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SHORT COMMUNICATION

Development of a multi-residue high-throughput UHPLC–MS/MS method for routine monitoring of SARM compounds in equine and bovine blood

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Abstract

Selective androgen receptor modulators (SARMs) are a group of anabolic enhancer drugs posing threats to the integrity of animal sports and the safety of animal-derived foods. The current research describes for the first time the development of a semi-quantitative assay for the monitoring of SARM family compounds in blood and establishes the relative stability of these analytes under various storage conditions prior to analysis. The presented screening method validation was performed in line with current EU legislation for the inspection of livestock and produce of animal origin, with detection capability (CCβ) values determined at 0.5 ng/mL (Ly2452473), 1 ng/mL (AC-262536 and PF-06260414), 2 ng/mL (bicalutamide, GLPG0492, LGD-2226, ostarine, S-1, S-6, and S-23), and 5 ng/mL (andarine, BMS-564929, LGD-4033, RAD140, and S-9), respectively. The applicability of the developed assay was demonstrated through the analysis of blood samples from racehorses and cattle. The developed method presents a high-throughput cost-effective tool for the routine screening for a range of SARM compounds in sport and livestock animals.

KEYWORDS

antidoping control, blood, food safety monitoring, SARMs, UHPLC–MS/MS

1 | INTRODUCTION

Anabolic-androgenic steroids (AAS) continue to be the most abused drugs in sports, both in- and out-of-competition,[1] reflecting advantages over other performance drugs providing long-lasting effects with reduced risks of detection.[2] An emerging class of "designer steroids" are selective androgen receptor modulators (SARMs) that act primarily as androgen receptor (AR) agonists in anabolic tissue, exhibiting only partial agonistic activity in androgenic tissues.[3] Various SARM compounds have undergone evaluation as human therapeutics[4] and whilst none have gained approval for clinical application, simplicity of use (oral administration)[3] and rapid metabolism reducing the window for detection[5,6] as well as widespread availability have facilitated significant SARM abuse in sports (human and animal) and raised the spectre of possible misuse in food-producing species. The use of SARMs in sports is banned by various bodies,[7–9] whereas adoption as anabolic growth promoting agents in animal husbandry is prohibited under EU Council Directive 96/22/EC.[10]

Whilst ideally both urine and blood should be sampled for antidoping and food safety control purposes, the extended window for detection of parent SARM compounds in feces has also been reported confirming that two SARM compounds with arylpropionamide pharmacophores (bicalutamide and ostarine (S-22)) are excreted in bovine feces.[11,12] However, the use of feces in routine testing remains restricted as it is neither a required matrix to be tested in the frame of EU residue control schemes nor is it authorized within antidoping

programs. The advantages of blood-based analysis include the relative short duration required for on-demand sampling during training, pre-race, or post-competition compared with that for urine collection.[13] Blood sampling is also less invasive than tissue-based analysis which can only feasibly occur post-mortem. Therefore, current efforts primarily remain focused on the development of analytical detection strategies utilizing urine and blood as test matrices of choice. Assays based on these complementary matrices rely on the detection of either parent compounds and/or respective metabolites[5,6,11,12,14] where compounds are rapidly metabolized, as is the case with many SARMs. However, metabolites can only be confirmed in a test matrix when their structure has been elucidated and where reference material (i.e. incurred samples) and/or analytical standards are available. As emerging drug compounds with metabolism pathways which can differ from species-to-species, reference materials for SARM metabolites are not readily available.

The majority of methods for SARM analysis in blood have been established to determine the pharmacokinetic profiles during pre-clinical studies involving rodents and humans. Within the antidoping arena only a limited number of procedures for analysis in blood have been reported for humans[15] and animals,[5,6,16] whilst within the food safety sphere no method has been reported for the detection of multiple SARMs. The current study therefore presents for the first time the development and validation of a high-throughput UPLC-MS/MS method for screening of 15 SARM residues in blood, focused on compounds reported to be used in human and animal sports and/or available as analytical standards. This semi-quantitative assay has been applied in a screening survey of samples sourced from horseracing and bovine livestock as a complementary test method to previously reported assay in urine.[14]

2 | MATERIALS AND METHODS

2.1 | Analytical reagents

The reagents used were as detailed elsewhere,[14,17] except for sodium chloride (NaCl, 99.5–100.5%, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent) sourced from VWR International (Ireland), and sources of reference standard materials listed within Supplementary data. Working quality control standard solution at a concentration of 8/16/32/80 ng/mL was prepared in acetonitrile (MeCN), with a working internal standard mix solution prepared at 80 ng/mL in acetonitrile-D (MeCN-D).

2.2 | Extracted matrix screen positive and recovery controls

Pooled blood (n = 10–20 equine plasma and bovine serum, respectively) was used for quality control (QC) purposes as described previously.[14] Extracted matrix screen positive controls were prepared by fortifying negative QC samples (n = 3) prior to extraction with 25 μL of quality control standard solution (8/16/32/80 ng/mL) to provide a screening target concentration of 0.5 ng/mL (Ly2452473), 1 ng/mL (AC-262536 and PF-06260414), 2 ng/mL (bicalutamide, GLPG0492, LGD-2226, ostarine, S-1, S-6 and S-23), and 5 ng/mL (andarine, BMS-564929, LGD-4033, RAD140, and S-9). To monitor for the loss of analytes during extraction, additional negative QC samples (n = 2) were spiked post-extraction with the quality control standard solution (17.5 μL).

2.3 | Sample preparation

Plasma and serum samples were stored at −80°C prior to analysis. Then 400 μL aliquots (in 2 mL micro tubes) were fortified with 25 μL of an 80 ng/mL internal standard mix solution and left to stand for 15 min. Then 1600 μL of 0.5 mM NH_4OH in acetonitrile (kept at −20°C overnight) was added and the contents vortexed for 60 s, and incubated at −20°C for 20 min to facilitate protein precipitation. Subsequently, 200 mg of NaCl was added to the resulting slurry and samples were centrifuged (21,380 × g, 10 min, 4°C). Afterwards, 1400 μL of the top organic layer was transferred into a 2 mL micro tube and 600 μL of *n*-hexane pre-saturated with acetonitrile added to enhance lipid removal, vortexed for 10 min and centrifuged (21,380 × g, 10 min, 4°C). The upper *n*-hexane layer was discarded and 1120 μL of the remaining extract transferred into a 2 mL micro tube and the solvent evaporated to dryness under nitrogen (≤ 5 Bar) at 40°C (Turbovap LV® system), reconstituted in H_2O:MeCN (4:1, v/v; 200 μL) with vortexing (5 min), and centrifuge filtered (PTFE 0.22 μm membrane, 9500 × g, 5 min, 10°C) prior to UHPLC–MS/MS analysis.

2.4 | UHPLC–MS/MS SARM compound analysis

Analysis by means of UHPLC–MS/MS was as described previously[14] with modifications, and the specific operating conditions as outlined in Tables 1 and 2. Stable isotope-labeled analogs of bicalutamide and S-1 (bicalutamide-D_4 and S-1-D_4) were used as internal standards for arylpropionamide residues as detailed in Table 2. The response factor calculated as a ratio between the analyte peak area and the internal standard peak area was obtained for arylpropionamides, with the peak area used as the response for other SARM pharmacophores.

2.5 | SARM screening method validation

"In-house" method validation in terms of selectivity, specificity, detection capability (CCβ), sensitivity, precision, limit of detection (LOD), absolute recovery as well as matrix effects and stability (presented in Supplementary data), was performed in line with the criteria stipulated for screening methods[18,19] for the inspection of food producing animals and produce of animal origin. Validation was undertaken at the screening target concentration (C_{val}) of 0.5 ng/mL (Ly2452473), 1 ng/mL (AC-262536 and PF-06260414), 2 ng/mL

TABLE 1 Analytical platform and respective conditions

Waters Acquity I-class UPLC®	
Column	Luna® Omega Polar C18 (100 × 2.1 mm, 100 Å, 1.6 μm) supplied with KrudKatcher™ ultra HPLC in-line filter, 45°C
Mobile phase A	0.1% (v/v) CH_3COOH in H_2O
Mobile phase B	0.1% (v/v) CH_3COOH in MeOH
Flow rate	0.40 mL/min
Run time	14 min
Injection volume	7.5 μL
Gradient profile	(1) 0.00 min 20% B, (2) 0.50 min 20% B, (3) 4.75 min 60% B, (4) 10.50 min 67.5% B, (5) 11.00 min 99% B, (6) 12.00 min 99% B, (7) 12.10 min 20.0% B, (8) 14.00 min 20% B
Flow diverted to waste	11.00–13.50 min
Needle wash	H_2O:MeOH (1:1, v/v)
Needle purge	H_2O:MeOH (4:1, v/v)
Seal wash	H_2O:MeOH (95:5, v/v)
Waters Xevo® TQ-MS	
Capillary voltage	2.50 kV (ESI+), 1.00 kV (ESI-)
Source temperature	120°C
Desolvation gas temperature	550°C
Desolvation gas flow	900 L/h
Collision gas flow	0.15 mL/min

(bicalutamide, GLPG0492, LGD-2226, ostarine, S-1, S-6, and S-23), and 5 ng/mL (andarine, BMS-564929, LGD-4033, RAD140, and S-9). The detection capability (CCβ)[18] was calculated by assessing threshold value (T) and cut-off factor (Fm)[19] through analysis of 43 blood samples (n = 22 equine plasma and n = 21 bovine serum), both blank and fortified at C_{val} as detailed elsewhere.[14] Both T-value and Fm were estimated for at least two transitions for each analyte, with the detection capability (CCβ) of the screening method validated when Fm > T. The method sensitivity ≥95% at C_{val}, expressed as a percentage based on the ratio of samples reported as positive in true positive samples (i.e. following fortification) means that the number of false-negative samples is truly ≤5%. The precision was calculated as coefficient of variation (CV%) of the response following fortification at C_{val}, thus not required to be determined for semi-quantitative methods.[18] The limit of detection (LOD) was estimated at a signal-to-noise ratio (S/N) of at least three measured peak-to-peak for respective diagnostic ions. Ruggedness was assessed utilizing 20 different blood plasma/serum samples (n = 10 per species), blank and fortified at C_{val}, and analyzed blindly on a different day and by a different analyst.[19] To evaluate matrix effects, 20 blank blood plasma/serum samples from different sources (n = 10 per species) were post-extraction spiked at a concentration equal to 2 × C_{val}. Matrix effects were calculated for each analyte as the percentage differences between the signal obtained when matrix extracts were injected and when a standard solution of equivalent concentration was injected, divided by the signal of the latter.

3 | RESULTS AND DISCUSSION

3.1 | SARM assay method development

In this study, SARM residues within blood were analyzed by means of UHPLC–MS/MS based on adaptions to previously described methodology[14] with chromatographic separation extended from 12 to 14 min and the elution gradient adjusted to improve separation of late eluting analytes from blood matrix interferences (Figure 1). Various protein precipitants were evaluated including water-miscible organic solvents (acetonitrile, acetone, and methanol) as well as the addition of a low volume of 1 M aqueous solutions of $ZnSO_4$, $(NH_4)_2SO_4$, and Na_2SO_4 followed by subsequent liquid–liquid extraction (LLE) with organic solvents (i.e. *tert*-butyl methyl ether (TBME), ethyl acetate, and dichloromethane (DCM)). Further approaches examined the efficacy of so called "double extraction" based on protein crash with acetonitrile followed by LLE with TBME, ethyl acetate, DCM, and diethyl ether. Unfortunately, none of the aforementioned approaches led to satisfactory results in terms of precision (0.0–47.1%), absolute recovery (6.0–174%), and matrix interference removal. Hence, the following features reported in previous studies were further investigated: (1) the impact of pH on the extraction efficiency of selected SARMs; (2) NaCl addition to the slurry following protein precipitation and residue extraction with acetonitrile, aiding removal of matrix contaminants through salt induced liquid–liquid partitioning; and, (3) *n*-hexane (pre-saturated with acetonitrile) assistance in removal of hydrophobic interferences such as lipids and/or phospholipids. The optimized sample extraction conditions described in Section 2.3 led to superior results in terms of recovery (80–91% for all SARMs, Supplementary data – Figure S1) and precision relative to above-mentioned approaches.

3.2 | SARM assay validation

Method specificity was examined by monitoring for interferences in acquired analyte and internal standard MS traces, with the absence of cross talk demonstrated by injection of analytes and internal standards singly. Method selectivity was verified through analysis of blood samples (n = 102) from different sources/species without observable interferences. Potential carry-over was investigated by the injection of blank solvent (MeOH) following the sample fortified at levels equal to 5 × C_{val} and was also monitored during routine analysis by injection of blank solvent following the sample fortified at C_{val} (screen positive control), with no analyte signal being detected. Evaluation of matrix effects (Figure 2 and Supplementary data – Table S1) revealed suppression effects in tested matrices with the greatest suppression observed for BMS-564929 in equine plasma (49.0%) and bovine serum (31.5%). Moreover, suppression was also significant for a

TABLE 2 UHPLC–MS/MS conditions for multi-residue SARM analysis in blood samples

Analyte	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	$C_{18}H_{10}D_4F_4N_2O_4S$	5.77	433.2 > 255.1	0.007	26	14	13	–	N/A
S-1-D_4	$C_{17}H_{10}D_4F_4N_2O_5$	7.58	405.2 > 261.1	0.020	34	20	10	–	N/A
AC-262536	$C_{18}H_{18}N_2O$	7.12	279.2 > 195.0[d]	0.015	36	22	1	+	N/A
			279.2 > 169.1			24			
			279.2 > 93.0			22			
Andarine (S-4)	$C_{19}H_{18}F_3N_3O_6$	5.73	440.1 > 150.0[d]	0.010	30	30	15	–	Bicalutamide-D_4
			440.1 > 261.1			20			
			440.1 > 205.0			34			
			440.1 > 107.0			46			
Bicalutamide	$C_{18}H_{14}F_4N_2O_4S$	5.78	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	$C_{14}H_{12}ClN_3O_3$	3.97	306.1 > 86.1[d]	0.350	30	24	3	+	N/A
			306.1 > 96.0			16			
			306.1 > 278.1			14			
GLPG0492	$C_{19}H_{14}F_3N_3O_3$	6.18	390.2 > 360.2[d]	0.017	34	20	5	+	N/A
			390.2 > 118.0			44			
			390.2 > 91.0			38			
LGD-2226	$C_{14}H_9F_9N_{2O}$	7.49	393.1 > 241.1[d]	0.015	60	38	6	+	N/A
			393.1 > 223.0			52			
			393.1 > 375.1			32			
			393.1 > 203.0			56			
LGD-4033	$C_{14}H_{12}F_6N_2O$	7.17	337.1 > 267.1[d]	0.020	28	10	8	–	N/A
			337.1 > 170.0			24			
			337.1 > 239.0			24			
Ly2452473	$C_{22}H_{22}N_4O_2$	6.84	375.2 > 272.1[d]	0.025	30	20	4	+	N/A
			375.2 > 289.2			18			
			375.2 > 93.0			38			
			375.2 > 180.0			38			
Ostarine (S-22)	$C_{19}H_{14}F_3N_3O_3$	6.21	388.1 > 118.0[d]	0.017	30	20	9	–	Bicalutamide-D_4
			388.1 > 269.1			18			
			388.1 > 90.0			54			
PF-06260414	$C_{14}H_{14}N_4O_2S$	4.74	303.1 > 168.2[d]	0.076	36	36	2	+	N/A
			303.1 > 232.1			24			
			303.1 > 210.1			26			
RAD140	$C_{20}H_{16}ClN_5O_2$	6.01	394.1 > 223.1[d]	0.005	20	10	7	+	N/A
			394.1 > 170.1			30			
			394.1 > 205.1			20			
			394.1 > 155.0			50			
S-1	$C_{17}H_{14}F_4N_2O_5$	7.62	401.1 > 261.0[d]	0.020	35	20	10	–	S-1-D_4
			401.1 > 205.0			26			
			401.1 > 111.0			24			
			401.1 > 289.0			20			
S-6	$C_{17}H_{13}ClF_4N_2O_5$	9.31	435.0 > 145.0[d]	0.050	30	25	14	–	S1-D_4
			435.0 > 289.0			20			
			435.0 > 205.0			30			

TABLE 2 (Continued)

Analyte	Formula	T_R[a] (min)	Transition (m/z)	Dwell time (s)	Cone (V)	CE[b] (eV)	SRM window[c]	ESI polarity	IS
			435.0 > 261.1			20			
S-9	$C_{17}H_{14}ClF_3N_2O_5$	8.86	417.1 > 127.0[d]	0.050	30	28	12	–	S1-D_4
			417.1 > 261.2			20			
			417.1 > 205.0			30			
S-23	$C_{18}H_{13}ClF_4N_2O_3$	8.58	415.1 > 145.0[d]	0.040	30	24	11	–	S1-D_4
			415.1 > 185.0			34			
			415.1 > 269.1			18			

[a]T_R, retention time.
[b]CE, collision energy.
[c]SRM 1 (6.80–7.40 min); SRM 2 (4.40–5.00 min); SRM 3 (3.40–4.50 min); SRM 4 (6.50–7.10 min); SRM 5 (5.85–6.45 min); SRM 6 (7.15–7.75 min); SRM 7 (5.70–6.30 min); SRM 8 (6.80–7.40 min); SRM 9 (5.90–6.50 min); SRM 10 (7.25–7.85 min); SRM 11 (8.20–8.80 min); SRM 12 (8.50–9.10 min); SRM 13 (5.45–6.05 min); SRM 14 (8.95–9.55 min); SRM 15 (5.40–6.00 min).
[d]Diagnostic ion.

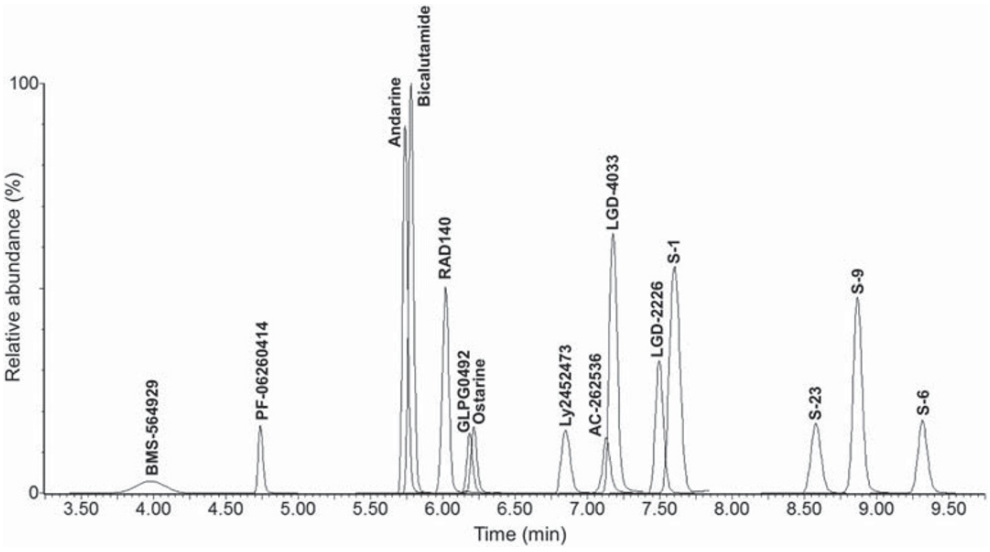

FIGURE 1 Overlay of UHPLC–MS/MS traces obtained following analysis of equine plasma fortified with 15 SARMs at 0.5/1/2/5 ng/mL

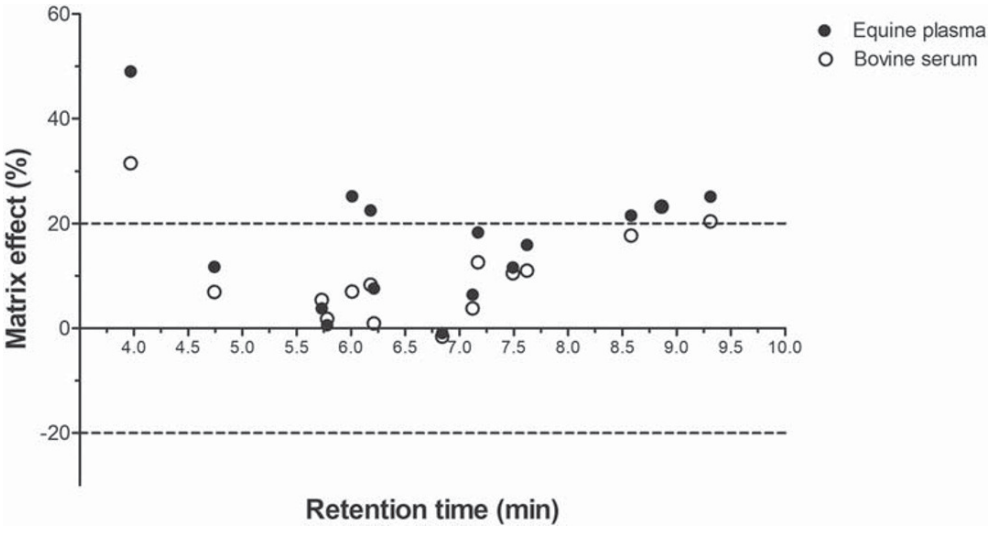

FIGURE 2 Ion suppression results for blood matrices based on the analysis of 20 samples (n = 10 per species) from different sources. ----- ±20% limit

TABLE 3 Validation results for analysis of SARM fortified blood samples (n = 22 equine plasma and n = 21 bovine serum)

Analyte	eLOD[b] (ng/mL)	C_{val}[c] (ng/mL)	CCβ	Relative cut-off factor (RFm)[d] (%)	Precision[e] (%)	Sensitivity[f] (%)
AC-262536	0.14	1	≤ C_{val}	89	6.5	95
Andarine (S-4)[a]	0.60	5	< C_{val}	56	26.9	100
Bicalutamide[a]	0.11	2	< C_{val}	58	25.4	100
BMS-564929	0.33	5	≤ C_{val}	89	6.4	95
GLPG0492	0.12	2	≤ C_{val}	82	10.9	95
LGD-2226	0.43	2	< C_{val}	79	12.8	100
LGD-4033	0.56	5	≤ C_{val}	81	11.6	95
Ly2452473	0.04	0.5	< C_{val}	87	7.6	98
Ostarine (S-22)[a]	0.08	2	< C_{val}	74	16.0	100
PF-06260414	0.04	1	< C_{val}	80	12.2	100
RAD140	0.44	5	< C_{val}	57	26.3	100
S-1[a]	0.09	2	< C_{val}	88	7.2	100
S-6[a]	0.23	2	≤ C_{val}	84	9.5	95
S-9[a]	0.11	5	< C_{val}	86	8.3	98
S-23[a]	0.07	2	< C_{val}	88	7.2	98

[a]Values calculated response-based.
[b]Estimated LOD (S/N ≥ 3).
[c]Screening target concentration.
[d]Calculated as 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.

number of arylpropionamide SARMs including S-1, S-6, S-9, and S-23 in both equine plasma (15.9–25.2%) and bovine serum (7.0–23.3%). If or when stable isotope-labeled analogs related to relevant SARM compounds are developed and/or become more affordable, they should be incorporated into the current method as internal standards to compensate for any signal loss resulting from matrix effects, thereby further enhancing accuracy and precision.

Since SARMs belong to a class of banned compounds for which a recommended concentration in blood has not yet been established in equine or bovine animals, and with no supporting experimental data from SARM-exposed livestock animals available, the screening target concentration was set based on the ALARA (as low as reasonably achievable) principle,[20] with validation performed at C_{val} levels as detailed in Section 2.5. Parent SARM compounds were included within the presented method as target residues based on reported testing of blood samples from SARM exposed equine animals[5,6] revealing the presence of respective metabolites, thus recommending the parent molecules as principle targets to be used in antidoping control with the corresponding metabolites employed as complementary ones. Although a single MS/MS transition was sufficient to fulfill the requirements of current legislation,[19] Fm > T was determined for a minimum of two transitions for all SARM compounds. The determined CCβ values were below or equal to C_{val} for at least two transitions for all analytes (Table 3 and Supplementary data Table S2), with sensitivity ≥95% for at least two transitions for all analytes and the determined ion ratios within ±30% tolerance range for all transitions of interest. The precision of the presented assay was satisfactory[18] for the majority of the analytes (Table 3, CV ≤ 16.0%), excluding andarine (26.9%), bicalutamide (25.4%) and RAD140 (26.3%). The relative cut-off factor (RFm) was calculated as percentage based on the ratio of the cut-off factor and the mean response of fortified samples[14] for each analyte (Table 3 and Supplementary data – Table S2), and was applied to screen positive controls (QC samples) during routine analysis. The ruggedness study of the current method resulted in an appropriate classification of all tested samples, with respective blank samples all "screen negative" and the corresponding fortified samples all "screen positive" (i.e. exceeding the cut-off factor).

Applicability of the developed method was demonstrated in the assessment of SARM compound stability (Supplementary data). A limited degree of instability was observed for SARMs in blood when stored for 12 weeks at −20°C, as well as after repeated freeze–thaw cycles. Furthermore, blood reconstitution solvent extracts were found to be sufficiently stable when stored over 4 weeks at −20°C and for 2 weeks at 4°C. The presented assay was employed to the routine testing for the presence of trace levels of SARM compounds in blood samples from racehorses (n = 50 equine plasma) and livestock abattoirs across Ireland (n = 52 bovine serum) – none of the tested samples were found to contain detectable levels of SARM residues.

4 | CONCLUSIONS

The objective of this study was to develop a fit-for-purpose, semi-quantitative method enabling screening in blood of 15 emerging SARM compounds belonging to nine different classes (arylpropionamide,

diarylhydantoin, hydantoin, indole, isoquinoline, phenyl-oxadiazole, quinolinone, pyrrolidinyl-benzonitrile, and tropanol) by means of UHPLC–MS/MS. The fully validated assay is amenable to high-throughput monitoring of SARMs in animal-based sport and food production systems, and is shown to be capable of detecting SARMs in equine blood at levels previously reported following routine testing[16] and/or within in vivo SARM exposure studies.[5,6] The presented methodology faciliates analysis of up to 50 test samples per day and can be readily adopted as a fast, simple and cost-effective tool in routine control testing programs focused on detecting the abuse of SARM compounds in animal sports and monitoring the safety compliance of livestock food production in line with respective regulations. New analytical targets (i.e. intact parent molecules and/or respective metabolites) that reveal exposure to existing SARMs in target species[5,6] can be readily incorporated into the presented method as can new emerging compounds whenever their use becomes evident. Whilst blood from SARM exposed animals was not available for analysis, the method was successfully applied to screen a range of routine test samples from target species (equine and bovine), and found to be suited for the detection of intact parent and/or metabolite molecules in LGD-4033, ostarine (S-22), and RAD140 exposed rodent animals (data not presented). Additionally, the described assay also offers the potential to be validated as a quantitative confirmatory method according to criteria stipulated in relevant legislation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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