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# Evaluation of nandrolone and ractopamine in the urine of veal calves: liquid chromatography-tandem mass spectrometry approach.

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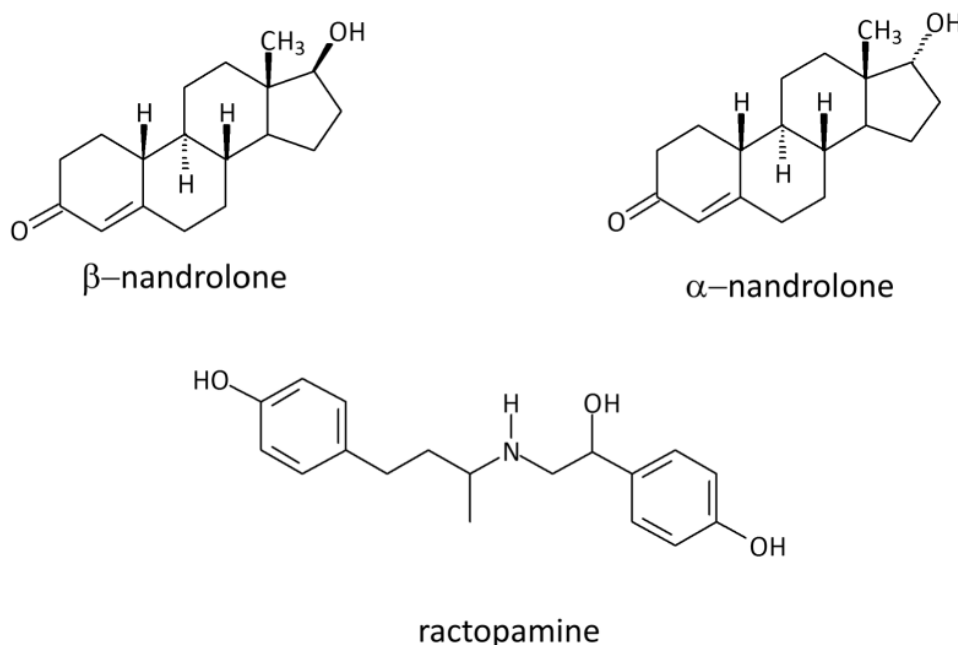
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## Abstract

Under European legislation, the use of growth promoters is forbidden in food-producing livestock. The application of unofficial protocols with diverse combinations of veterinary drugs, administered in very low concentrations, hinders reliable detection and subsequent operative prevention. It was observed that nandrolone (anabolic steroid) and ractopamine ( $\beta$ -adrenergic agonist) are occasionally administered to animals, but little is known about their synergic action when they are administered together. Two specific analytical methods based on liquid chromatography-tandem mass spectrometry have been developed, both of which include hydrolysis of the corresponding conjugates. For the nandrolone method, solid-phase extraction was necessary for the complete elimination of the interferences, while employment of the Quantitation Enhanced Data-Dependent scan mode during MS acquisition of ractopamine enabled the utilization of simple liquid-liquid extraction. The nandrolone method was linear in the range of 0.5-25 ng/mL, while the ractopamine calibration curve was constructed from 0.5 to 1000 ng/mL. The corresponding coefficients of correlations were  $>0.9907$ . The lower limit of quantification for both methods was 0.5 ng/mL, followed by overall recoveries  $>81\%$ . Precisions expressed as relative standard deviations were  $<17\%$ , while matrix effects were minimal. Urine samples taken at the slaughterhouse from veal calves enrolled in an experimental treatment consisting of intramuscular administration of  $\beta$ -nandrolone-phenylpropionate accompanied with a ractopamine-enriched diet were analysed. Those methods might be useful for studying the elimination patterns of the administered compounds along with characterization of the main metabolic pathways

## Introduction

Demethylation of testosterone in position 19 leads to the formation of 19-nortestosterone ( $\beta$ -nandrolone) (Figure 1). The absence of a methyl group from the 19th C atom, although considered a modest structural modification, has a significant impact on pharmacological effects:  $\beta$ -nandrolone, unlike testosterone, exhibits stronger anabolic activity, while its influence on androgenic characteristics is reduced.[1] Since 1950, when the first  $\beta$ -nandrolone synthesis took place, the pharmaceutical industry has produced norsteroids for humans and animals for substitutive (hypogonadism), complementary (osteoporosis and haematological diseases), and contraceptive treatments. Nandrolone has been used among athletes as a doping agent to accelerate muscle growth and strength, increase body mass, and speed up recovery.[2] Moreover, nandrolone could be used in cattle breeding and medical veterinary practices for the impediment of degenerative processes and promoting tissue repair. Unfortunately, despite the fact that its use was prohibited in the EU for food-producing livestock (CE/96/22),[3] nandrolone has been one of the most illegally applied anabolic steroids, either as a growth promoter or for the augmentation of feed efficiency.



**Figure 1. Chemical structures of target analytes.**

Nandrolone was once thought to be a solely synthetic steroid, but its metabolites have been detected in untreated injured males and pregnant females.[4, 5] The endogenous appearance of nandrolone-related compounds could be associated with an increase in adrenal steroid output during extreme stress or due to high oestrogen production during pregnancy. In addition, the formation of 19-norsteroids by the demethylation of testosterone in stored human urine samples was also observed.[6] Consequently, procedures that would distinguish between the occurrence of nandrolone residues due to illicit treatment and natural production in the animal's body, at the same time eliminating the possibility of artificial formation, are highly necessary and considered indispensable.

Usually, nandrolone is distributed in the ester form as phenylpropionate,[7] laureate,[8-10] or decaonate.[11] This type of administration decreases the rate of absorption of the drug, leading to a prolonged anabolic effect. Following absorption,  $\beta$ -nandrolone esters are rapidly decomposed to

free  $\beta$ -nandrolone. In cattle, the metabolism of  $\beta$ -nandrolone involves the epimerization to  $\alpha$ -nandrolone (Figure 1), which, as a sulphate or glucuronide conjugate, is the main urinary metabolite in this species.[4] This is analogous to the metabolism of testosterone, which is excreted in the urine of many species as a conjugate of  $17\alpha$ -testosterone. A number of other minor metabolites of  $\beta$ -nandrolone have been reported in cattle, namely  $5\alpha$ -estrane- $3\beta$ ,  $17\alpha$ -diol,  $5\beta$ -oestrane- $3\alpha$  and  $17\beta$ -diol,[12] or 19-noretiocholanolone and 19-norepiandrosterone.[13]

$\beta$ -nandrolone may be administered alone or as part of a cocktail along with other growth-promoting substances in so-called smart combinations. This is commonly done to hinder the detection of abuse, since the concentration of each individual drug given in combination with others is substantially lower than that which would be required to induce the same anabolic effect if administered on its own.

Ractopamine (Figure 1), a  $\beta$ -adrenergic agonist, is mainly used as a bronchodilator or tocolytic agent for therapeutic purposes in human medicine. However, it is also used as a growth promoter in food-producing animals due to its anabolic effects when administered in higher doses.[14] These properties are due to ractopamine's ability to alter the distribution and metabolism of nutrients favouring muscle deposition in relation to fat reduction. The use of this substance as a feed additive is authorized in the USA[15] for the growth promotion of fattening pigs and cattle. However, ractopamine has been banned in the EU (CE/96/22),[3] and intense debate is still ongoing regarding this issue (European Food Safety Authority).[16] The EU remains strongly opposed to the adoption of maximum residue limits (MRLs) for ractopamine, since outstanding safety concerns remain to be elucidated.

At the farm level, the misuse of banned medicines in living animals is generally monitored by analyses of the animals' urine. Several analytical procedures have been developed for the efficient clean-up of biological matrices, such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE). In the protocols reported in the literature, the above-mentioned pre-treatment procedures are used for the successful screening of nandrolone and/or ractopamine, almost exclusively with other substances from their classes.[17, 18] Gas chromatography coupled to mass spectrometry (GC-MS) is a subtle and highly sensitive technique with an indispensable, although sometimes laborious, derivatization step.[8] The combination of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) offers a simplified, specific, and sensitive alternative to GC-MS methods involving adequate extraction procedures.[19, 20] Therefore, the development of analytical procedures for the determination of veterinary drugs, such as nandrolone and ractopamine, in biological matrices must always be taken into account, and any improvement of existing techniques is more than supportive. The aim of this study was to develop two LC-MS/MS methods that would be specific for each of the cited substances. The final objective was to apply those procedures to the urine samples of calves that were subjected to simultaneous nandrolone and ractopamine experimental administration.

## Materials and methods

### Materials and reagents

All solvents were of high performance liquid chromatography (HPLC) or analytical grade and were purchased from Fluka (Sigma-Aldrich, St Louis, MO, USA). Formic acid 98–100% was obtained from Riedel-de Haën (Sigma-Aldrich, St Louis, MO, USA). Water was purified by a Milli-Q System.  $\alpha$ - and  $\beta$ -nandrolone and their internal standard  $d_3$ - $17\beta$ -nandrolone were purchased from LGC Standards (Teddington, UK), while ractopamine and its internal standard isoxsuprine were obtained from Sigma-Aldrich (St Louis, MO, USA). Beta-glucuronidase from *Escherichia coli* K

12 (EC 3.2.1.31) and  $\beta$ -glucuronidase/arylsulfatase from *Helix pomatia* (EC 3.2.1.31/ EC 3.1.6.1) were supplied by Roche Diagnostics GmbH (Boehringer Mannheim, Germany).

The veterinary medicament Nandrosol (AST Farma B.V. Oudewater, the Netherlands), consisting of nandrolone phenylpropionate 50 mg/mL and ractopamine (Unibrom Corp., Weifang, PR China), was used for administration to veal calves.

### **Sample preparation for nandrolone determination**

A 1 mL aliquot of filtered urine was diluted to 2 mL with phosphate buffer (0.2 M, pH = 6.2) and incubated with 40  $\mu$ L  $\beta$ -glucuronidase from *E. coli* at 55 °C for 4 h. After cooling to room temperature, 20  $\mu$ L of 0.1  $\mu$ g/mL d3- $\beta$ -nandrolone (internal standard, IS) was added to a final concentration of 2 ppb. Each sample was extracted using Oasis HLB cartridge (3 mL, 60 mg, Waters, Milford, MA, USA) with an extraction vacuum manifold (Supelco, etc.). The following extraction procedure was optimized. The column was conditioned by passage of methanol followed by water. The sample was deposited on the column and washed first with 2 mL of 10% methanol and then with 1 mL of 2% ammonia in 40% methanol. The elution to recover the compounds of interest was performed with 2 mL of methanol. The eluate was evaporated under the steam of nitrogen and reconstructed in 1000  $\mu$ L of mobile phase.

### **Sample preparation for ractopamine determination**

The following extraction procedure was performed for the ractopamine. Three mL of acetate buffer (1 M, pH-5) was added to 2 mL aliquots of urine sample. The deconjugation was performed at 45 °C for 4 h with 20  $\mu$ L of  $\beta$ -glucuronidase/sulphatase from *Helix Pomatia*. After cooling at room temperature, isoxsuprine as IS (2 ng/mL, final concentration) was added, with a subsequent pH adjustment (8.5–9.5) with 1 M NaOH (1.2 mL + a few drops). Then, the urine samples were treated with 5 mL of an organic solutions mixture that consisted of *tert*-butyl methyl ether and ethyl acetate (4: 1 v/v). After mixing on a horizontal rotary shaker for 20 min, the samples were subjected to centrifugation (15 min, 3500 g) in order to separate the organic from the aqueous phase. The supernatant (organic phase) was dried with a rotary evaporator at 40 °C. The residue was resuspended in 500  $\mu$ L of initial mobile phase (methanol and 0.1% formic acid in aqueous solution, 10:90 v/v) and transferred to the autosampler vials.

### **Instrumentation**

Both instrumental LC-MS/MS procedures were carried out with a Thermo Finnigan HPLC system (Thermo Fisher, San José, CA, USA) comprised of a Surveyor MS equipped with a degasser and a Surveyor AS autosampler equipped with a column oven and a rheodine valve. The TSQ Quantum triple quadrupole of Thermo Finnigan utilized an electrospray ionization source (ESI) as the interface (Thermo Fisher, San José, CA, USA). The mass spectrometer was operated in the positive ion electrospray ionization (ESI+) mode. The column eluate was directed through a programmable divert valve, which enabled time-dependent switching of the valve guiding the flow of the direction of waste. Data were analysed with the Xcalibur software (Thermo Fisher, San José, CA, USA).

#### ***LC-MS/MS conditions for nandrolone determination***

Analytical separations were achieved using a Synergi Hydro RP reverse-phase HPLC column 150 x 2.0 mm, i.d. 4  $\mu$  80 A (Phenomenex, Torrance, CA, USA). The column temperature was set at 30 °C. Binary gradient profiles were developed using a water solution of 0.1% formic acid (A) and methanol (B) at a flow rate of 250  $\mu$ L/min. The chromatographic conditions for phase B were as

follows: from 25% to 70% for 0–20 min, achieving 95% in the 21st min, holding for 3 min, returning to starting values at the 25th min, and equilibrating up to 30 min. A sample aliquot (10  $\mu$ L) was injected into the system with the autosampler. The column eluate was directed into the ionization source within 5 – 25 min.

Acquisition parameters were optimized in the ESI+ mode with direct continuous pump-syringe infusion of standard solutions of the analytes at the concentration of 1  $\mu$ g/mL. A flow rate of the syringe and pump flow rate in the ion source of the mass spectrometer were 10  $\mu$ l/min and 100  $\mu$ l/min, respectively. The optimized parameters were: the capillary voltage set at 3000 V; the ion transfer capillary temperature set a 340  $^{\circ}$ C; and the heath, auxiliary (nitrogen) gas fixed at 30 and 10 arbitrary units, respectively. The collision gas was argon at 1.5 mTorr, and the peak resolution of 0.70 Da FWHM was used on Q1 and Q3. The signal acquisition was performed by the multiple reaction-monitoring mode (MRM) with corresponding transitions. Precursor ions (as pseudomolecular species  $[M + H]^{+1}$ ), are shown in Table 1 together with the product ions, tube lens voltages and collision energies.

Table 1. MS/MS condition for the MRM acquisitions of analytes and relative internal standards

Compound	Tube lens	Quantification transition ( $m/z$ ) <sub>CE</sub>	Confirmation transitions ( $m/z$ ) <sub>CE</sub>
1. CE: collision energy, expressed in volts			
<b>Nandrolone assay</b>			
$\alpha/\beta$ -nandrolone	102	275 $\rightarrow$ 109 <sub>25</sub>	275 $\rightarrow$ 145 <sub>20</sub> , 275 $\rightarrow$ 199 <sub>19</sub> , 275 $\rightarrow$ 239 <sub>15</sub> , 275 $\rightarrow$ 257 <sub>16</sub>
d3- $\beta$ -nandrolone (IS)	118	278 $\rightarrow$ 109 <sub>19</sub>	278 $\rightarrow$ 202 <sub>19</sub> , 278 $\rightarrow$ 242 <sub>16</sub> , 278 $\rightarrow$ 260 <sub>15</sub>
<b>Ractopamine assay</b>			
Ractopamine	85	302 $\rightarrow$ 164 <sub>15</sub>	302 $\rightarrow$ 91 <sub>38</sub> , 302 $\rightarrow$ 107 <sub>30</sub> , 302 $\rightarrow$ 121 <sub>22</sub> , 302 $\rightarrow$ 284 <sub>11</sub>
Isoxsuprine (IS)	84	302 $\rightarrow$ 150 <sub>21</sub>	302 $\rightarrow$ 77 <sub>33</sub> , 302 $\rightarrow$ 105 <sub>33</sub> , 302 $\rightarrow$ 107 <sub>29</sub> , 302 $\rightarrow$ 284 <sub>14</sub>

#### LC-MS/MS conditions for ractopamine determination

Reverse-phase liquid chromatography was performed using the same column as for the nandrolone assay. The flow rate was 200  $\mu$ L/min and the elution solvents were 0.1% formic acid in water (A) and acetonitrile (B). The mobile phase gradient used (A:B; v/v) was 95:5 at the start, then 45:55 up to 10 min, altered to 10:90 at 12 min, held until 15 min, before returning to the initial conditions in the 19th min, followed by an equilibration period up to the end of the run (22 min). The flow rate was set at 200  $\mu$ L/min, while the injection volume was 10  $\mu$ L. The divert valve was programmed to lead the eluent towards the detector in the time interval from 5 to 12 min.

Acquisition parameters were optimized in the same manner as for nandrolone. The potential set on the capillary was 4200 V while the ion transfer capillary temperature was fixed at 360 °C. Nitrogen flow as sheath and auxiliary gas was set at 40 and 6 arbitrary units, while collision gas pressure (Ar) was 1.5 mTorr. A peak resolution of 0.70 Da FWHM was used on Q1 and Q3. The signal acquisition was performed by MRM with corresponding transitions, as presented in Table 1. Additional confirmation was obtained by Quantitation Enhanced Data-Dependent (QED) mode. When an MRS transition reached 10 000 counts (signal threshold), the QED scan detection was activated to collect full MS spectra applying a ramped collision energy (CE) from 15 to 35 V.

## Assay validation

The validations of both procedures were performed in order to evaluate the methods in terms of specificity, linearity, sensitivity, precision, recovery, matrix effects, and stability.

### Specificity

We analyzed an appropriate number of representative blank samples (n = 20) and checked for any interferences (signals, peaks, ion traces) in the region of interest where the target analyte was expected to elute. The occurrence of possible interferences from closely related substances (isomers, metabolites, degradation products, endogenous substances, matrix constituents) was tested by monitoring the MRM profiles of investigated compounds at the retention time interval expected for their elution. A signal-to-noise (S/N) ratio greater than 3 was considered satisfactory in order to verify the method specificity.

### Calibration curves preparation, linearity, and sensitivity

Standard stock solutions (1 mg/mL) of nandrolone and ractopamine were prepared in methanol. Standard spiking solutions at concentrations of 1 µg/mL were obtained by dilution of the stock standard solutions. The appropriate amount of standard spiking solution was added to 2 mL of urine to prepare calibration standards in the concentration ranges 0.5–25 ng/mL and 0.5–1000 ng/mL for nandrolone and ractopamine, respectively. The linear calibration curves (including point zero) were established by employing the IS calibration method: the ratio between the peak area of the analytes and the peak area of IS was plotted on the Y-axis with scalar concentration levels of standards plotted on the X-axis, leading to the equation  $Y = m * X + b$ . The fitting of linearity was verified by squared correlation coefficients ( $R^2$ ). Sensitivity was determined as a lower limit of quantification (LLOQ). This parameter was calculated from the lowest point on the calibration curve under the following conditions: (1) the analyte response at the LLOQ should be at least 5 times the response compared to blank response, and (2) the analyte peak should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% (FDA, Guidance 2001).<sup>[21]</sup>

### Precision and repeatability

*Instrumental precision* was evaluated by six injections of the analyte (nandrolone or ractopamine) at concentrations of 10 ng/mL according to the optimal operative conditions. In this way, the repeatability of the instrumental system, expressed as relative standard deviations (RSD), was acquired. *Method precision* as intra-day precision, presented also as RSD, of the assay was determined by replicate analyses of blank urine samples (n = 6) fortified with 2, 5, and 10 ng/mL of each compound. The same samples were used for determination of the intermediate method precision, which was obtained from injections over 3 consecutive days.



## Matrix effects

The matrix effect (ME) was examined by comparing the mean peak areas of the analytes and the IS between two different series. The first series consisted of blank urine samples spiked after extraction with 5 ng/mL of  $\alpha/\beta$ -nandrolone or ractopamine, while the second series consisted of reference standards (5 ng/mL of each analyte, including the corresponding ISs). The ME was defined as follows:  $ME\% = 100 \times \text{series 1} / \text{series 2}$ , and indicated the possibility of ionization suppression or enhancement for analytes and IS; an endogenous matrix effect was implied if the ratio was less than 85% or more than 115%.[\[22\]](#)

## Recovery

The recoveries for the concentration levels 2.5, 5, and 10 ng/mL for nandrolone and 5, 25, and 100 ng/mL for ractopamine were determined on fortified blank specimens. The resulting peak area ratios (analyte/IS) were compared with those obtained by processing the corresponding area ratios of the standards.

## Urine collection from control and treated veal calves

Urine samples were collected from 26 three-month-old male Friesian veal calves, from which 20 had not been exposed to any treatment while 6 were used treated with nandrosol and ractopamine. Nandrosol was given at a dose of 150 mg/day/animal *im* four times in a two-month period, every 15th day. Ractopamine was administered continuously *per os* 80 mg/animal daily from the 25th to the 55th day. The animals were sacrificed three days after the end of experimental treatment. The urine from the six animals enrolled in an experimental study was taken before the treatment (after spontaneous micturition, taking care to prevent faecal contamination) and directly from the bladder at the slaughterhouse. The samples were divided into aliquots and stored at  $-80\text{ }^{\circ}\text{C}$  until the analysis was performed.

## Results and discussion

At the beginning of this study, the basic idea was to develop and validate a unique method that would be capable of monitoring alterations in the concentrations of nandrolone and ractopamine during the experimental treatment of animals. In the literature, many studies deal with multi-residual and multi-class determination of veterinary drugs.[\[17-20\]](#) Most of them are screening methods that represent a compromise in terms of sample preparation, purification, and any possible hydrolysis. Leporati *et al.*[\[18\]](#) successfully found a compromise regarding the simultaneous screening of 30 veterinary drugs including  $\alpha$ -nandrolone and ractopamine, but not  $\beta$ -nandrolone, as some interference from blank urine samples obstructed its evaluation. On the other hand, Zhang *et al.*[\[19\]](#) determined  $\beta$ -nandrolone and ractopamine, without any reference to  $\alpha$ -nandrolone. Nevertheless, we are unaware of any studies that provide the simultaneous determination of  $\alpha$ - and  $\beta$ -nandrolone together with ractopamine, which we particularly intended to evaluate. However, from the beginning, we faced different problems regarding the integrated extraction and deconjugation procedure that could be applied for all of the analytes in question. Subsequently, we realized that ESI-MS/MS conditions were also different. Consequently, our ultimate aim was to set up highly specific analytical procedures for both compounds, which would be used in pharmacokinetic purposes and in monitoring the elimination rate, not just for the screening. Therefore, in order to reach the optimal analytical conditions for either nandrolone or ractopamine, we decided to perform two separate methods. Each method was validated and was shown to be specific for the analyte in question.

## Method validation

### Nandrolone assay

The first issue in nandrolone/ractopamine determination was setting up a deconjugation procedure. Initially, many efforts were made to develop unique deconjugation processes that would be suitable for either nandrolone or ractopamine. The deconjugation procedures were studied separately and based on our experience regarding screening the steroid hormones in general.[23, 24] Principally, two enzymes,  $\beta$ -glucuronidase from *Escherichia coli* K 12 (EC 3.2.1.31) and  $\beta$ -glucuronidase/arylsulphatase from *Helix pomatia*, were considered in order to find the best solution that would accomplish our purpose. Furthermore, when preliminary examinations of few samples originating from animals enrolled in experimental treatment with nandrolone/ractopamine were performed, an enormous difference in urinary concentration of two compounds was observed. All this led us to conclude that it would be better to use two separate deconjugation methods.

As far as bovine urine is concerned, the greater part of anabolic hormones is excreted predominantly as glucuronide conjugates.[25] For this reason, we used selective glucuronide conjugate hydrolysis by recombinantly produced  $\beta$ -glucuronidase enzyme from *Escherichia coli* K 12 (EC 3.2.1.31) to measure the concentrations of combined free and glucuronide forms of both nandrolone isomers. The final conditions of enzymatic hydrolysis (concentration of the enzyme, incubation period and temperature) were adjusted after performing a series of experiments, followed by LC-MS/MS determination of the released unconjugated form. When the concentration of the free forms reached a plateau, efficiency was recognized to be 100%. The results are presented in the Supporting Information (Figure S1). The temperature of the enzymatic hydrolysis was adjusted at 55 °C, as lower temperatures (37 °C) required very long incubation times. We observed that an enzyme concentration of 3.2 U/mL was adequate to achieve complete hydrolysis of the nandrolone conjugates after 4 h of incubation at 55 °C; those conditions were adopted as final.

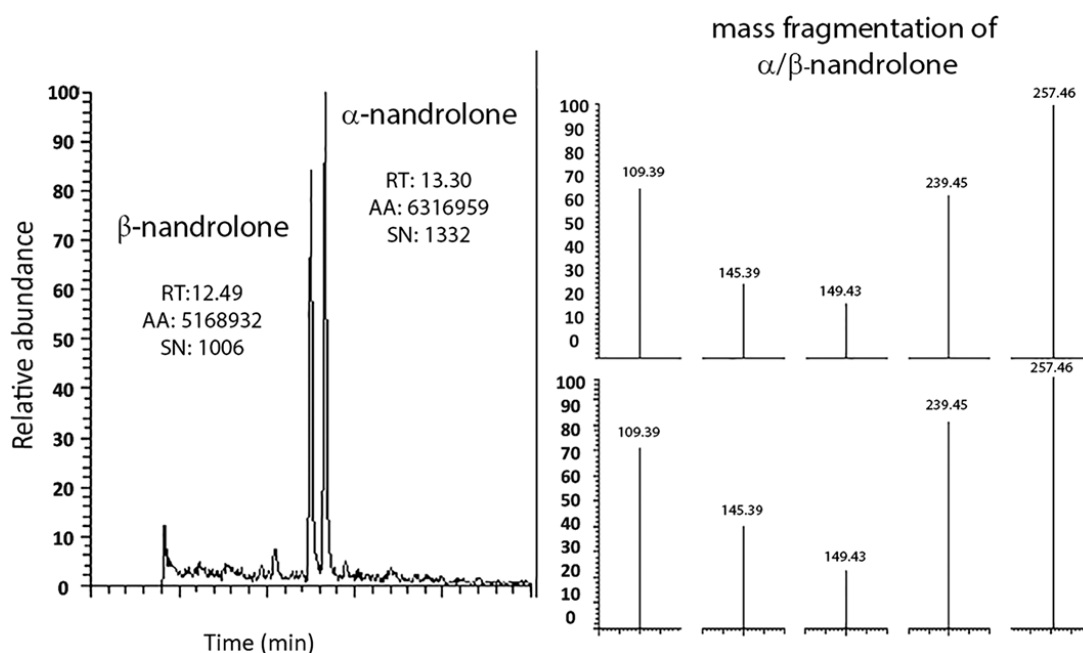
The reason for the employment of pure, recombinantly produced  $\beta$ -glucuronidase enzyme is an important point of clarification, as it can be speculated that alternative methods (using *Helix pomatia* juice) that hydrolyse both glucuronide and sulphate conjugates may result in higher concentrations than those reported here. For this reason, during preliminary experiments, the simultaneous cleavage of glucuronides and sulphate conjugates was performed by applying the *Helix pomatia* juice. The concentration did not significantly change (data not shown), but heterogeneous properties of *Helix pomatia* mixture provoked more pronounced matrix effects and a lower S/N ratio.

As far as sample purification is concerned, different possibilities were considered starting from final conditions of deconjugation process that immediately preceded. Initially, we tried with the mixture *tert*-butyl methyl ether/ethyl acetate (4: 1 v/v) in attempt to establish the simple liquid-liquid extraction (LLE) for both analytes. This kind of purification was adequate for ractopamine with recovery more than 87% (explained in details in ractopamine assay section) while gave just a moderate recovery (not higher than 52%) in the case of  $\alpha$ - and  $\beta$ -nandrolone. Moreover, nandrolone efficient determination was endangered by the fact that LLE did not remove the interferences that triggered pronounced ion suppression (matrix effects were 62% and 71% for  $\beta$ -nandrolone and  $\alpha$ -nandrolone, respectively). It became evident that final pH (6.2) after deconjugation process does not favour LLE of nandrolone. Moreover, under this pH, formation of emulsion was occurred, affecting reproducibility and accuracy. This practically eliminate the possibility to use LLE in nandrolone assay, directing us towards solid phase extraction (SPE), which indeed turned out to be indispensable for nandrolone.

Recently, a new class of very efficient hydrophilic-lipophilic balanced (HLB) polymers has been induced as stationary phase in SPE. The main characteristics of these polymers are the ability to remain wetted and to retain analytes. The use of these polymeric phases permits more reproducible and less tedious extraction processes than with the conventional silica-based SPE cartridges. Therefore, we applied Oasis HLB cartridge in order to quantitatively extract nandrolone while minimizing interferences present in the urine matrix. The sample volume was set at 1 mL, and it was noticed that any further increase in sample volume led to cartridge overload. A dual washing step with 10/90 methanol in water followed by 2% ammonia in a 40/60 methanol/water mixture was performed. This washing procedure proved to enhance clean-up without eluting nandrolone. Employment of weak alkaline conditions was of particular importance, because this step eliminated visible bilirubin interferences, which resulted in clean chromatograms with acceptable matrix effects values: 94% for  $\alpha$ - and 87% for  $\beta$ -nandrolone. Acetone, acetonitrile, and methanol were tested as the elution solvents. Finally, methanol provided the highest extraction recovery (87%) and was selected as the most efficient eluent.

To obtain satisfactory separation and high sensitivity of the target analytes, optimization of the liquid chromatography and mass spectrometry conditions was performed by the injection of standard solutions of a mixture of two nandrolone isomers.

After preliminary trials, in full-scan mode from 50 to 500  $m/z$ , the five product ions with the higher S/N ratio for each analyte and IS were chosen for identification. The CE was adjusted in the MRM mode for each transition monitored in order to reach the highest sensitivity and specificity for the two isomers. The five diagnostic product ions, among which is the ion for the quantification, the relative intensities, and CEs are reported in Table 1. The  $m/z$  values of the selected precursor and fragment ions used for MS detection are generally in agreement with those previously reported in the literature, especially when it concerns quantifier ions for  $\alpha$ - and  $\beta$ -nandrolone (transition 275  $\rightarrow$  109) and d3- $\beta$ -nandrolone (transition 278  $\rightarrow$  109).<sup>[7]</sup> The transition 275  $\rightarrow$  257 used in confirmation purposes for  $\alpha$ - and  $\beta$ -nandrolone is analogous to the former MS/MS determination,<sup>[21]</sup> while other fragmentation patterns appeared to be specific for MS/MS technique that we applied. However, no attempt was made to acquire different fragmentation patterns and thus to distinguish  $\alpha$ - from  $\beta$ -nandrolone. Therefore, the gradient elution programme of 0.1% formic acid in water and methanol with suitable flow rate was carefully optimized until it permitted the complete separation of the two isomers. The Synergi Hydro RP column was our first choice as we used it successfully for anabolic hormone determination,<sup>[23, 24, 26]</sup> Also here, in the case of nandrolone, it showed good performances and satisfactory peak shapes and baseline separation. The MRM chromatogram of a sample spiked with 5 ng/mL  $\alpha$ - and  $\beta$ -nandrolone is presented in Figure 2.



**Figure 2. LC-MS/MS chromatographic profiles and corresponding MS fragmentation of  $\beta$ - and  $\alpha$ -nandrolone, standards (5 ng/mL, each), and internal standard (d3- $\beta$ -nandrolone, 2 ng/mL) in blank urine sample.**

Specificity and selectivity were evaluated as described in the materials and methods section, applying the Commission Decision 2002/657/CE.[27] Blank samples (n = 20) were analysed and did not show any interference (signals, peaks, ion traces) in the region of interest where the target analytes were expected to be. The relative retention time of the analyte corresponds to that of the standard, with a tolerance of  $\pm 2.5\%$ . Relative intensities of the five product ions, expressed as a percentage of the intensity of the most intense ion, correspond to those of the reference analyte. The ion ratios of the two product ions with relative intensities greater than 50% did not exceed the tolerance of  $\pm 20\%$ .

Five concentration levels with three replicates each were used to build the regression line by means of the least square method. The equations of calibration curves were used to calculate unknown concentrations in real urine samples of bovines enrolled in the experimental study. Regression coefficients of the curves indicated very good linearity for both compounds. The LLOQ values (Table 2) indicated that this method could be used for confirmation purposes, as the recommended concentration for both isomers is set at 1 ng/mL.[28] Precision, defined as the closeness of agreement between independent test results obtained under stipulated conditions, is herein expressed as repeatability. The repeatability (intra- and inter-day) was satisfactory, with the relative standard deviation (RSD) ranging from 6% to 17%, as shown in Table 2. Instrumental precision was 9% and 5% for  $\alpha$ -nandrolone and  $\beta$ -nandrolone, respectively. These results show a good repeatability, indicating a stable instrumental response as far as the performance of the LC-MS/MS-ESI system is concerned.

Table 2. Validation results for  $\alpha/\beta$ -nandrolone and ractopamine

Analyte	Calibration curve (n = 3)	R <sup>2</sup>	LLOQ	Spiked level (ng/mL)	Recovery (% n = 18)	Repeatability	
						intra-day (RSD; n = 6)	inter-day (RSD; n = 18)
$\alpha$ - Nandrolone	Y = 0.4714 ( $\pm$ 0.0088) X + 0.1787 ( $\pm$ 0.0991)	0.9944	0.5	2.5	87	13	17
				5	86	12	10
				10	94	7	10
$\beta$ - Nandrolone	Y = 0.4063 ( $\pm$ 0.0098) X + 0.1703 ( $\pm$ 0.1107)	0.9907	0.5	2.5	81	14	15
				5	92	10	12
				10	95	11	6
Ractopamine	Y = 0.0792 ( $\pm$ 0.0078) X + 0.5258 ( $\pm$ 0.1471)	0.9913	0.5	5	74	14	17
				25	84	7	12
				100	108	14	13

## Ractopamine assay

As already mentioned, obtaining a unique deconjugation for both compounds would be much better from a practical point of view, but from the outset it was too difficult to find a compromise. Applying an already adopted procedure for anabolic hormones to ractopamine resulted in a number of problems: we noticed that using  $\beta$ -glucuronidase from *Escherichia coli* led to incomplete hydrolysis of ractopamine conjugates, while  $\beta$ -glucuronidase/arylsulphatase from *Helix pomatia* increased the yield. The results are presented in the Supporting Information ([Figure S2](#)).

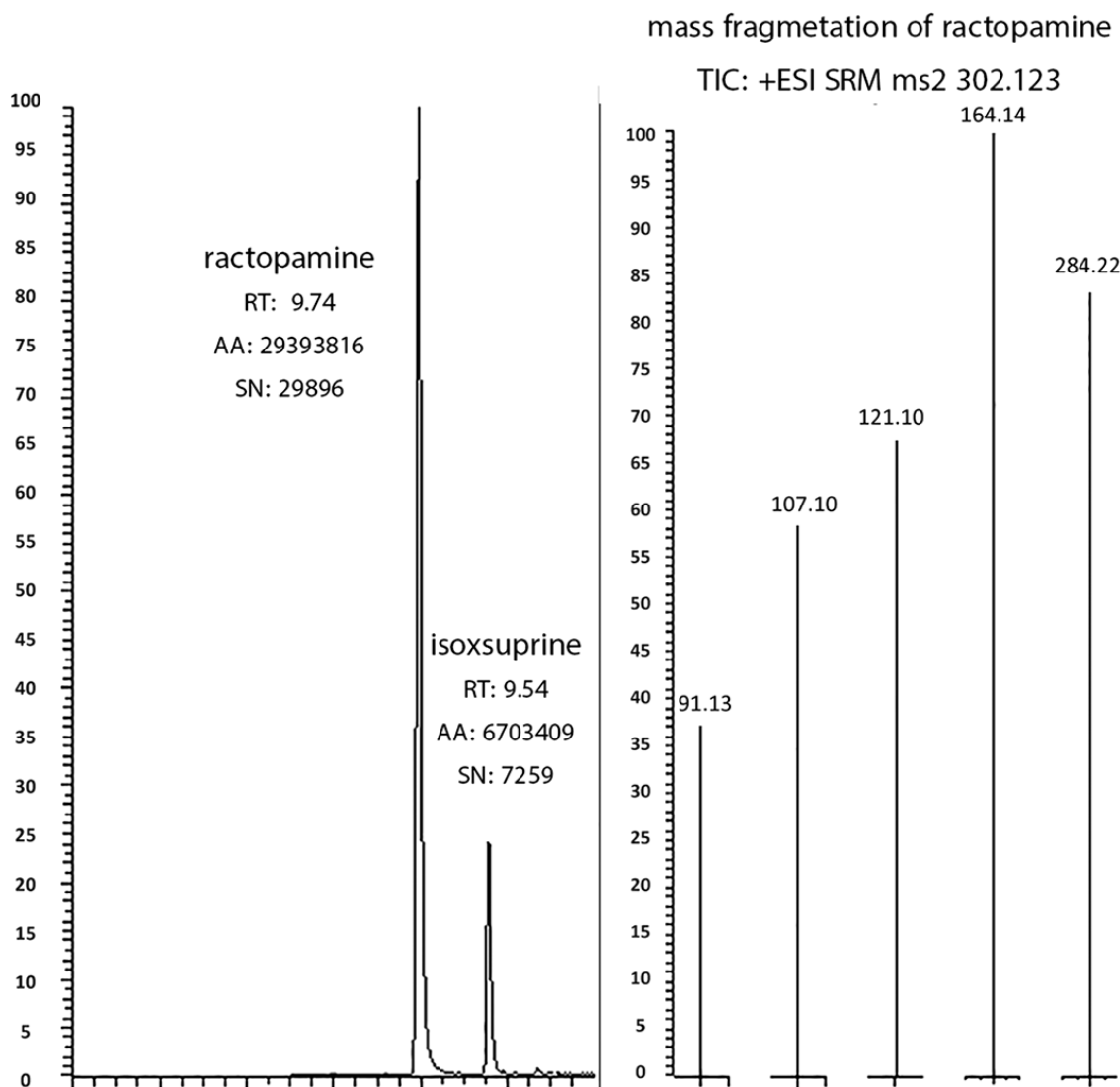
Since we aimed to determine the total ractopamine content in bovine urine, the analytical strategy required a hydrolysis step to release the ractopamine from its sulphate and/or glucuronic acid conjugates.  $\beta$ -glucuronidase/sulphatase from *Helix pomatia* was chosen after a series of experiments documented it as an appropriate enzyme source. The sulphatase activity in addition to glucuronidase activity was essential, as ractopamine is present in both conjugated forms. The amount of *Helix pomatia* juice, temperature, and duration of hydrolysis were optimized as 20  $\mu$ L of enzyme solution at 45 °C for 4 h. No substantial enhancement in ractopamine yield was observed when any of these parameters increased ([Figure S2](#)).

After hydrolysis, the urine pH was adjusted to approximately 8.5–9.5. This was fundamental considering that ractopamine contains one secondary amine group, two acidic phenolic moieties, and one tertiary alcohol function ([Figure 1](#)). The ionic forms of those functional groups, which exist

under weakly basic conditions, are favourable for the subsequent extraction by organic solvent. Simple LLE was selective enough for purification. Various experimental conditions of LLE were compared by selecting different organic solvents with the best recovery results achieved for a mixture of *tert*-butyl methyl ether and ethyl acetate (1:4, v/v). This combination was especially advantageous for the extraction of isoxsuprine (extraction recovery 92%), which possesses the aromatic ether function and is thus readily dissolved in an organic mixture (Table 2).

In order to accomplish the highest possible sensitivity and to find the specific MS/MS transitions, the instrument parameters were optimized by direct continuous pump infusion of standard in both full-scan and product ions mode. Experiments were carried out under positive and negative polarity with different compositions of mobile phase additives in order to improve ionization efficiency. The best results were obtained by operating in positive ion mode using formic acid (0.1% in aqua) and acetonitrile. The possibility of using a 0.1% aqueous solution of ammonium-formiate was also examined. It gave a better absolute response, but the chromatographic separation was not satisfactory with pronounced background noise. An acetonitrile and formic acid aqueous solution combination was found to provide acceptable resolution and symmetrical peak shapes for ractopamine and its IS, and it was therefore adopted as the mobile phase.

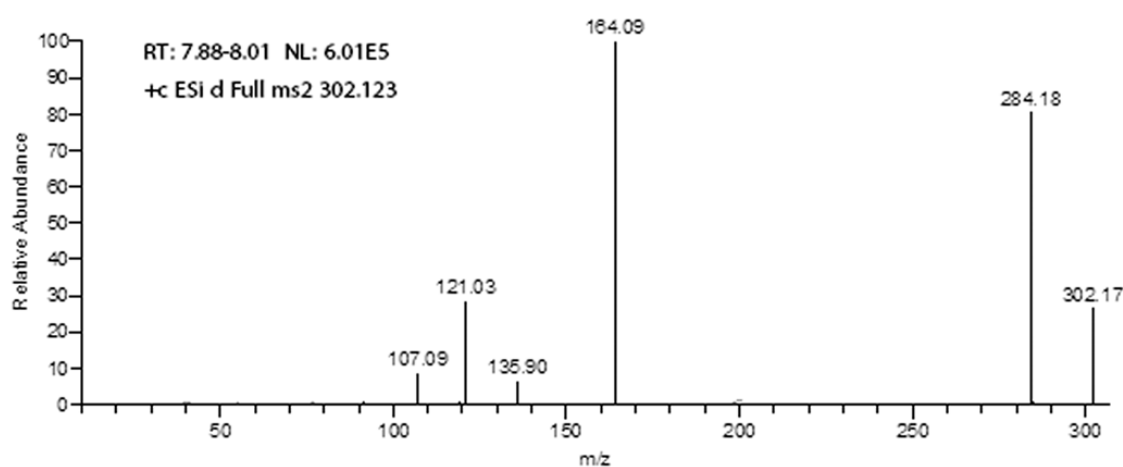
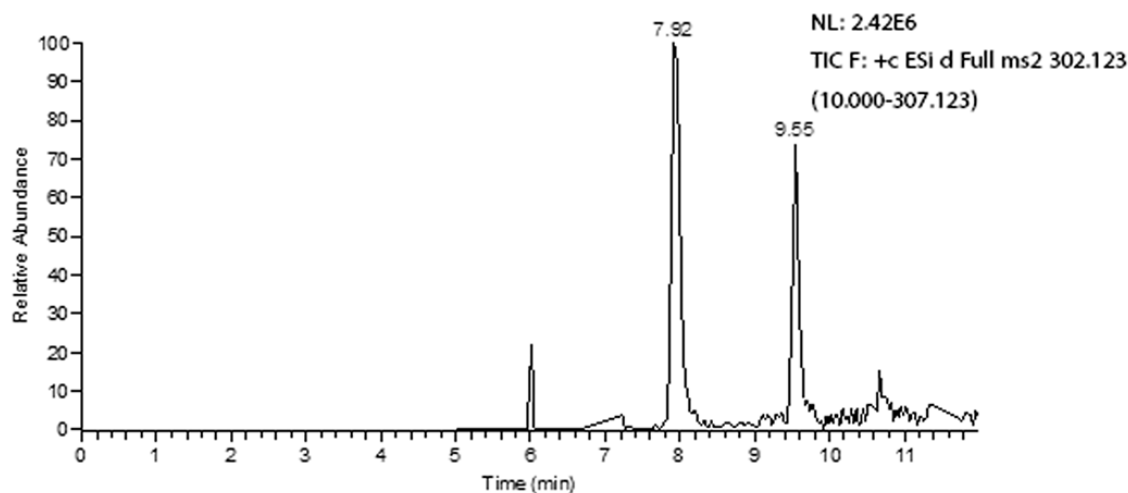
Ractopamine and isoxsuprine (IS) are structural isomers; they possess the same phenethylamine residue, yet differ in ether and phenolic function. This causes a significant difference among their polarities, but both of them provide equal pseudo-molecular ions ( $m/z$  302). Consequently, it was crucial to separate them chromatographically (Figure 3) and to optimize their fragmentation patterns. The obtained product ions are consistent with results published elsewhere.[29, 30] Table 1 shows the optimized fragment voltages for the  $[M + 1]^+$  ions, while exemplifying total ion current chromatograms of ractopamine and isoxsuprine in real urine sample are present in Figure 3. Furthermore, both compounds share the same fragment ions (284, 107), with fundamental differences in fragment 164 that originate from ractopamine and 150 emerging from isoxsuprine. This is why those two product ions were chosen for quantification (Table 2). The ratios between the quantitative ion and the qualifier ions were within  $\pm 20\%$  in order to meet the criterion for a positive result. Preliminary direct infusion tests revealed that it was also possible to use ion 284 as a precursor ion for ractopamine characterization. This ion can be formed in Q1 by so-called instrument-induced dehydration.[31] The formation of this 'pseudo-precursor' ion can be preferential if the higher transfer tube temperature is applied. The problem with this species arose from the findings that an interference with similar fragmentation pattern and at the retention time very close to ractopamine appeared during the specificity test. This unknown compound appeared only after the hydrolysis of blank samples, while examination of the same sample without enzymatic deconjugation did not revealed its presence.[32]



**Figure 3.**

LC-MS/MS chromatographic profiles and corresponding MS fragmentation of ractopamine (7 ng/mL) and internal standard (isoxsuprine, 2 ng/mL) in real urine sample.

Keeping in mind these findings, in order to eliminate the influence of minor contaminants, along with commonly used MRM, we introduced the so-called QED scan in mass spectrometry acquisition. When using QED, a ‘full-scan MS/MS’ mass spectrum is obtained by data-dependent scanning for confirmatory analysis during the single reaction monitoring (SRM) experiment, which is used for routine quantitation. Once a particular SRM transition reaches a ‘user set’ intensity threshold, the instrument automatically triggers QED. The QED scan generates a highly sensitive, fragment-rich full MS/MS spectrum that can be used to positively confirm the existence of a compound (Figure 4). This feature allows additional positive confirmation data, providing the required number of identification points needed for the positive presence of a compound.[27] While excellent linearity and quantitative results were gathered using MRM transitions, additional QED data were collected during each run.



**Figure 4.**

The full ion spectra of ractopamine by using the QED-MS/MS technique. The spectra have fragment information, which facilitates distinction from possible interference.

Employment of the QED mode allows significant time and cost savings by giving priority to the simple LLE over techniques based on SPE. On the other hand, the matrix effect is an important issue in electrospray ion sources, especially when straightforward LLE purification is practiced. Large amounts of endogenous compounds may potentially co-elute with target analytes and affect the efficiency of the ionization process. In this study, 86% (ractopamine) and 114% (IS) alternations in signal intensity were observed when comparing the matrix-matched standards with the standard solution. This implies that plain LLE clean-up is effective in removing interferences.

The selectivity was investigated by analyzing 20 blank samples in order to verify the absence of interfering substances. The obtained chromatograms (MRM and QED) demonstrated that no interfering peaks appeared within the 2.5% margin of the relative retention time of ractopamine.

Validation in terms of linearity, recovery, and repeatability was performed in the same manner as for nandrolone, as explained in the materials and methods section. Satisfactory results were obtained (Table 2), indicating good method performance. Apart from the validation linearity, the quantification calibration curve was constructed, too. The reasons are that during the real sample analysis, the concentrations found in the majority of them were above the highest point of validation calibration curve. The concentrations were extrapolated from calibrations that cover the entire



concentration range (0–1000 ng/mL), with eight scalar levels included. Satisfactory linearity was obtained ( $R^2=0.9891$ ).

The achieved LLOQ (0.5 ng/mL) is highly acceptable, and is comparable with one of the most sensitive methods published so far,[\[33\]](#) where the authors described a method based on a biosensor for the monitoring of ractopamine residues in urine.

## Application of methods on real urine sample

In order to confirm the applicability of both methods and draw attention to the fact that each veterinary medicine should be monitored very carefully, the developed methods were applied on real urine samples from control and from veal calves enrolled in an experimental treatment consisting of the intramuscular administration of  $\beta$ -nandrolone-phenylpropionate accompanied with ractopamine given as a dietary supplement. Before implementation of the protocol, the urinary presence of the test substances was not discovered in any of the animals involved. This is particularly important for nandrolone, since under certain physiological conditions, it is considered pseudo-endogenous.[\[4, 33-35\]](#) The quantitative analysis of target compounds initially produced questionable results, particularly when it came to differences in the order of magnitude of the obtained concentration: maximum levels were up to 5.2 ng/mL for  $\alpha$ -nandrolone while the amount of ractopamine was several hundred ng/mL. However, this could be expected given the diverse treatment schedule with the distinct distribution mode. Moreover, those drugs have completely different metabolic pathways and elimination outcomes.

$\beta$ -nandrolone-phenylpropionate was exclusively eliminated as conjugated  $\alpha$ -nandrolone, which confirms that this species is a predominant metabolite of the parent drug.[\[9, 13, 36\]](#) All real trial samples were analyzed with and without hydrolysis, and the free forms of both nandrolone isomers were not detected in any of them. The drug was intramuscularly administered four times at the dose of 150 mg/animal, which resulted in the detectable accumulation of its main metabolite in the urine taken at slaughterhouse. It should be emphasized that the veal calves enrolled in this study ( $n = 6$ ) excreted detectable residues of  $\alpha$ -nandrolone ( $3.5 \pm 1.5$  ng/mL) 11 days after the last treatment, which is very important when considering the suggested recommended concentration for nandrolone.[\[28\]](#)

Unlike  $\beta$ -nandrolone, ractopamine was administered in a completely different way: over 31 consecutive days, *per os* at the dose of 80 mg/day. The samples were also analyzed before and after hydrolysis, and the free ractopamine was detected but never exceeded 5% of the total amount obtained after hydrolysis. Those results are in line with the results achieved by Smith and Shiver,[\[27\]](#) indicating considerable ractopamine accumulation and postponed excretion pattern, to some extent comparable with the results obtained by Liu *et al.*[\[30\]](#) After a short withdrawal period (3 days) at a slaughterhouse, a substantial urinary amount was detected ( $252 \pm 144.5$  mg/mL), which is consistent with previously published data.[\[37, 38\]](#) In addition, a study carried out on heifers reported detectable ractopamine residues in urine 5 to 7 days after the last exposure to dietary ractopamine, pointing out that the hydrolysis of ractopamine metabolites may extend the period in which it is detectable in cattle.[\[32\]](#) This trend was confirmed by a study that was recently published by Tang *et al.*,[\[39\]](#) who investigated ractopamine depletion in cows.

## Conclusions

With this paper, we want to emphasize that the screening methods applied commonly in routine analysis, are not always the best solution for the evaluation of levels of veterinary drug administered to animals in very low dose. The reported research provides some useful insights into the

mechanisms governing the persistence of  $\alpha$ -nandrolone and ractopamine in veal calves' urine following experimental treatment with corresponding parent growth-promoter substances. The highly specified procedures reported herein makes possible to proceed with studies of the combined physiological effect of anabolic steroids and  $\beta$ -adrenergic agonists administered together.

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