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A downscaled multi-residue strategy for detection of anabolic steroids in bovine urine using gas chromatography tandem mass spectrometry (GC–MS^3)

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Abstract

Within the scope of the European Community member states' residue monitoring plan, illicit administration of anabolic steroids is monitored at slaughterhouse level as well as on living animals. At farm level, urine is one of the target matrices to detect possible abuse of anabolic steroid growth promoters. Optimisation of the routinely applied analysis method resulted in a procedure for which high performance liquid chromatographic (HPLC) fractionation prior to GC–MS^n analysis was no longer required. Analytical results could be obtained within 1 day and only 5 mL urine was needed tot carry out the screening procedure. Using the downscaled methodology, all validation criteria described in the European Commission document 2002/657/EC could be fulfilled, and the minimum required performance limits (MRPLs) established for anabolic steroids in urine, could be achieved.

A higher GC–MS technique's specificity was achieved by detecting the steroids using GC–MS^3. Nevertheless, it was decided to screen routinely sampled urine with GC–MS^2 whereas GC–MS^3 was applied to confirm the presence of anabolic steroid residues in suspected sample extracts.

Keywords: Gas chromatography; Tandem mass spectrometry; Bovine urine; Sex steroid hormones; Anabolic steroid residues; Minimum required performance limits

1. Introduction

By nature, steroidal hormones are produced by the male and female sex organs (testes, ovaries), the adrenal cortex and the placenta. As they are involved in the development of reproductive structures and secondary sexual characteristics, sex hormones are generally applied in veterinary medicine to regulate rut and improve fertility [1]. Next to endogenous steroids, many semi-synthetic and synthetic analogues have been produced and administered to animals.

Based upon pharmacological effects steroids can be divided into three principal groups: estrogens, gestagens and androgens (EGAs) [2]. Because of their anabolic effects, EGAs have been used in animal husbandry to increase the weight of meat-producing animals. Enhanced nitrogen retention and build-up of proteins result in improved muscle growth, a higher carcass quality (lean meat) [3–5] and a higher feed efficiency. However, based upon results of pharmacological/toxicological studies, the use of steroidal hormones for cattle fattening purposes has been forbidden in the European Community (EC) since 1988 [6–8]. Since then, analytical laboratories are involved to analyse the samples taken by the inspection services. For that reason, many analytical procedures have been developed to screen and confirm the presence of EGAs in several matrices.

In 2002, the EC has proposed to establish minimum required performance limits (MRPLs) which all EC member state accredited analytical laboratories must achieve in order to ensure the quality of analysis carried out on official governmental inspection services' order (2002/657/EC) [9]. In Belgium, the Federal Agency for the Safety of the Food Chain (FAVV-AFSCA) has setup a residue monitoring plan and national MRPLs have been established for substances for which no maximum residue level (MRL) has been imposed (Group A substances).

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Table 1
HPLC fractionation of anabolic steroids

Component	Fraction 3–4[a]	Fraction 5–6	Fraction 7–8[a]
Hexestrol	x		
Diethylstilbestrol	x		
Dienestrol	x		
α/β-Nortestosterone	x (β)	x (α)	
α/β-Boldenone	x		
Methylandrostanediol			x
Methandriol		x	
Ethylestranediol			x
Methylboldenone	x		
Methyltestosterone		x	
Ethinylestradiol	x		
α/β-Zeranol	x		
α-Trenbolone	x		
Norgestrel		x	
Norethandrolone			x
Chloroandrostenedione		x	
Fluoxymesterone	x		

[a] Fractions 1, 2 and 9 were of no interest (matrix and hydrolysis enzyme residual components).

Illegal steroid administration is being monitored at various stages in the food chain. At farm level, misuse of EGAs in living animals is being monitored by analysis of the animal's excreta (urine, faeces). Out of analytical point of view, urine is preferred to faeces because of its homogeneity. Furthermore, after administration, EGAs are metabolised into more hydrophilic structures to advance elimination out of the animal's body, by which detection of EGAs' residues and their degradation products in aqueous matrices becomes an option [10].

Development of procedures for the determination of residual substances in urine has always been a challenge, as urine is a reservoir of the body's waste products possibly affecting unequivocal detection of the target analytes. Prior to this study, an anabolic steroid residue analysis of urine was performed by hydrolysis of 25 mL bovine urine with *Helix pomatia* juice (62 ± 2 °C, 120 min), followed by a diethyl ether liquid–liquid extraction and fractionation with high performance liquid chromatography (HPLC). Selectively chosen HPLC fractions were combined afterwards to be evaporated and derivatized with $MSTFA^{++}$ (Table 1). Finally, three GC–MS^2 analyses (one run for each combined HPLC fraction) were required to obtain the results for only one urine sample. It took at least 48 h until the analytical results could be passed to the inspection services. And, as urine is sometimes hard to sample resulting in little urine volumes, analysis could not be resumed because of a lack of laboratory sample volume.

In this experimental setup, the extraction and clean-up part of the conventional procedure was optimised because of its rate-limiting part in the conventional methodology.

2. Experimental

2.1. Chemical reagents and reference standards

Reference steroid standards, i.e. hexestrol, diethylstilbestrol, dienestrol, α-nortestosterone, β-nortestosterone, α-boldenone, β-boldenone, methylandrostanediol, methandriol, ethylestranediol, methylboldenone, methyltestosterone, ethinylestradiol, α-zeranol, β-zeranol, α-trenbolone, norgestrel, norethandrolone, chloroandrostenedione, fluoxymesterone, androsterone and equilinine, were obtained from Steraloids (Wilton, NY, USA), Sigma (St. Louis, MO, USA) or the National Reference Laboratory (WIV, Brussels, Belgium). Equilinine and androsterone were used as respectively internal and external reference standard. The EGAs' stock solutions (200 μg mL^{-1} anabolic steroid in absolute ethanol) and a working solution containing all EGAs at National MRPL concentration level were stored at 4 °C when frequently used. If not, storage at −18 °C was recommended.

All reagents and solvents used were of analytical grade quality and provided by Merck (Darmstadt, Germany). Sodium acetate buffer (pH 5.2 ± 0.5) was made out of 150 mL acetic acid 0.4 M and 1 L sodium acetate 0.4 M. Sodium carbonate solution (pH 10.2 ± 0.5) was prepared by mixing 100 mL $NaHCO_3$ (10%, w/v in water) and 500 mL Na_2CO_3 (10%, w/v in water). Both the sodium acetate buffer and the sodium carbonate solution were adjusted to the desired pH with hydrochloric acid 2 M or sodium hydroxide 5 M. Abalone acetone powder from abalone entrails (glucuronidase activity 286,000 units g^{-1}; sulphatase activity 18,500 units g^{-1}) was purchased from Sigma (St. Louis, MO, USA). The derivatization reagent $MSTFA^{++}$, needed to obtain GC–MS^n suitable EGAs (enol-trimethylsilyl ethers), was prepared by dissolving 100 mg ammonium iodide (NH_4I) (Sigma, St. Louis, MO, USA) and 0.2 mL ethanethiol (Acros, Geel, Belgium) in 5 mL *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), followed by dilution of 1.5 mL of this solution with 10 mL MSTFA.

3. Apparatus and materials

3.1. Extraction and clean-up

Following devices were used for extraction and clean-up: a balance, a mini-shaker, a centrifuge, a rotary vacuum evaporator, a water bath, a vacuum sample processing station and a nitrogen evaporator.

Next to Nunc tubes (Nalge Nunc International, Rochester NY, USA) and amber 0.7 mL autosampler vials, glassware and other recipients were selectively chosen to be suitable in each step of the procedure. Solid phase extraction columns were purchased at IST International (Mid Glamorgan, UK): Isolute C_{18} reversed phase columns (500 mg–6 mL) and Isolute aminopropyl (NH_2) columns (100 mg–1 mL).

3.2. GC–MS^n apparatus

A POLARIS ion trap mass spectrometer, coupled to a ThermoQuest CE Trace GC gas chromatograph (Thermo Finnigan, Austin, TX, USA) with a split/splitless injector, was used to perform the GC–MS^n analyses. Samples were injected using a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium or hydrogen gas was used as GC carrier gas at a flow-rate of 1 mL min^{-1}. The hydrogen carrier gas was made out

of ultrapure water using a hydrogen generator (Packard, Meriden, USA).

3.3. Conditions for GC–MS^n analysis [11]

3.3.1. GC parameters

Non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 GC-column (25 m × 0.22 mm, ID 0.25 μm) (SGE Incorporated, Austin, TX, USA), injector temperature of 250 °C with a split vent flow of 60 mL min^{-1}, injection in splitless mode (split valve closed at −0.10 min, open at 1.00 min).

Temperature program (hydrogen as carrier gas): initial 100 °C (hold 1 min); to 250 °C (30 °C min^{-1}); to 290 °C (2.5 °C min^{-1}); to 300 °C (10 °C min^{-1}) (hold 1.5 min).

Temperature program (helium as carrier gas): initial 100 °C (hold 1 min); to 250 °C (17 °C min^{-1}); to 300 °C (2 °C min^{-1}) (hold 1 min).

3.3.2. MS^n acquisition method parameters [11]

Ion source temperature at 200 °C, transfer line temperature at 275 °C, MS^n measurements in electron impact mode, helium as collision gas in the ion trap at a supply pressure of 3 bar, the electron ionisation energy at 70 eV, several scan segments with scan events depending on the EGAs to be analysed and mass range depending on the selected precursor ion (in GC–MS^3 two precursor ions must be selected and the mass range is determined on basis of the fragmentation pattern of the second precursor ion).

3.3.3. GC–MS^n interpretation

Xcalibur™ software (Thermo Finnigan, Austin, USA) version 1.2.

4. Method

4.1. Extraction

After centrifugation (9000 rpm, 10 min, 4 ± 1 °C), a-5 mL aliquot of bovine urine was diluted with 5 mL ultrapure water and checked for pH 7 ± 0.5 with hydrochloric acid 2 M or sodium hydroxide 5 M. In case of routine analysis, the same steps were carried out for a compliant and a non-compliant control sample, this latter being fortified with 50 μL of the 10 times diluted EGAs working solution. Subsequently, 50 μL of a 0.5 ng μL^{-1} solution of equilinine was added to each urine sample.

Extraction of EGAs out of the crude urine was established with solid phase extraction (SPE). The C_{18}-column was conditioned with 5 mL methanol and equilibrated with 5 mL ultrapure water. The urine sample was quantitatively passed (at 1 mL min^{-1}) on top of the column and allowed to drain in, thereby using a vacuum sample processing station (e.g. VacMaster®). The SPE column was washed with 5 mL ultrapure water. The EGAs were eluted with 2 mL × 4 mL methanol.

4.2. Hydrolysis

After evaporation of the primary methanol extract (rotary vacuum evaporator, medium speed), the dry residue was dissolved with 10 mL of ultrapure water and 2 mL of sodium acetate solution (0.2 M, pH 5.2), and the pH was adjusted to pH 5.2 ± 0.5 with hydrochloric acid 2 M or sodium hydroxide 5 M. Subsequently, hydrolysis was carried out by incubation (62 ± 2 °C, 120 min) with 12,000 units of abalone acetone powder.

4.3. Clean-up

Initial clean-up was performed by a liquid–liquid extraction with 40 mL diethyl ether. The diethyl ether phase containing the EGAs, was washed with 5 mL sodium carbonate and 2 mL × 5 mL ultrapure water, and evaporated until dry (rotavapor, 60 ± 2 °C). The residue was resolved in 4 mL dichloromethane/isopropanol (2:1, v/v).

Further clean-up was realized by SPE with an NH_2-column. Therefore, the SPE column was conditioned with 2 mL dichloromethane/isopropanol (2:1, v/v). The extract was quantitatively passed on the top of the column and collected immediately. After addition of the GC–MS^n reference standard (5 μg L^{-1} androsterone, i.e. 50 μL of a 0.5 ng μL^{-1} solution of androsterone), the elute was evaporated until dry under a nitrogen flow (40 ± 2 °C) in an autosampler GC–MS vial.

4.4. GC–MS^2 and GC–MS^3 analysis

Twenty-five microliters of $MSTFA^{++}$ was added to the residue and, after closure, the solution was mixed thoroughly using a vortex mixer. After incubation (60 min, 60 ± 2 °C), 1 μL was injected into the GC.

Since a GC–MS^n run of a mixture of standard EGAs was performed before the sample to verify the optimum status of the GC–MS device, a-50 μL aliquot of the 10 times diluted EGAs working solution was evaporated and derivatized under the same conditions as the test sample.

5. Results and discussions

In urine, steroids can be present in free, glucuronic acid and sulphate forms due to metabolisation after administration. In order to obtain a complete view on the administered dose, enzymatic hydrolysis of the glucuronic acid and sulphate conjugates into the free form is essential. For the downscaled methodology abalone acetone powder was preferred to *H. pomatia* juice because of less steroid conversion and less chromatographic interferences during chromatographic analysis [12]. Optimum deconjugation conditions could be achieved at pH 5.2 ± 0.5 with 12,000 units abalone acetone powder at 60 ± 2 °C for 120 min. After a liquid–liquid extraction with diethyl ether, according to the conventional method but with reduced solvent volumes, a clean sample extract containing all (deconjugated and free) EGAs was obtained.

In the conventional method, HPLC fractionation was needed for further clean-up of the primary extract. This led to a low

analytical response during GC–MS detection because of a loss of components during the fractionation step. As hydrolysis with abalone acetone powder resulted in a cleaner sample extract, HPLC was left out and a switch towards SPE was made. A C_{18} phase was chosen for extraction of EGAs out of the crude urine, and a final SPE with NH_2 was carried out to remove polar interferences at the end of the extraction and purification process. The whole procedure resulted in one extract enabling a urine sample to be analysed by GC–MS^n ($n = 2$ or 3) within solely one run.

Notwithstanding the quality of the conventional method, the downscaled procedure should be preferred because only 5 mL urine – instead of 25 mL – was needed to carry out the analytical procedure enhancing the opportunity to re-analyse the sample if needed. Furthermore, as the whole analytical procedure could be carried out within 1 day, a significant gain in sample capacity was achieved and analytical results were passed to the inspection services within 24 h. Finally, analytical costs could be reduced because of far less needed solvent volumes and less reference standard amounts. Originally, six internal EGAs standards were required to ensure the quality of the HPLC fractionation because the sample extract was separated into six different fractions. For the downscaled methodology, equilinine was selected as exclusive internal standard.

6. Validation study

The optimised method was validated according to the regulating EC quality criteria (concerning the performance of analytical methods and the interpretation of results: 2002/657/EC).

As analytical results were obtained by a mass spectrometric technique, the analytical approach was considered suitable for screening as well as confirmation analyses. Criteria for chromatographic separation (analyte's relative retention time corresponding to that of the calibration solution within ±0.5%) were fulfilled, as gas chromatographic retention times are stable under unchanged GC conditions. Concerning mass spectrometric detection, 4 identification points (IPs) could be achieved for each EGAs investigated, as the GC–MS^2 and GC–MS^3 analysis resulted in a sufficient number of diagnostic ions (Table 2) within the maximum permitted tolerances. As for methylandrostanediol, for example, interpretation of the GC–MS^2 mass spectrum resulted in 7 IPs ($1.0_{\text{precursor}} + (4 \times 1.5_{\text{transition product}})$), while 8.5 IPs ($1.0_{\text{precursor}} + 1.5_{MS^2\ \text{transition product}} + (4 \times 1.5_{MS^3\ \text{transition product}})$) were obtained when applying GC–MS^3 (Fig. 1). However, despite the GC–MS^3's higher mass spectrometric specificity, GC–MS^2 should be preferred when quantitative interpretation must be performed.

Although for banned substances – such as EGAs – quantification is not necessary, it may be useful to have an estimation of the EGAs' residual concentration present in the suspected sample. During the confirmation analysis the control sample taken along will be fortified with the particular analyte at a concentration level equal to its concentration found during the screening procedure, and the response of the analyte present in the suspect sample will be compared with the analyte response in the control sample.

Further performance characteristics, i.e. specificity/selectivity, applicability/ruggedness/stability, detection limit (CCβ) and decision limit (CCα), have been determined according to the guidelines described in 2002/657/EC. For specificity and selectivity, GC–MS^2 and surely GC–MS^3 are sufficiently specific analytical techniques to differentiate between target analytes and interferences. When analysing 20 blank urine samples originating from different animals, no significant matrix effects, i.e. interfering peaks with signal-to-noise ratio higher than 3, were observed.

The method's ruggedness was tested during development also. Although incorporation of minor pH condition's changes during hydrolysis, extraction and clean-up did not result in sig-

Table 2
Specific diagnostic ions of EGAs when analysed in full scan GC–MS, GC–MS^2 and GC–MS^3 (precursor ions in bold)

Component	GC–MS	GC–MS^2	GC–MS^3
Hexestrol	163-179-191-**207**	149-164-**179**-191	73-105-149-163
Diethylstilbestrol	217-383-397-**412**	217-231-**383**-397	201-217-354-368
Dienestrol	379-381-395-**410**	244-379-381-**395**	291-305-379-380
α/β-Nortestosterone	182-194-403-**418**	182-313-328-**403**	181-197-287-313
α/β-Boldenone	**206**-325-415-430	163-175-183-**191**	163-175-183-193
Methylandrostanediol	255-270-345-**435**	199-213-**255**-345	145-173-199-213
Methandriol	**253**-268-343-358	169-183-**197**-211	155-167-169-182
Ethylestranediol	157-241-**331**-421	159-185-199-**241**	145-159-185-199
Methylboldenone	206-339-429-**444**	206-297-**339**-429	229-243-269-283
Methyltestosterone	301-341-356-**446**	251-**301**-341-356	169-171-185-286
Ethinylestradiol	232-285-**425**-440	**193**-231-281-407	91-135-145-165
α/β-Zeranol	307-335-389-**433**	295-309-**389**-415	265-279-291-305
α-Trenbolone	303-318-397-**412**	369-370-383-**397**	310-325-369-381
Norgestrel	194-301-316-**456**	194-301-**316**-427	179-194-287-301
Norethandrolone	287-300-356-**446**	287-299-300-**356**	285-299-327-341
Chloroandrostenedione	429-449-456-**464**	234-359-**429**-449	221-245-283-339
Fluoxymesterone	319-407-462-**552**	319-357-407-**462**	247-337-427-447
Androsterone	239-329-419-**434**	239-329-344-**419**	169-239-329-361
Equilinine	280-305-395-**410**	280-320-381-**395**	279-289-305-380

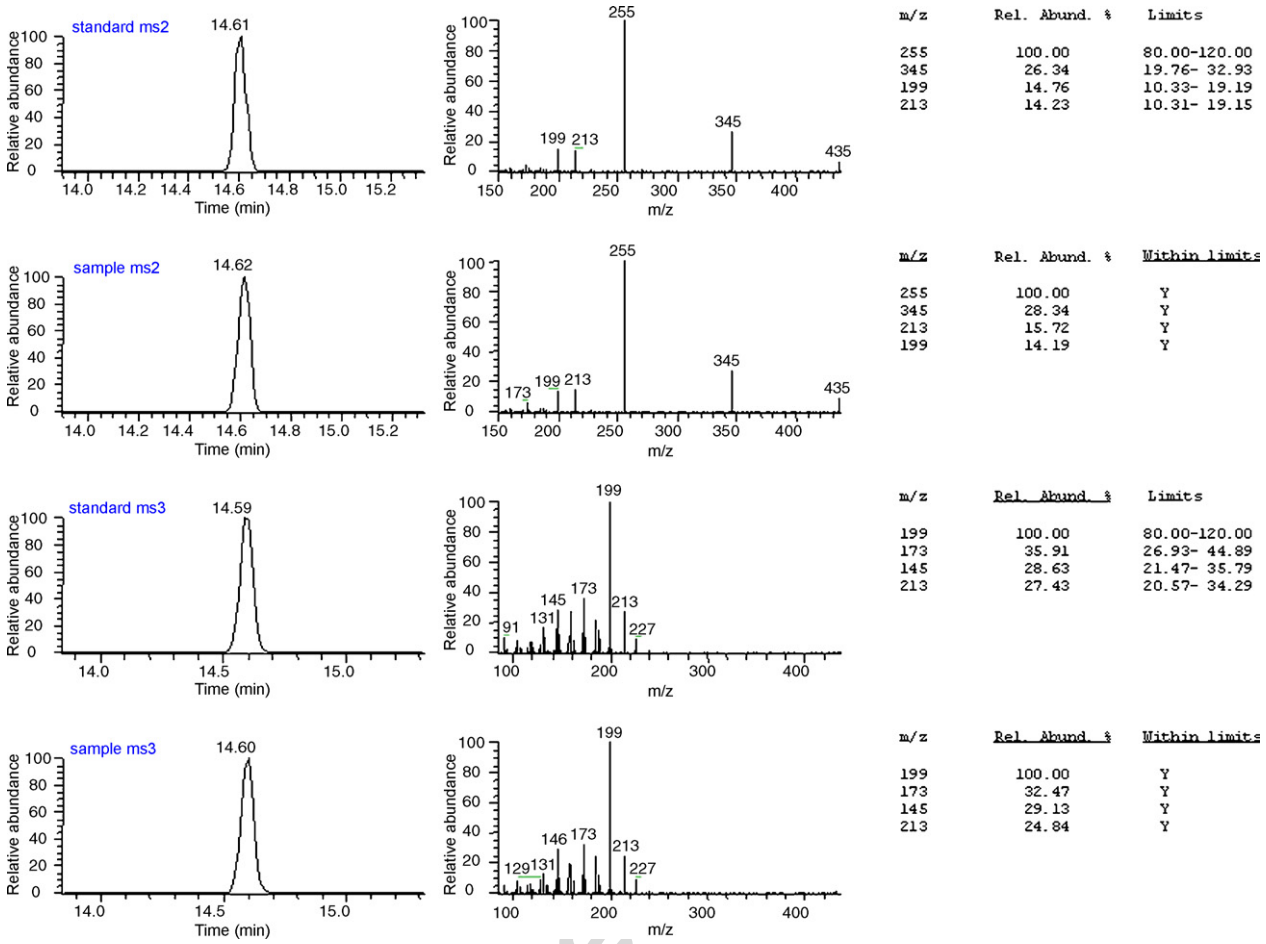

Fig. 1. Methylandrostanediol at National MRPL level (5 μg L^{-1}) in bovine urine when analysed by GC–MS^2 and GC–MS^3.

nificant differences, the directives described above should be respected as optimum results must be preferred. Furthermore, it was important to start out of centrifuged urine (precipitation of proteins eventually disturbing efficient EGAs extraction). Variations in the abalone hydrolysis duration time indicated 2 h as being necessary to obtain optimum deconjugation results, but longer duration times sketched no loss of steroids.

Table 3
Detection capability (CCβ) for all investigated steroids (2002/657/EC)

Component	Technique		
	National MRPL (μg L^{-1})	GC–MS^2, CCβ (μg L^{-1})	GC–MS^3, CCβ (μg L^{-1})
Hexestrol	5	2.5	5
Diethylstilbestrol	2	1	2
Dienestrol	2	1	2
α/β-Nortestosterone	2	1	2
α/β-Boldenone	5	2.5	5
Methylandrostanediol	5	2.5	5
Methandriol	5	2.5	5
Ethylestranediol	2	1	2
Methylboldenone	3	1.5	3
Methyltestosterone	2	1	2
Ethinylestradiol	2	1	2
α/β-Zeranol	2	1	2
α-Trenbolone	2	*10*	*10*
Norgestrel	2	1	2
Norethandrolone	2	1	2
Chloroandrostenedione	5	2.5	5
Fluoxymesterone	5	2.5	5

CCβ could be determined out of the analysis of fortified samples (20 blank urine samples fortified respectively at MRPL and at half MRPL concentration level). For all investigated EGAs – except for α-trenbolone – all identification criteria could be fulfilled at national MRPL when using GC–MS^3, and at half that level when analysed with GC–MS^2, in more than 95% of the analysed samples. As in the blank samples no peaks could be detected in each steroid's window, it was concluded that CCα was equal or lower to the CCβ concentration levels (Table 3). For α-trenbolone, the MRPL of 10 μg L^{-1} could not be blamed to the sample preparation, but was owing to the analytical instrument's insufficient detection capacity under routine conditions. Therefore, one third of the final sample extract (before derivatization with $MSTFA^{++}$) was analysed with high performance thin layer chromatography, and when suspected of containing α-trenbolone, the sample was re-analysed using the conventional method or other analytical approaches [13,14].

7. Conclusion

The method developed during this experimental setup was suitable to screen for the presence of steroidal residues in bovine urine samples. Only 5 mL urine was needed to carry out the procedure consisting of a primary SPE with reversed phase C_{18} cartridges, hydrolysis with abalone entrails, a liquid–liquid diethyl ether extraction and a final SPE with an NH_2 column. The extract was analysed with GC–MS^2 and/or GC–MS^3, resulting in detection capabilities at half National MRPLs for most of the investigated steroids using GC–MS^2 and at National MRPLs when applying GC–MS^3. Therefore, GC–MS^2 was chosen to be used for screening purposes, and, in case of confirmation of a sample a suspected sample of containing EGAs, an appeal for confirmation was made to GC–MS^3.

This newly designed easy-to-apply analytical approach should be preferred to the conventional routine method because of significant reduction in analysis duration time and a decrease in analytical costs.

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