Journal of Chromatography A, xxx (2019) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

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

# Development and validation of a semi-quantitative ultra-high performance liquid chromatography-tandem mass spectrometry method for screening of selective androgen receptor modulators in urine

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ARTICLE INFO

*Article history:*
Received 25 January 2019
Received in revised form 16 April 2019
Accepted 17 April 2019
Available online xxx

*Keywords:*
Selective androgen receptor modulators
UHPLC-MS/MS
Urine
Doping control
Residue and food safety

ABSTRACT

A semi-quantitative method was developed to monitor the misuse of 15 SARM compounds belonging to nine different families, in urine matrices from a range of species (equine, canine, human, bovine and murine). SARM residues were extracted from urine (200 μL) with *tert*-butyl methyl ether (TBME) without further clean-up and analysed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). A 12 min gradient separation was carried out on a Luna Omega Polar C18 column, employing water and methanol, both containing 0.1% acetic acid (*v/v*), as mobile phases. The mass spectrometer was operated both in positive and negative electrospray ionisation modes (ESI±), with acquisition in selected reaction monitoring (SRM) mode. Validation was performed according to the EU Commission Decision 2002/657/EC criteria and European Union Reference Laboratories for Residues (EU-RLs) guidelines with CCβ values determined at 1 ng mL$^{-1}$, excluding andarine (2 ng mL$^{-1}$) and BMS-564929 (5 ng mL$^{-1}$), in all species. This rapid, simple and cost effective assay was employed for screening of bovine, equine, canine and human urine to determine the potential level of SARMs abuse in stock farming, competition animals as well as amateur and elite athletes, ensuring consumer safety and fair play in animal and human performance sports.

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## 1. Introduction

Investigation of alternative pharmacophores to anabolic-androgenic steroids (AAS) which can separate anabolic effects on muscle and bone from androgenic activity in other tissues such as the prostate and seminal vesicles [1], has led to the emergence of selective androgen receptor modulators (SARMs), a class of non-steroidal agents with affinity for the androgen receptor (AR) similar to that of dihydrotestosterone (DHT) [2]. As a heterogeneous group of molecules incorporating a range of pharmacophores that lack the steroid nucleus of testosterone and dihydrotestosterone [2], SARMs behave as partial AR agonists in androgenic tissues (prostate and seminal vesicle) but act mainly as full AR agonist in anabolic tissue (muscle and bone) [1,3]. The structural modification of known AR antagonists, such as the nonsteroidal antiandrogens bicalutamide, flutamide, hydroxyflutamide and nilutamide [2,4], resulted in the initial generation of novel nonsteroidal AR agonists with an arylpropionamide-nucleus, namely SARM S-1 and andarine (S-4), for potential use as therapeutics in benign prostatic hyperplasia (BPH) and androgen-deficiency related disorders [5–7]. Since then, several classes of chemical scaffolds with SARM-like properties have been developed exhibiting strong anabolic activity and high tissue selectivity, elevated absorption rates via oral administration, and reduced undesirable androgenic side-effects [8–11]. Potential pharmacologic applications of SARMs have been focused towards

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https://doi.org/10.1016/j.chroma.2019.04.050
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conditions involving muscle and bone wasting disorders following cancer and other chronic diseases, as well as in hypogonadism, hormone replacement therapy, male contraception, benign prostatic hyperplasia, breast and prostate cancer [8,9].

Ease of availability, simplicity of use, advantageous biological effects [12] and short detection windows [13,14, 23–25, 15–22] are key features increasing the potential for SARM misuse, and consequently they are widely recognised as drugs of abuse in both human and animal (e.g. equine and canine) sports, and as emerging candidates for illicit use in food-producing species [19]. Although many SARM compounds are currently undergoing evaluation in various studies, as yet none are approved for pharmaceutical use [8], there is widespread SARM availability via black- and grey-market sources. Recently, various SARMs (e.g. S-4, S-22 and LGD-4033) have been identified within black-market products [26–29], online vendors [30–34], and confiscated goods [35]. SARMs have gained particular popularity in professional sports and are banned by the World Anti-Doping Agency (WADA) [36], the International Agreement on Breeding, Racing and Wagering (IABRW) [37] and Fédération Equestre Internationale (International Equestrian Federation, FEI) [38], with many reports of positive findings from routine testing [39–42]. More recently, 65 adverse analytical findings (AAFs) for a range of SARMs (e.g. andarine, ostarine, LGD-4033 and RAD140) were reported in human sport in 2017 alone [43]. The potential for SARMs to be further adopted for use in food-producing animals (e.g. in cattle livestock) to increase muscle growth and reduce fat mass also remains a distinct threat [44].

Advanced and reliable screening and confirmatory analytical assays are required to detect SARM use for doping practices in sport and monitor for potential misuse in stock farming. A number of SARM compounds have been successfully included into human anti-doping control [45–52] with some assays developed for equine racing animals [14,17,18,53]. However, to date only a limited number of analytical procedures covering solely arylpropionamides have been established for food safety analysis [15,16,54,55]. LC-MS and occasionally GC-MS-based approaches have been applied to elucidate the metabolic pathways of some emerging SARMs in various species to support the development of detection assays for these compounds [14,19,22,56]. Moreover, the detection of SARMs and associated metabolites in canine [57,58], rodents [57–62], as well as human specimens [63–67] were conducted to support SARM clinical studies. Whilst urine and blood are common matrices of choice, faeces have been proposed as an alternative matrix for the analysis of arylpropionamide-derived compounds in bovine [15,16], canine [57] and rats [57,62]. However, the reported screening and/or confirmatory assays are typically capable of analysis of either a single SARM compound or a limited number of SARMs and related metabolites in a single specimen (Table 1).

In the present study, an innovative fast, simple and cost-effective semi-quantitative multi-residue UPLC-MS/MS screening assay was developed for a group of 15 key SARM compounds with different physicochemical properties, chosen based upon their reported use in human and animal sports and availability as certified analytical standards. Target SARM compounds included AC-262536, andarine (S-4), bicalutamide, BMS-564929, GLPG0492, LGD-2226, LGD-4033, Ly2452473, ostarine (S-22), PF-06260414, RAD140, S-1, S-6, S-9 and S-23 (Fig. 1). The developed method has been validated in urine matrices from a range of species (equine, canine, human, bovine and murine) in accordance with the EU Commission Decision 2002/657/EC criteria [68] and European Union Reference Laboratories for Residues (EU-RLs) guidelines [69]. The assay was employed to screen for SARM residue presence in urine sourced from racing animals (equine and canine), amateur and elite athletes, as well as farm (bovine) and experimentally treated animals.

**Table 1**
Comparison of the actual method with other methods for urine analysis reported in literature.

| Compound group | Analyte | Method | Species | Sample volume (mL) | Sample preparation | Detection limits | Method performance | Reference |
|---|---|---|---|---|---|---|---|---|
| Arylpropionamide | Andarine (S-4), bicalutamide, ostarine (S-22) | HPLC-MS/MS | Bovine | 1.0 | Enzymatic hydrolysis followed by SPE (Strata-X) | CCα 0.315–0.491 ng mL$^{-1}$<br>CCβ 0.401–0.724 ng mL$^{-1}$ | Linearity 0.0–5.0 ng mL$^{-1}$ RSDr 2.2–13.6 % $RSD_{RL}$ 6.5–47.4 % Accuracy 89.2–103.3 % | [54] |
| | Andarine (S-4) | UHPLC-MS/MS | Human | 0.1 | "Dilute-and-shoot" | LOD 0.5 ng mL$^{-1}$<br>LOQ 2.5 ng mL$^{-1}$ | Linearity 2.5–250 ng mL$^{-1}$<br>Intra-day accuracy 92–106 %<br>Inter dayaccuracy 94–107 % | [52] |
| | Andarine (S-4), bicalutamide, hydroxyflutamide, ostarine (S-22) | UHLC-MS/MS | Bovine | 3.0 | SPE (Oasis HLB), ammonium acetate buffer (pH 4.8, 0.25 M) | Sensitivity 0.25 ng mL$^{-1}$ | Linearity 0.25–30 ng mL$^{-1}$<br>Precision 0.6–17.6 %,<br>Recovery 71–119 % | [16] |
| | | | | 3.0 | Enzymatic hydrolysis followed by SPE (Oasis HLB) | | | |

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Table 1 (*Continued*)

| Compound group | Analyte | Method | Species | Sample volume (mL) | Sample preparation | Detection limits | Method performance | Reference |
|---|---|---|---|---|---|---|---|---|
| | Ostarine (S-22) | UHPLC-MS/MS | Bovine | 3.0<br>3.0 | SPE (Oasis HLB), acetate buffer (pH 5, 0.2 M)<br>Enzymatic hydrolysis followed by SPE (as above) | eLOQ 0.1 ng $mL^{-1}$ | N/A | [15] |
| | Andarine (S-4), bicalutamide, hydroxyflutamide, ostarine (S-22) | UHPLC-MS/MS | Bovine | 3.0 | Enzymatic hydrolysis followed by SPE (Oasis HLB) | LOD 0.015–0.142 ng $mL^{-1}$ | Linearity 0.25–25 ng $mL^{-1}$ | [55] |
| | | USFC-Q-IM-ToF (mode: $MS^E$) | | | | LOD 0.0018–0.0406 ng $mL^{-1}$ | | |
| | Andarine (S-4), ostarine (S-22) | UHPLC-HRMS (modes: MS, DDA) | Equine | 3.0 | Enzymatic hydrolysis followed by on-line SPE (Oasis HLB) | eLOD 1.25 ng $mL^{-1}$ (S-22), 5 ng $mL^{-1}$ (S-4) | Inter-day precision 9.4–11.7 % Recovery 11–15 % | [53] |
| | Andarine (S-4), ostarine (S-22), ostarine glucuronide, S-23, S-24 | HPLC-HRMS | Human | 0.09 | "Dilute-and-shoot" | LLOD <0.1 ng $mL^{-1}$ | Intra-day precision 3.2–7.7 % Inter-day precision 4.4–14.5 % | [45] |
| | Andarine (S-4), bicalutamide, ostarine (S-22) | UHPLC-MS/MS | Bovine | 2.0 | Enzymatic hydrolysis followed by LLE (TBME, $K_2CO_3$/$NaHCO_3$ buffer) | CCα 0.025 ng $mL^{-1}$ CCβ 0.025-0.05 ng $mL^{-1}$ | Linearity 0.0–2.0 ng $mL^{-1}$ Accuracy 89-105 % $RSD_r$ 2.6–10.4 $RSD_{RL}$ 2.9–12.2 % | [19] |
| | Andarine (S-4), ostarine (S-22) | UHPLC-HRMS | Human | | Enzymatic hydrolysis followed by SPE (Bond-Elut Plexa PCX), 2% aq. HCOOH | LOD < 5 ng $mL^{-1}$ | | [46] |
| | Andarine (S-4), M5 metabolite of S-4, ostarine (S-22) | HPLC-MS/MS | Human | N/A | Enzymatic hydrolysis followed by alkaline LLE (pentane and diethyl ether) | LOD 0.1 ng $mL^{-1}$ (S-4, S-22) | N/A | [41] |
| | Andarine (S-4), metabolite of S-4 | HPLC-MS/MS | Human | 0.09 | Dilute-and-shoot¨ | LOD 1.0 ng $mL^{-1}$ | Intra-day precision 5.5–10.3 % Inter-day precision 0.38-4.7 % | [47] |
| | Andarine (S-4), S-1, S-9, S-24 | HPLC-MS/MS | Human | 2.0 | SPE (PAD-1) | LLOD 1.0 ng $mL^{-1}$ | Intra-day precision 7.6–11.6 % Inter-day precision 9.9–14.4 % Recovery 85-105 % | [48] |
| | M1 metabolite of S-1 | HPLC-MS | Rats | N/A | LLE (ethyl acetate) | LOQ 10 ng $mL^{-1}$ | Linearity 10–10,000 ng $mL^{-1}$ Relative recovery 89% | [62] |
| Arylpropionamide, pyrrolidinyl-benzonitrile | Andarine (S-4), ostarine (S-22), S-1, LGD-4033, metabolites: *O*-dephenyl andarine, *O*-dephenyl ostarine | GC-EI-Q-ToF (modes: MS and MS/MS by continuous switching) | Human | 0.5 | Enzymatic hydrolysis followed by LLE (TBME $NaHCO_3$/$K_2CO_3$ buffer (pH 9.5)) and derivatisation (MSTFA/ethanethiol/$NH_4I$) | LLOD 0.2–10 ng $mL^{-1}$ | N/A | [75] |
| Bicyclic hydantoin, quinolinone | BMS-564929, LGD-2226 | GC-MS | Human | N/A | Enzymatic hydrolysis followed by LLE and derivatisation | LLOD 0.2 ng $mL^{-1}$ (LGD-2226) LLOD 10 ng $mL^{-1}$ (BMS-564929) | Intra-day precision 6.8–16.6 % Inter-day precision 12.7–17.7 % Recovery 83–85 % | [80] |

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Table 1 (*Continued*)

| Compound group | Analyte | Method | Species | Sample volume (mL) | Sample preparation | Detection limits | Method performance | Reference |
|---|---|---|---|---|---|---|---|---|
| Bicyclic hydantoin, benzimidazole | BMS-564929, 5,6-dichloro-benzimidazole derivatives ($n = 4$) | HPLC-MS/MS | Human | 2.0 | SPE (PAD-1) | LLOD 1.0 ng mL$^{-1}$, 20 ng mL$^{-1}$ (BMS-564929) | Intra-day precision 2.4–13.2 % Inter-day precision 6.5–24.2 % Recovery 89–106 % | [79] |
| Indole | GSK2881078 | UHPLC-MS/MS | Human | N/A | LLE | LLOQ 0.05 ng ml$^{-1}$ HLOQ 50 ng mL$^{-1}$ | N/A | [64] |
| | Ly2452473 | UHPLC-HRMS (modes: MS, MS/MS) | Human | N/A | SPE (Bond Elute C18) | N/A | Recovery 98 % | [67] |
| Isoquinoline | PF-06260414 | HPLC-MS/MS | Human | 0.15 | N/A | LLOQ 0.01 ng ml$^{-1}$ HLOQ 10 ng mL$^{-1}$ | Precision ≤ 6.5 % Accuracy ≤ 9.8 % | [65] |
| Quinolinone | US 6,462,038, LG-121071 | GC-MS | Human | 3.0 | Enzymatic hydrolysis followed by LLE (TBME, pH 9.6, carbonate buffer (0.1 M)) and derivatisation (MSTFA/$NH_4I$/dithiothreitol) | LOD 1.0 ng mL$^{-1}$ | Linearity 5.0–500 ng mL$^{-1}$ Intra-day precision 8.1–14.8 % Inter-day precision 9.5-16.2 % Recovery 97-101% | [76] |
| | LGD-2226, 6-alkylamino-2-quinolinones ($n = 2$) | GC-μAPPI-MS/MS | Human | 1.0 | Enzymatic hydrolysis followed by SPE (Oasis HLB) and derivatisation (MSTFA) | LOD 0.01–1.0 ng mL$^{-1}$ LOQ 0.03–3.0 ng mL$^{-1}$ | Linearity LOQ-100 ng mL$^{-1}$ Intra-day repeatability 5–9 % Recovery 92–111 % | [77] |
| | LGD-2226, 6-alkylamino-2-quinolinones ($n = 3$) | HPLC-MS/MS | Human | 2.0 | Enzymatic hydrolysis followed by LLE (TBME, pH 9.6, $K_2CO_3$/$NaHCO_3$ buffer) | LLOD 0.01-0.2 ng mL$^{-1}$ | Intra-day precision 3.2-8.5 % Inter-day precision 6.3-16.6 % Recovery 81-98 % | [51] |
| Pyrrolidinyl-benzonitrile | LGD-4033 | UHPLC-Q-ToF (mode: $MS^E$) | Equine | 2.0 | Enzymatic hydrolysis followed by SPE (Oasis HLB) | LOD 2.6 ng mL$^{-1}$ $[M-H]^-$, 0.5 ng mL$^{-1}$ $[M+HCOOH-H]^-$ | N/A | [14] |
| Tetrahydroquinolinone | LG121071 | HPLC-MS/MS | Human | 1.0 | Enzymatic hydrolysis followed by LLE (TBME, pH 9.6, carbonate buffer (20%)) | LLOD 0.5 ng mL$^{-1}$ | Linearity 0.5–5.0 ng mL$^{-1}$, 1–200 ng mL$^{-1}$ Intra-day precision 2.3–8.5 % Inter-day precision 7.2–11.7 % Recovery 40 % | [49] |
| Tricyclic tetrahydroquinoline | Tricyclic tetrahydroquinoline derivatives ($n = 3$) | HPLC-MS/MS | Human | 2.0 | Enzymatic hydrolysis followed by LLE (TBME, pH 9.6, $K_2CO_3$/$NaHCO_3$ buffer, $Na_2SO_4$) | LLOD 0.2–0.6 ng mL$^{-1}$ | Intra-day precision 6.4–15.1 % Inter-day precision 11.3–21.8 % Recovery 92–97 % | [50] |
| 9 pharmacophores | 15 analytes | UHPLC-MS/MS | Equine, bovine, canine, human, murine | 0.2 | LLE (TBME, $NH_4OH$ aq. (50 mM, pH 10.5)) | CCβ 1 ng mL$^{-1}$, 2 ng mL$^{-1}$ (S-4), 5 ng mL$^{-1}$ (BMS-564929) eLOD 0.01–0.75 ng mL$^{-1}$ (equine) | Precision 9.8–33.6 % (equine) Sensitivity 95–100 % Recovery 74–94 % | Actual method |

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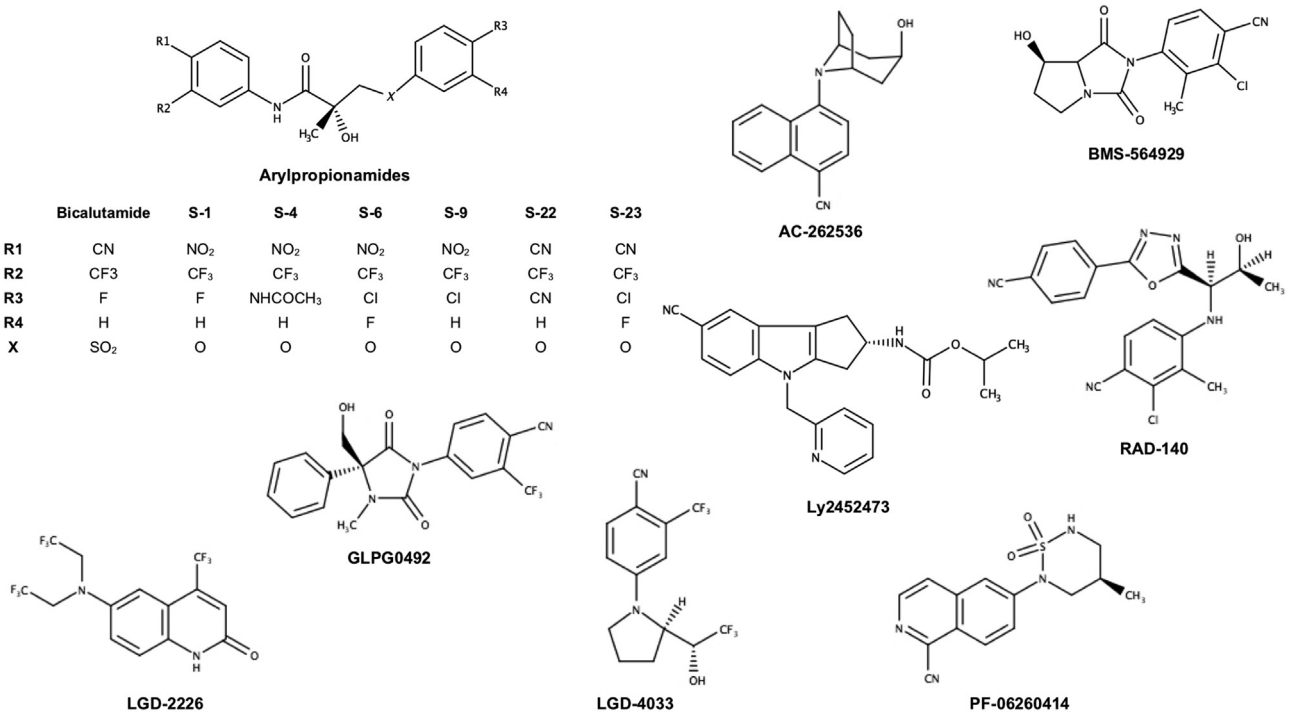


**Fig. 1.** Chemical structures of SARMs included in the actual UHPLC-MS/MS method.

## 2. Experimental

### 2.1. Reagents and apparatus

Ultra-pure water (18.2 MOhm) was generated in house using a Millipore water purification system (Millipore, Cork, Ireland). Methanol (MeOH) and acetonitrile (MeCN), both Chromasolv™ LC–MS grade, as well as ammonium hydroxide solution, ≥25% in $H_2O$ and acetic acid, both eluent additives for LC–MS, were sourced from Honeywell (VWR International, Dublin, Ireland). LiChrosolv® LC grade *tert*-butyl methyl ether (TBME), ethanol (puriss. p.a., ACS reagent, absolute alcohol, without additive, ≥99.8%), dimethyl sulfoxide (ACS reagent, ≥99.9%) and acetonitrile-D, 99.5% (MeCN-D) were sourced from Sigma-Aldrich (Dublin, Ireland). SafeSeal polypropylene micro tubes (2 mL) were obtained from Sarstedt (Nümbrecht, Germany). A DVX-2500 multi-tube vortexer (VWR International, Dublin, Ireland), a Hettich Micro 200R centrifuge from Davidson & Hardy (Belfast, UK) and a Turbovap LV evaporator from Caliper Life Sciences (Mountain View, USA) were used during sample preparation. In this study, the density of urine was measured through specific gravity (SG) of the urine samples using a pocket refractometer PAL-USG (CAT) from Atago (Tokyo, Japan).

AC-262536 (P/N 96443-25MG), andarine (S-4, P/N 78986-25MG), bicalutamide (P/N PHR-1678-1 G), LGD-2226 (P/N 07682-25MG), Ly2452473 (P/N CDS025139-50MG), PF-06260414 (P/N PZ0343-5MG), S-1 (P/N 68114-25MG), S-6 (P/N 79260-25MG) and S-23 (P/N 55939-25MG) were purchased from Sigma-Aldrich (Dublin, Ireland). LGD-4033 (P/N CAY9002046-50 mg), ostarine (S-22, P/N MK-2866) and RAD140 (P/N CAY18773-1 mg) were purchased from Cambridge Bioscience Ltd. (Cambridge, UK). BMS-564929 (10 mM solution in DMSO, P/N HV-12111) and GLPG0492 (10 mM solution in DMSO, P/N HY-18102) were purchased from MedChem Express (Sollentuna, Sweden). S-9 (P/N D289535), Bicalutamide-$D_4$ (P/N B382002) and S-1-$D_4$ (P/N D289532) were purchased from Toronto Research Chemicals (TRC; Toronto, Canada). All standards and internal standards stock solutions were prepared at a concentration of 1 mg mL$^{-1}$ in MeCN, DMSO, EtOH and MeCN-D, respectively. Intermediate mixed standard solutions were prepared at the following concentrations: 20/40/100, 1/2/5 and 0.1/0.2/0.5 μg mL$^{-1}$ in MeCN by serial dilutions. Working quality control standard solution at a concentration of 10/20/50 ng mL$^{-1}$ was prepared in MeCN. Intermediate internal standard mix solutions were prepared at 20 and 1 μg mL$^{-1}$, respectively, using MeCN-D as the diluent. A working internal standard mix solution was prepared at 50 ng mL$^{-1}$ in MeCN-D. All standards and internal standards stock solutions were found to be stable for at least one year when stored at −20 °C during 'in-house' stability studies. Working quality control standard and working internal standard mix solutions were found to be stable for at least 3 months when stored at −20 °C.

### 2.2. Preparation of extracted matrix screen positive and recovery control checks

Negative quality control (QC) samples were obtained by pooling aliquots ($n$ = 5–10) of negative urine samples. Extracted matrix screen positive controls were prepared by fortifying three negative QC samples (200 μL) prior to extraction with 20 μL of quality control standard solution to give a screening target concentration of 1 ng mL$^{-1}$ in urine for all analytes excluding andarine and BMS-564929 giving a concentration of 2 and 5 ng mL$^{-1}$, respectively. Additionally, two blank QC samples were spiked after extraction with quality control standard solution (20 μL) to monitor for loss of analytes during extraction.

### 2.3. Sample preparation

All sampling and analysis were performed under the guidance and approval of local ethical regulations. Urine samples were stored at −80 °C prior to analysis. Urine samples were centrifuged at 4500 × $g$ for 10 min at 4 °C, and following checking of pH and specific gravity (SG), aliquoted (200 μL) into 2 mL micro tubes. Samples were fortified with 20 μL of a 50 ng mL$^{-1}$ internal standard mix solution and left to stand for 15 min, and a 200 μL volume of

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50 mM aqueous $NH_4OH$ pH 10.5 added to each sample. Tube contents were vortexed for 60 s and 1.2 mL of TBME was subsequently added. Following vortexing for 15 min, samples were centrifuged at 15,000 rpm (21,380 × *g*) for 10 min at 4 °C, and supernatants transferred into clean empty 2 mL micro tubes and evaporated to dryness under flow of nitrogen (≤ 5 bar) at 40 °C on a Turbovap LV system. Samples were reconstituted in $H_2O$:MeCN (4:1, *v/v*; 100 μL) by vortexing (5 min) and 9 μL of extracts were injected onto the UHPLC-MS/MS system.

### 2.4. UHPLC-MS/MS conditions

Separations were performed using a Waters (Milford, MA, USA) Acquity I-Class UPLC® system comprising of a stainless steel Luna® Omega Polar C18 analytical column (100 × 2.1 mm, 100 Å, 1.6 μm) (Phenomenex, P/N 00D-4748-AN) equipped with KrudKatcher™ Ultra HPLC in-line filter (Phenomenex, P/N AF0-8497) maintained at a temperature of 45 °C and the pump was operated at a flow rate of 0.40 mL $min^{-1}$. A binary gradient system was used to separate analytes comprising of mobile phase A, 0.1% (*v/v*) acetic acid in water and mobile phase B, 0.1% (*v/v*) acetic acid in MeOH. The gradient profile was as follows: (1) 0.0 min 20% B, (2) 0.5 min 20% B, (3) 9.0 min 99% B, (4) 10.0 min 99% B, (5) 10.1 min 20% B, (6) 12.0 min 20% B. The injection volume was 9 μL. After each injection the needle was washed and purged with $H_2O$:MeOH (1:1, *v/v*) and $H_2O$:MeOH (4:1, *v/v*) solutions, respectively. A divert valve was used to reduce source contamination (8.50–11.50 min a flow was diverted to waste).

SARM residues were detected using a Waters Xevo® TQ-MS triple quadrupole mass analyser (Manchester, UK) operating both in positive and negative electrospray ionisation modes (ESI±). The UHPLC-MS/MS system was controlled by MassLynx™ software and data was processed using TargetLynx™ software (both from Waters). The electrospray voltage was set at 2.5 kV (ESI+) and 1.0 kV (ESI−), respectively. The desolvation and source temperatures were set at 550 and 120 °C, respectively. Nitrogen was employed as the desolvation and cone gases, which were set at 900 L $h^{-1}$ and 50 L $h^{-1}$, respectively. Argon was employed as the collision gas, at a flow rate of 0.15 mL $min^{-1}$, which typically gave pressures of $2.5 \times 10^{-3}$ mbar. The MS conditions were optimised using IntelliStart by infusion of 1 μg $mL^{-1}$ standard solutions and 50% mobile phases A and B at flow rates of 5 μL $min^{-1}$ and 0.2 mL $min^{-1}$, respectively. The cone voltage was optimised for each precursor ion and two to four most abundant product fragment ions were selected. The selected reaction monitoring (SRM) windows were time-sectored, and dwell time and inter-channel delays were set to get maximum response for the instrument. These conditions are outlined in Table 2. Inter scan delay was set to 5 ms between successive SRM windows, inter-channel delay was set to 5 ms and polarity switching 20 ms. Dwell times ranged from 0.005 to 0.300 s (Table 2). Available stable isotope-labelled analogues of bicalutamide and S-1 (bicalutamide-$D_4$ and S-1-$D_4$) were used as internal standards for arylpropionamide residues (Table 2). The response factor was obtained for arylpropionamides as a ratio between analyte peak area and internal standard peak area, while in the case of the other SARM residues, peak area was used as the response.

### 2.5. Method validation

The method was validated according to the EU Commission Decision 2002/657/EC criteria and European Union Reference Laboratories for Residues (EU-RLs) 20/1/2010 guidelines for screening assays. The following performance studies were carried out to prove the suitability of the method in achieving the goal for which it was developed: selectivity, specificity, detection capability (CCβ), sensitivity, precision, limit of detection (LOD) and absolute recovery as well as applicability, ruggedness and matrix effects. Validation was carried out at the screening target concentration ($C_{val}$) of 1 ng $mL^{-1}$ excluding andarine and BMS-564929 validated at 2 and 5 ng $mL^{-1}$, respectively. The detection capability (CCβ), defined in 2002/657/EC, was calculated in accordance with the EU-RLs 20/1/2010 guidelines, by assessing threshold value (T) and cut-off factor (Fm). To determine the T-value, 61 blank equine urine samples of different origins were analysed using the method described above on a number of occasions by two different analysts to obtain total of 61 data points. The T-value was estimated for at least two transitions for each analyte as a sum of a mean response and 1.64 times the standard deviation of noise levels acquired for 61 blank samples. To determine the cut-off factor (Fm), 61 blank equine urine samples of different origins were fortified at the screening target concentration ($C_{val}$) on numerous occasions and the samples were analysed by two different analysts. This gave a total of 61 independent data points for each analyte at the targeted concentration of 1 ng $mL^{-1}$ excluding andarine (2 ng $mL^{-1}$) and BMS-564929 (5 ng $mL^{-1}$), respectively. The cut-off factor (Fm) was estimated for at least two transitions for each analyte as a mean response decreased by 1.64 times the standard deviation of response acquired for 61 fortified samples. According to the European Union Reference Laboratories for Residues (EU-RLs) 20/1/2010 guidelines, the detection capability (CCβ) of the screening method is validated when the cut-off factor is greater than the threshold value (Fm > T). It can then be deduced that CCβ is truly below the validation level. Since the very first requirement expected from a screening method is to avoid false negative (also called "false compliant") results, the detection capability of the method was estimated as the concentration level where ≤5% of false-negative results remain.

The sensitivity of the method was expressed as the percentage based on the ratio of samples detected as positive in true positive samples i.e. following the fortification [70]. A sensitivity ≥ 95% at the screening target concentration ($C_{val}$) means that the number of false-negative samples is truly ≤ 5%. Despite being a required performance characteristic to be determined solely for quantitative methods [68], precision was calculated as the coefficient of variation (CV) of the response following fortification at the screening target concentration ($C_{val}$). Limit of detection (LOD) was estimated at a signal-to-noise ratio (*S/N*) at least three measured peak to peak.

Following initial determination of the detection capability (CCβ) for equine urine, the developed method was applied to the same matrix type from four different species - bovine, canine, human and murine urine, respectively. Murine urine was included as a matrix within the validation process in recognition that many SARM metabolism *in vivo* studies utilise experimental rodent models and as such the developed method may find application in such studies. The applicability of the screening method was evaluated by analysing 20 blank urine samples ($n = 5$ per species) and the same 20 blank urine samples ($n = 5$ per species) fortified at the screening target concentration ($C_{val}$) used previously for equine urine. Animal species were included in the ruggedness study as factors that could influence the results. Moreover, to investigate the ruggedness of the developed assay, 15 different blank urine samples ($n = 5$ per species) and the same 15 blank urine samples ($n = 5$ per species) fortified at the screening target concentration were analysed at a different day and by a different operator that executed the applicability study [69]. To evaluate matrix effects in equine, bovine, canine, human and murine urine, 25 blank samples from different sources of each matrix ($n = 5$) were post-extraction spiked at the concentration equal to two times the screening target concentration ($2 \times C_{val}$), namely 2 ng $mL^{-1}$ excluding andarine (4 ng $mL^{-1}$) and BMS-564929 (10 ng $mL^{-1}$), respectively. Matrix effects for each analyte were calculated as percentage differences between the signals obtained when matrix extracts were injected and when a

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**Table 2**
UHPLC-MS/MS conditions for urine samples.

| Analyte | Pharmacophore | Others names | Formula | $T_R$[a] (min) | Transition (*m/z*) | Dwell time (s) | Cone (V) | CE[b] (eV) | SRM window[c] | ESI polarity | IS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Bicalutamide-$D_4$ | Arylpropionamide | | $C_{18}H_{10}D_4F_4N_2O_4S$ | 5.88 | 433.2 > 255.1 | 0.007 | 26 | 14 | 13 | – | N/A |
| S-1-$D_4$ | Arylpropionamide | | $C_{17}H_{10}D_4F_4N_2O_5$ | 6.87 | 405.2 > 261.1 | 0.005 | 34 | 20 | 10 | – | N/A |
| AC-262536 | Tropanol | | $C_{18}H_{18}N_2O$ | 6.73 | 279.2 > 195.0[d] | 0.005 | 36 | 22 | 1 | + | N/A |
| | | | | | 279.2 > 169.1 | | | 24 | | | |
| | | | | | 279.2 > 93.0 | | | 22 | | | |
| Andarine | Arylpropionamide | S-4, GTX-007 | $C_{19}H_{18}F_3N_3O_6$ | 5.83 | 440.2 > 150.0[d] | 0.005 | 30 | 30 | 15 | – | Bicalutamide-$D_4$ |
| | | | | | 440.2 > 261.1 | | | 20 | | | |
| | | | | | 440.2 > 205.0 | | | 34 | | | |
| | | | | | 440.2 > 107.0 | | | 46 | | | |
| Bicalutamide | Arylpropionamide | | $C_{18}H_{14}F_4N_2O_4S$ | 5.90 | 429.2 > 255.0[d] | 0.007 | 24 | 16 | 13 | – | Bicalutamide-$D_4$ |
| | | | | | 429.2 > 185.0 | | | 46 | | | |
| | | | | | 429.2 > 173.0 | | | 24 | | | |
| BMS-564929 | Hydantoin | | $C_{14}H_{12}ClN_3O_3$ | 4.06 | 306.1 > 86.1[d] | 0.300 | 30 | 24 | 3 | + | N/A |
| | | | | | 306.1 > 96.0 | | | 16 | | | |
| | | | | | 306.1 > 278.1 | | | 14 | | | |
| GLPG0492 | Diarylhydantoin | DT-200 | $C_{19}H_{14}F_3N_3O_3$ | 6.18 | 390.2 > 360.2[d] | 0.009 | 34 | 20 | 5 | + | N/A |
| | | | | | 390.2 > 118.0 | | | 44 | | | |
| | | | | | 390.2 > 91.0 | | | 38 | | | |
| LGD-2226 | Quinolinone | | $C_{14}H_9F_9N_{20}$ | 6.82 | 393.1 > 241.1[d] | 0.005 | 60 | 38 | 6 | + | N/A |
| | | | | | 393.1 > 223.0 | | | 52 | | | |
| | | | | | 393.1 > 375.1 | | | 32 | | | |
| | | | | | 393.9 > 203.1 | | | 56 | | | |
| LGD-4033 | Pyrrolidinyl-benzonitrile | VK5211 | $C_{14}H_{12}F_6N_2O$ | 6.70 | 337.1 > 267.2[d] | 0.005 | 28 | 10 | 8 | – | N/A |
| | | | | | 337.1 > 170.0 | | | 24 | | | |
| | | | | | 337.1 > 239.1 | | | 24 | | | |
| Ly2452473 | Indole | CDS025139, TT-701 | $C_{22}H_{22}N_4O_2$ | 6.51 | 375.2 > 272.1[d] | 0.025 | 30 | 20 | 4 | + | N/A |
| | | | | | 375.2 > 289.2 | | | 18 | | | |
| | | | | | 375.2 > 92.8 | | | 38 | | | |
| | | | | | 375.2 > 180.0 | | | 38 | | | |
| Ostarine | Arylpropionamide | S-22, EnoboSarm, GTx-024, MK-2866 | $C_{19}H_{14}F_3N_3O_3$ | 6.20 | 388.1 > 118.0[d] | 0.009 | 30 | 20 | 9 | – | Bicalutamide-$D_4$ |
| | | | | | 388.1 > 269.1 | | | 18 | | | |
| | | | | | 388.1 > 90.0 | | | 54 | | | |
| PF-06260414 | Isoquinoline | | $C_{14}H_{14}N_4O_2S$ | 4.82 | 303.1 > 210.1[d] | 0.076 | 36 | 26 | 2 | + | N/A |
| | | | | | 303.1 > 232.1 | | | 24 | | | |
| | | | | | 303.1 > 168.2 | | | 36 | | | |
| RAD140 | Phenyl-oxadiazole | | $C_{20}H_{16}ClN_5O_2$ | 6.06 | 394.1 > 223.1[d] | 0.005 | 20 | 10 | 7 | + | N/A |
| | | | | | 394.1 > 170.1 | | | 30 | | | |
| | | | | | 394.1 > 205.1 | | | 20 | | | |
| S-1 | Arylpropionamide | | $C_{17}H_{14}F_4N_2O_5$ | 6.88 | 401.1 > 261.1[d] | 0.005 | 35 | 20 | 10 | – | S-1-$D_4$ |
| | | | | | 401.1 > 205.0 | | | 26 | | | |
| | | | | | 401.1 > 111.0 | | | 24 | | | |
| | | | | | 401.1 > 289.1 | | | 20 | | | |
| S-6 | Arylpropionamide | | $C_{17}H_{13}ClF_4N_2O_5$ | 7.36 | 435.1 > 145.0[d] | 0.009 | 30 | 25 | 14 | – | Bicalutamide-$D_4$ |
| | | | | | 435.1 > 289.1 | | | 20 | | | |
| | | | | | 435.1 > 205.0 | | | 30 | | | |
| | | | | | 435.1 > 261.1 | | | 20 | | | |
| S-9 | Arylpropionamide | 4-Desacetamido-4-chloro andarine | $C_{17}H_{14}ClF_3N_2O_5$ | 7.26 | 417.2 > 127.0[d] | 0.009 | 30 | 28 | 12 | – | Bicalutamide-$D_4$ |
| | | | | | 417.2 > 261.2 | | | 20 | | | |
| | | | | | 417.2 > 205.0 | | | 30 | | | |
| S-23 | Arylpropionamide | | $C_{18}H_{13}ClF_4N_2O_3$ | 7.16 | 415.2 > 145.0[d] | 0.007 | 30 | 24 | 11 | – | Bicalutamide-$D_4$ |
| | | | | | 415.2 > 185.0 | | | 34 | | | |
| | | | | | 415.2 > 269.1 | | | 18 | | | |

[a] TR, retention time.
[b] CE, collision energy.
[c] SRM 1 (6.45–7.05 min); SRM 2 (4.50–5.10 min); SRM 3 (3.60–4.50 min); SRM 4 (6.20–6.80 min); SRM 5 (5.90–6.50 min); SRM 6 (6.55–7.15 min); SRM 7 (5.75–6.35 min); SRM 8 (6.40–7.00 min); SRM 9 (5.90–6.50 min); SRM 10 (6.60–7.20 min); SRM 11 (6.90–7.50 min); SRM 12 (7.00–7.60 min); SRM 13 (5.60–6.20 min); SRM 14 (7.10–7.70 min); SRM 15 (5.55–6.15 min).
[d] Diagnostic ion.

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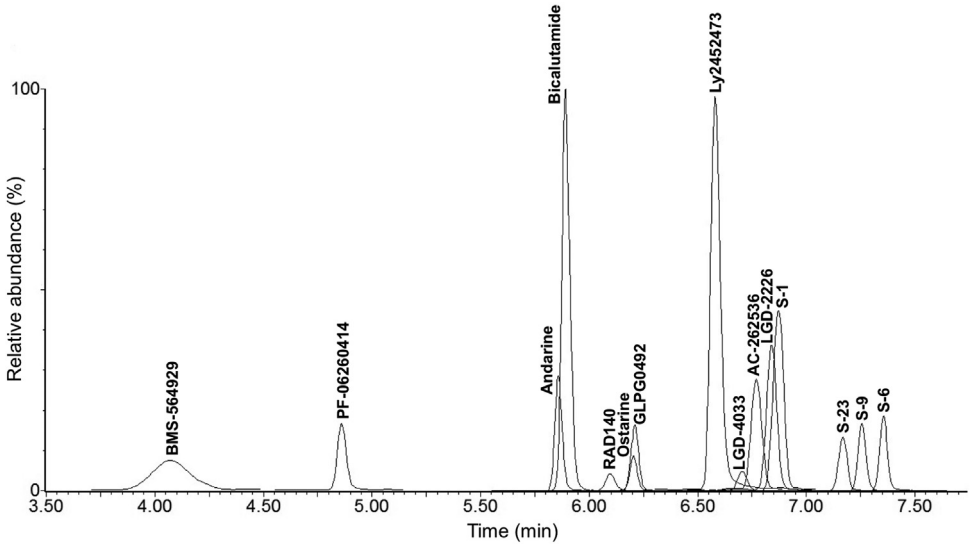


**Fig. 2.** Overlay of UHPLC-MS/MS traces of equine urine fortified with 15 SARMs at 1/2/5 ng $mL^{-1}$.

standard solution of equivalent concentration was injected, divided by the signal of the latter [71].

### 2.6. Application of the method

The method developed in this study has been applied to routine screening for the presence of SARM residues in bovine urine samples ($n = 51$) from abattoirs across Ireland, equine urine samples ($n = 61$) donated by the Irish Equine Centre (IEC), canine urine samples ($n = 109$) provided by the Irish Greyhound Board and human urine samples donated by non-professional volunteer athletes ($n = 22$) as well as urine samples from athletes ($n = 20$) supplied by the WADA accredited Anti-Doping Laboratory of Rome (Italy), selected among those already reported as negative, and after anonymization.

## 3. Results and discussion

### 3.1. Method development

#### 3.1.1. UHPLC-MS/MS conditions

In this study, SARM residues were analysed by electrospray ionisation mass spectrometry (ESI-MS) using both positive and negative ionisation modes. Data acquired in SRM mode by monitoring protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecules, respectively. Diagnostic ions obtained were in agreement with those reported in the literature. At least two most abundant product fragment ions were monitored for each SARM compound yielding at least four identification points [68]. The electrospray voltage, desolvation and source temperatures, desolvation, cone and collision gas flow rates were optimised to get maximum response for the instrument. SRM windows were time sectored and adequate conditions were established through effective set-up of dwell times, inter-scan and inter-channel delay as well as polarity switching. A total of 12–15 data points were typically obtained across a peak to attain reproducible integration and thus achieve highly repeatable analysis.

A number of different mobile phases and additives including volatile buffer (ammonium formate) and acid (formic, acetic) were assessed with a range of UHPLC column chemistries, namely Acquity UPLC®: HSS T3 and CSH $C_{18}$, Cortecs®: $C_{18}$ and T3 (all from Waters), Kinetex: F5, EVO $C_{18}$, and Luna Omega Polar $C_{18}$ (all from Phenomenex). Comparison of column type and mobile phase performance were made based on peak shape (Supplementary data - Fig. 1) and relative abundance of analytes (Supplementary data - Fig. 2 and 3). Optimal LC conditions were identified as that based on mobile phases comprised of water and methanol both containing 0.1% (*v/v*) acetic acid employing a Luna Omega Polar C18 column. Gradient conditions and flow rate were adjusted in order to achieve most favourable chromatographic separation, and as presented in Fig. 2, all analytes were separated within the first 7.70 min of the chromatographic run.

#### 3.1.2. Sample preparation

One of the main goals of this study was to develop a rapid, simple and cost-effective sample preparation procedure that would be suitable for all the 15 SARMs of interest in urine matrix from five different animal species: equine, bovine, canine, human and murine, respectively. Liquid-liquid extraction (LLE) procedures have been successfully employed in both human and equine sport drug testing, as well as in food control applying a range of organic solvents e.g. *tert*-butyl-methyl ether (TBME), ethyl acetate (EA) and diethyl ether [25,42,51]. Nevertheless, to the best of the authors' knowledge, no multi-residue analytical method based on a LLE has been proposed that covers all the 15 SARM compounds included in the current study. This research investigated the impact of a range of extraction parameters, such as volume of equine urine sample (0.2–2.0 mL) and organic solvent (ratio 1:3, 1:6, *v/v*), pH (3.0, 5.0, 9.0 and 11.0), salt addition (sodium and ammonium sulphates), and concentration factor (2, 4, 13.3) in order to achieve satisfactory recovery of all the 15 analytes. The pH had a significant impact both on the extraction of all the analytes and matrix coextractive interferences. Overall, a pH of 5.0 worked adequately for all the analytes providing with higher absolute recovery values (78–108 %) in equine urine, but on the other hand it led to the unacceptable signal suppression for some of the SARMs (e.g. BMS-564929, GLPG0492 and RAD140) in comparison to a LLE at pH 9.0. Consequently, the optimum results were achieved by the addition of 200 μL of a buffer solution (50 mM aqueous $NH_4OH$, pH 10.5) to 200 μL of equine urine, setting the pH value around 9.0 prior to a liquid-liquid extraction with 1.2 mL of TBME.

Moreover, supported liquid extraction (SLE) in equine urine was tested employing the Isolute SLE+ cartridges (1 and 2 mL) and 96-well plate (400 μL). A range of parameters were evaluated,

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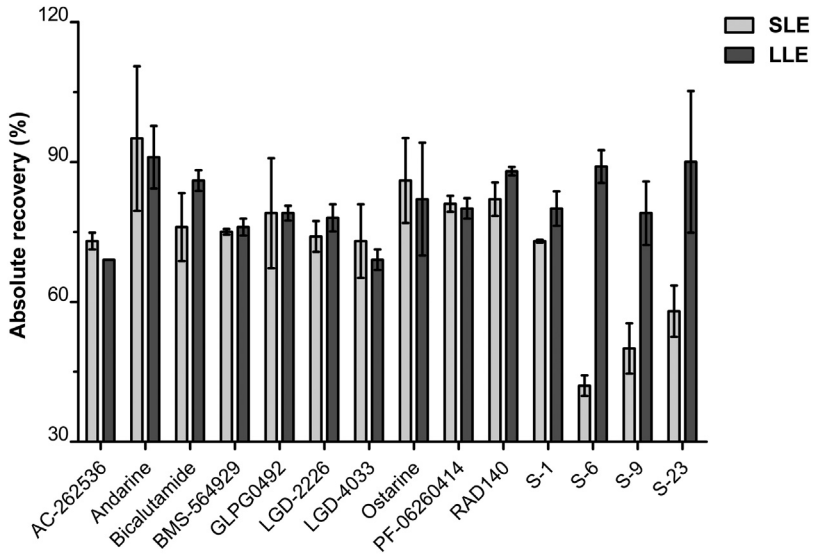


**Fig. 3.** Average absolute recoveries (and standard deviations, shown by error bars) obtained applying SLE and LLE in equine urine fortified at 1 ng mL$^{-1}$ ($n$ = 2).

including urine sample volume (0.2–1 mL), pH value, as well as organic eluent (TBME, EA and DCM as recommended by the manufacturer). Among the SLE protocols, recovery and precision were the best working with 200 μL urine and 400 μL 96-well plate under alkaline conditions (200 μL 50 mM $NH_4OH$ pH 10.5) with TBME. Nevertheless, SLE was not determined to be a procedure of choice due to absolute recoveries lower (42–95 %) than those obtained for the above-mentioned LLE with TBME (69–91 %) as outlined in Fig. 3.

Following extraction (LLE and SLE), the organic solvent (TBME) was evaporated at 40 °C to dryness under a stream of nitrogen. It was found that evaporation of solvent to dryness did not lead to any significant loses of analytes and consequently the use of dimethyl sulfoxide (DMSO) as a "keeper" was avoided. Moreover, a range of different reconstitution solvents was investigated, and $H_2O$:MeCN (4:1, *v/v*), was found to provide satisfactory sensitivity with acceptable peak shapes of all the analytes. Finally, the optimum conditions described in Section 2.3 provided with average absolute recoveries, calculated at the screening target concentration, in the range of 74–94 % for all SARMs of interest in all tested urine matrices (Table 4).

### 3.2. Method validation

#### 3.2.1. Selectivity, specificity, and matrix effect studies

The specificity of the method was investigated through monitoring for interferences in the UHPLC-MS/MS traces for the analytes and internal standards. The absence of cross talk was verified by injecting analytes and internal standards singly. The selectivity of the method was established through testing 263 urine samples from different sources coming from five different species (bovine, canine, equine and murine animals as well as humans) without observed interferences. Carry-over was assessed during the validation study by injecting blank solvent (MeOH) following the sample fortified at the concentration equal to five times the screening target concentration ($5 \times C_{val}$) and it was also monitored during a routine analysis by injecting blank solvent (MeOH) following the sample fortified at the screening target concentration (screen positive control). No analyte signal appeared in blank solvent (MeOH). Matrix effects evaluation (Table 4) highlighted both suppression and enhancement effects in five matrices, namely equine, bovine, canine, human and murine species, respectively. The greatest amount of suppression was observed for BMS-564929 in equine (72%) and human (47%) urine, both BMS-564929 and RAD140 in bovine (50%) and canine (57%) urine, and RAD140 in murine (81%) urine matrix, respectively. On the other hand, the greatest amount of enhancement was observed for bicalutamide in equine (29%) and murine (29%) urine matrix, respectively. Alternatively, in the event that other isotope-labelled analogues related to SARM compounds of interest are developed and/or become more affordable, they can be implemented as internal standards into the method to compensate for signal loss resulting from matrix effects so as to improve accuracy and precision.

#### 3.2.2. Detection capability (CCβ)

Since a recommended concentration for SARMs in urine has not been established [38,72], the screening target concentration was based on their anabolic properties and set at levels of exogenous anabolic androgenic steroids and other anabolic agents [72,73]. Validation was performed at the screening target concentration ($C_{val}$) set at 1 ng mL$^{-1}$ excluding andarine (2 ng mL$^{-1}$) and BMS-564929 (5 ng mL$^{-1}$), respectively, and a single MS/MS transition was sufficient to ensure the screening of the analyte according to the current legislation [69]. However, the cut-off factors (Fm) were above T-values for at least two transitions for all SARMs of interest. The determined CCβ values were below or equal the validation levels for at least two transitions for all analytes (Table 3, Table 4 and Supplementary data Table 1). The sensitivity as highlighted in Table 3 (and Supplementary data - Table 1) was ≥ 95% for at least two transitions for all SARMs. Moreover, the determined ion ratios were within ± 30% tolerance range for all transitions of interest [74]. To conclude, all SARMs of interest can be detected in equine urine by applying this screening assay with a risk of a false-negative rate ≤5% as required by the current legislation [68,69]. In accordance with the EU Commission Decision 2002/657/EC, precision expressed as CV, in the case of a quantitative method, should be as low as possible (analyte concentration below 100 ng mL$^{-1}$). The precision of the current screening assay was observed to be in the range of 9.8–30.9% in equine urine (Table 3), whereas in the case of all other species was found to range from 6.4 to 48.2% (Supplementary data – Table 2).

Relative cut-off factor (RFm) was calculated for each analyte (Table 3) (and Supplementary data – Table 1) as the percentage based on the ratio of the cut-off factor and the mean response of fortified samples, and was applied to screen positive controls (QC samples) during routine application of this screening test.

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**Table 3**
Validation results for fortified equine urine samples ($n = 61$).

| Analyte | eLOD[b] (ng mL$^{-1}$) | $C_{val}$ [c] (ng mL$^{-1}$) | CCβ | Relative cut-off factor (RFm)[d] (%) | Precision[e] (%) | Sensitivity[f] (%) |
|---|---|---|---|---|---|---|
| AC-262536 | 0.06 | 1 | <$C_{val}$ | 63 | 22.6 | 97 |
| Andarine[a] | 0.18 | 2 | <$C_{val}$ | 52 | 29.0 | 100 |
| Bicalutamide[a] | 0.10 | 1 | <$C_{val}$ | 80 | 12.2 | 98 |
| BMS-564929 | 0.44 | 5 | ≤$C_{val}$ | 61 | 23.8 | 95 |
| GLPG0492 | 0.14 | 1 | <$C_{val}$ | 71 | 17.8 | 97 |
| LGD-2226 | 0.08 | 1 | <$C_{val}$ | 69 | 18.9 | 97 |
| LGD-4033 | 0.04 | 1 | <$C_{val}$ | 49 | 30.9 | 97 |
| Ly2452473 | 0.01 | 1 | <$C_{val}$ | 68 | 19.7 | 97 |
| Ostarine[a] | 0.09 | 1 | <$C_{val}$ | 76 | 14.8 | 97 |
| PF-06260414 | 0.05 | 1 | ≤$C_{val}$ | 51 | 29.6 | 95 |
| RAD140 | 0.50 | 1 | ≤$C_{val}$ | 45 | 33.6 | 95 |
| S-1[a] | 0.11 | 1 | ≤$C_{val}$ | 84 | 9.8 | 95 |
| S-6[a] | 0.04 | 1 | ≤$C_{val}$ | 60 | 24.6 | 95 |
| S-9[a] | 0.75 | 1 | <$C_{val}$ | 76 | 14.5 | 98 |
| S-23[a] | 0.06 | 1 | <$C_{val}$ | 67 | 20.0 | 98 |

[a] Values calculated response-based.
[b] Estimated LOD (S/N≥3).
[c] Screening target concentration.
[d] Calculated as the percentage based on the ratio of the cut-off factor and the mean response of fortified samples.
[e] Calculated as coefficient of variation (CV) of the response following fortification.
[f] Expressed as percentage based on the ratio of samples detected as positive in true positive samples, following fortification.

**Table 4**
Recovery and matrix effect data.

| Analyte | Recovery (%)[a] | RSD (%)[a] | Ion supression/enhancement (%) ± SD (%) in matrix[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | Equine | Bovine | Canine | Human | Murine |
| AC-262536 | 74 | 18.9 | 17.4 ± 7.3 | 2.5 ± 4.2 | 3.7 ± 7.4 | 3.6 ± 3.1 | 13.8 ± 2.6 |
| Andarine | 88 | 13.4 | −8.8 ± 4.8 | −2.7 ± 8.2 | −2.8 ± 5.6 | −2.6 ± 6.1 | −8.9 ± 7.1 |
| Bicalutamide | 94 | 11.5 | −28.9 ± 27.8 | −9.5 ± 12.5 | −1.4 ± 8.1 | −2.0 ± 11.0 | −28.9 ± 18.7 |
| BMS-564929 | 81 | 13.7 | 72 ± 10.9 | 49.7 ± 12.4 | 57 ± 6.0 | 46.6 ± 14.1 | 72 ± 6.3 |
| GLPG0492 | 87 | 10.4 | 55 ± 12.1 | 27.9 ± 11.4 | 40.4 ± 4.7 | 33.2 ± 9.3 | 48.8 ± 5.5 |
| LGD-2226 | 81 | 15.1 | 35.9 ± 9.7 | 21.8 ± 2.7 | 27.2 ± 8.5 | 17.8 ± 3.8 | 29.8 ± 3.8 |
| LGD-4033 | 80 | 15.3 | 13.5 ± 11.4 | 10.1 ± 7.4 | 11.9 ± 9.4 | 5.5 ± 6.4 | 18.3 ± 6.2 |
| Ly2452473 | 88 | 8.4 | 11.0 ± 3.6 | 0.9 ± 1.4 | 4.4 ± 3.5 | 0.9 ± 5.1 | 8.0 ± 3.7 |
| Ostarine | 93 | 9.9 | −12.6 ± 12.5 | −3.1 ± 10.5 | 1.5 ± 4.9 | −3.5 ± 7.4 | −11.9 ± 15.4 |
| PF-06260414 | 87 | 10.1 | 52 ± 13.6 | 27.4 ± 9.4 | 30.4 ± 10.1 | 30.0 ± 4.8 | 51 ± 12.0 |
| RAD140 | 87 | 12.7 | 66 ± 7.7 | 50 ± 11.7 | 57 ± 10.7 | 36.9 ± 11.6 | 81 ± 5.5 |
| S-1 | 81 | 12.6 | 7.3 ± 12.0 | 3.5 ± 4.0 | 7.9 ± 6.9 | 7.5 ± 7.4 | 4.8 ± 7.4 |
| S-6 | 75 | 21.4 | 37.8 ± 24.3 | 19.7 ± 2.9 | 23.3 ± 2.1 | 17.4 ± 4.1 | 17.2 ± 3.4 |
| S-9 | 77 | 18.8 | 19.1 ± 22.1 | 11.1 ± 2.2 | 15.2 ± 2.3 | 7.5 ± 5.6 | 11.1 ± 7.2 |
| S-23 | 81 | 17.0 | 9.3 ± 4.6 | 10.7 ± 2.8 | 10.5 ± 1.4 | 7.4 ± 5.8 | 11.3 ± 5.5 |

[a] Recovery was determined by comparing results from fortified samples to those of negative samples spiked post-extraction at the screening target concentration ($C_{val}$, $n = 2$). Recovery is based on data collected from routine application of the method in five species of interest over 15 month period ($n = 25$ analytical runs).
[b] Ion suppression results for urine matrices are based on the analysis of 25 samples ($n = 5$ per species) from different sources. Values calculated as described in Section 2.5. Negative values indicate matrix enhancement.

Results from on-going QC samples (negative controls (pooled blank urine) and screen positive controls fortified at the screening target concentration) are being recorded continuously and the data utilised to verify that the screening assay performs reliably and robust.

### 3.2.3. Extension of validation: application to bovine, canine, human and rat urine

Following initial validation of the developed assay in equine urine, an extension of validation was performed on the same matrix type from four different species - bovine, canine, human and murine urine, respectively. The validation study was carried out on two consecutive days on a series of 20 blank urine samples ($n = 5$ per species) and the same 20 blank urine samples ($n = 5$ per species) fortified at the screening target concentration ($C_{val}$), the same as for equine urine, 1 ng mL$^{-1}$ excluding andarine (2 ng mL$^{-1}$) and BMS-564929 (5 ng mL$^{-1}$), respectively. The sensitivity as highlighted in Supplementary data (Tables 2–3), was ≥95% for at least two transitions for all SARMs in all matrices of interest and there was maximum one result below the cut-off factor established initially for equine urine. In such a case it can be concluded that the developed screening assay is applicable to the new species, namely bovine, canine, human and murine urine, with the same detection capability (CCβ) values for all target analytes as the original matrix.

The ruggedness study of the developed assay resulted in correct classification of all tested samples. In detail, respective 15 blank samples ($n = 5$ per species) were all "screen negative" whereas the corresponding fortified ones were all "screen positive" (i.e. exceeded the cut-off factor).

### 3.2.4. Application of method to analysis of urine from SARM exposed animals

Due to the unavailability of a suitable proficiency test, an inter-laboratory study was performed in conjunction with RIKILT (Wageningen, the Netherlands). Three bovine urine samples provided by RIKILT, collected within the frame of an ostarine (S-22) administration study in a steer calf [19], were tested blindly. All samples were identified correctly as follows: one sample was screened negative (collected before the treatment) and another two were screened positive (collected 2 h and 3 days, respectively, following an oral administration of ostarine) - Fig. 4.

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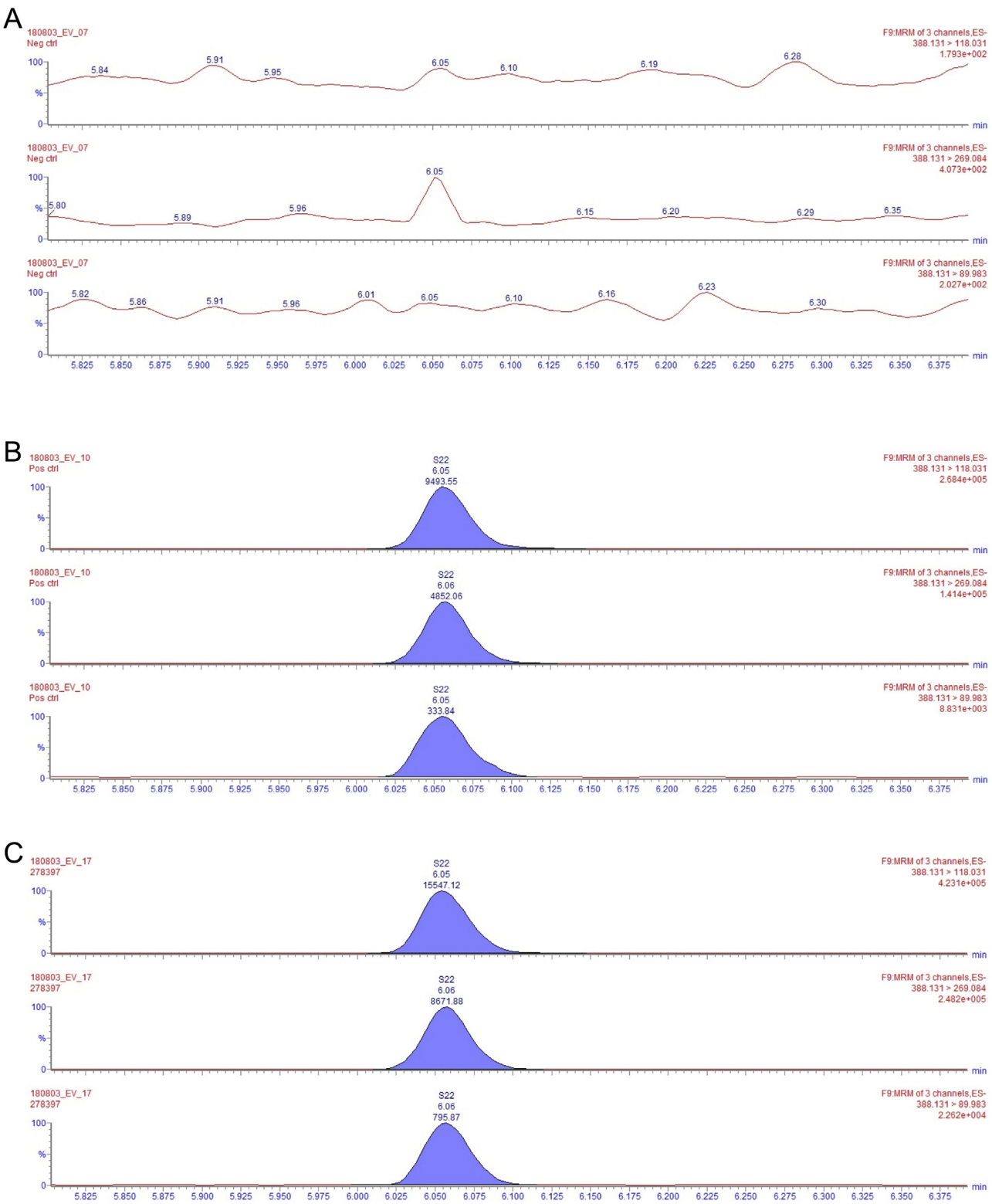


**Fig. 4.** UHPLC-MS/MS traces of (a) blank bovine urine sample, (b) fortified at 1 ng mL$^{-1}$ with ostarine (S-22), (c) bovine urine sample screened positive (collected 3 days following an oral administration of ostarine).

### 3.2.5. *Sample survey*

The assay developed in this study has been used to monitor for the presence of trace levels of SARM residues in urine samples. A total of 263 urine samples were analysed and none of the samples tested contained detectable quantities of SARM residues.

## 4. Comparison with other existing methods

A range of LC- and occasionally GC–MS-based [75–77] screening and/or confirmatory analytical assays have been published for the detection and/or quantification of SARMs in urine sam-

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ples, with application in both anti-doping drug analysis and food testing (Table 1). Among these, most of them were only single-analyte or multi-residues analytical methods with up to four SARM compounds belonging to the same class. Only three multi-residue analytical methods were developed to cover more than one SARM group, but they were restricted only to human doping control. Sobolevsky et al. [78] included four arylpropionamides (S-1, S-4, S-9, S-22), one phenyl-oxadiazole (RAD140) and one pirrolydinil-benzonitrile (LGD-4033) within the same method. Thevis et al. proposed two analytical assays covering one bicyclic hydantoin and one benzimidazole in a first method [79], namely BMS-564929 and a 5,6-dichloro-benzimidazole-derivate, and one bicyclic hydantoin (BMS-564929) and one quinolinone (LGD-2226) in a second method [80], respectively. All the above-mentioned assays required a considerable amount of urine sample (1–7.5 mL), and consequently high volume of organic solvents to undertake a standard purification via LLE or SPE. In contrast, the analytical method presented in this study is advantageous in comparison with existing analytical assays allowing for screening of a wider range of SARM residues in urine relative to other published methods. To the best of our knowledge, this is the first screening method able to analyse 15 different emerging SARM compounds belonging to nine different SARM classes, such as arylpropionamide, diarylhydantoin, hydantoin, indole, isoquinoline, phenyl-oxadiazole, quinolinone, pyrrolidinyl-benzonitrile and tropanol, in five different species with a reasonably short chromatographic run of 12 min. Low amount of sample volume (0.2 mL) and organic solvent (1.6 mL) required by the current assay make it fast, simple, cost effective, environmentally friendly as well as providing for a rapid sample turnaround.

## 5. Conclusions

The present study describes a fit-for-purpose, semi-quantitative screening method for the determination of 15 emerging SARM compounds by UHPLC-MS/MS, in five different urine matrices: equine, canine, human, bovine and murine. The extraction procedure of the target analytes is based on a simple LLE with TBME, and the analytical assay was fully validated according to the EU Commission Decision 2002/657/EC criteria and European Union Reference Laboratories Residues (EU-RLs) guidelines. Detection capability (CCβ) for all analytes was determined at 1 ng $mL^{-1}$, except for andarine (S-4) and BMS-564929 at 2 and 5 ng $mL^{-1}$, respectively. This high-throughput method allows the analysis of 50 test samples in one day. The applicability of the assay was demonstrated by analysis of a range of routine samples (>260) from different species as well as by the analysis of bovine urine samples collected within the frame of ostarine (S-22) administration study. In summary, the method presented in this study can be adopted and implemented by laboratories as a fast, simple and cost-effective tool to detect the abuse of SARM compounds in animal and human sport competitions and to monitor the safety of food commodities from cattle livestock, in compliance with respective regulations, and also offers the opportunity in the future to incorporate additional SARM compounds as and when their use becomes evident.

## Acknowledgments

The authors Emiliano Ventura and Anna Gadaj contributed equally to the work in this paper. The research was supported by funding from the European Union's Horizon 2020 research and innovation programmeunder the Marie Skłodowska-Curie grant agreement No. 642380.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.04.050.

## References

[1] S. Segal, R. Narayanan, J.T. Dalton, Therapeutic potential of the SARMs: revisiting the androgen receptor for drug discovery, Expert Opin. Investig. Drugs 15 (2006) 377–387, http://dx.doi.org/10.1517/13543784.15.4.377.
[2] J.T. Dalton, A. Mukherjee, Z. Zhu, L. Kirkovsky, D.D. Miller, Discovery of nonsteroidal androgens, Biochem. Biophys. Res. Commun. 244 (1998) 1–4, http://dx.doi.org/10.1006/bbrc.1998.8209.
[3] J. Chen, Discovery and therapeutic promise of selective androgen receptor modulators, Mol. Interv. 5 (2005) 173–188, http://dx.doi.org/10.1124/mi.5.3.7.
[4] D. Yin, Key structural features of nonsteroidal ligands for binding and activation of the androgen receptor, Mol. Pharmacol. 63 (2003) 211–223, http://dx.doi.org/10.1124/mol.63.1.211.
[5] W. Gao, P.J. Reiser, C.C. Coss, M.A. Phelps, J.D. Kearbey, D.D. Miller, J.T. Dalton, Selective androgen receptor modulator treatment improves muscle strength and body composition and prevents bone loss in orchidectomized rats, Endocrinology. 146 (2005) 4887–4897, http://dx.doi.org/10.1210/en.2005-0572.
[6] W. Gao, J.D. Kearbey, V.A. Nair, K. Chung, A.F. Parlow, D.D. Miller, J.T. Dalton, Comparison of the pharmacological effects of a novel selective androgen receptor modulator, the 5α-reductase inhibitor finasteride, and the antiandrogen hydroxyflutamide in intact rats: new approach for benign prostate hyperplasia, Endocrinology. 145 (2004) 5420–5428, http://dx.doi.org/10.1210/en.2004-0627.
[7] D. Yin, Pharmacodynamics of selective androgen receptor modulators, J. Pharmacol. Exp. Ther. 304 (2002) 1334–1340, http://dx.doi.org/10.1124/jpet.102.040840.
[8] M. Thevis, D.A. Volmer, Mass spectrometric studies on selective androgen receptor modulators (SARMs) using electron ionization and electrospray ionization/collision-induced dissociation, Eur. J. Mass Spectrom. Chichester (Chichester) 24 (2018) 145–156, http://dx.doi.org/10.1177/1469066717731228.
[9] R. Narayanan, C.C. Coss, J.T. Dalton, Development of selective androgen receptor modulators (SARMs), Mol. Cell. Endocrinol. 465 (2018) 134–142, http://dx.doi.org/10.1016/j.mce.2017.06.013.
[10] S.M. Choi, B.-M. Lee, Comparative safety evaluation of selective androgen receptor modulators and anabolic androgenic steroids, Expert Opin, Drug Saf. 14 (2015) 1773–1785, http://dx.doi.org/10.1517/14740338.2015.1094052.
[11] W. Gao, J.T. Dalton, Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs), Drug Discov. Today 12 (2007) 241–248, http://dx.doi.org/10.1016/j.drudis.2007.01.003.
[12] W. Gao, J. Kim, J.T. Dalton, Pharmacokinetics and pharmacodynamics of nonsteroidal androgen receptor ligands, Pharm. Res. 23 (2006) 1641–1658, http://dx.doi.org/10.1007/s11095-006-9024-3.
[13] A.G. Fragkaki, P. Sakellariou, P. Kiousi, N. Kioukia-Fougia, M. Tsivou, M. Petrou, Y. Angelis, Human in vivo metabolism study of LGD-4033, Drug Test. Anal. (2018) 1635–1645, http://dx.doi.org/10.1002/dta.2512.
[14] A. Hansson, H. Knych, S. Stanley, E. Berndtson, L. Jackson, U. Bondesson, M. Thevis, M. Hedeland, Equine in vivo-derived metabolites of the SARM LGD-4033 and comparison with human and fungal metabolites, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1074–1075 (2018) 91–98, http://dx.doi.org/10.1016/j.jchromb.2017.12.010.
[15] N. Cesbron, A. Sydor, M. Penot, S. Prevost, B. Le Bizec, G. Dervilly-Pinel, Analytical strategies to detect enobosarm administration in bovines, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 34 (2017) 632–640, http://dx.doi.org/10.1080/19440049.2016.1258122.
[16] D. Rojas, G. Dervilly-Pinel, N. Cesbron, M. Penot, A. Sydor, S. Prévost, B. Le Bizec, Selective androgen receptor modulators: comparative excretion study of bicalutamide in bovine urine and faeces, Drug Test. Anal. 9 (2017) 1017–1025, http://dx.doi.org/10.1002/dta.2113.
[17] A. Hansson, H. Knych, S. Stanley, M. Thevis, U. Bondesson, M. Hedeland, Investigation of the selective androgen receptor modulators S1, S4 and S22 and their metabolites in equine plasma using high-resolution mass spectrometry, Rapid Commun. Mass Spectrom. 30 (2016) 833–842, http://dx.doi.org/10.1002/rcm.7512.
[18] A. Hansson, H. Knych, S. Stanley, M. Thevis, U. Bondesson, M. Hedeland, Characterization of equine urinary metabolites of selective androgen receptor modulators (SARMs) S1, S4 and S22 for doping control purposes, Drug Test. Anal. 7 (2015) 673–683, http://dx.doi.org/10.1002/dta.1768.
[19] E. de Rijke, M.L. Essers, J.C.W. Rijk, M. Thevis, T.F.H. Bovee, L.A. van Ginkel, S.S. Sterk, Selective androgen receptor modulators: in vitro and in vivo metabolism and analysis, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 30 (2013) 1517–1526, http://dx.doi.org/10.1080/19440049.2013.810346.
[20] M. Thevis, A. Thomas, I. Möller, H. Geyer, J.T. Dalton, W. Schänzer, Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator S-22 to identify potential targets for routine

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doping controls, Rapid Commun. Mass Spectrom. 25 (2011) 2187–2195, http://dx.doi.org/10.1002/rcm.5100.
[21] M. Thevis, A. Thomas, G. Fußhöller, S. Beuck, H. Geyer, W. Schänzer, Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator andarine (S-4) for routine doping control purposes, Rapid Commun. Mass Spectrom. 24 (2010) 2245–2254, http://dx.doi.org/10.1002/rcm.4637.
[22] M. Thevis, E. Gerace, A. Thomas, S. Beuck, H. Geyer, N. Schlörer, J.D. Kearbey, J.T. Dalton, W. Schänzer, Characterization of in vitro generated metabolites of the selective androgen receptor modulators S-22 and S-23 and in vivo comparison to post-administration canine urine specimens, Drug Test. Anal. 2 (2010) 589–598, http://dx.doi.org/10.1002/dta.211.
[23] T. Sobolevsky, M. Dikunets, G. Rodchenkov, Metabolism study of the selective androgen receptor modulator LGD-4033, in: 33rd Col, Work. Dope Anal. Manfred-Donike Institute, Col. Ger. (2015) 75–79.
[24] T. Sobolevsky, M. Dikunets, G. Rodchenkov, In Vitro and in Vivo Metabolism of RAD140, a Novel Non-steroidal SARM, 31st Col. Work. Dope Anal. Manfred-Donike Institute, Col. Ger., 2013, pp. 121–124.
[25] T. Sobolevsky, M. Dikunets, I. Sukhanova, G. Rodchenkov, Applicability of Routine Analytical Procedures to Detect Andarine and Ostarine. A Comparative Study, 30th Col. Work. Dope Anal. Manfred-Donike Institute, Col. Ger., 2012, pp. 116–119.
[26] L. Geldof, O.J. Pozo, L. Lootens, W. Morthier, P. Van Eenoo, K. Deventer, In vitro metabolism study of a black market product containing SARM LGD-4033, Drug Test. Anal. 9 (2017) 168–178, http://dx.doi.org/10.1002/dta.1930.
[27] O. Krug, A. Thomas, K. Walpurgis, T. Piper, G. Sigmund, W. Schänzer, T. Laussmann, M. Thevis, Identification of black market products and potential doping agents in Germany 2010-2013, Eur. J. Clin. Pharmacol. 70 (2014) 1303–1311, http://dx.doi.org/10.1007/s00228-014-1743-5.
[28] M. Kohler, A. Thomas, H. Geyer, M. Petrou, W. Schänzer, M. Thevis, Confiscated black market products and nutritional supplements with non-approved ingredients analyzed in the cologne doping control laboratory 2009, Drug Test. Anal. 2 (2010) 533–537, http://dx.doi.org/10.1002/dta.186.
[29] M. Thevis, Detection of the arylpropionamide-derived selective androgen receptor modulator (SARM) S-4 (Andarine) in a black-market product, Drug Test. Anal. 1 (2009) 387–392, http://dx.doi.org/10.1002/dta.91.
[30] K. Tsarouhas, N. Kioukia-Fougia, P. Papalexis, A. Tsatsakis, D. Kouretas, F. Bacopoulou, C. Tsitsimpikou, Use of nutritional supplements contaminated with banned doping substances by recreational adolescent athletes in Athens, Greece, Food Chem. Toxicol. 115 (2018) 447–450, http://dx.doi.org/10.1016/j.fct.2018.03.043.
[31] A.Z. Temerdashev, A.A. Azaryan, A.V. Labutin, M.A. Dikunets, I.O. Zvereva, I.I. Podol'skii, G.T. Berodze, I.A. Balabaev, Application of chromatography–mass spectrometry methods to the control of sport nutrition and medicines marketed via internet, J. Anal. Chem. 72 (2017) 1184–1192, http://dx.doi.org/10.1134/S1061934817110090.
[32] R.M. Van Wagoner, A. Eichner, S. Bhasin, P.A. Deuster, D. Eichner, Chemical composition and labeling of substances marketed as selective androgen receptor modulators and sold via the internet, JAMA - J. Am. Med. Assoc. 318 (2017) 2004–2010, http://dx.doi.org/10.1001/jama.2017.17069.
[33] M. Thevis, A. Lagojda, D. Kuehne, A. Thomas, J. Dib, A. Hansson, M. Hedeland, U. Bondesson, T. Wigger, U. Karst, W. Schänzer, Characterization of a non-approved selective androgen receptor modulator drug candidate sold via the Internet and identification of in vitro generated phase-I metabolites for human sports drug testing, Rapid Commun. Mass Spectrom. 29 (2015) 991–999, http://dx.doi.org/10.1002/rcm.7189.
[34] M. Thevis, H. Geyer, A. Thomas, W. Schänzer, Trafficking of drug candidates relevant for sports drug testing: detection of non-approved therapeutics categorized as anabolic and gene doping agents in products distributed via the Internet, Drug Test. Anal. 3 (2011) 331–336, http://dx.doi.org/10.1002/dta.283.
[35] C. Weber, M. Kamber, V. Lentillon-Kaestner, O. Krug, M. Thevis, Seizures of doping substances at the Swiss Border - a descriptive investigation, Forensic Sci. Int. 257 (2015) 359–368, http://dx.doi.org/10.1016/j.forsciint.2015.10.001.
[36] The World Anti-Doping Agency (WADA), The World Anti-Doping Agency Code, The 2019 Prohibited List International Standard, 2019 https://www.wada-ama.org/sites/default/files/wada_2019_english_prohibited_list.pdf.
[37] International Federation of Horseracing Authorities (IFHA), International Agreement on Breeding, Racing and Wagering (IABRN), Article 6A, Prohibited Substances, 2018 https://www.ifhaonline.org/resources/ifAgreement.pdf.
[38] Equine Prohibited Substances List, Fédération Equestre Int., 2018, Equine Prohibited Substances List.pdf https://inside.fei.org/sites/default/files/2018.
[39] H.D. Cox, D. Eichner, Detection of LGD-4033 and its metabolites in athlete urine samples, Drug Test. Anal. 9 (2017) 127–134, http://dx.doi.org/10.1002/dta.1986.
[40] A.T. Cawley, C. Smart, C. Greer, M. Liu Lau, J. Keledjian, Detection of the selective androgen receptor modulator andarine (S-4) in a routine equine blood doping control sample, Drug Test. Anal. 8 (2016) 257–261, http://dx.doi.org/10.1002/dta.1867.
[41] B. Starcevic, B.D. Ahrens, A.W. Butch, Detection of the selective androgen receptor modulator S-4 (Andarine) in a doping control sample, Drug Test. Anal. 5 (2013) 377–379, http://dx.doi.org/10.1002/dta.1466.
[42] E. Grata, L. Perrenoud, M. Saugy, N. Baume, SARM-S4 and metabolites detection in sports drug testing: a case report, Forensic Sci. Int. 213 (2011) 104–108, http://dx.doi.org/10.1016/j.forsciint.2011.07.014.
[43] World Anti-Doping Agency (WADA), 2017 Anti-Doping Testing Figures Report, 2017 https://www.wada-ama.org/sites/default/files/resources/files/2017_anti-doping_testing_figures_en_0.pdf.
[44] J.T. Dalton, D.D. Miller, J.D. Kearbey, SARMs and method of use thereof, US009278914B2, United States Patent, (2016). https://patentimages.storage.googleapis.com/5e/d2/77/51ddcb0a4e882e/US9278914.pdf.
[45] C. Görgens, S. Guddat, A. Thomas, P. Wachsmuth, A.K. Orlovius, G. Sigmund, M. Thevis, W. Schänzer, Simplifying and expanding analytical capabilities for various classes of doping agents by means of direct urine injection high performance liquid chromatography high resolution/high accuracy mass spectrometry, J. Pharm. Biomed. Anal. 131 (2016) 482–496, http://dx.doi.org/10.1016/j.jpba.2016.09.015.
[46] A. Musenga, D.A. Cowan, Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control, J. Chromatogr. A 1288 (2013) 82–95, http://dx.doi.org/10.1016/j.chroma.2013.03.006.
[47] S. Guddat, E. Solymos, A. Orlovius, A. Thomas, G. Sigmund, H. Geyer, M. Thevis, W. Schänzer, High-throughput screening for various classes of doping agents using a new 'dilute-and-shoot' liquid chromatography-tandem mass spectrometry multi-target approach, Drug Test. Anal. 3 (2011) 836–850, http://dx.doi.org/10.1002/dta.372.
[48] M. Thevis, M. Kamber, W. Schänzer, Screening for metabolically stable aryl-propionamide-derived selective androgen receptor modulators for doping control purposes, Rapid Commun, Mass Spectrom. Tokyo (Tokyo) 20 (2006) 870–876, http://dx.doi.org/10.1002/rcm.2389.
[49] A. Knoop, O. Krug, M. Vincenti, W. Schänzer, M. Thevis, In vitro metabolism studies on the selective androgen receptor modulator LG121071 and its implementation into human doping controls using liquid chromatography-mass spectrometry, Eur. J. Mass Spectrom. Chichester (Chichester) 21 (2015) 27–36, http://dx.doi.org/10.1255/ejms.1328.
[50] M. Thevis, M. Kohler, A. Thomas, N. Schlörer, W. Schänzer, Doping control analysis of tricyclic tetrahydroquinoline-derived selective androgen receptor modulators using liquid chromatography/electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 2471–2478, http://dx.doi.org/10.1002/rcm.3637.
[51] M. Thevis, M. Kohler, J. Maurer, N. Schlörer, M. Kamber, W. Schänzer, Screening for 2-quinolinone-derived selective androgen receptor agonists in doping control analysis, Rapid Commun. Mass Spectrom. 21 (2007) 3477–3486, http://dx.doi.org/10.1002/rcm.3247.
[52] E.V. Dmitrieva, A.Z. Temerdashev, A.A. Azaryan, E.M. Gashimova, Determination of andarine (S-4), a selective androgen receptor modulator, and ibutamoren (MK-677), a nonpeptide growth hormone secretagogue, in urine by ultra-high performance liquid chromatography with tandem mass-spectrometric detection, J. Anal. Chem. 73 (2018) 674–678, http://dx.doi.org/10.1134/S1061934818070031.
[53] W.H. Kwok, T.L.S. Choi, Y.Y.K. Tsoi, G.N.W. Leung, T.S.M. Wan, Screening of over 100 drugs in horse urine using automated on-line solid-phase extraction coupled to liquid chromatography-high resolution mass spectrometry for doping control, J. Chromatogr. A 1490 (2017) 89–101, http://dx.doi.org/10.1016/j.chroma.2017.02.020.
[54] K.S. Schmidt, J. Mankertz, In-house validation of a liquid chromatography–tandem mass spectrometry method for the determination of selective androgen receptor modulators (SARMS) in bovine urine, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 35 (2018) 1292–1304, http://dx.doi.org/10.1080/19440049.2018.1471222.
[55] L. Beucher, G. Dervilly-Pinel, N. Cesbron, M. Penot, A. Gicquiau, F. Monteau, B. Le Bizec, Specific characterization of non-steroidal selective androgen peceptor modulators using supercritical fluid chromatography coupled to ion-mobility mass spectrometry: application to the detection of enobosarm in bovine urine, Drug Test. Anal. 9 (2017) 179–187, http://dx.doi.org/10.1002/dta.1951.
[56] M. Thevis, W. Schänzer, Detection of SARMs in doping control analysis, Mol. Cell. Endocrinol. 464 (2018) 34–45, http://dx.doi.org/10.1016/j.mce.2017.01.040.
[57] M.A. Perera, D. Yin, D. Wu, K.K. Chan, D.D. Miller, J. Dalton, In vivo metabolism and final disposition of a novel nonsteroidal androgen in rats and dogs, Drug Metab. Dispos. 34 (2006) 1713–1721, http://dx.doi.org/10.1124/dmd.106.009985.
[58] W. Gao, Interspecies differences in pharmacokinetics and metabolism of S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethylphenyl)-propionamide: the role of N-acetyltransferase, Drug Metab. Dispos. 34 (2005) 254–260, http://dx.doi.org/10.1124/dmd.105.007120.
[59] J. Kim, C.C. Coss, J.T. Dalton, Effect of para halogen modification of S-3-(phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamides on metabolism and clearance, Arch. Pharm. Res. 37 (2014) 1464–1476, http://dx.doi.org/10.1007/s12272-013-0258-4.
[60] J. Kim, R. Wang, K.A. Veverka, J.T. Dalton, Absorption, distribution, metabolism and excretion of the novel SARM GTx-024 [(S)- *N* -(4-cyano-3-(trifluoromethyl)phenyl)-3-(4-cyanophenoxy)-2-hydroxy-2-methylpropanamide] in rats, Xenobiotica 43 (2013) 993–1009, http://dx.doi.org/10.3109/00498254.2013.788233.
[61] J.D. Kearbey, D. Wu, W. Gao, D.D. Miller, J.T. Dalton, Pharmacokinetics of S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide in rats, a non-steroidal selective

Please cite this article in press as: E. Ventura, et al., Development and validation of a semi-quantitative ultra-high performance liquid chromatography-tandem mass spectrometry method for screening of selective androgen receptor modulators in urine, J. Chromatogr. A (2019), https://doi.org/10.1016/j.chroma.2019.04.050

androgen receptor modulator, Xenobiotica. 34 (2004) 273–280, http://dx.doi.org/10.1080/00498250410008962.
[62] D. Wu, Z. Wu, J. Yang, V.A. Nair, D.D. Miller, J.T. Dalton, Pharmacokinetics and metabolism of a selective androgen receptor modulator in rats: implication of molecular properties and intensive metabolic profile to investigate ideal pharmacokinetic characteristics of a propanamide in preclinical study, Drug Metab. Dispos. 34 (2006) 483–494, http://dx.doi.org/10.1124/dmd.105.006643.
[63] A. Fragkaki, P. Sakellariou, P. Kiousi, M. Tsivou, M. Petrou, Y. Angelis, Human in Vivo Metabolism Study of LGD-4033, Drug Test. Anal. (29) (2018) 4033, http://dx.doi.org/10.1002/dta.1930.
[64] R.V. Clark, A.C. Walker, S. Andrews, P. Turnbull, J.A. Wald, M.H. Magee, Safety, pharmacokinetics and pharmacological effects of the selective androgen receptor modulator, GSK2881078, in healthy men and postmenopausal women, Br. J. Clin. Pharmacol. 83 (2017) 2179–2194, http://dx.doi.org/10.1111/bcp.13316.
[65] I. Bhattacharya, S. Tarabar, Y. Liang, V. Pradhan, J. Owens, B. Oemar, Safety, Pharmacokinetic, and Pharmacodynamic Evaluation after Single and Multiple Ascending Doses of a Novel Selective Androgen Receptor Modulator in Healthy Subjects, Clin. Ther. 38 (2016) 1401–1416, http://dx.doi.org/10.1016/j.clinthera.2016.03.025.
[66] S. Basaria, L. Collins, E.L. Dillon, K. Orwoll, T.W. Storer, R. Miciek, J. Ulloor, A. Zhang, R. Eder, H. Zientek, G. Gordon, S. Kazmi, M. Sheffield-Moore, S. Bhasin, The safety, pharmacokinetics, and effects of LGD-4033, a novel nonsteroidal oral, selective androgen receptor modulator, in healthy young men, Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 68 (2013) 87–95, http://dx.doi.org/10.1093/gerona/gls078.
[67] P. Yi, J.F. Rehmel, K. Cassidy, C. Hadden, K. Campanale, N. Patel, J. Johnson, Disposition and metabolism of LY2452473, a selective androgen receptor modulator, in humans, Drug Metab. Dispos. 40 (2012) 2354–2364, http://dx.doi.org/10.1124/dmd.112.047613.
[68] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and interpretation of results, Off. J. Eur. Communities. L 221 (2002) 8–36 https://publications.europa.eu/en/publication-detail/-/publication/ed928116-a955-4a84-b10a-cf7a82bad858/language-en.
[69] [CRL] Community Reference Laboratory, Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines (initial Validation Transfer) (2010), 2010 https://ec.europa.eu/food/sites/food/files/safety/docs/cs_vet-med-residues_guideline_validation_screening_en.pdf.
[70] T. Fawcett, An introduction to ROC analysis, Pattern Recognit. Lett. 27 (2006) 861–874, http://dx.doi.org/10.1016/j.patrec.2005.10.010.
[71] H. Trufelli, P. Palma, G. Famiglini, A. Cappiello, An overview of matrix effects in liquid chromatography-mass spectrometry, Mass Spectrom. Rev. 30 (2011) 491–509, http://dx.doi.org/10.1002/mas.20298.
[72] CRL Guidance Paper (7 December 2007), CRLs View on State of the Art Analytical Methods for National Residue Control Plans, 2007 https://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf.
[73] Technical Document–TD2018MRPL, The World Anti-Doping Agency (WADA), 2018 https://www.wada-ama.org/sites/default/files/resources/files/td2018mrpl_v1_finaleng.pdf.
[74] Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticide Residues and Analysis in Food and Feed, SANTE/11813/2017, 2017 https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2017-11813.pdf.
[75] M. Polet, W. Van Gansbeke, P. Van Eenoo, Development and validation of an open screening method for doping substances in urine by gas chromatography quadrupole time-of-flight mass spectrometry, Anal. Chim. Acta 1042 (2018) 52–59, http://dx.doi.org/10.1016/j.aca.2018.08.050.
[76] E. Gerace, A. Salomone, F. Fasano, R. Costa, D. Boschi, A. Di Stilo, M. Vincenti, Validation of a GC/MS method for the detection of two quinolinone-derived selective androgen receptor modulators in doping control analysis, Anal. Bioanal. Chem. 400 (2011) 137–144, http://dx.doi.org/10.1007/s00216-010-4569-8.
[77] L. Luosujärvi, M. Haapala, M. Thevis, V. Saarela, S. Franssila, R.A. Ketola, R. Kostiainen, T. Kotiaho, Analysis of selective androgen receptor modulators by gas chromatography-microchip atmospheric pressure photoionization-mass spectrometry, J. Am. Soc. Mass Spectrom. 21 (2010) 310–316, http://dx.doi.org/10.1016/j.jasms.2009.10.019.
[78] T. Sobolevsky, G. Krotov, M. Dikunets, M. Nikitina, E. Mochalova, G. Rodchenkov, Anti-doping analyses at the Sochi Olympic and Paralympic Games 2014, Drug Test. Anal. 6 (2014) 1087–1101, http://dx.doi.org/10.1002/dta.1734.
[79] M. Thevis, M. Kohler, A. Thomas, J. Maurer, N. Schlörer, M. Kamber, W. Schänzer, Determination of benzimidazole- and bicyclic hydantoin-derived selective androgen receptor antagonists and agonists in human urine using LC-MS/MS, Anal. Bioanal. Chem. 391 (2008) 251–261, http://dx.doi.org/10.1007/s00216-008-1882-6.
[80] M. Thevis, M. Kohler, N. Schlörer, W. Schänzer, G. Fußhöller, Screening for two selective androgen receptor modulators using gas chromatography-mass spectrometry in doping control analysis, Eur. J. Mass Spectrom. Chichester (Chichester) 14 (2008) 153, http://dx.doi.org/10.1255/ejms.917.

Please cite this article in press as: E. Ventura, et al., Development and validation of a semi-quantitative ultra-high performance liquid chromatography-tandem mass spectrometry method for screening of selective androgen receptor modulators in urine, J. Chromatogr. A (2019), https://doi.org/10.1016/j.chroma.2019.04.050