable at 325 °C for 4 min. Electron ionization (EI) was performed using 40 eV of electron energy with a source temperature of 250 °C. The emission current was optimized by the AutoTune function in the Q-Exactive Tune software. Full scan acquisition mode was applied using a mass range of *m/z* 200–670 and a resolving power of 120,000 at *m/z* 200. Internal mass calibration was conducted during the measurement using three different background ions originating from the column bleed (*m/z* 207.0324, 281.0511, and 355.0699) and a mass search window of ± 2 ppm.

2.4. Library building

Reference analytical standards were analyzed for building an in-house library for targeted data analysis. The theoretical molecular weights of analytes were calculated and extracted from the chromatograms to detect the compounds in the standards with Xcalibur (version 4.5.474.0 Thermo Finnigan LLC, San Jose, CA, USA). Compound spectra were extracted, and *m/z* values were exported to a database in Excel and converted to a library in TraceFinder software (Version 4.1 Thermo Finnigan LLC, San Jose, CA). The TraceFinder in-house library consisted of three main parts: (i) compound name, (ii) *m/z* of three ions (when available), and (iii) retention time. The ions that were monitored for each compound are given in Table S2.

2.5. Data processing

For data processing, the acquired data of samples were imported in TraceFinder (version 4.2, Thermo Finnigan LLC, San Jose, CA, USA), and post-target data analysis was performed with the created library (mass tolerance 5 ppm). The resulting signals were exported to an Excel *.CSV format. This Excel file was imported into an Excel assemble spreadsheet, formatting the data for further automatic processing.

In all cases, for a non-conform screening result, the retention time and mass tolerance of the analyte corresponded to that of the calibration standard with a maximum tolerance of ± 0.1 min and 5 ppm, respectively. Additionally, the signal-to-noise (S/N) ratio of all diagnostic ions of the analyte was greater than, or equal to, three.

2.6. Method validation

Method validation was performed according to qualitative screening criteria described in Regulation (EU) 2021/808 [26]. The following performance characteristics were determined: CCβ, specificity, and ruggedness. STCs were determined during method optimization and based on the EURLs' recommended concentrations, and can be found in Table S3. [27]. Performance characteristics of synthetic hormones were experimentally assessed. Performance characteristics of natural hormones were assessed on their synthetic counterparts that were most similar in retention time, as indicated in Table S4. The CCβ was determined based on fortified bovine (n = 20) and porcine (n = 20) urine samples from different individuals at and above the STC. The CCβ consequently equals the concentration level where ≤ 5 % false compliant results remain. The specificity of the method was assessed by analyzing representative blank bovine (n = 20) and porcine (n = 20) urine samples of different individual animals to determine if any interferences of signals, peaks, or ion traces were present in the region where the target analyte was expected to elute. The ruggedness was evaluated using fortified blank porcine (n = 3) urine samples at the STC under different experimental conditions that could occur in routine testing. The tested deviations were: (i) 5 min additional evaporation of the TBME layer and (ii) full evaporation of the combined n-pentane layer and reconstitution in 1.0 mL n-pentane before transfer into derivatization vials.

3. Results & discussion

3.1. Method optimization

The method aims to detect a broad selection of growth promotors with different physiochemical properties; therefore, a compromise in recovery from sample cleanup, total run time, sensitivity, and specificity are inevitable. In daily practice, we run a broad HRMS analysis parallel to current targeted MS/MS-based methods in order to make sure that we will directly confirm the most common growth promotors. However, GC-HRMS has the option to go beyond this common list. Consequently, these earlier-mentioned compromises in recovery, sensitivity, and specificity are acceptable [7]. In order to optimize compromised settings for a broad scope, different GC-HRMS parameters were evaluated for performance of the overall method. For this optimization, a set of 20 steroids, a representative set for the whole scope, was evaluated (Table S5). Instrumental parameters on GC-Q-Orbitrap were optimized based on peak shape, signal intensity, peak area, signal–noise ratio, and number of data points per peak.

3.1.1. Generic sample preprocessing

Urine contains many substances since it is the main excretion route for the majority of polar substances [28]. Without any purification, the target class of compounds will be disturbed, affecting the performance and sensitivity of the method. However, the purification steps should allow for broad detection and, therefore, cannot be too extensive. Based on previous protocols for steroid and generic analysis [29–31], sample cleanup was performed by a combination of liquid–liquid extraction with methyl *tert*-butyl ether (TBME), Oasis hydrophilic-lipophilic balanced solid-phase extraction (HLB SPE), and n-pentane liquid–liquid extraction under alkaline conditions before derivatization.

3.1.2. GC separation

Many matrix compounds are introduced because the cleanup is kept as generic as possible. As a result, signals of the matrix compounds overlap with signals of the analytes, which cannot only be solved by using the Orbitraps high resolutions up to 120,000. Furthermore, this massive amount of ions introduced will limit the filling time of the C-trap, resulting in a loss of sensitivity. Also, some analytes have very high structural similarities. Therefore, optimization of separation was desirable. The GC program was varied to obtain optimal separation of the different estranediol isomers (5α-estran-3ß, 17ß-diol; 5α-estran-3α, 17ß-diol and 5α-estran-3α, 17α-diol). A slower GC-gradient of 2 °C/min resulted in a chromatographic resolution of 148. While a faster GC program (20 °C/min) resulted in a lower resolution of 48 (Fig. S1), it improved the throughput since it was quicker and improved the sensitivity by up to 4-fold, and was therefore selected. A longer GC column, 60 m instead of the initial 30 m, was used in the final method to obtain optimal separation and maintain a good sensitivity for the whole scope of the method.

3.1.3. Ionization energy

The ionization energy was optimized in terms of maximizing intensity of the highest *m/z* ion of each compound. As an example, Fig. 2 shows an overview of the sum of *m/z* ion intensities for diethylstilbestrol (DES). The highest intensities obtained were at an electron voltage of 40 eV (Fig. 2). Compared to the intensities at 70 eV, this was an increase of 4 times.

The commonly used electron voltage for electron ionization is 70 eV, which results in extensive fragmentation for steroids, especially in many fragments at lower masses at the cost of the higher *m/z* fragments. Urine contains many endogenous steroids with closely related structures producing similar low-mass ions. Also, since the ion density at low *m/z* is high and an Orbitrap uses a variable filling of the trap based on the number of ions present, the sensitivity for the desired analytes will be lower. By reducing the electron energy, less fragmentation will occur and increase the intensity of higher mass ions. As an example, Fig. 3 shows the two mass spectra of the higher mass ions of the steroid 17β-chlorotestosterone (TMS-derivative), at 40 eV (Fig. 3A) and 70 eV (Fig. 3B). When comparing both spectra, the *m/z* fragments of 466.2485 and 451.2243 (assigned as diagnostic ions, Table S2) have a higher relative intensity at 40 eV than 70 eV. Also, the absolute intensity of the base peak is a factor of 2.5 higher. Our data, and also others, show that a lower electron ionization energy increases the sensitivity for the higher *m/z* and improves the method's specificity, while at the same time, only minor differences in characteristic diagnostic fragments of mass spectra are present between 40 eV and 70 eV ionization energies [32]. Therefore, the expected impact on future retrospective or untargeted applications or the use of online libraries would be limited. While more selective molecular ion species are sometimes also obtained using chemical ionization [33], for the here-included compounds this would limit the comprehensive detection, due to the positive and negative ionization mode being required for androgens and estrogens, respectively, and was therefore not considered within this research.

After increasing the sensitivity for higher mass ions by lowering the eV, the lower mass ions, including any still possible remaining derivatization mixture, were removed by using the quadrupole as a mass filter, thereby increasing the filling time of the C-trap. In Fig. 4, an example is given of the chromatographic peak of methylboldenone, using two mass ranges of the quadrupole. After the quadrupole removed the lower mass ions (Fig. 4B), the peak intensity increased, and the peak shape improved. By removing lower mass ions, the injection time of

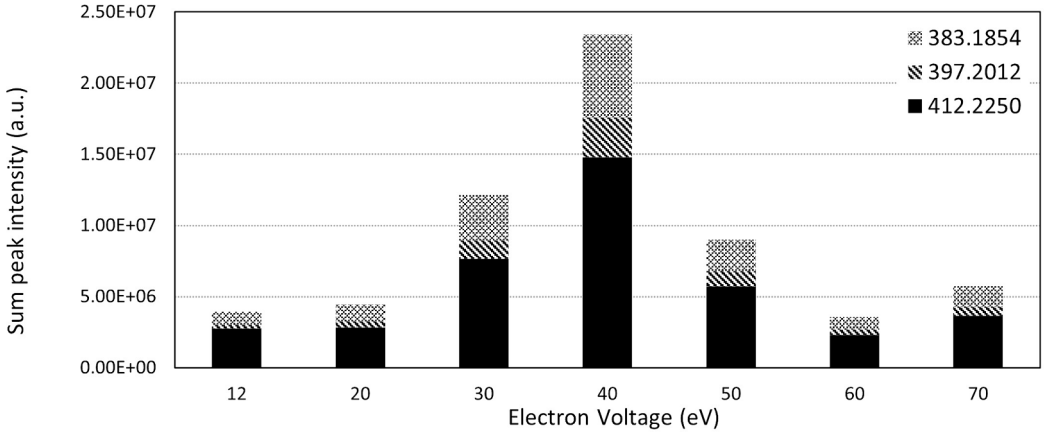

Fig. 2. Effect of the electron voltage on the intensity of three ions of diethylstilbestrol (DES, TMS-derivative) (x-axis electron voltage, y-axis summation of the peak intensity, the legend shows *m/z* of the three ions measured).

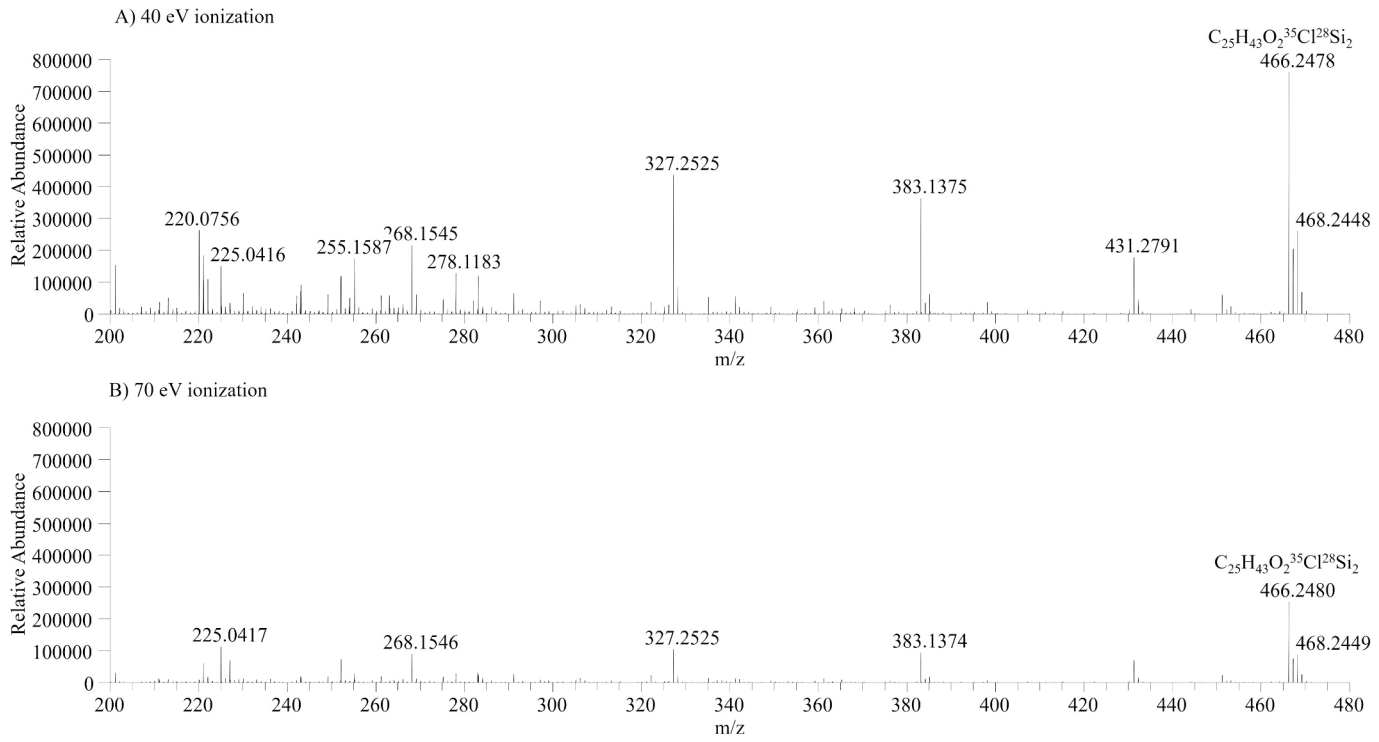

Fig. 3. Mass spectra the higher mass ions of 17β-chlorotestosterone. (A) Electron ionization energy of 40 eV. (B) Electron ionization energy of 70 eV.

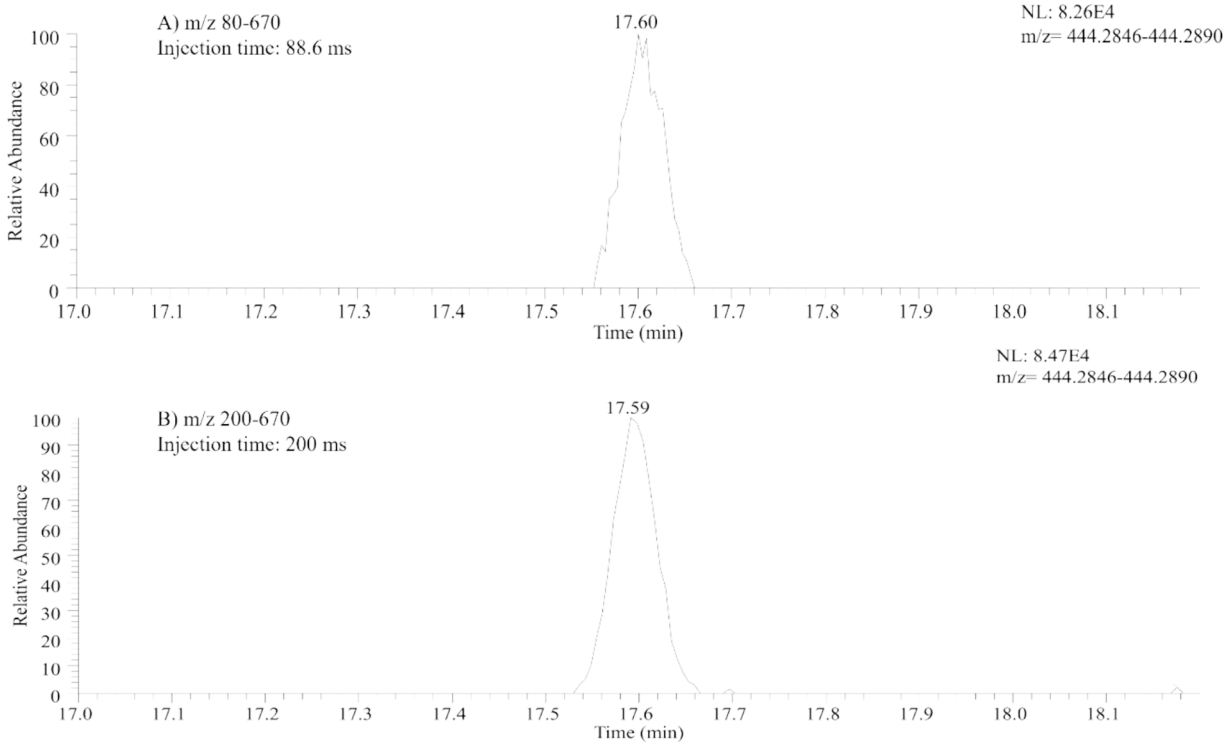

Fig. 4. Extracted traces of methylboldenone *m/z* 444.2868. (A) Quadrupole mass range *m/z* 80–670. (B) Quadrupole mass range *m/z* 200–670.

methylboldenone increased from 88.6 to 200 ms. This increase demonstrates the overcoming of post-interface ion suppression associated with the saturation of the C-trap by co-eluting interferences, as has been reported for other residue analysis methods using Q-Orbitrap detection [34,35]. A drawback of this approach is also removing, for example, steroid skeletal masses lower than *m/z* 200. The loss of these masses may, for other applications in the future, complicate untargeted steroid searching. However, as the higher steroid masses are broadly acquired with good resolution and specificity, general steroid fragments and typical steroid neutral losses in high resolution can be used for (semi-)

untarged steroid detection [36]. Another option to overcome the limitation may be to add an extra scan event that covers the whole *m/z* range, and for this event use a low resolution. The low resolution of this event may ensure minimizing of time constraints of the Orbitrap, and the low-resolution (but full scan) data could be used for additional identification purposes in case an unknown substance is detected. This option was not evaluated in this research. All in all, the increase in sensitivity by omitting $m/z < 200$ was decisive in the methodological choice.

3.1.4. Resolution

The HRMS Q-Orbitrap instrument enables resolution variation, which is an important parameter for identification purposes. Furthermore, increasing the resolution minimizes interferences of co-eluting matrix artifacts and other compounds, thus increasing specificity for the included analytes.. However, due to the nature of Orbitrap analysis, resolution and scan speed are inversely correlated [37]. The method was tested at 60,000 and 120,000 FWHM at *m/z* 200. At 120,000 resolution, the scan speed is approximately 8 scans per second compared to 12 scans per second for 60,000 (Fig. S2A). Based on extracted ion chromatograms of analytes, it was assessed that at 120,000, there was still a sufficient number of scans per peak (Fig. S2B). Therefore, a final resolution of 120,000 was chosen for this method.

3.2. Method validation

The final optimized method was validated for qualitative screening of bovine and porcine urine according to Regulation (EU) 2021/808 [26]. Table 1 shows the evaluation of detection capability (CCβ) of the synthetic substances. Endogenous substances are included in Table S4. For a total of 47 out of 51 synthetic steroidal substances, the criterion of

Table 1
The screening target concentrations (STC) of the synthetic steroidal substances and the number of spiked bovine and porcine urine samples detected by the method with an S/N > 3 out of 20 fortified samples.

Substance	STC	Bovine urine, #detects out of 20 fortified samples (#/20)	Porcine urine, #detects out of 20 fortified samples (#/20)
17α-chlorotestosterone	0.25	20/20	20/20
17α-ethyl-5β-estran-3α,17β-diol	0.25	18/20	20/20
17α-nortestosterone	0.25	19/20	16/20
17β-chlorotestosterone	0.25	20/20	20/20
17β-nortestosterone	0.25	20/20	20/20
19-norepiandrosterone	0.25	20/20	20/20
Dienestrol (DE)	0.25	20/20	20/20
Diethylstilbestrol	0.25	20/20	20/20
Dromostanolone	0.25	20/20	20/20
Ethinylestradiol	0.25	20/20	20/20
Hexestrol	0.25	20/20	20/20
Methylboldenone	0.25	20/20	19/20
Methyltestosterone	0.25	20/20	16/20
Norgestrel	0.25	20/20	20/20
Norethandrolone	0.25	20/20	18/20
Normethandrolone	0.25	20/20	10/20
1,3,5(10)-Estratrien-3-ol	1	20/20	20/20
1,4-androstadiene-3,17-dione	1	0/20	20/20
4-chloro-androst-4-ene-3,17-dione	1	20/20	20/20
19-norandrostenedione	1	20/20	20/20
19-nor-4-androstenediol	1	20/20	16/20
19-norandrosterone	1	20/20	20/20
19-noretiocholanolone	1	20/20	20/20
5α-estran-17β-ol-3-one	1	20/20	20/20
5β-estran-17α-ol-3-one	1	20/20	20/20
5β-estran-17β-ol-3-one	1	20/20	20/20
9-Dehydromethyltestosterone	1	20/20	0/20
formestane	1	20/20	20/20
Mesterolone	1	20/20	20/20
Mestranol	1	20/20	20/20
Methenolone	1	20/20	16/20
1α-methylandrosterone	5	18/20	20/20
2α-methylandrosterone	5	20/20	20/20
5α-androst-1-ene-3β,17β-diol	5	20/20	20/20
5α-estran-17α-ol-3-one	5	20/20	20/20
Androsta-1,4,6-triene-3,17-dione	5	20/20	19/20
4-chloro-19-norandrostane-17α-ol-3-one	10	20/20	20/20
19-nor-5-androstenediol	10	20/20	20/20
5-androsten-3β,7β-diol-17-one	10	20/20	19/20
5α-estran-3,17-dione	10	20/20	20/20
Androstenediol	10	20/20	19/20
exemestane	10	20/20	20/20
fluoxymesterone	10	20/20	20/20
MEAD-I	10	20/20	20/20
MEAD-II	10	20/20	20/20
Methandriol	10	20/20	20/20
Methasterone	10	20/20	20/20
α-zearalanol	10	20/20	20/20
α-zearalenol	10	20/20	20/20
β-zearalanol	10	20/20	20/20
β-zearalenol	10	17/20	20/20

≤ 5 % false compliant results was met at the tested standard screening concentration (STC) in bovine urine samples. For these 47 substances, the CCβ equals the STC. The substances 17α-ethyl-5β-estran-3α,17β-diol (detected in 18 out of 20 fortified samples (18/20)), 1α-methyl androsterone (18/20), 1,4-androstadiene-3,17-dione (0/20), and β-zearalenol (17/20) did not fulfill the criterion of ≤ 5 % false compliant results at the STC.

In porcine urine, a total of 44 synthetic steroidal substances fulfilled the criterion of ≤ 5 % false compliant results. 17α-Nortestosterone (detected in 16 out of 20 fortified samples (16/20)), methyltestosterone (16/20), norethandrolone (18/20), normethandrolone (10/20), 19-nor-4-androstenediol (16/20), 9-dehydromethyltestosterone (0/20), and methenolone (16/20) did not fulfill the criterion of ≤ 5 % false compliant results at the tested STC.

Benzestrol, zearalenone, and 17α-1-testosterone were also tested for possible inclusion in this method but did not provide signals with a minimum S/N of three at the desired STC.

As expected, the CCβ values span a wider range compared to routinely operated dedicated MS/MS methods. However, full scan acquisition enables future retrospective, trend, or unknown steroidal analysis besides the currently fixed library scope using *e.g.* the subclass-specific fragmental patterns associated with TMS-derivatized steroids [36], whether or not in combination with machine learning tools [38]. Ultimately, the developed HRMS method can be used as screening method, with suspect findings being introduced to confirmation analyses performed using targeted MS/MS-based methods so misuse of the most common growth promoters will be detected.

The CCβ values of 53 natural hormones were determined by projection of the validation results of the synthetic steroids. As blank materials (i.e. urine samples) for these 53 analytes were unavailable. Natural hormones were therefore matched to the closest eluting synthetic steroids (Table S4). The option to work with synthetic urine blank materials for validation was excluded, ensuring realistic validation circumstances. Since bovine and porcine urines are complex matrices, and extensive sample cleanup was undesirable due to the broad purpose of the method, real urine samples from monitoring programs would give relatively high and highly variable matrix background signals, which was also reflected in current validationstudy.

3.2.1. Specificity

Manual inspection of 20 chromatograms of blank samples excluded the presence of significant interferences. Solely, significant interferences were found for steroidal substances that are documented to have an endogenous origin in bovine and/or porcine urine, including 17α-/17β-nortestosterone and its documented metabolites 19-noretiocholanolone [39], 1,4-androstadiene-3,17-dione [40], formestane [41], and α-/β-zearalenol [42]. Therefore, the method's specificity was established fit for qualitative screening purposes.

3.2.2. Ruggedness

Ruggedness was evaluated by 2 deviations during the sample preparation for STC-fortified porcine urine samples. In all the deviations tested, the CCβ levels were still visible with a S/N > 3 for all analytes. The steroidal substances 17α-ethyl-5α-androstane-3α,17β-diol, 9-dehydromethyltestosterone, benzestrol, tetrahydrogestinone, zearalenone, and zearalanone could not be detected with a S/N > 3 for any of the ruggedness samples. However, Table 1 shows that these components were also undetected in any fortified porcine urine samples under standard procedure. Based on these results, the deviations to the method did not affect the screening of the validated exogenous/synthetic growth promoters at the selected STCs.

3.3. Application to bovine urine samples

The performance of the validated method was further assessed by analyzing 5 urine samples from previous animal studies and 125 bovine urine samples from the Dutch National Residue Control Plan. For urine samples from previous animal studies, separate animals had been treated with nortestosterone phenylpropionate, 17β-1-testosterone, methyltestosterone, dromostanolone, and norethandrolone; these samples were analyzed in random order unknown to the technician. During data analysis, the presence of these administered steroidal substances (or their known metabolites) were all detected and correctly flagged as suspects using the developed method and data evaluation. As example, Fig. 5 illustrates the results following nortestosterone phenylpropionate treatment. Shown are chromatograms of the 17α-nortestosterone ion traces (Fig. 5A and B) and 5α-estran-3β,17α-diol ion traces (Fig. 5C and D), that correspond to the urine sample of the nortestosterone phenylpropionate treated cow (Fig. 5A and C) and a blank bovine urine sample (Fig. 5B and D). In our study, 17α-nortestosterone and the endogenous marker 5α-estran-3β,17α-diol were detected in the urine of the treated cow, which aligns with the findings of Sauer et al. [39]. Besides these residues, we also detected other documented metabolites of nortestosterone including 19-norepiandrosterone and 19-noretiocholanolone (data not shown) [39,43].

The urine samples from the animals treated with synthetic androgenic steroids 17β-1-testosterone, methyltestosterone, dromostanolone, and norethandrolone showed residues for 17α-1-testosterone, methyltestosterone, dromostanolone, and norethandrolone respectively, all with signals with S/N > 3. The urine sample of an animal treated with methyltestosterone also demonstrated residues of the methylandrostandiols MEAD-I and MEAD-II and 2α-methylandrosterone (S/N > 3), of which the methylandrostandiols are known metabolites following methyltestosterone treatment [44,45].

For the bovine urine samples from the Dutch National Residue Control Plan, the method detected 13 suspect screening results for synthetic steroidal substances in 10 out of 125 bovine urine samples. These screening results are summarized in Table 2. The suspected samples (i.e., positively screened) were further confirmed. In total, 3 (of the 10) bovine urine samples demonstrated residues of 17α-nortestosterone, 19-noretiocholanolone, and 19-norepiandrosterone (Table 2). The marker 5α-estran-3β,17α-diol, previously reported as a marker of exogenous use, was neither detected in the GC-HRMS screening nor confirmed by the GC–MS/MS confirmatory testing, demonstrating that the confirmed residues are likely of an endogenous origin [39].

For the bovine urine samples, no confirmatory analysis was performed to the findings of the aromatase inhibitor formestane. As tandem-MS confirmatory analysis would solely confirm that formestane is present at residue levels. Current knowledge is still missing as to the origin of formestane and to distinguish natural origin from illegal administration. Formestane can be from natural origin in bovine, porcine, and human urine samples [41,46]. Therefore, further research, outside the scope of this paper, would be required to follow-up these findings and to confirm the origin of these residues.

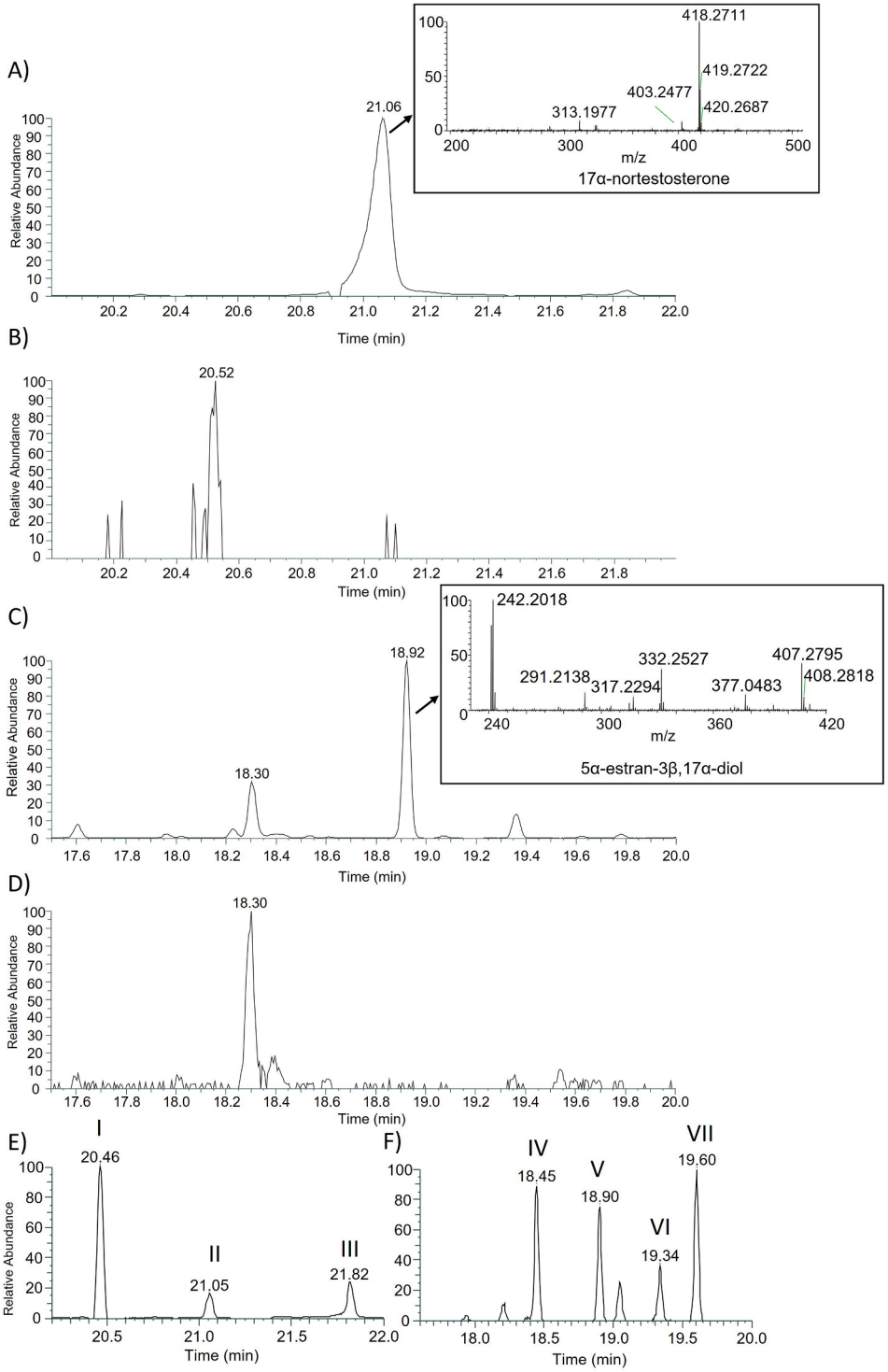

Fig. 5. Ion traces of exact masses of 17α-nortestosterone (A and B, *m/z* 418.2718, XIC 2.20E8 and 2.12E7 respectively) and 5α-estran-3β,17α-diol (C and D, *m/z* 407.2798, XIC 2.42E4 and 2.07E5 respectively) in a bovine urine sample following nortestosterone phenylpropionate treatment (A and C), and a blank bovine urine sample (B and D). Ion traces of bovine urine sample fortified with analytical standards at CCβ level; E) *m/z* 418.2718, XIC 6.77E6, I 5α-estran-3,17-dione, II 17α-nortestosterone, III 17β-nortestosterone; F) *m/z* 407.2798; XIC 1.04E6, IV 5α-estran-3α,17β-diol, V 5α-estran-3β,17α-diol, VI 5β-estran-3α,17β-diol, and VII 5α-estran-3β,17β-diol.

Table 2
Positively screened synthetic steroidal substances in bovine urine samples, which originated from the Dutch National Residue Control Plan, and the confirmation of the bovine urine samples.

Bovine urine sample	GC-HRMS screening result	Confirmatory analysis result (amount detected in μg/L)
1	17α-nortestosterone	17α-nortestosterone (1.0 μg/L)
2	17α-nortestosterone, 19-noretiocholanolone, formestane	17α-nortestosterone (6.2 μg/L), 19-noretiocholanolone (8,1 μg/L), 19-norepiandrosterone (1.5 μg/L)
3	17β-nortestosterone, 4-estren-3,17-dione	Not detected
4	5-estren-3β,17β-diol	Not detected
5	17α-nortestosterone	17α-nortestosterone (3.4 μg/L), 19-noretiocholanolone (0.30 μg/L), 19-norepiandrosterone (0.27 μg/L)
6	19-norepiandrosterone	Not detected
7	formestane	Not analyzed
8	formestane	Not analyzed
9	formestane	Not analyzed
10	formestane	Not analyzed

4. Conclusions

There is an urgent request for risk-based monitoring approaches. For a better understanding of unexpected or new steroids in food systems, the use of HRMS approaches can help. However, GC-HRMS is currently limitedly used, due to a still limited availability of knowledge, methods and instrumentation in official control laboratories [7]. Our study aimed to contribute to methodology and knowledge for risk-based HRMS approaches, and therefore developed a validated method for broad monitoring of steroids in urine by GC-Q-Orbitrap HRMS, using a generic sample preparation and an in-house library containing 51 synthetic steroidal substances and 53 naturally occurring steroidal substances. Our method was fully validated according to Commission Implementing Regulation (EU) 2021/808 and is fit for purpose as a qualitative screening method, with most CCβs at relevant levels for forbidden compounds, satisfactory specificity, and ruggedness. Our method evaluated bovine urine samples from Dutch NRCP and previous animal studies. The results demonstrated performance in detecting steroid abuse in cattle urine samples. Given this effectiveness, which also provides a broad and untargeted detection of steroids, the method is an instrumental screening tool for steroidal substances for official food control laboratories in risk-based monitoring programs. The presented method can effectively monitor and detect synthetic steroids and their metabolites, allowing for efficient instrumental screening. This screening tool also has the potential for additional future applications, such as metabolite and profiling studies and retrospective analysis (for trend analyses).

Funding

This project was financially supported by the EC DG SANTE (European Union Reference Laboratory Residues of veterinary medicines and contaminants in food of animal origin) and the Dutch Ministry of Agriculture, Nature and Food Quality (LNV) under projects WOT-02–003-067, WOT-02–003- 007.

CRediT authorship contribution statement

Josha Jager: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Marco Blokland:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Rachelle Linders:** Validation, Investigation, Formal analysis. **Paul Zoontjes:** Validation, Software, Formal analysis, Data curation. **Eric van Bennekom:** Validation, Investigation. **Saskia Sterk:** Supervision, Project administration. **Esmer Jongedijk:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors are grateful to Lisa Dral for her technical contribution with GC-HRMS. The authors would like to thank Dr. J.L. Banach from Wageningen Food Safety Research for her comments on an earlier version of this paper and for improving the English.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.110916.

References

[1] Kuipers, H. *anabolic steroids: side effects*. Encyclopedia of Sports Medicine and Science 1998 [cited 2022 7 Feb 2022]; Available from: https://www.sportsci.org/encyc/anabstereff/anabstereff.html.
[2] A.A.M. Ruokonen, N. Bolton, R. Vihko, Response of serum testosterone and its precursor steroids, SHBG and CBG to anabolic steroid and testosterone self-administration in man, J. Steroid Biochem. 23 (1) (1985) 33–38.
[3] European Commission, *Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists.* Official Journal of the European Communities, 1996. **OJ L 125, 23.5.1996**: p. 3.
[4] European Commission, *Commission Delegated Regulation (EU) 2022/1644 of 7 July 2022 supplementing Regulation (EU) 2017/625 of the European Parliament and of the Council with specific requirements for the performance of official controls on the use of pharmacologically active substances authorised as veterinary medicinal products or as feed additives and of prohibited or unauthorised pharmacologically active substances and residues thereof* Official Journal of the European Communities, 2022. **OJ L 248, 26.9.2022**: p. 3-17.
[5] W. Abushareeda, et al., Comparison of gas chromatography/quadrupole time-of-flight and quadrupole Orbitrap mass spectrometry in anti-doping analysis: I. Detection of anabolic-androgenic steroids, Rapid Commun. Mass Spectrom. 32 (23) (2018) 2055–2064.
[6] R. Pratiwi, et al., Recent advances in the determination of veterinary drug residues in food, Foods 12 (18) (2023) 3422.
[7] E. Jongedijk, et al., Use of high-resolution mass spectrometry for veterinary drug multi-residue analysis, Food Control 145 (2023) 109488.
[8] S. Belarbi, et al., Comparison of new approach of GC-HRMS (Q-Orbitrap) to GC–MS/MS (triple-quadrupole) in analyzing the pesticide residues and contaminants in complex food matrices, Food Chem. 359 (2021) 129932.
[9] Z. Meng, et al., Rapid screening of 350 pesticide residues in vegetable and fruit juices by multi-plug filtration cleanup method combined with gas chromatography-electrostatic field orbitrap high resolution mass spectrometry, Foods 10 (7) (2021) 1651.

[10] Y. Sapozhnikova, A. Nuñez, J. Johnston, Screening of chemicals migrating from plastic food contact materials for oven and microwave applications by liquid and gas chromatography - Orbitrap mass spectrometry, J. Chromatogr. A 1651 (2021) 462261.
[11] S. Uclés, et al., Shifting the paradigm in gas chromatography mass spectrometry pesticide analysis using high resolution accurate mass spectrometry, J. Chromatogr. A 1501 (2017) 107–116.
[12] Z. Kaabia, et al., Comprehensive steroid profiling by liquid chromatography coupled to high resolution mass spectrometry, J. Steroid Biochem. Mol. Biol. 183 (2018) 106–115.
[13] S. Liesenfeld, et al., Comprehensive LC-HRMS metabolomics analyses for the estimation of environmental inputs of altrenogest in pig breeding, Chemosphere 287 (2022) 132353.
[14] S. Liesenfeld, P. Steliopoulos, G. Hamscher, Comprehensive metabolomics analysis of nontargeted LC-HRMS data provides valuable insights regarding the origin of veterinary drug residues, J. Agric. Food Chem. 68 (44) (2020) 12493–12502.
[15] A. Kaufmann, et al., High-resolution mass spectrometry–based multi-residue method covering relevant steroids, stilbenes and resorcylic acid lactones in a variety of animal-based matrices, Anal. Chim. Acta 1054 (2019) 59–73.
[16] A. Kaufmann, et al., High resolution mass spectrometry-based detection and quantification of beta-agonists at relevant trace levels in a variety of animal-based food matrices, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 38 (8) (2021) 1350–1363.
[17] A. Temerdashev, et al., Determination of oxprenolol, methandienone and testosterone in meat samples by UHPLC-Q-ToF, Heliyon 9 (2) (2023).
[18] T. Higashi, K. Shimada, Derivatization of neutral steroids to enhance their detection characteristics in liquid chromatography–mass spectrometry, Anal. Bioanal. Chem. 378 (4) (2004) 875–882.
[19] O.J. Pozo, et al., Ionization of anabolic steroids by adduct formation in liquid chromatography electrospray mass spectrometry, J. Mass Spectrom. 42 (4) (2007) 497–516.
[20] W. Abushareeda, et al., Gas chromatographic quadrupole time-of-flight full scan high resolution mass spectrometric screening of human urine in antidoping analysis, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1063 (2017) 74–83.
[21] A. Bileck, et al., A comprehensive urinary steroid analysis strategy using two-dimensional gas chromatography - time of flight mass spectrometry, Analyst 143 (18) (2018) 4484–4494.
[22] M.K. Kioussi, et al., A generic screening methodology for horse doping control by LC-TOF-MS, GC-HRMS and GC-MS, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 941 (2013) 69–80.
[23] G.M. Randazzo, et al., Steroid identification via deep learning retention time predictions and two-dimensional gas chromatography-high resolution mass spectrometry, J. Chromatogr. A 1612 (2020) 460661.
[24] A.L. Silveira, et al., Multiresidue determination of the anabolic-agent residues steroids, stilbenes, and resorcylic acid lactones in bovine urine by GC-MS/MS with microwave-assisted derivatization, J. Agric. Food Chem. 66 (32) (2018) 8630–8638.
[25] L. Wang, et al., Rapid and simultaneous determination of anabolic andro-genic steroids in livestock and poultry meat using one-step solid-phase extraction coupled with UHPLC–MS/MS, Molecules 29 (1) (2024) 84.
[26] European Commission, *Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 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 Communities, 2021. **OJ L 180, 21.5.2021**: p. 84–109.
[27] E. Verdon, J. Polzer, S. Sterk, EURL Guidance on Minimum Method Performance Requirements (MMPRs). For Specific Pharmacologically Active Substances in Specific Animal Matrices, 2020.
[28] C.G. Regårdh, Factors contributing to variability in drug pharmacokinetics. IV. renal excretion, J. Clin. Pharm. Ther. 10 (4) (1985) 337–349.
[29] M. Doué, et al., Supercritical fluid chromatography applied to the highly selective isolation of urinary steroid hormones prior to GC/MS analysis, J. Chromatogr. B 1086 (2018) 97–104.
[30] M.H. Blokland, et al., Endogenous steroid profiling by gas chromatography-tandem mass spectrometry and multivariate statistics for the detection of natural hormone abuse in cattle, Food Additives & Contaminants: Part A 29 (7) (2012) 1030–1045.
[31] F. van Tricht, et al., A fast quantitative multi-analyte method for growth promoters in bovine meat using bead-disruption, 96-well SPE clean-up and narrow-bore UHPLC-MS/MS analysis, Food Anal. Methods 11 (8) (2018) 2206–2217.
[32] P.Q. Tranchida, et al., Comprehensive two-dimensional gas chromatography-mass spectrometry using milder electron ionization conditions: A preliminary evaluation, J. Chromatogr. A 1589 (2019) 134–140.
[33] P. Van Renterghem, et al., Validation of an ultra-sensitive detection method for steroid esters in plasma for doping analysis using positive chemical ionization GC-MS/MS, J. Chromatogr. B 1141 (2020) 122026.
[34] S. Moretti, et al., Multiclass method for the determination of 62 antibiotics in milk, J. Mass Spectrom. 51 (9) (2016) 792–804.
[35] G. Saluti, et al., Simultaneous determination of aminoglycosides and colistins in food, Food Chem. 266 (2018) 9–16.
[36] A.S. Kollmeier, M.K. Parr, Mass spectral fragmentation analyses of isotopically labelled hydroxy steroids using gas chromatography/electron ionization low-resolution mass spectrometry: A practical approach, Rapid Commun. Mass Spectrom. 34 (12) (2020) e8769.
[37] A. Kaufmann, High-resolution mass spectrometry for bioanalytical applications: Is this the new gold standard? J. Mass Spectrom. 55 (9) (2020) e4533.
[38] M. Ljoncheva, et al., Machine learning for identification of silylated derivatives from mass spectra, J. Cheminf. 14 (1) (2022) 62.
[39] M.J. Sauer, et al., Residues and metabolism of 19-nortestosterone laurate in steers†, Analyst 123 (12) (1998) 2653–2660.
[40] M. Van Puymbroeck, et al., Identification of some important metabolites of boldenone in urine and feces of cattle by gas chromatography-mass spectrometry†, Analyst 123 (12) (1998) 2681–2686.
[41] T. Meijer, et al., Determination and confirmation of selective estrogen receptor modulators (SERMs), anti-estrogens and aromatase inhibitors in bovine and porcine urine using UHPLC-MS/MS, Food Additives & Contaminants: Part A 34 (4) (2017) 641–651.
[42] D.G. Kennedy, et al., Zeranol is formed from Fusarium spp. toxins in cattle in vivo, Food Addit. Contam. 15 (4) (1998) 393–400.
[43] T. Piper, W. Schanzer, M. Thevis, Revisiting the metabolism of 19-nortestosterone using isotope ratio and high resolution/high accuracy mass spectrometry, J. Steroid Biochem. Mol. Biol. 162 (2016) 80–91.
[44] C. Gómez, et al., Alternative long-term markers for the detection of methyltestosterone misuse, Steroids 78 (1) (2013) 44–52.
[45] M.C. Dumasia, In vivo biotransformation of 17α-methyltestosterone in the horse revisited: identification of 17-hydroxymethyl metabolites in equine urine by capillary gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 17 (4) (2003) 320–329.
[46] X. de la Torre, et al., Detection of formestane abuse by mass spectrometric techniques, Drug Test. Anal. 6 (11–12) (2014) 1133–1140.