THESE DE DOCTORAT DE

ONIRIS

ECOLE DOCTORALE N° 605
Biologie Santé
Spécialité : « *SANTE PUBLIQUE* »

Par
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Study of some aryl-propionamide SARMs metabolism in food producing animals. Highlighting biomarkers of exposure and effects for control purposes.

Thèse présentée et soutenue à la faculté des Sciences Vétérinaires, Université de Buenos Aires, Argentine, le 25 juin 2020
Unité de recherche : UMR 1329 LABERCA

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Thesis

Convention de Cotutelle Internationale de Thèse Doctorale entre

ONIRIS – Universidad de Buenos Airs

STUDY OF SOME ARYL-PROPIONAMIDE SARMS METABOLISM IN FOOD PRODUCING ANIMALS. HIGHLIGHTING BIOMARKERS OF EXPOSURE AND EFFECTS FOR CONTROL PURPOSES.

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Dedication

To Vivi, my mother and father for their love, company and encouragement...

Acknowledgements

To my family and my colleagues (from INTA, from Facultad de Ciencias Veterinaria de la UBA y from LabERCA).

Special thanks for your help to Andrea Calzetta, Gaud Dervilly, Bruno LeBizec, Ariel Miranda, Mariana Galicio, Maria Juana Ruiz, Alba Castro, Susana Rojas, Diego Cristos, Diego Fernandez, Gaston Pelissier, Martin Paladino, Alexis Sosa, Alexander Sydor, Zied Kaabia, Gerome Laparre, Tao Peng, Berenger Marais, Kristine Hennig, Margarida Cardoso, Natalia Pesquero, Gabriela Denoya, Juan Manuel Rojas, Geronimo Ortigosa, Fernanda Martinez, Juan Carlos Fernandez, Claudia Gonzalez, Marcelo Masana y Sergio Vaudagna.

Abstract

At present, the chemical industry produces veterinary drugs that are used in livestock production for animal health care or to improve zootechnical aspects that increase production yield. However, the misuse of these substances may lead to residues that may remain in food derived from treated animals. Finally, these chemical residues could be ingested by the human consumer and it could eventually cause some negative effect on their health.

Depending on the toxicity of such residues in food, veterinary drugs are regulated by voluntary implementing regulations or mandatory legislation. These regulations may change according to the criteria considered by each country and consequently international food trade may be affected by these differences in tolerances for waste between countries.

Growth promoters are substances that are ingested, injected or implanted in animals that improve production performance. There are a large number of substances that increase weight gain, feed efficiency and protein percentage in the carcass. The mechanism of action of these substances to gain weight can go from medicines that eliminate parasites favoring the correct assimilation of the ingested nutrients to chemicals with mechanism of action at the cellular level of the skeletal muscle that stimulate protein synthesis.

In livestock, the growth promoters traditionally used are anabolic androgenic steroids, b2-agonists and Growth hormone (GH). Other anabolic substances used include zeranol, anabolic antimicrobials, stylebens and thyrostatics substances.

Emerging anabolic practices include GH secretagogues, IGF-1 analogues (Insulin Growth Factor-1) and Selective Androgen Receptor Modulators (SARMs).

Selective modulators of androgen receptors are a new family of non-steroids substances, which has the ability to bind to androgen receptors (AR). Ideally they have selectivity in their effect (anabolic or androgenic) depending on the tissue, they have low toxicity (hepatic, cardiovascular, etc.) and they possess favorable pharmacological properties such as high digestive absorption facilitating oral

administration and show a long average life. For these reasons, they have been already sold on the Internet and cases have been detected in sport (animal and human). With this background, it is to be hoped that the SARMs will follow the same path as other anabolic compounds until they reach animal production.

In Argentina since 1961, substances with anabolic effect have been regulated and restrictions have increased over time. Establishing the importance of ensuring food quality and strengthening international trade.

In Europe, as economic community, control and restriction for the use of anabolic agents in livestock farming began in 1981. The employment of growth promoters is currently prohibited in food producing animals.

The study of the metabolism of these chemicals is essential when controlling livestock practices. This is because after administration, a substance suffers metabolic reactions within the body. Thus, the concentration of the administered drug decreases at the expense of the increase of the products of these metabolic reactions (metabolites). Therefore, some metabolites could remain as indicators of the use of the substance in higher concentrations and for longer periods of time than the administered drug.

The general objective of this paper is to provide tools for the control of SARMs in livestock production, developing analytical methods and generating information about their metabolism in food producing animals. In the study liquid chromatography coupled to mass detectors was applied, which is a confirmation method and provide structural data for unknown substances.

Enobosarm, Andarine, Bicalutamide and Hidroxyflutamide were studied in different stages of this work because of the accessibility to get each of them.

The analytical methods developed for the SARMs were evaluated to fullfill Argentine and European normative criteria. A method was achieved for the control of Enobosarm, Andarine, Bicalutamide and Hidroxyflutamide in urine and faeces, while for serum a method was developed for Bicalutamide and Andarine.

Two animal trials were conducted to learn about metabolic aspects of these substances. The first with Bicalutamide administration to a bovine (Montbeliard

breed), taking urine and faeces. In the second test cattle (Angus breed) and pigs (Chetapuy breed) were used, worked as follows: 6 cattle in 2 groups of 3 animals: treated with Bicalutamide and control (untreated); 3 pigs in three groups: treated with Bicalutamide, treated with Andarine and control (no treatment). Urine, faeces and blood were collected after administration, as they were easily accessible for routine work. The urinary and fecal elimination profiles with the corresponding serum level curves were established.

Bicalutamide was found to have rapid absorption and slow elimination, with detectable concentrations in excreta for more than one week (9 – 11 days). In contrast, Andarine showed rapid elimination (48 hs.) together with rapid absorption.

Phase I and Phase II metabolites were found in animal excreta. The results showed 5 metabolites in cattle treated with Bicalutamide but only 4 of them in the pig treated with the same substance. In contrast, the porcine treated with Andarine presented 12 metabolites. Based on the information provided by the mass spectrometry it was possible to propose the chemical structure of 16 metabolites found, leaving only one metabolite of Andarine without structure proposed.

After analysing the results in cattle treated with a single dose of Bicalutamide, it can be concluded that the lyophilized fecal matter is the best matrix for control. The markers in faeces that showed the highest signal were M3 followed by Bicalutamide (without modification). In urine the best marker was M2 followed by M5. Despite the large signal obtained for these metabolites, Bicalutamide (without modification) was the substance that remained most detectable, 7 days in urine and 11 days in faeces. For the control of Bicalutamide in pigs with a single dose, Bicalutamide was selected in both matrices based on intensity and signal duration. The control of Andarine and its metabolites in pigs poses a difficulty that lies in the short period that remains detectable.

This work is the first scientific study carried out with oral administration of Bicalutamide in food producing animals. It is the first work done with Andarine in

pigs of Chetapuy breed. In addition, it is the first work to report 2 of the 5 proposed Bicalutamide metabolites.

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ABREVIATURAS

SARMs: Selective Androgenic Receptor Modulator

APA: Aryl-Propionamide

APA-SARMs: Aryl-Propionamide SARMS

AES: Anabólicos Esteroides Androgénicos

GH: Growth Hormone

IGF-1: Insulin Growth Factor-1

AR: Androgen Receptors

AcH: acetic acid

ACN: acetonitrile

ADR: Adverse Drug Reaction

APCI: Atmospheric Pressure Chemical Ionization

API: Atmospheric Pressure Ionization

APPI: Atmospheric Pressure Photoionization

CYP: Cytochrome P450 Enzyme

DMSO: Dimetil Sulfoxide

ECVAM: European Centre for the Validation of Alternative Methods

EMS: Enhanced Full Mass Scan

EPI: Enhanced Product Ion Scan

ER: Enhanced Resolution Scan

ESI: Electrospray Ionization

FA: formic acid

TBME: Methyl t-butyl ether

FWHM: Full Width at Half Maximum

GC: gas chromatography

GSH: Glutathione (reduce form)

GST: Glutathione S-transferase

HLH: Human Liver Homogenate

HLM: Human Liver Microsomes

HPLC: High-Performance Liquid Chromatography

HRMS: High-Resolution Mass Spectrometry

IADR: Idiosyncratic Adverse Drug Reaction

IDA: Information Dependent Acquisition

IT: Ion Trap

KCN: Potassium Cyanide

LC: Liquid Chromatography

LLE: Liquid-Liquid Extraction

MDF: Mass Defect Filter

MeOH: methanol

MRM: Multiple Reaction Monitoring

MS: Mass Spectrometry

MS/MS: Tandem Mass Spectrometry

m/z: mass-to-charge ratio

NADPH: Nicotinamide Adenine Dinucleotide Phosphate(forma reducida)

SRM: Single Reaction Monitoring

STD: standard

ISTD: internal standard

SPE: solid phase extraction

AA: ammonium acetate

AF: acid formic

AcH: acetic acid

FA: ammonium formiate.

Hypotheses and Objectives.

HYPOTHESES

- The study of the metabolism of aryl-propionamide SARMs (APA-SARMs) is necessary for the development of analytical methods of control of livestock practices.
- APA-SARMs and its metabolites in urine and faeces samples can be used to monitor abuse in livestock practices.

GENERAL OBJECTIVES

- Study APA-SARMs levels in urine, faeces, and blood in production animals.
- Develop analytical chemistry tools for residue monitoring to implement APA-SARMs control in production animals.

PARTICULAR OBJETIVES

- Develop a Liquid Chromatography coupled to tandem mass spectrometry method for the study of APA-SARMs.
- Identify useful metabolites for the APA-SARMs control in livestock practices.
- Study APA-SARMs and its metabolites excretion in urine and faeces in swine and bovine.

Introduction

FOOD SAFETY AND VETERINARY DRUG

Use of veterinary drugs in livestock could leave residues that may remain in food derived from treated animals. Finally, these chemical residues could be ingested by the human consumer and it could eventually cause some negative effect on their health.

Depending on the toxicity of such residues in food, veterinary drugs are regulated by voluntary implementing regulations or mandatory legislation. These regulations may change according to the criteria considered by each country and consequently international food trade may be affected by these differences in tolerances for waste between countries.

Argentine Legislation

The Argentine legislation proposes to ensure the supply of safe and quality food to domestic consumers and international buyers. Moreover, as a member of the WTO (World Trade Organization), it complies with the SPS Agreement (Agreement on the Application of Sanitary and Phytosanitary Measures) and bases its legislation on scientific evidence.

In Argentina since 1961, substances with anabolic effect have been regulated and use of growth promoters during livestock production is currently prohibited. However, the therapeutic use of oestrogen is permitted.

European Union Legislation

EU legislation seeks to ensure safe food for EU consumers, regardless of whether they were produced in the EU or in third countries.

In relation to the employment of growth promoters in livestock production, European legislation is based on the *precaution principle* established in the SPS Agreement (substances for which the full toxic effect is not known) and based on scientific information showing that under certain

circumstances and doses these substances have toxic effects on the animal and the consumer of food derived from treated animals [1, 2].

In Europe, as an economic community, control and restriction for the use of anabolic agents in livestock farming began in 1981. In that year, its regulations (Directive 81/602/EEC) prohibited certain substances (diethylstilbestrol, other stilbenes and thyrostatics). Then in 1988, the European Community (EC) banned the use of growth promoters in food producing animals [3]. However, the therapeutic use of oestrogen is permitted.

GROWTH PROMOTERS IN LIVESTOCK PRODUCTION

Growth promoters are substances that are ingested, injected or implanted in animals that improve production performance. There are a large number of substances that increase weight gain, feed efficiency and protein percentage in the carcass [4].

In livestock, the growth promoters traditionally used are anabolic androgenic steroids, β2-agonists and Growth hormone (GH). Other anabolic substances used include zeranol, anabolic antimicrobials, stylebens and thyrostatics substances [3].

Emerging anabolic practices include GH secretagogues, IGF-1 analogues (Insulin Growth Factor-1) and Selective Androgen Receptor Modulators (SARMs) [3].

ANDROGENS AND ITS RECEPTORS

Androgens are substances known to develop male sexual characteristics and should also be able to bind and activate androgen receptors (AR) [5]. Testosterone (T), Dihydrotestosterone (DHT) and Androstenodione are natural androgens. Androgens are produced in different organs and a part of T is converted into DHT in tissues (Figure 1). After entering the cells of the target tissues, a part of the Testosterone is transformed into DHT, especially into organs such as the prostate and genitals. Some functions depend on this transformation, that is to say depend on DHT levels. The basic intracellular mechanism of the anabolic effect is attributed to increased protein synthesis in the target cell. [6].

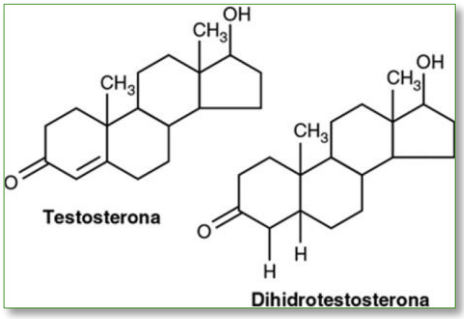


Figure 1. Chemical structure of Testosterone and Dihydrotestosterone. [6]

Androgen Receptors

The androgen receptor (AR) is a member of the superfamily of nuclear steroid receptors. It is a soluble protein, which works as an intracellular transcription factor. Figure 2 show it has three regions : DNA-Binding Domain (DBD), Ligand Binding Domain (LBD) and N-terminal domain (NTD) [7].

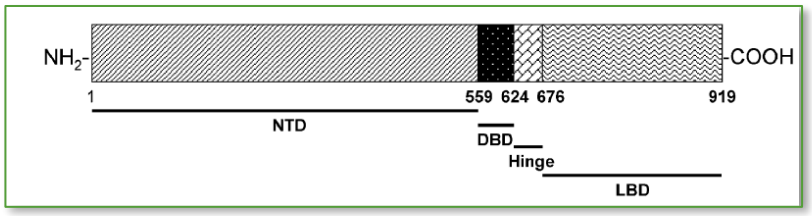


Figure 2. Structure of the human Androgen Receptor [7]

AR remains in the cytoplasm attached to HSP 70 and HSP 90 (Heat Shock Protein, HSP). However, in the presence of an agonist ligand that binds at the LBD region, triggering conformational changes through which it travels inside the Cellular Nucleus, convening cofactors and activating the transcriptional machinery in order to activate the dependent genes of AR under stimulation of agonist ligands (Figure 3) Effects on reproductive tissues (prostate, testicles, seminal vesicles and accessory structures) are known as *androgenic effects* while effects on muscles and bones are called *anabolic effects* [7].

In the last decade progress was made on the mechanism of action of T in the different tissues. There are three types of actions: (a) T can activate AR directly where *5α-reductase* is not expressed; (b) T is transformed to DHT before activating AR (5 % to 10 %); (c) about 0,2 % T is attacked by an aromatase that transforms T into an oestrogen by activating *Oestrogen Receptors* [3, 7].

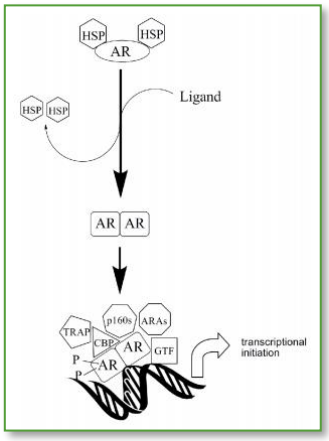


Figure 3. Mechanism of action of the androgen receptor [8]

The formation of DHT is important because it has greater affinity for RA than T, giving a more powerful response in "*DHT-dependent*" tissues such as prostate, testicles, seminal vesicles and accessory structures (androgenic effects). Oestrogens generated by T transformation would be strong regulators of metabolic processes related to behavior and cognition, cardiovascular activity, sexual function including libido and bone turnover processes in the male. However, the highest proportion of T would act without changes in "*DHT-Independent*" tissues or where 5α-reductase is poorly expressed, acting directly on the growth of skeletal muscle, bone tissue formation, the distribution of adipose tissue and sexual function [7, 9].

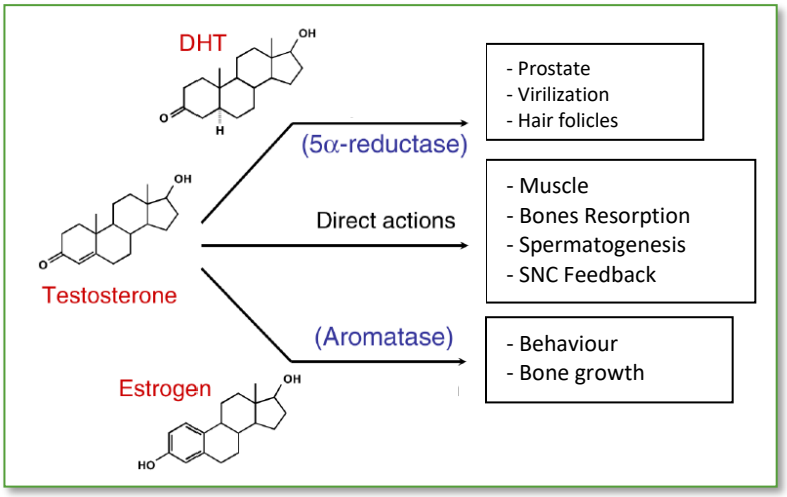


Figure 4. Effects of Testosterone on tissues [3, 9].

T can bind to androgen receptors (*AR*) directly. In some cases, according to the enzymes present in the tissue, T can be transformed by *5α-reductase* to join the AR with greater affinity than T. In other tissues, an *Aromatase* is present capable of transforming T and enabling its binding to oestrogen receptors (*ER*).

SELECTIVE ANDROGEN RECEPTOR MODULATORS (SARMS)

Anabolic androgenic steroids have the disadvantage of simultaneously producing anabolic and androgenic effects. In addition, its therapeutic use is limited due to low oral bioavailability, cross reactivity with other steroid receptors, liver toxicity and other undesirable side effects in the prostate and cardiovascular system [5, 10-12].

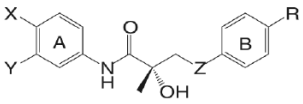
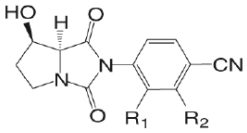
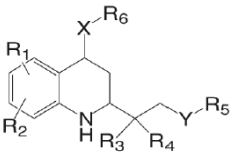
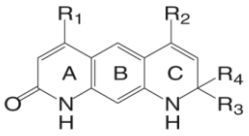
The term selective androgen receptor modulators SARMs and its tissue selectivity was first reported in 1998. SARMs are a new family of non-steroids substances, which has the ability to bind to androgen receptors (AR) [13, 14]. Ideally they have selectivity in their effect (anabolic or androgenic) depending on the tissue, they have low toxicity (hepatic, cardiovascular, etc.) and they possess positives pharmacological properties such as high digestive absorption facilitating oral administration and show a long average life[7, 8, 15, 16].

The mechanism of action of the SARMs is not fully established. However, the probable hypotheses are not susceptible to the action of the *5α-reductase* that makes possible the action of steroid androgens in some tissues (Figure 4) or generate a different conformational changes on AR in different tissues, modifying the recruitment of cofactors at the genomic level (Figure 3).

(Table 1), MRSA can be classified into at least four classes: 1) arylpropionamide (APA), 2) bicyclic hydantoin, 3) quinoline, and 4) analogs of tetrahrahrahroquinolein.

Considering their chemical structures, SARM can be classified into at least four classes as show Table 1: 1) arylpropionamide (APA), 2) bicyclic hydantoin, 3) quinoline, and 4) Analogues of tetrahrahrahroquinolein.

Table 1. Non steroid SARMs Families [17].

Chemical Families	General Structure
Analogues of Aryl-propionamide	
Analogues of Bicyclic Hydantoin	
Analogues of Tetrahydroquinolein	
Analogues of Quinolein	

The first successful development of a SARM is Bicalutamide (Figure 5), belonging to the derivatives of aryl-propionamide (APA) [18, 19]. Despite being used as an antiandrogen, by blocking AR, in human it produces an increase in hormone levels: testosterone, oestradiol, luteinizing hormone and stimulating follicle hormone [20-26].

Bicalutamide metabolism studies had reported metabolites producted by Phase I (hydroxylation) and Phase II (glucuronide and sulfate conjugation) reactions. In addition, fecal and urine elimination profiles of Bicalutamide and its metabolites highlighted the importance of these matrices as a quantitatively important route of excretion [27, 28].

Bicalutamide metabolism studies had reported metabolites produced by Phase I (hydroxylation) and Phase II (glucuronide and sulfate conjugation) reactions [18, 19]. In addition, fecal and urine elimination profiles of Bicalutamide and its metabolites highlighted the importance of these matrices as a quantitatively important route of excretion [27, 28].

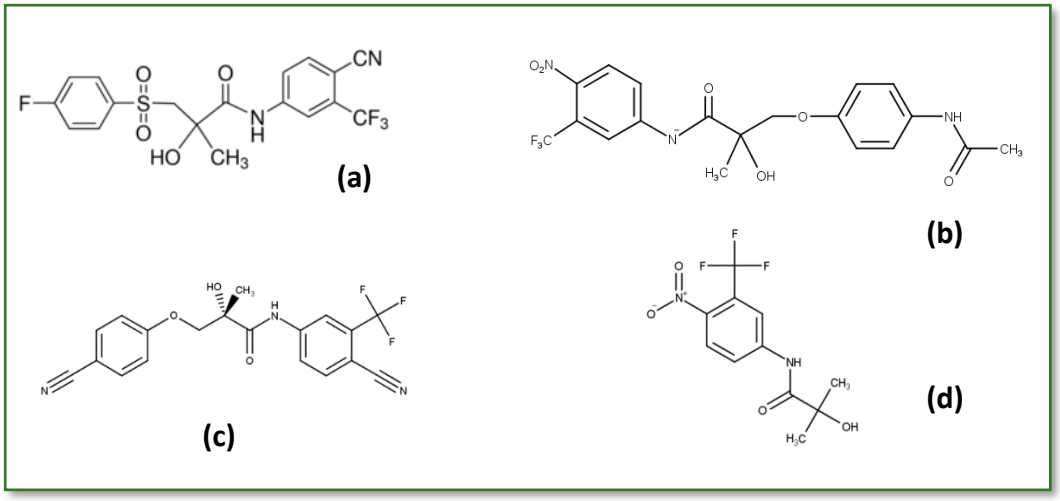


Figure 5. Chemical structure of Bicalutamide (a); Andarine (b); Ostarine (c); Hidroxyflutamide (d).

SARMs are in different stages of clinical study [7, 29, 30]. There are also patents for the use of these substances in animal production [31] due to their AR agonist capacity [32].

In recent years, a growing number of studies have been published on the analytical and metabolic aspects of SARMs. The methods employed are mostly liquid chromatography coupled to high resolution mass spectrometry detectors [33-35]. Metabolic studies had confirmed Phase I (hydroxylation, dephenylation, nitrorreduction, etc.) and Phase II (glucuronide and sulfate conjugation) reactions for in-vitro and in-vivo studies [36-39].

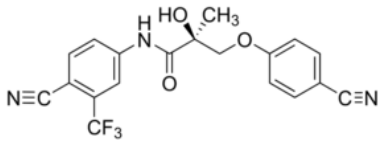
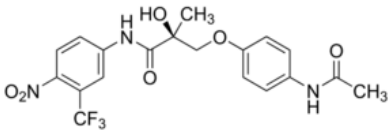
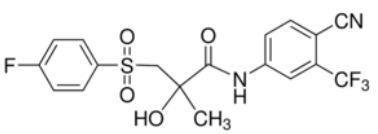
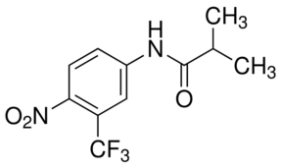
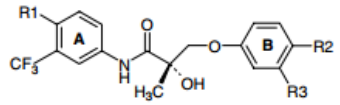
For the above mentioned SARMS constitute a risk of abuse in sport and animal production. For this reason, since 2008, the use of SARMs in sports was prohibited by the World Drug Enforcement Agency [40, 41]. In addition, there have been cases of Internet illegal sales and their use in pig production has been reported [42, 43]. Cases of abuse by SARMs in sports have been published [44] and 13 detections have been reported in 2013 [45]. In this way, SARMs began to travel a path from sports doping to livestock production as has happened before with other anabolic agents [38].

Materials and Methods

Table 2 presents a summary of the studied SARMs, their structures and mass spectrometry fragmentation.

Samples preparation was based on previous work carried out in the Laboratoire d'étude des résidus et Contaminants dans les Aliments [3, 14] and other studies summarised in the Table 3,Table 4, Table 5, Table 6 and Table 7.

Table 2. Analytical characteristics of the compounds studied.

Compound	Synonyms	CAS	Formula	Structure	MW (g/mol)	Metabolites	Fragments (m/z)	Elimination
Ostarine	(*S*)-3-(4-Cyanofenoxi)-*N*- [4-cyano-3-(trifluoromethyl)phenyl]-2-hidroxy-2-methylpropionamide **S22, MK-2866 ENOBOSARM GTx**	841205-47-8	$C_{19}H_{14}F_3N_3O_3$		389.33	dephenylation Hidroxylation with/without glucuronidation Sulfonation Carboxylation	Ions (± 0.5): 388.1; 118.1; 184.9; 241.1; 269.1	$t_{1/2}$ Urine= 4hs till 6 days $t_{1/2}$ plasma= 1.4hs till 16.3h
Andarine	(S)-3-(4-Acetylaminofenoxi)-2-hidroxy-2-methyl-N-(4-nitro-3-trifluoromethylphenyl) propionamide **S4**	401900-40-1	$C_{19}H_{18}F_3N_3O_6$		441.36	O-dephenylantion	Ions (± 0.5): 440.0; 150.1; 205.0; 261.1; 289.1	$t_{1/2}$ plasma= 2,6 hs till 5.3hs
Bicalutamide *Casodex ®*	N- [4-Cyano-3-(trifluoromethyl)phenyl]-3- [(4-fluorophenyl)sulfonyl]-2-hidroxy-2-methylpropionamide	90357-06-5	$C_{18}H_{14}F_4N_2O_4S$		430.37	Glucuronidation Dephenylation Hidroxylation	Ions (± 0.5): 428.9; 185.1; 255.1	$t_{1/2}$ plasma= 1 week (rats 1 day, dogs/humans 6-7 days)
Flutamide *Flucinom*	2-Methyl-N-(4-nitro-3-[trifluoromethyl]phenyl) Propionamide	13311-84-7	$C_{11}H_{11}F_3N_2O_3$		276.21	2-Hidroxyflutamide	Ions (± 0.5): 275.1; 229.1; 205.0; 202.0; 186.0 **2OH** Ions (± 0.5): 291.1; 205.0; 175.0; 159.0; 155.0	-Flutamida: variable -2-Hidroxyflutamide: $t_{1/2}$ plasma= 5.2hs
Arylpropionamides	**X**	X	X		X	Ions (± 0.5): 289 ; 261 ; 241 ; 205		x

STD: standard; **ISTD:** internal standard; **LLE:** liquid-liquid extraction**; SPE:** solid phase extraction**; TBME**: Methyl t-butyl ether; **AA**: ammonium acetate; **AF**: acid formic; **AcH**: acetic acid; **FA**: ammonium formiate.

Table 3. Methods considered for the analysis of APA-SARMs in Urine.

Compound	Method	
Enobosarm *[38]*	**LLE** • 2 mL Urine + ISTD (Bicalutamide) + Buffer Na_2HPO_4/NaH_2PO_4 (1: 2, v/v); (pH = 7) • **Hydrolysis** *β-glucuronidase* 1 h (50 °C) *+ sulfatase* • Cool a Room temp.+ 100 mg $K_2CO_3/NaHCO_3$ (2: 1, v/v) + vortex • **Extraction** *head-over-head* con 4 mL TBME 15min • Centrifugation 4000 x g 10 min + dry supernatant (40 °C) with N_2 • Adding STD APA (0,01 mg/L) to blank extracts for *Matrix Match calibration* • Evaporation to dry → 40 °C with N_2 →Reconstitute 250 µL H_2O /ACN 4: 1 (v/v), vortex	**SPE** • 3 mL de Urine + ISTD + 3 mL Buffer AA 0,25M, pH = 5 • Conditioning 12 mL MeOH + 6 mL H_2O • Load sample(strata-X) • Wash 1° 6 mL MeOH/H_2O (4: 6, v/v) + 1 % AcH • Wash 2° 6 mL MeOH/H_2O (2: 8, v/v) + 0,25 % NH_4^+ • Elution 6 mL MeOH • Evaporation to dry → 40 °C with N_2 • Reconstitute 100 µL MeOH + 400 µL AF 20 mM
Enobosarm *[46]*	Centrifugation a 17 500 x g durante 5 min with recuperation del supernatant	
Enobosarm *[47, 48]*	**SPE** • 2 mL Urine + ISTD → Conditioning 2 mL MeOH + 2 mL H_2O → Load sample→ Wash 2 mL H_2O→Elution 2 mL MeOH →Evaporation to dry → Reconstitute the residuesiduo with 100 µL MeOH /0,1 % AF	
Andarine *[49]*	**Screening** • Urine thawed (37 °C) • Centrifugation 1350 x g (5 min) 1ra aliquot: 2 mL + ISTD (mefuside)→ saturation with potassium carbonate→ extraction with basic pH /4 mL of ethyl acetate 2da aliquot: 2 mL + ISTD (mefuside) → saturation with NaH_2PO_4→ extraction a pH acid/4 mL de ethyl acetate • Centrifugation a 1350 x g (5 min) → collect organic phases and evaporate • Reconstitute 125 µL de Buffer FA 10 mM pH 4/ACN (80/20)	**Confirmation** • Urine thawed (37 °C) • Centrifugation 1350 x g (5 min) 2 mL Urine + ISTD (mefuside) + 1 mL Buffer phosphate 0,8 M pH 7 **Hydrolysis** β-glucuronidase a 50 °C (1 h) • + Buffer $Na_2CO_3/NaHCO_3$→ pH 8,5-9 • **LLE** with 5 mL TBME • collect organic phase • Evaporation to dry → with flow of air (50 °C) • Reconstitute 125 µL Buffer FA 10 mM pH = 4/ACN (80/20)
Andarine *[50]*	• 3 mL Urine (**Hydrolysis** : 1 mL buffer phosphate pH = 7 + 30 µL glucuronidase (1 h) a 56 °C) →Cool- room temp. + $NaHCO_3/K_2CO_3$ (2 : 1)→**LLE** with 5 mL diethyl ether→Centrifuge and evaporate the organic phase →Reconstitute 100 µL MeOH/H_2O (30/70)	

STD: standard; **ISTD:** internal standard; **LLE:** liquid-liquid extraction**; SPE:** solid phase extraction**; TBME**: Methyl t-butyl ether; **AA**: ammonium acetate; **AF**: acid formic; **AcH**: acetic acid; **FA**: ammonium formiate; **H2O:** water.

Table 4. Methods considered for the analysis of APA-SARMs in faeces.

Compound	Protocols
Acetotiolutamide *[51]*	Faeces (6-10.5 g) + 10 mL H2O→ blender→ Extract 3 times with MeOH/AE 2: 1 (v/v) → Collect liquid phases of extraction and vaporate to low Pressure→ Reconstitute with methanol and filter 300 µL with membrane (0.45um).
Andarine *[52]*	• Faeces with adding double volume with H2O→ blender→ freezing to -20 °C→Thawed, centrifuge 3000 x g (10 min) + ISTD → Filter supernatant (0.22 µm)→ Inject 20 µL - HPLC/MS • Extract x 4 times (Solid with ethyl acetate/methanol 2:1 (v/v))→centrifuge 3000 x g (10 min) → Mix and evaporate the supernatants under N_2→ Reconstitute with 500 µL de methanol/H2O, 1:1 (v/v)→ Filter supernatant (0.22 µm) → Inject 20 µL -HPLC/MS
SARM LY2452473 *[53]*	Faeces: 3 x Extraction with 5 volumes of ACN/AA 10 mM (1:1, v:v)→ Evaporate and then Reconstitute extracts - MeOH/AA 10 mM (1:2, v:v).
S-26 *[54]*	Faeces: 3x extraction with 10 mL de MeOH/AE 2: 1. (v/v) →Mix and evaporate liquid phases → Dilute the residues with MeOH.
Enobosarm *[46]*	Homogenate faeces with 3 times volume H2O → Extraction: 5 g de faeces 2 x (15 mL de ACN)→ Vortex 2 min→ Centrifuge to 1400 x g. 10 min) → Mix supernatant and evaporate to 20 °C with N_2→Reconstitute 1 mL de ACN/H2O (1: 1, v/v)→ Centrifuge to 17500 x g. 5 min
Bicalutamide [19]	Extraction de faeces homogenised (5-10g) with ACN (30 mL) → Evaporate to dry under low Pressure →Reconstitute with 1 mL MeOH→**Analysis by TLC**
Bicalutamide [18]	Extraction faeces homogenised (5g) with 3-6 volume MeOH → Evaporate to dry dry under low Pressure → Reconstitute with 1-2 mL MeOH →**Analysis by TLC**

MeOH: Methanol; **AE:** Ethyl acetate; **ACN:** Acetonitrile; **AA:** Ammonium acetate; **ISTD:** Internal Standard (internal standard); **TLC:** Thin Layer Chromatography; **H2O:** water.

Table 5. Methods considered for the analysis of APA-SARMs in Blood.

Compound	Protocols
Bicalutamide *[55]*	• 50 µL Plasma + 950µL MeOH (ISTD) → Shake 30"→ Centrifuge 14000 rpm (5 min) → 50 µL supernatant + 950 µL ACN al 70% → Shake 30"
Ostarine *[46]*	• 3 x Extractions: Plasma + 3 volumes ACN →Took and mix 3 supernatants → Evaporate to dry 20 °C y N_2→Reconstitute 0.7-0.9 mL of H_2O:ACN (1+1)→ Centrifuge 17500 x g (5 min)
Andarine *[56]*	• Plasma + ACN (ISTD) same volumes of plasma → 16000 g x 30 min (4 °C)→ Recuperation of supernatant
S-1 *[57]*	• 100 µL Plasma + 30ul ISTD + 1 mL ACN→ Centrifuge 16100 x g (10 min)→ supernatant + 1 mL H2O +7.5 mL AE→ shake 10 min → Centrifuge 1540 x g (10 min) → Evaporate the organic phase to dry with N_2→Reconstitute en 150 µL de H_2O: ACN (1+1) → Centrifuge 16100 x g (10 min)
Bicalutamide *[58]*	• 50 µL Plasma + 200 µL ACN (ISTD) → Shake 15"→ Centrifuge 14000 x g (10 min) → Recover supernatant
Andarine *[52]*	• 500 µL Plasma + ISTD + 1 mL ACN → Centrifuge 2000 x g (5 min) → + 7 mL AE shake 40 min→ Centrifuge 2000 x g (5 min) → Evaporate the organic phase to dry with N_2→Reconstitute 100 µL ACN: Agua (35: 65 v/v)
Hidroxyflutamide *[59]*	• 200 µL Plasma + 20 µL MeOH (ISTD) + 20 µL MeOH → Vortex → + 600 µL MeOH → Vortex (60") → Centrifuge 1200 rpm (5 min)→ Recover supernatant

MeOH: Methanol; **AE:** Ethyl acetate; **ACN:** Acetonitrile; **ISTD:** Internal Standard (internal standard); **H2O:** water.

Table 6. Chromatographic Conditions considered for the analysis of APA-SARMs.

Compounds	Chromatographic Conditions	
Ostarine *[38]*	Column BEH C18 (150x2.1 mm; 1.7 µm); (50 °C) V_{inj} = 5 µL Flow 0.2 mL/min - Grad: A= H_2O + AF 5 mM; B= ACN+AF 5 mM 10 % B to 0 y 1 min→to 100 % in 15 min→hold (2 min) →100 % to 10 % B in 1min → 10 % B (8 min) = 28 min	Column BEH C18 (100x2.1mm; 1.7µm) (50 °C) – V_{inj}= 5µL Flow 0.6 mL/min (Split 1: 1 to MSD) - Grad.: A= H_2O; B= ACN 0 % B hold 0-0.3min→to 20 % B at 0.3-1min→ to 70%B at 1-5 min→ to 100 % B at 5-5.5min→ 100 % B (1min) → 100% to 10 % B in 0.5min→ 0 % B (2min) = 9 min
Ostarine ***[46]***	Column Sepax C18 (250x4.6 mm; 5 µm) – Flow 0.8 mL/min – Grad.: A= H_2O+AF 0.1% (ESI +) /A= H_2O + AF 0.1% (ESI -) + FA 20 mM B= ACN + AF 0.1% – 10 % to 80 % B in 20min → 90 % B in 1min → hold (4min) → 10% B in 8min	
Ostarine *[48]*	Column Kinetics Phenomenex (100x2.1 mm; 2.7 µm) – Flow 0.2 mL/min – Grad.: A= H_2O+AA 5 mM – pH = 3.5; B= ACN 5 % to 100 % B in 12min → hold (2min)	
Andarine *[49]*	Column Sunfire C8 (100x2.1 mm; 3.5 µm) with Filtro (0.2 µm) – Flow 0.35 mL/min (25 °C); Vol. Injection= 10 µL Grad.: A= H_2O+FA 10mM pH=4; B= ACN; 5 %B (0 to 0.75 min)→to 80 % in 4.25 min→ hold (1min)→5 % B in 1.5 min	
Andarine *[50]*	Column BEH C18 (100x2.1 mm; 1.7 µm) – Precolumn BEH C18 (50x2.1 mm; 1.7 µm) (30 °C) – Flow 0.4 mL/min; Vol. Injection= 10 µL Grad.: A= H_2O + FA 0.1 %; B= ACN + FA 0.1 % 5 % to 40 % B in 15min → 95% in 2min → 5 % B in 3min	
APA-SARMs *[47. 48]*	Column monolithique RP C18(100x 3um) Vol. Injection= 50µL Grad.: A= H_2O ; B= MeOH;30% to 90% de B in 10min with Flow de 0.9 mL/min	
Hidroxyflutamide *[59]*	Column Ultimate C18 (50x2.1 mm; 5 µm) (30 °C) - Pre-Column Phenomenex C18 (4x3.0 mm; 5µm) - Flow 0.2 mL/min; Vol. Injection= 5µL Hold: H_2O/ACN 2: 1 (v/v) 4min	

V_{inj}: injected volume; **ESI:** electrospray ionization; **AA:** ammonium acetate; **AF:** acid formic; **FA:** ammonium formiate; **MeOH:** Methanol; **ACN:** Acetonitrile; **MSD:** Mass Spectrometer Detector.

Table 7. Parameters of mass spectrometry detectors considered for analysis of APA-SARMs.

SARMs	CondicIons de MS	
Ostarine *[38]*	MicroTof-Q y Triple Quadrupole Full scanESI (-/+) y SRM MS/MS; Capillary Voltage 2,5 kV; Source 120 °C; Desolvat. Temp 450 °C; Cone flow450 L/h; Gas de Desolvat. Flow 150 L/h; Gas CID: argon $2{,}2.10^{-3}$ mbar	
Ostarine ***[46]***	LTQ	
	ESI (-): sheath gas 50 u; Spray 3,2 kV; Capillary Voltage -35V (300 °C) Lens tube -65V	ESI (+) (glucuronidated) sheath gas 50 u; Spray 3,2kV; Capillary Voltage 35V (300 °C) Lens tube 65V
Ostarine *[48]*	QTrap MS/MS ESI (-) Source 350 °C, -4 kV; Ionisation -3 kV; Capillary Voltage 290 °C; Full scan m/z 50 - 2000; Resolution 50000; Full scan 50 - 2000 m/z; Resolution 25000; HCD 20 V; Full scan 50 - 2000 m/z; Resolution 25000; HCD 40 V	
Andarine *[49]*	Screening: TSQ ESI (-) Colis. offset volt.: 20 V; Q1 y Q3 en 0,7 amu mass resolution Source temp 320 °C; spray 3 kV; Lens/tube 110 V; Q2 Gas Colis.: argon 0,2 Pa	
Andarine *[50]*	QTOF-MS ESI (-); m/z 100 - 1000: Desolvat. Gas flow 850 L/h (360°C); Capillary Voltage 2,4 kV; Voltage Cone 40 V; Micro-channel plates 1750 V; Source temp. 120 °C; Cone flow10 L/h; Gas Colis. 0,25 mL/min	
APA-SARMs *[47, 48]*	LTQ orbitrap APCI (+) full scan 100 - 500 m/z; tps scan: 1s; Tension decharge 5 kV; Desolvat. 300 °C; Cone voltage 30 V; Gas Nebul. (N_2): 1 L/min; Resolution 60000 FWHM	
Hidroxyflutamide *[59]*	Q Trap ESI (-): -4200/-4500 V; Full scan m/z 70 – 1000; Source 275 – 350 °C; Gas Colis.: (N_2) $6{,}10^{-3}$ Pa	

CONTROL METHOD FOR SARMS IN LIVESTOCK PRODUCTION

Reagents and solutionscIons

Methanol, Ethanol and Acetonitrile HPLC grade were purchased from Fisher scientific (Pittsburgh, USA). Ultra-pure water (> 18.4 MΩ/cm) was obtained from Barnstead® NANOpure® ultrapure water purification system (Thermo Scientific). Acid formic was purchased from LGC Standards (Teddington, UK). β-Glucuronidase/Arylsulfatase (from Helix pomatia) stabilized aqueous solution for biochemistry (EC3.2.1.31+EC3.1.6.1) and Ammonium Acetate for analysis were purchased from Merck (Darmstadt, Germany).

Standards of Enobosarm, Andarine, Bicalutamide and Hidroxyflutamide were purchased from Sigma Aldrich (Saint-Louis, USA) and Bicalutamide-D4 was purchased from LGC Standards (Teddington, UK). Oasis® HLB extraction cartridges (3cc, 60 mg, 30 um) were purchased from Waters (Milford, USA).

Standard Solutions

Primary stock standards solutions of each SARMs compound in ethanol at 1 mg/ml were prepared with a gravimetric procedure. Bicalutamide-D4 was used as internal standard (ISTD). Mixture and dilutions of Enobosarm, Andarine, Bicalutamide and Hidroxyflutamide were made to obtain properly concentrations. Standards solutions were stored to -20 °C.

Instrumentation: Control Method for SARMs (LabERCA, ONIRIS)

Chromatographic separation was carried out on Acquity system UPLC (Waters, Milford, MA, USA). A column ACQUITY UPLC® BEH (2.1x100 mm; 1.7 µm) was used as stationary phase. Mass spectrometric analysis was performed using a triple quadrupole mass detector XEVO TQ-S (Waters, Milford, MA, USA) equipped with an electrospray ionization source operating in negative mode (ESI-). Data acquisition was performed using MassLynx 4.0 software (Waters,

Milford, MA, USA). A centrifuge Heraeus Multifuge X3FR (Thermo Scientific) was used for sample preparation (Annex I; page 105).

Sample Preparation

Urines samples were treated by a solid phase extraction (SPE) and faeces samples preparation involves a combination of liquid-liquid extraction (LLE) follow by SPE. Phase II metabolites were studied applying an enzymatic hydrolysis step (see Annex II ; page 110).

Method Performance

Parameters of *selectivity*, *linearity*, *accuracy*, *precision* and *sensibility* were studied in order to evaluate the performance of the method.

Blank samples were taken from non-treated animals and it were used for the construction of calibration curves and for recuperation assays.

The blank samples were spiked with different amounts of standard solutions and then were processed with a batch samples. Calibration curves were constructed for each compound using chromatograms areas ratio (***S***=analytes/ISTD) as a function of the concentration. The samples concentrations were determined by interpolation therefore calibration curves were made to cover different ranges of concentration for urines 0.25 – 30 ng/ml and for faeces 0.5 – 1600 ng/g.

Concentrations (***C***) were calculated as follow:

$$\boldsymbol{C = \frac{(S - b)}{a}}$$

S: area ratio; ***b***: intercept and ***a***: slope of the equation of linear regression.

Linearity: was evaluated by *determination coefficient* (r^2) from the equation of linear regression of the calibration curves.

Accuracy: was evaluated as recovery (***R %***) at different concentrations levels in the calibration curves range, for urines between 0.25 – 30 ng/ml and for faeces 0.5 – 1600 ng/g. Another calibration curves were constructed adding different amounts of stock standard solutions to blank samples extracts. The recovery was calculated for each compound and to different level of

concentrations to evaluate the accuracy of the method. The recovery of the method was calculated as follow:

$$\boldsymbol{R}\% = \frac{\boldsymbol{C}}{\boldsymbol{C_{spiked.}}} x100$$

$\boldsymbol{C}$: concentration calculated; $\boldsymbol{C_{spiked}}$: spiked concentration.

Precision: was evaluated as relative standard deviation (RSD %) calculated from the triplicate analysis of spiked samples (urines: 0.25 – 30 ng/mL and faeces: 0.5 – 1600 ng/g).

Sensibility: was determined at the lowest level of calibration curves 0.25 ng/ml for urine and 0.5 ng/g for faeces. A ratio signal-to-noise more than 10 and recovery between 70 % – 120 % were fixed as acceptability criteria for each compound in both matrices.

Selectivity: was given by the HPLC-Ms/Ms method applying a Multiple Reaction Monitoring mode, relative retention time to the ISTD and the relative abundance ions. Blank sample chromatograms showed no interferences for the studied compounds.

BICALUTAMIDE AND ANDARINE IN ANIMAL TRIALS

The metabolism study was carried out through two animal trials described below (ver *First Animal Trial*, page 34 y en *Second Animal*, page 35).

First Animal Trial (Oniris-LabERCA)

In 10 mL of ethanol were dissolved 100 mg of Bicalutamide (CASODEX®) and then four times the volume of commercial apple juice was added. The oral dose was administrated by a syringe.

Sampling

Bicalutamide elimination study was approved by French Committee of Ethics (Protocol Nº 02323.01). Urines and faeces samples of a healthy calf (race: Montbeliard, weight: 190 kg, age: 8.5 months) were taken before and after an oral dose of 100 mg of Bicalutamide at day 0. Sampling of faeces and urine started 3 days before the administration, every day until Day 7 (Figure 2). In addition, during Day 0: 3samples were taken 8 a.m. (0 hours) 12 a.m. (4 hours) and 4 a.m. (8 hours).

Urines samples were stored at freezer (- 20 °C) until process, the faeces were immediately freeze-dried.



Figure 6. Animal N°4382 - calf (1st animal trial)

An oral dose of 100 mg of Bicalutamide was given to the calf. (breed Montbeliard, weight: 190 kg, age: 8.5 months).

Metabolites Study

The study of the metabolites was carried out by mixing extracts of matrices (urine and faeces) from the days: 1; 3; 5 and 7.

The strategy for this test was to combine the information obtained from different Scan modes (Precursor Ion Scan, Product Ion Scan, Neutral Losses Scan and Full Scan). After postulating structures candidate for metabolites, tests were performed on Multiple Reaction Monitoring and Single Reaction Monitoring to obtain more confidence on the structures proposed.

Second Animal trial (INTA – Anguil)

In this trial 3 pigs, Chetapuy breed (females) and 6 Angus cattle, had recived the detailed doses in Table 8. The animals were in good health and the whole test was performed under veterinary supervision.

Sampling procedures based on the OIE Manual [60] were carried out by veterinarians of the Estation Experimental Agropecuaria Anguil (INTA Anguil).

Oral doses of Bicalutamide and Andarine (dissolved in ethanol and diluted in apples juice) were administrated with syrenges.

Table 8. Doses administrated on second animal trial

Animal N°	Weight (kg)	Treatment	Doses	Species
379	320	*CDX*	50 mg durante 5 days	Steer
421	340	*CDX*	200 mg	Steer
404	336	CTR	-	Steer
323	329	CTR	-	Steer
326	306	CTR	-	Steer
443	338	*CDX*	100 mg	Steer
334	108	*CDX*	100	Porcine
330	103	AND	100	Porcine
329	95	CTR	-	Porcine

CDX: Casodex ®; AND: Andarine; CTR: control (no doses).



Figure 7. Steers (Angus) treated by Casodex ® (2nd animal trial).

Table 9. Sampling on the second animal trial (2nd animal trial).

	Steers			Porcines		
Day	**Sangre**	**Faeces**	**Urine**	**Sangre**	**Faeces**	**Urine**
-3	X	X	X	X	X	X
-2	X	X	X	X	X	X
-1	X	X	X	X	X	X
0	X	X	X	X	X	X
0,17	X	X	X	X	X	X
0,33	X	X	X	X	X	X
1	X	X	X	X	X	X
2	X	X	X	X	X	X
3	X	X	X	X	X	X
4	X	X	X	X	X	X
5	X	X	X	X	X	X
6	X	X	X	X	X	X
7	X	X	X	No	no	No
8	no	No	No	X	X	X
9	X	X	X	No	no	No
10	no	No	No	X	X	X
11	X	X	X	No	no	No

Sampling

Urine, faeces and blood samples were taken before and after drugs administration (day 0) as shown in

Table 9. On day 0 extra samples were taken, at 4 and 8 hours after dosing.

The urine and faeces were preserved (- 20 °C) until they were processed in the laboratory. The blood was incubated at 25 °C for 1 hour, then centrifuged and finally separated the serum which was stored at - 20 °C.

Reagents and solutions

Methanol, Ethanol and Acetonitrile HPLC grade were purchased from Fisher scientific (Pittsburgh, USA). Ultra-pure water (> 18.4 MΩ/cm) was obtained from PureLab Classic ultrapure water purification system (Elga). Acid formic was purchased from Sigma-Aldrich. β-Glucuronidase/Arylsulfatase (from *Helix pomatia*) stabilized aqueous solution (EC3.2.1.31+EC3.1.6.1) was obtained from Roche and Ammonium Acetate for analysis were purchased from Sigma Aldrich.

Standards of Andarine, Bicalutamide and Hidroxyflutamide were purchased from Sigma Aldrich (Saint-Louis, USA). Oasis® HLB extraction cartridges (3cc, 60 mg, 30 µm) were purchased from Waters (Milford, USA).

Preparation of standards solution

Primary stock standards solutions of each SARMs compound in ethanol at 1 mg/ml were prepared with a gravimetric procedure. Hidroxyflutamide was used as internal standard (ISTD). Mixture and dilutions of Andarine, Bicalutamide and Hidroxyflutamide were made to obtain adequate concentrations. Standards solutions were stored to -20 °C.

Sample preparation

Serum

Serum samples were thawed at room temperature. Serum (100 uL) were spiked with 50 uL of ISTD 2.5 ng/mL in water:acetonitrile (7:3; 0.1 % Formic acid) solution. To precipitate serum proteins a methanol:acetonitrile (1:1) solution (200 uL) was added and 15 seconds vortex mixed was carried on. Then samples were centrifuged at 19000 rcf for 20 min and the clear supernatant was injected.

Instrumentation (INTA Castelar)

Chromatographic separation was carried out on Acquity system UPLC (Waters, Milford, MA, USA). A C18 column XBridge (2.1x100 mm; 3.5 µm) was

used as stationary phase (Waters). Mass spectrometric analysis was performed using a single quadrupole mass detector XEVO SQD (Waters, Milford, MA, USA) equipped with an electrospray ionization source operating in negative mode (ESI-). Data acquisition was performed using MassLynx 4.0 software (Waters, Milford, MA, USA).

Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using a mobile phase of solution A (water with 0.1 % acid formic) and solution B (acetonitrile-methanol, 50:50 v/v, with 0.1 % acid formic). A gradient at a flow rate of 0.25 ml/min was employed starting at 90 % A (0-1 min); to 65 % A (1-2 min), to 45 % A (11 min), to 0 % A (11.01 min) and holding at 0 % A (12.5 min); to 90 % A (13 - 15 min). The injection volume was 10 µl. The autosampler temperature was set at 20 °C and the column was kept at 55 °C.

Mass spectrometer source temperature was set at 150 °C. Nitrogen was used as desolvation gas at a temperature of 350 °C and at a flow of 750 l/h. Capillary voltage was set at -2.9 kV. Andarine, Bicalutamide and Hidroxyflutamide were detected by Single Ion Monitoring (SIM) mode (Table 15).

The study of metabolites was performed using a mobile phase with Solution A: water with 0.1 % Formic Acid and 0.1 % Ammonium Formiate and Solution B: acetonitrile-methanol, 50:50 v/v, with 0.1 % acid formic. The flow rate was 0.25 ml/min and the gradient employed was 90 % A (0 – 1 min), 90 % to 70 % A (1 – 3 min), 70 % to 20% A (3 – 11 min), 20 % to 0 % A (11 – 13 min), hold at 0 % A (13 – 15 min), to 90 % B (15 – 16 min) and hold at 90 % A (16 – 18 min). The injection volume used was 10 µl. The autosampler temperature was set at 20 °C and the column was kept at 55 °C.

Table 10. Parameters of mass spectrometry used for study Bicalutamide and Andarine.

RT (min)	Compound	Ion	Cone (V)
8.01	Hidroxyflutamide	291.1 (Q) 205.0 (q)	40
8.60	Andarine	261.0 (q) 440.1 (Q)	40
9.20	Bicalutamide	429.1 (q) 255.1 (Q)	30

Ions and retention times (RT) used for Andarine, Bicalutamide e Hidroxyflutamide. (Q) Ion de quantification, (q) Ion de qualification.

Performance Method (2° animal trial)

Parameters of *selectivity*, *linearity*, *accuracy*, *precision* and *sensibility* were studied in order to evaluate the performance of the method.

Blank urines, faeces and serum samples were obtained from non-treated animals and to perform the calibration curves for each matrix blank samples from different animals were mixed to have a representative a blank sample.

Calibration curves were constructed using drawing signal (***S***) obtained as areas ratio (***S***= $Area/Area_{ISTD}$) and the spiked concentration. The covered ranges were: urines 0.5 – 200 ng/mL, faeces 2 – 3500 ng/g and serum 2 – 3000 ng/mL.

Linearity: was evaluated by *determination coefficient* (r^2) from the equation of linear regression of the calibration curves.

Accuracy: was evaluated as recovery (***R %***) at different concentrations levels in the calibration curves range, for urines between 0.5 – 200 ng/mL, for faeces 2 – 3500 ng/g and for serum 2 – 3000 ng/mL. Another calibration curves were constructed adding different amounts of stock standard solutions to blank samples extracts. The recovery was calculated for each compound and to different level of concentrations helped by *matrix matched calibration [61,62]*)

Precision: was evaluated as the relative standard deviation (RSD %). The compounds concentration of each spiked sample was determined 3 times at

these levels to calculate the RSD%: urines 0.25; 0.5; 1; 3 y 10 ng/mL and for faeces and serum 1; 2; 5; 10 y 20 ppb (serum: ng/mL and faeces: ng/g).

Sensibility: was determined at the lowest level of calibration curves a ratio signal-to-noise more than 3 and recovery between 70 % – 120 %.

Selectivity: was based on relative retention time of each analytes to ISTD and relative abundance of the ions described on Table 10. Blank sample chromatograms showed no interferences for the studied compounds.

SARMs Metabolites Study (INTA – EEA CONCORDIA)

Metabolites were studied with the collaboration of the Laboratory of INTA – Concordia (To Ríos, Argentina). In this study, urines and faeces samples of the second animal trial were treated as mentioned before XXX.

Instrumentation

Chromatographic separation was carried out on Acquity system UPLC (Waters, Milford, MA, USA). A column ACQUITY UPLC® BEH (2.1x100 mm; 1.7 µm) was used as stationary phase and mobile phase used was constituted by a gradient of solution A (water, 10% Acetonitrile and 0.1% formic acid) and solution B (Acetonitrile 0.1% formic acid). The gradient flow rate was 0.45 mL/min and starts with 100 % of solution A by 2.5 min then 0 % of solution A at 7.5 min, holding this condition by 1 min then initial condition in 1.5 min. Mass spectrometric analysis was performed using a triple quadrupole mass detector XEVO TQ-D (Waters, Milford, MA, USA) equipped with an electrospray ionization source operating in negative mode (ESI-). Data acquisition was performed using MassLynx 4.0 software (Waters, Milford, MA, USA). A centrifuge Heraeus Multifuge X3FR (Thermo Scientific) was used for sample preparation.

Results and discussion

CONTROL METHOD FOR SARMS IN LIVESTOCK PRODUCTION

Urines

Frozen samples were thawed at room temperature then to 3 ml of sample and 20 µl of ISTD 0.1 ng/µl 3 ml ammonium acetate buffer (0.25 M; pH 4.8) were added in order to adjust the pH to 5 ± 0.3. The pH was checked with pH indicator strip sand, if necessary, adjusted with acetic acid or sodium hydroxide. SPE cartridges were preconditioned with 2 x 3 ml methanol and 3 ml water. Samples were transferred to the cartridges. Immediately after, the SPE phases were washed with 3 ml solution water/methanol (95:5) followed 3 ml solution water/methanol (80:20). Analytes were eluted from the SPE cartridges with 4 ml methanol. Evaporation to dryness under a gentle stream of nitrogen at 45 °C was performed. Evaporation was followed by reconstitution in 0.2 ml of a 70:30 (v/v) water/acetonitrile with 0.1 % acid formic mixture. The method is presented in Figure 8.

Phase II metabolite were treated by an enzymatic hydrolysis. Urines (3 ml) were put in a tube and then 20 µl of ISTD 0.1 ng/µL, 3 ml ammonium acetate buffer (0.25 M; pH 4.8) was added in order to adjust the pH to 5.0 ± 0.3. The pH was checked with pH indicator strip sand, if necessary, adjusted with acetic acid or sodium hydroxide. Then 20 µl β-Glucuronidase/Arylsulfatase was added before overnight (10 h) incubation at 50 ± 5 °C. Subsequent purification steps were performed as described above (Annex II; page 110)

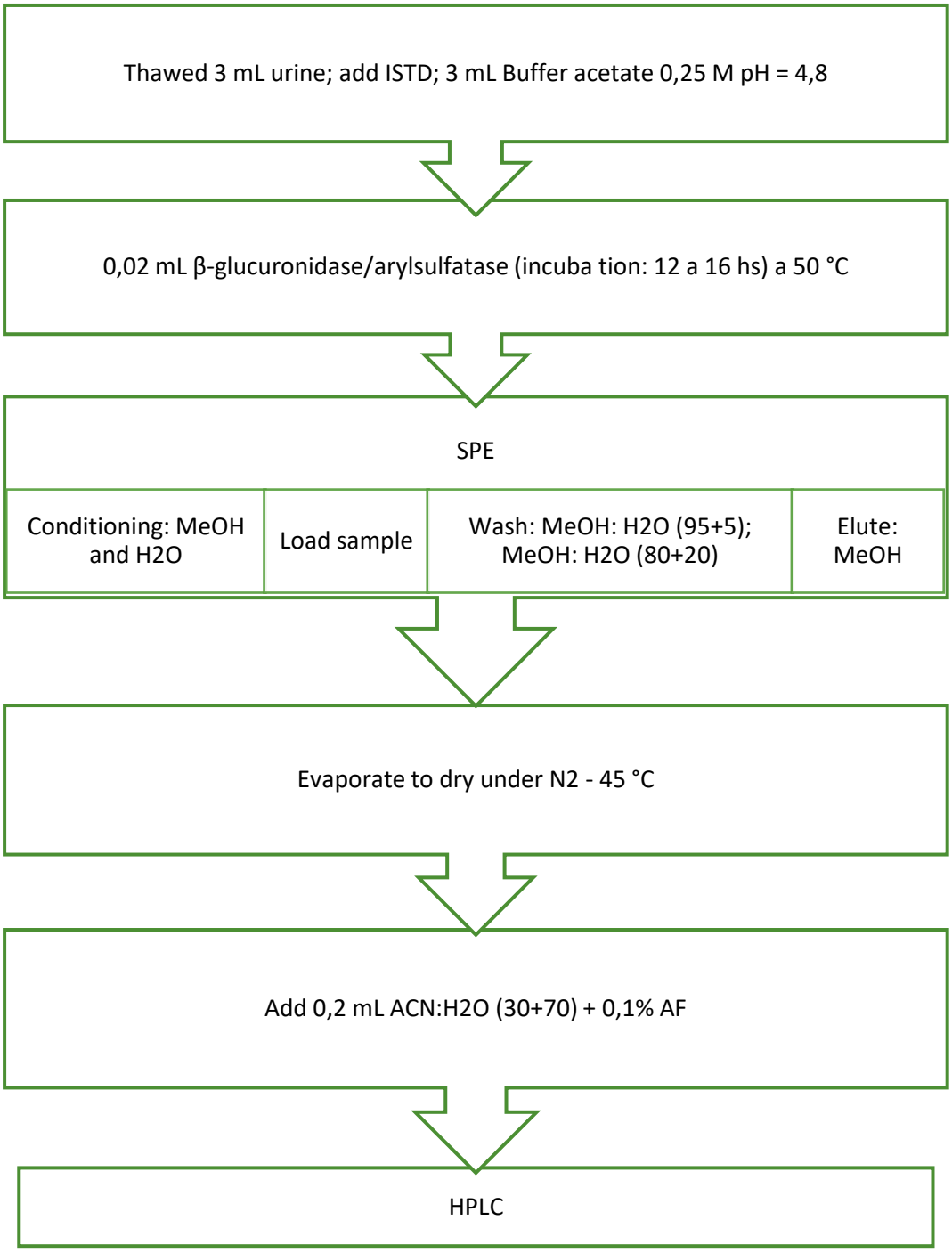


Figure 8. Method for Urines samples.

Faeces

Bicalutamide elimination study was carried out with the lyophilized samples. In a tube, 0.5 g homogenized portions were weighted, 4 ml ammonium acetate buffer (0.25 M; pH 4.8) and 20 µl of ISTD 10 ng/µl were added. The pH was checked with pH indicator strip sand, if necessary, adjusted with acetic acid or sodium hydroxide. Next an agitation of 30 min followed by a centrifugation was made (20 min, 5500 r.p.m. and 5 °C). The liquid phase was centrifuged again (5 min, 2500 r.p.m., 5 °C) to avoid solids residues. Liquid phase was taken and was evaporated to dryness under a stream of nitrogen at 45 °C. The residues were resuspended with 2 ml ammonium acetate buffer (0.25 M; pH 4.8).

SPE cartridge was preconditioned with 2 x 3 ml methanol and 3 ml water. Samples were transferred to the cartridges. Immediately after the SPE cartridges were washed with 3 ml solution water/methanol (95:5) followed 3 ml solution water/methanol (80:20). Analytes were eluted from the SPE cartridges with 4 ml methanol. Next, evaporation to dryness under a gentle stream of nitrogen at 45 °C was perfomed. Evaporation was followed by reconstitution in 0.2 ml of a 70:30 (v/v) water/acetonitrile with 0.1 % acid formic mixture. Finally, the purified were filtered 30 min with centrifugal Polyethersulfone membrane (10 K) at 17000 x g (Figure 9).

Phase II metabolites were deconjugated by an enzymatic hydrolysis. The lyophilized and homogenized samples were weighted in a tube (0.5 g) and 4 ml ammonium acetate buffer (0.25 M; pH 4.8) and 20 µl of ISTD 10 ng/µl were added. The pH was checked with pH indicator strip sand, if necessary, adjusted with acetic acid or sodium hydroxide. Then an agitation of 30 min followed by a centrifugation was made (20 min, 5500 r.p.m. and 5 °C). The liquid phase was centrifugated again (5 min, 2500 r.p.m., 5 °C). Liquid phase was taken and was evaporated to dryness under a stream of nitrogen at 45 °C. The residues were resuspended with 2 ml ammonium acetate buffer (0.25 M; pH 4.8). The tube was incubated overnight (10 h) with 20 µl of β-Glucuronidase/Arylsulfatase added. Subsequent purification steps were performed as described above (see Annex II; page 110).

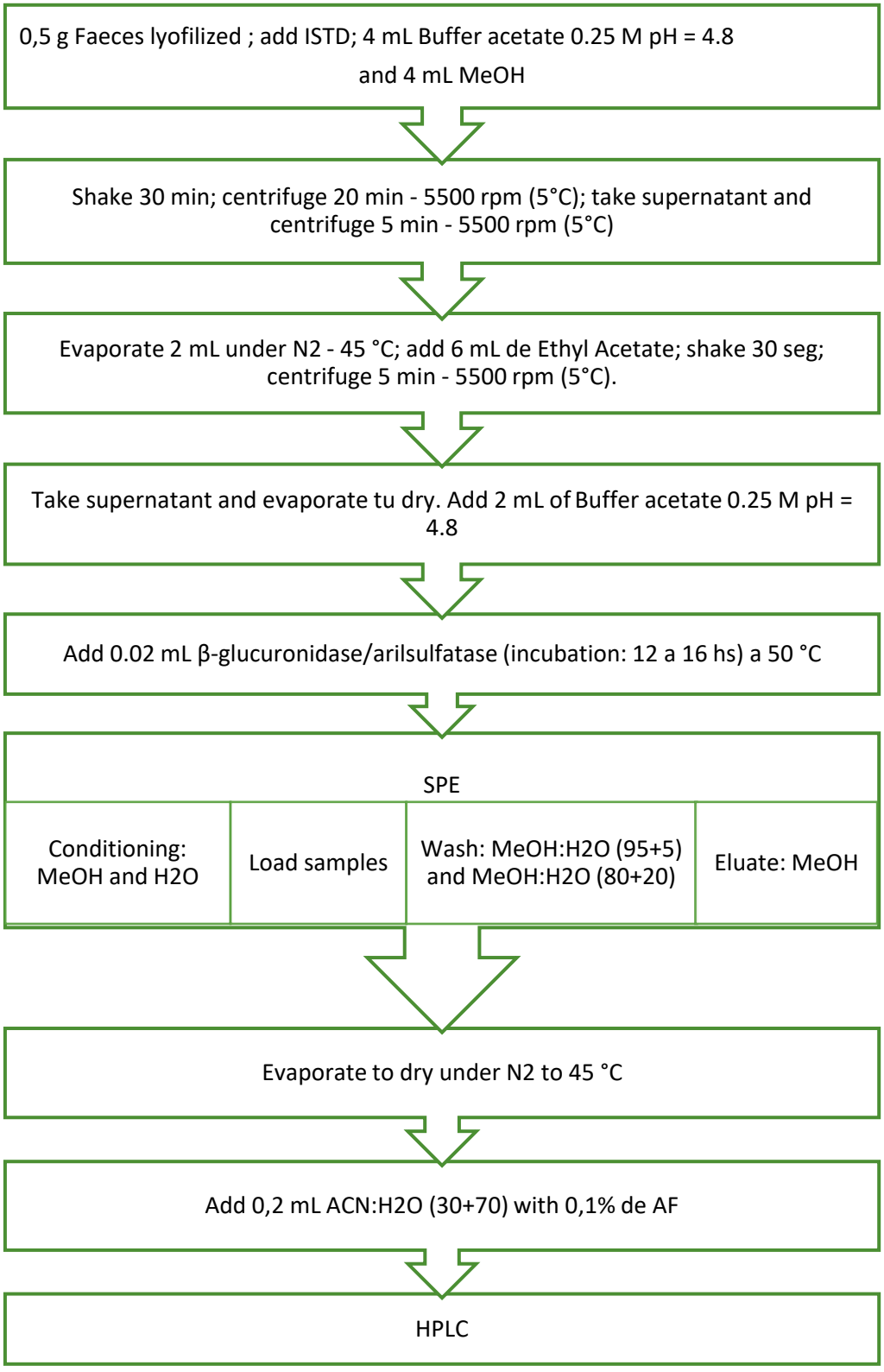


Figure 9. Method for faeces samples.

Evaluation of method performance

Table 11 presents the chromatographic parameters employed for the performance evaluation, more information are presented in Annex I. Annex III (page 114) presents data used for the performance evaluation.

Table 11. Parameters used for SARMs during method development.

RT (min)	Analyte	Transitions	Cone (V)	Collision (eV)
2.81	Hidroxyflutamide	291.1>205.0 (Q)	40	24
		291.1>175.0 (q)		32
		291.1>155.0 (q)		36
3.02	Andarine	440.1>150.0 (Q)	40	26
		440.1>261.0 (q)		18
		440.1>205.0 (q)		32
3.44	Bicalutamide- D4	433.1 > 255.0 (Q)	30	16
		433.1 > 185.0 (q)		44
		433.1 > 177.0 (q)		24
3.46	Bicalutamide	429.1 > 255.1 (Q)	30	16
		429.1 > 185.0 (q)		44
		429.1 > 173.0 (q)		24
3.96	Ostarine	388.1>118.0 (Q)	30	18
		388.1>269.0 (q)		18
		388.1>185.0 (q)		38

Enobosarm, Andarine, Bicalutamide, Bicalutamide-D4 y Hidroxyflutamide, (Q) Transitions of quantification, (q) transitions of qualification.

Table 12 shows the parameter obtained during the performance evaluation. Results show proper performance of the method (120 % > R% >70 %; r^2 > 0.99; RSD% < 20 %) in the range of concentrations studied 0.25 – 30 ng/mL para Urine y 0.5 – 1600 ng/g para faeces (Decision 2002/657/EC) [38, 45].

Sensitivity expressed as limit of quantification (LQ or LOQ) was 0.25 ng/mL for urines and 0.5 ng/g for faeces. These levels of concentration are appropriate, taking into account that the European recommendation (CRL Guidance Paper 2007) for anabolic steroid substances in urine was 0.5 ng/mL [38]. In the case of faecal matter, there are no recommendations [38] for steroid anabolic agents.

Table 12. Parameters obtained during the performance evaluation.

Urine				
	Hidroxyflutamide	Enobosarm	Bicalutamide	Andarine
a	1.4176	0.9815	0.9604	0.3593
b	0.1774	0.0859	0.1529	0.0471
r^2	0.998 ± 0.002	0.998 ± 0.002	0.999 ± 0.007	0.999 ± 0.006
RSD (%)	0.6 - 11.6	2.4 - 7.1	1.8 - 6.6	4.4 - 17.9
R%	97 – 118	73 - 118	95 – 111	71 – 107

Faeces				
	Hidroxyflutamide	Enobosarm	Bicalutamide	Andarine
a	0.7532	0.5833	0.6086	0.2108
b	0.7926	0.3069	0.4191	0.1650
r^2	0.996 ± 0.003	0.993 ± 0.005	0.996 ± 0.003	0.996 ± 0.002
RSD (%)	1.6 - 10.9	2.8 - 13.9	1.6 - 10.9	0.6 - 18
R%	88 – 115	85 - 117	83 - 109	74 - 108

Figure 10 presents chromatograms showing sensitivity, specificity and selectivity achieved by the method at low concentrations. The absence of interference (peaks) in blank samples and also a very good signal at the lowest tested concentrations can be observed.

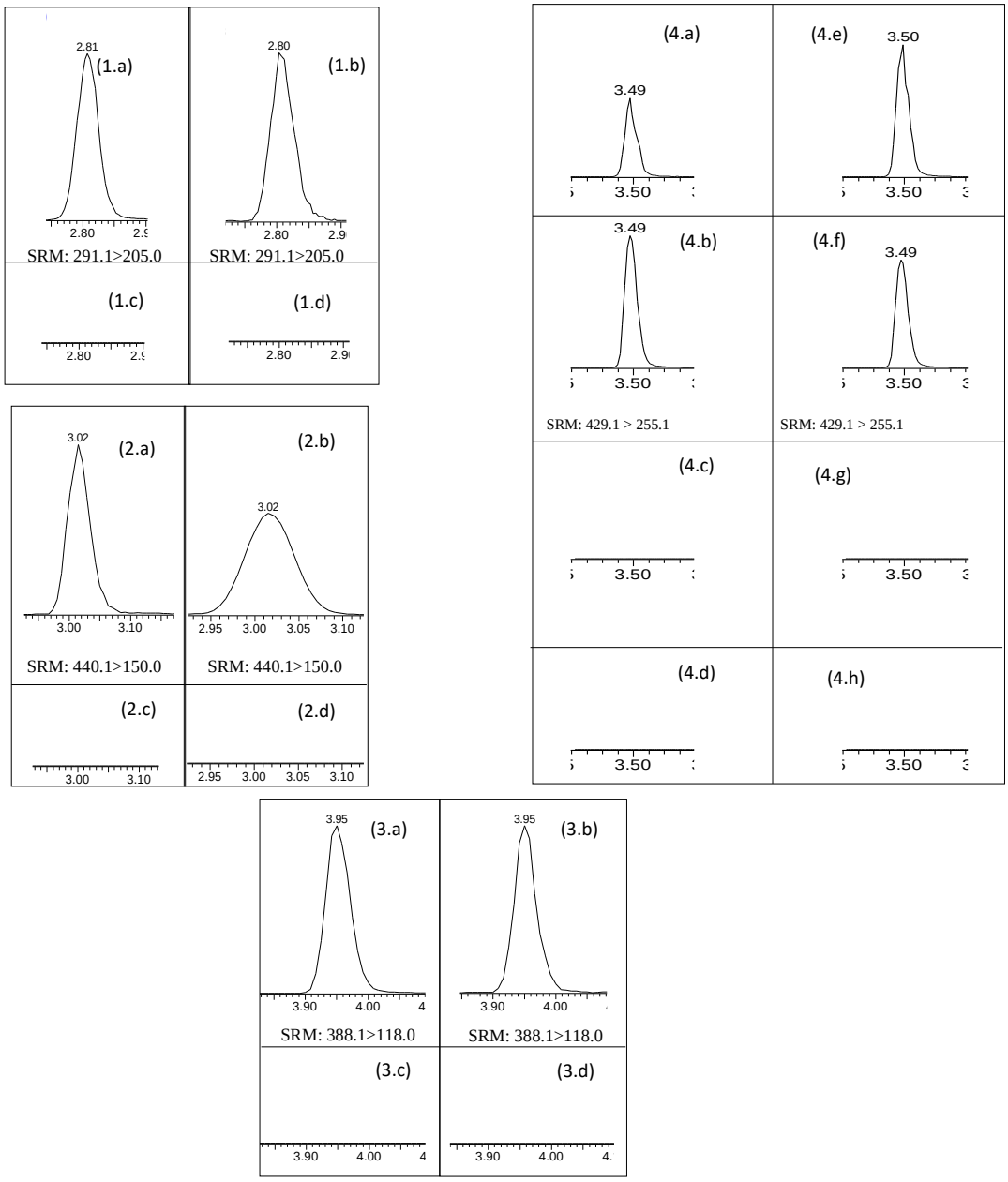


Figure 10. Cromatogramas SRM used for quantification of SARMs.

(1.a) Extract of Blank Urine with addition of Hidroxyflutamide (0.25 ng/mL); (1.b) Extract of blank faeces with addition of Hidroxyflutamide (0.5 ng/g); (1.c) Extract of Blank Urine; (1.d) Extract of blank faeces; (2.a) Extract of Blank Urine with addition of Andarine (0.25 ng/mL); (2.b) Extract of blank faeces with addition of Andarine (0.5 ng/g); (2.a) Extract of Blank Urine; (2.d) Extract of blank faeces; (3.a) Extract of Blank Urine with addition of Enobosarm (0.25 ng/mL); (3.b) Extract of blank faeces with addition of Enobosarm (0.5 ng/g); (3.c) Extract of Blank Urine; (3.d) Extract of blank faeces ; (4.a) Extract of Urine of treated calf, after administration of Casodex (day 1); (4.b) Extract of Blank Urine with addition of Bicalutamide; (4.c) Extract of Blank Urine; (4.d) Extract of Urine of treated calf, before administration of Casodex (day -3); (4.e) Extract of Urine of treated calf, after administration of Casodex (day 1); (4.f) Extract of Blank Urine with addition of Bicalutamide; (4.g) Extract of Blank Urine; (4.h) Extract of Urine of treated calf, before administration of Casodex (day -3).

Conclusions Partial:

- An analytical method for the study of Enobosarm, Hidroxyflutamide, Bicalutamide and Andarine was developed in urine and bovine faeces, The limit of quantification was 0.25 ng/mL for urine and 0.5 ng/g for faeces.
- The proposed method of processing for urine is simpler than for faeces. This was expected considering that faecal matter is a more complex matrix (heterogeneous and more content of protein, lipid, etc.). As a consequence faeces requires more work to eliminate possible interference or substances that could contaminate/deteriorate the instrumental equipment. For this reason the time taken for faeces analysis is double, especially due to evaporation stages.
- An analyst on a working day of 8 hours, applying the proposed protocols, could process up to 12 stool samples or up to 24 urine samples. In the case of enzymatic hydrolysis, we should consider overnight incubation.
- The proposed methods showed satisfactory performance for regulatory requirements in terms of linearity, sensitivity, precision, accuracy, selectivity and specificity. Consequently, the methodology developed is capable of being applied in the bovine production with the purpose of controlling use of Bicalutamide, Ostarine, Hidroxyflutamide and Andarine, in urine and faeces.
- It should be noted that this is the first work that studies the elimination of Bicalutamide in cattle and uses the faecal material as a matrix for its control in livestock production.

METABOLITE STUDY OF BICALUTAMIDE AND ANDARINE

First animal trial (Bicalutamide)

Bicalutamide Quantification in urines and faeces

The results obtained for Bicalutamide in urine and faeces during the 7 days of the test are presented in Figure 14 and more details are presented in the tables of Annex IV (page121). As expected, no Bicalutamide was detected in the samples before the treatment. Four hours after oral administration, Bicalutamide was already detected in urine and faeces. In urine, Bicalutamide concentration increased quickly to show the maximum at day 1 as follows: free form, 13.15 ng/ml and for the addition of free and deconjugated forms total: 17.36 ng/ml, attesting for the metabolisation of the drug under phase II metabolites. Both Bicalutamide forms (free and total) exhibited similar elimination profiles, they increase quickly to day 1 and the decreasing was slow afterwards. In faeces, Bicalutamide elimination was observed in greater proportion than in urine. Indeed, at day 1 the concentrations reached the ppm level (free= 1254.11 ng/g; total= 1346.66 ng/g). In faeces also, conjugated forms could be highlighted.

Studies obtained in some laboratory animals describe an excretion similar to that found in this work with a greater elimination of Bicalutamide in the first days of post-administration [18, 63].

The concentrations found in faeces were much higher than in urine. However, the relative increase for day 1 was higher in urine (4hs= 19 %; 8hs= 49 %) than in faeces (4hs=1 %; 8hs=19 %). Early urinary augmentation could be explained because Bicalutamide has 2 isomers (R and S) of which the S isomer has little biological activity and would be rapidly eliminated with minimal metabolic changes. Then the elimination would start quickly with the S isomer by urine [19, 63]. In contrast, R-Bicalutamide excretion is slow because of its metabolism that includes enterohepatic recirculation with biliary excretion [27]. This behaviour would probably be applicable to the bovine studied since

in Figure 11 (a) and (b) it can be seen that during the first 3 days we have the highest concentration values.

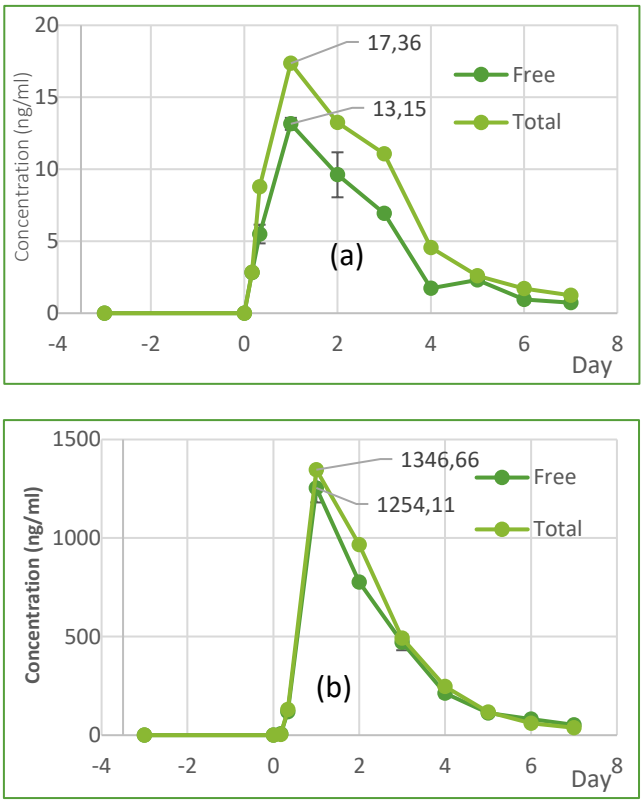


Figure 11.Excretion profile of Bicalutamide in faeces (a) y Urine (b).

Metabolites study

Figure 11 (a) y (b) present the concentration results for Bicalutamide *free* and Bicalutamide *total* (free + released, by enzymatic action). *Total* Bicalutamide curve is parallel to the *free* and during the whole test is above but always very close. It would be explain because the free (released without modifications) is eliminated in greater amount than conjugate (such as glucuronide, sulfate, etc.) in both matrices (urine and faeces). It is also possible to observe conjugated Bicalutamide in urine and faeces, performing subtraction of less free total. The presence of Bicalutamide *free* without modification in faeces could also be explained because a part of conjugated Bicalutamide is excreted in bile and the digestive flora would release the Bicalutamide from its conjugation [27].

Table 13 shows the results of the analysis carried out for the search of Bicalutamide metabolites with the mass spectrometry detector, searching with the different SCAN modes and confirming in SRM mode. The quasi-molecular ions [M-H$^-$] can be observed with the related ions and the transitions confirmed by SRM are indicated.

The search focused on previous works identifying characteristic ions [18, 19, 38], m/z = 185, 255, 159, 173, 621, 445, 525, 411 (Annex IV; page 121).

Table 13. Bicalutamide Metabolites found in the treated calf.

	M1	M2	M3	M4	M5
[M-H$^-$]	605	621	445	509	525
Related Ions (m/z)	587	603	427	491	507
	561	445*	409	411	445*
	543	427*	185*	173*	427*
	429*	365*	255*	185*	255*
	411*	185*	189*	255*	185*
	193*	255*		429*	175
	185	175			189
	255	189*			
	159*	271*			
	173	201*			
		173			
		159			

Figure 12 presents a composite chromatogram constructed by the sum of the SRM (total current ion, TIC) obtained in the analysis of the extracts mixture prepared as described in Metabolites Study (page 35).

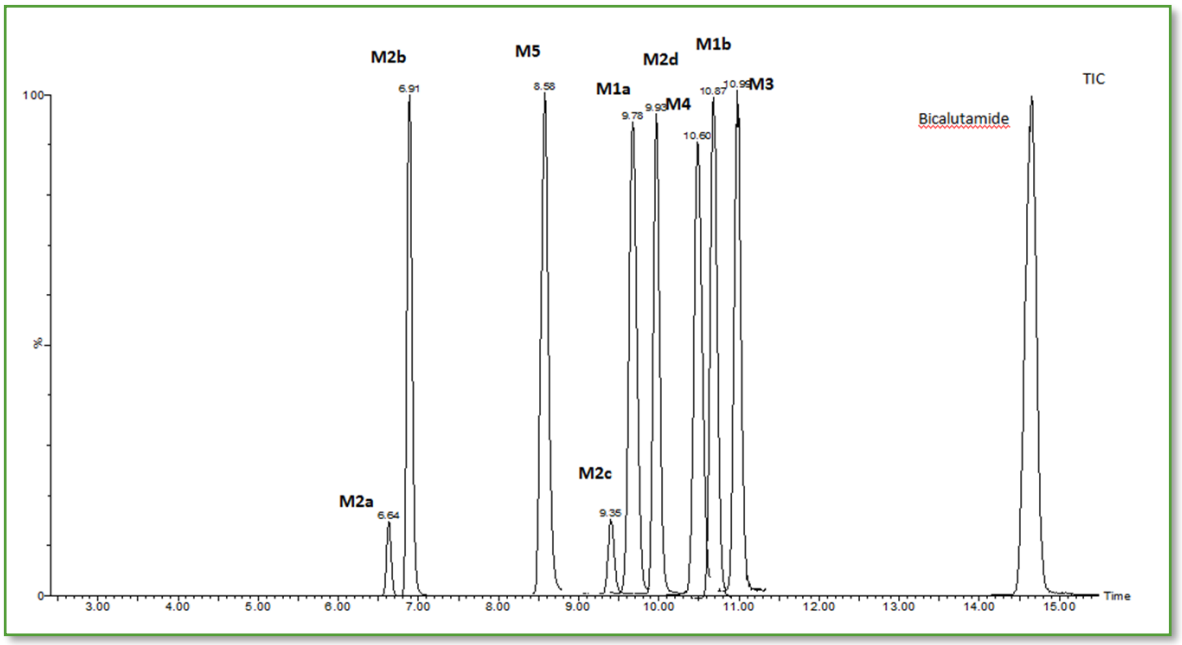


Figure 12. Composite Chromatogram constructed by TICs (1° trial)

TICs: M1a – M1b: 605>159 + 605>193 + 605>411; M2a – M2b: 621>185 + 621>255 + 621>445; M2c – M2d: 621>201 + 621>445 + 621>271; M3: 445>185 + 445>189 + 445> 255; M4: 509>173 + 509>185 + 509>255; M5: 525>185; 525>255; 525>445; Bicalutamide: 429,1>173,02 + 429,1>185,04 + 429,1>255,1.

After analyzing mass spectrometry information and bibliographic data, the metabolites found were 5. The following structures were proposed:

Metabolite 1 (M1): It has been attributed to glucuronated Bicalutamide observed under its deprotonated form (Figure 13). It showed relations with the following fragment ions: a) M1 losing water, m/z = 587; b) M1 loosing formiate, m/z = 561, followed by a loss of water, m/z = 543. It also showed: Bicalutamide characteristic fragments, m/z = 429, 411 and fragment: m/z = 193, which is consistent with glucuronides moieties. M1 generated fragments corresponding to both Bicalutamide rings without changes which is consistent with the glucurunidation at the tertiary alcohol of Bicalutamide as follows: a) ring tri-fluoro-methyl substituted, m/z = 185, 255 and b) ring mono-fluoro substituted, m/z = 159, 173. M1 has already been observed and reported in previous studies [18, 19, 63].

Figure 12 shows two chromatographic peaks for M1 SRM transitions, M1a (9.78 min) and M1b (10.87 min), but this study has no evidence to confirm

which peak correspond to R and S Bicalutamide forms. Figure 13 presents a probable structure for M1, but the present work could not indicate the real position of glucuronic acid (Gluc).

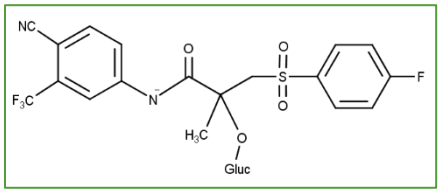


Figure 13. Proposed structure for M1 (m/z = 605).

Metabolite 2 (M2): It corresponds to hydroxylated and glucuronated Bicalutamide deprotonated form, M2 (Figure 14). It showed relations with the follow fragments: a) Bicalutamide hydroxylated, m/z = 445; b) Bicalutamide hydroxylated loosing water, m/z = 427. It was also found m/z = 643, consistent with the sodium adducted of M2. M2 was reported in previous studies. [18, 19, 28, 63]. Figure 12 shows four peaks (M2a, M2b, M2c and M2d) for M2 - MRM transitions. It could suggest mono-hydroxylation with glucuronidation could happen at each ring for the isomer R and S. The limitations of this study not allow suggesting wich of these peak correspond to S or R forms. M2a (6.6 min) and M2b (6.9 min): showed fragments corresponding to the tri-fluorate- methyl substituted ring without changes, m/z = 185, 255 and fragments consistent with the mono-fluorate substituted ring hydroxilated (m/z = 175, 189) and glucuronated (m/z = 365). M2c (9.3 min) and M2d (9.9 min): showed fragments corresponding to the mono-fluorate substituted ring without changes m/z = 159, 173 and fragments consistent with the tri-fluorate- methyl substituted ring hydroxilated (m/z = 201, 271).

Figure 14 shows 2 possible location alternatives for glucuronic acid (Gluc). This Figure does not propose an exact structure since the information provided by the mass spectrometry detector is not sufficient.

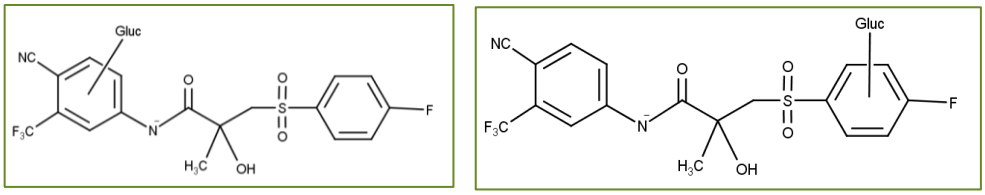


Figure 14. Proposed structure for M2 (m/z = 621).

Metabolite 3 (M3): Corresponding to the deprotonated form of Bicalutamide hydroxylated, M3, [M-H] = 445(Figure 15). It was reported in previous studies [19, 63].

M3 showed relations with the follow fragments: M3 loosing water molecules, m/z = 427 y m/z = 409. M3 also showed relations with the unchanged tri-fluorated-methyl ring, m/z = 185, 255 and fragments consistent with the hydroxylated monofluorate ring (m/z = 189).

Figure 15 shows a probable chemical structure of M3 consistent with the mass spectra information obtained. Hydroxyl can be assumed to be in the monofluorineted ring but the exact position cannot be known.

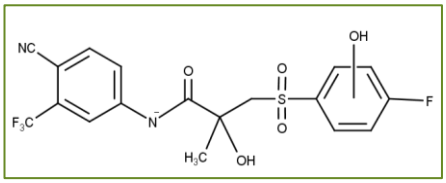


Figure 15. Proposed structure for M3 (m/z = 445).

Metabolite 4 (M4): Corresponding to the deprotonated form of Bicalutamide sulfated, M4 (Figure 16). It showed a fragment consistent with loosing water, m/z = 491, and characteristics fragments of Bicalutamide, m/z = 429 y 411. It showed relations consistent to the both unchanged rings of Bicalutamide, m/z = 173, 185 y 255, it suggest the sulfate is located at the tertiary alcohol of Bicalutamide.

Figure 16 shows a probable chemical structure of M4 consistent with the mass spectra information obtained.

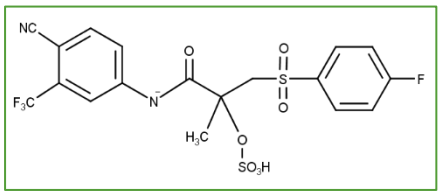


Figure 16. Proposed structure for M4 (m/z = 509).

Metabolite 5 (M5): Corresponding to the deprotonated form of hydroxylated and sulfated Bicalutamide, M5 (Figure 17). It showed fragments consistent with: a) M5 loosing water, m/z = 507; b) deprotonated form of Bicalutamide hydroxylated, m/z = 445 and c) the deprotonated form of Bicalutamide hydroxylated loosing water, m/z = 427. M5 also showed fragments corresponding to the tri-fluorated-methyl ring without changes m/z = 185, 255 and fragments consistent with the monofluorate ring hydroxilated (m/z = 175, 189) suggesting sulfate was located at the monofluorate ring hydroxylated. Figure 17 shows a probable chemical structure of M5 consistent with the mass spectra information obtained.

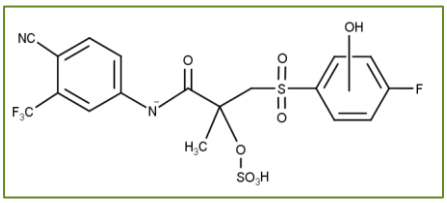


Figure 17. Proposed structure for M5 (m/z = 525).

Metabolites elimination

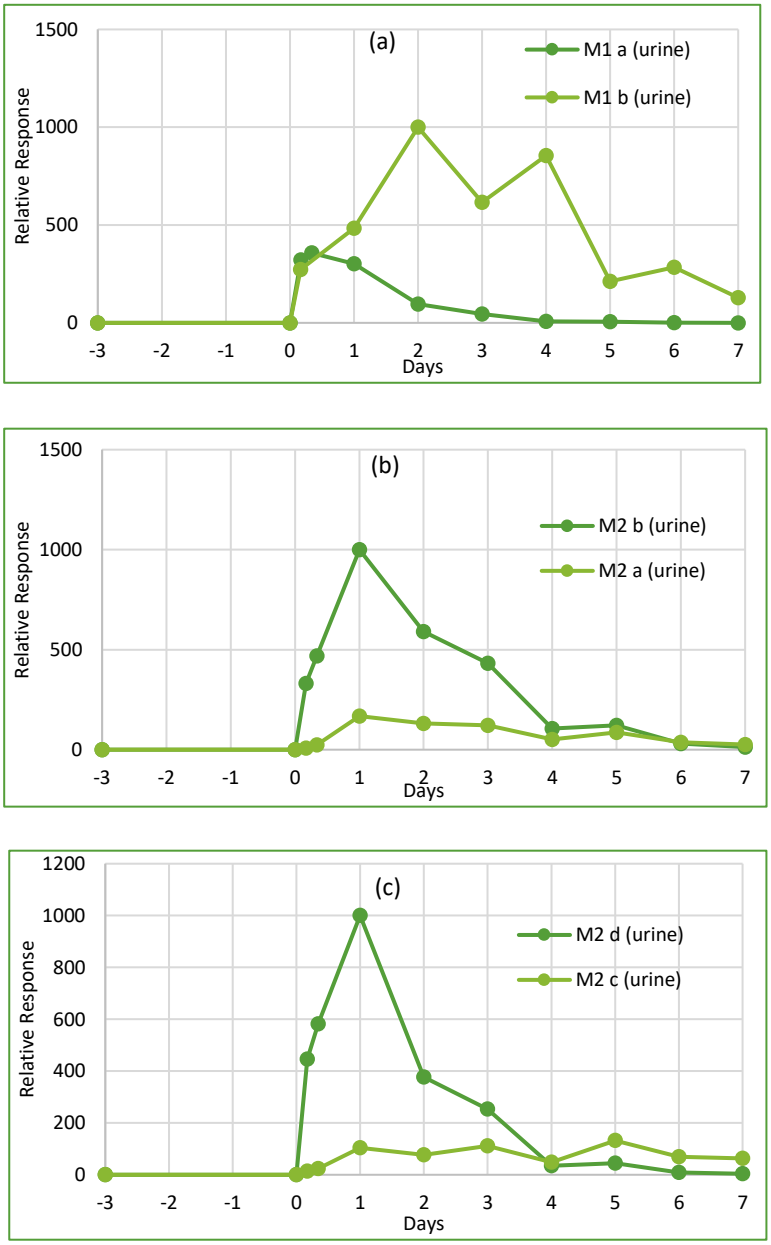
Table 14 shows the MRM used to establish the time windows for each metabolite found in the samples.

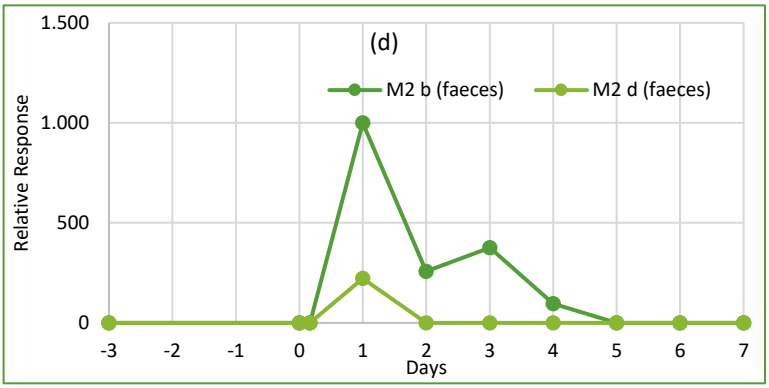
Table 14. Transitions used for the construction of excretion profiles.

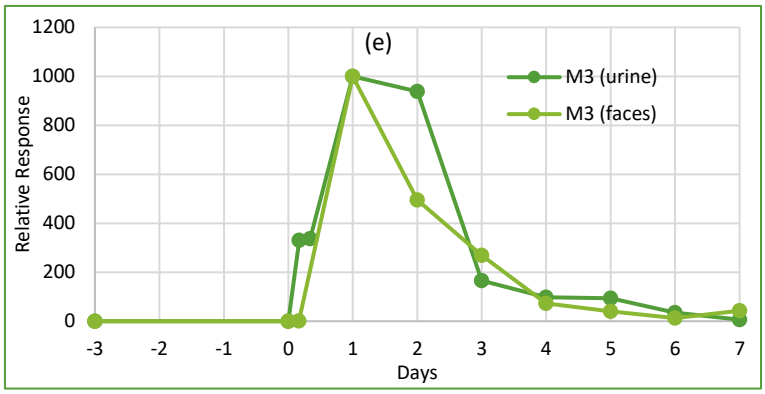
	[M-H$^-$]	MRM (m/z)		
M1	605	605>411	605>193	605>159
M2	621	621>185	621>255	621>189
M3	445	445>185	445>255	445>189
M4	509	509>173	509>185	509>255
M5	525	525>445	525>255	525>185

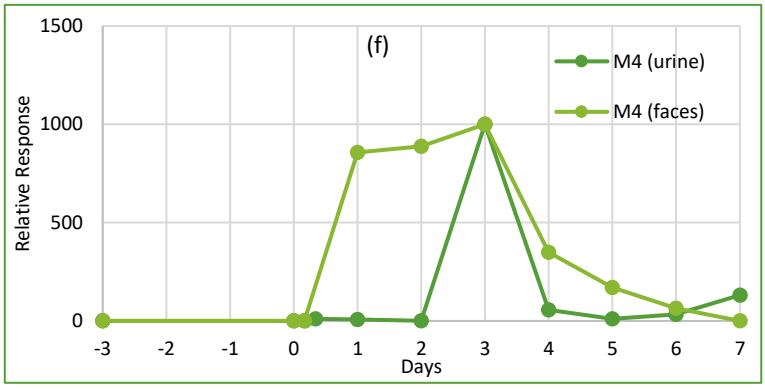
Below are the metabolite excretion profiles obtained from chromatographic analysis (Figure 18). In the absence of standards for each metabolite, the signal cannot be transformed into units of concentration. For this reason the following figures are constructed on an arbitrary basis, where the signal of each Figure is relative to the maximum metabolite signal. However, these Figures are very important since they show which metabolites are characteristic of each matrix and in addition, how long each metabolite is detectable by determining the temporal window.

Figure 18. Excretion profile of Bicalutamide metabolites.




(d)
M2 b (faeces)
M2 d (faeces)
Relative Response
1.500
1.000
500
0
-3 -2 -1 0 1 2 3 4 5 6 7
Days


(e)
M3 (urine)
M3 (faces)
Relative Response
1200
1000
800
600
400
200
0
-3 -2 -1 0 1 2 3 4 5 6 7
Days


(f)
M4 (urine)
M4 (faces)
Relative Response
1500
1000
500
0
-3 -2 -1 0 1 2 3 4 5 6 7
Days

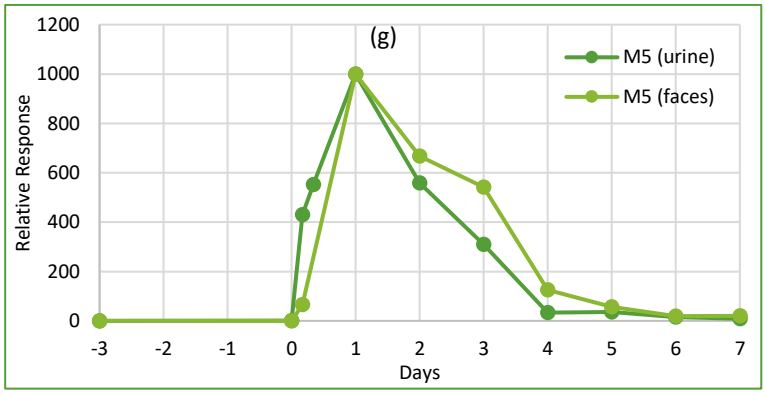

(g)
M5 (urine)
M5 (faces)
Relative Response
1200
1000
800
600
400
200
0
-3 -2 -1 0 1 2 3 4 5 6 7
Days

Figure 18 (a), (b), (c), (d), (e), (f) y (g) show the results obtained in this part of the study, presenting the variation of the relative response during the trial (days).

M1: In urine, M1a and M1b start its quick increasing 4 hs after the administration of Bicalutamide (Figure 18 (a)). The maximum for M1a was at 8 hs after the administration follow by a slow and marked decreasing. M1b show the maximum to day 1, its decreasing was the slower than M1a. In faeces, M1a and M1b were not found.

M2: In urine, M2a and M2c show a slow increasing until day 1 and then they maintain the response. M2b and M2d show a quick and big increasing with a maximum at day 1 follow by a slow decreasing even with a response similar to M2a and M2c at day 4 and lower at day 6 and 7 (Figure 18 b y c).

In faeces, M2d was found just at day 1 with a 22 % of the signal of M2c. M2c shows his first value at day 1 then decrease and since day 5 was not detected (Figure 18 d).

M3: In both matrices, the increasing starts 4 hours after the Bicalutamide administration and the maximums were found at day 1. The decreasing was slower than the increasing (Figure 18 e).

M4: In urine, the relative response was low during the trial and it shows a peak at day 3. In faeces, the increasing starts 4 hours after the Bicalutamide administration and it shows the peak at day 3 (Figure 18 f).

M5: In both matrices, the maximum were at day 1. In urine, the peak was almost symmetric while in faeces it increased quickly and it decreased slowly (Figure 18 g).

The Bicalutamide and its metabolites behaviour as observed in this studied are in agreement with observations reported in rat, mouse and rabbit, where the maximum Bicalutamide concentration was recovered in 48hs in rat mouse and rabbit (25). The relative responses found in this study between different peaks, mainly M2, were consistent with enantioselective metabolism of Bicalutamide in others animal (25) and men (24).

The results found demonstrate that main metabolites of Bicalutamide in calf result from hydroxylation and the phase II reactions. The observed metabolic pathways are similar to other APA-SARMs (10, 16, 29, 31, 32).

Because of the low aqueous solubility of Bicalutamide (< 5 mg/L) a low occurrence of the drug in excreta would be expected. However, as noted in the results presented and previous studies show a slow elimination explained by an intense absorption of the drug.

The results indicate an intense metabolism of the drug that includes oxidative modifications, sulfated and glucuronated derivatives. The metabolites found can be explained by described reactions for other APA-SARMs [34, 36, 46, 49, 54].

There was also a large excretion of the drug without changes in urine and faeces. These results are confirmed by previous work in laboratory animals and humans [18, 19, 63]. However, in laboratory animals other authors highlight the appearance of a majority urinary metabolite that was not found in this trial. Also, the metabolites from the clivage of amide bond, described by other authors were not found in this study [18, 19, 63].

Maximum concentrations found in faeces and urine within the first 24 hours are consistent with previous work performed on humans and laboratory animals [18, 63]. In rat, mouse and rabbit the recovery of most part of the administered drug is obtained in the first 48 hs [18, 19, 63].

In addition, the data obtained (especially for M2) support the hypothesis of a metabolism with enantio-selectivity as reported in other species [18], including humans [63]. The signal intensity of M1 and M2 in faeces would refer more to contamination (during sampling or laboratory manipulation of the sample) than to a true faecal excretion of these glucuronides [18, 19, 63].

The metabolites found in urine and the prolonged time of excretion would support the existence of an intense intestinal reabsorption proposed by other authors [18, 19, 63]. This explains much of the persistence attributed to this drug.

Higher concentrations of Bicalutamide in faeces may be related to the removal of faecal water during the lyophilisation process although some

previous work in laboratory animals indicates a significant excretion of Bicalutamide in faeces [18, 27]. In our case it is possible that both effects are simultaneous.

For metabolites M4 and M5 no previous bibliographic description was found. The observed differences could be explained by the metabolism of the animal and the studied breed or by the methodological differences used [38] [64].

Partial Conclusions:

- The variation in Bicalutamide concentration in urine and faeces was determined after oral administration of 100 mg of Bicalutamide. In both matrices a rapid increase is observed, reaching a maximum on day 1 (13.15 ng/mL in urine and 1254.11 ng/g in faeces) and a slow decrease in concentration until day 7 (0.72 ng/mL in urine and 52.90 ng/g in faeces).
- These data support the use of urine and faeces as useful matrices for the control of this drug and probably for other APA-SARMs in livestock practices.
- Both studied matrices presented metabolites from Phase I and Phase II reactions. The metabolites found come from hydroxylation and conjugation reactions. Some metabolites found were previously described on in-vivo trials in humans and laboratory animals (rat, dog, rabbit and mice).
- The metabolites found can be explained by described reactions for other APA-SARMs.
- The largest variety of metabolites were found in urine showing their maximum within 48 hours after administration of the drug. Only M4 (derived sulphate) showed its peak on day 3 for urine and faecal matter. After day 4 the metabolites are close to 10 % of its maximum intensity, except Bicalutamide, M1b, M2b and M2c which are kept above (in urine).
- Based on the results, the analytical control of Bicalutamide should be carried out by studying in urine M2, M5 and M3; in faeces M3. However, Bicalutamide without modification is the best candidate since it presents large

signal, it is detectable until the end of the test and it is possible to quantify because of it has a standard.

- This work constitutes the first study of Bicalutamide elimination in bovine animals, the first to propose the faeces as matrix of analysis and the first to describe the presence of M4 and M5.

Second animal trial

Excretion study of Bicalutamide, Andarine and its metabolites

Chromatographic equipment for the analysis of excretion on this animal trial was different from the first trial and for this reason the performance was evaluated by: *Linearity*, *Accuracy*, *Precision*, *Sensibility* and *Selectivity*.

Method Performance (INTA – Castelar)

Table 15 shows chromatographic and mass spectrometric parameters used for the identification and quantification of the APA-SARMs. Annex VI presents the development of this parameters (page 124).

Table 16 presents a summary of the results of the parameters calculated for the 3 matrices.

The parameters obtained for the study were satisfactory in all cases, in a wide range of tested concentrations (urine: 0.5 - 200 ng/mL; faeces: 2 - 3500 ng/g; serum: 2– 3000 ng/mL), for Bicalutamide and Andarine:

- Coefficients of determination (r^2) > 0.98
- Recoveries between 70 % - 120 %
- Relatives Standard Deviation (RSD %) < 20 %
- Interferences were not observed on the 3 studied matrices.

Annex VII (page 127) presents tables and more information about this evaluation.

Figure 19 presents SIM (Single Ion Monitoring) chromatograms of spiked blank samples using stock standards solutions (5 ng/mL) urines 0.5 ng/mL, faeces: 2 ng/g and serum: 2 ng/mL.

Table 15. Parameters of mass spectrometry used for SARMs (2° trial).

RT (min)	Analitos	Ions	Cono (V)
8.01	Hidroxyflutamide	291.1 (Q)	40
		205.0 (q)	
8.6	Andarine	261.0 (q)	40
		440.1 (Q)	
9.2	Bicalutamide	429.1 (q)	60
		255.1 (Q)	

Single Ion Monitoring used for Andarine, Bicalutamide and Hidroxyflutamide. (Q) Ion of quantification, (q) Ion of qualification.

Table 16. Parameters obtained the performance evaluation (2nd trial).

	Urine	
	Bicalutamide	Andarine
a	0.0626	0.0099
b	-0.5630	-2.6763
r^2	0.9987 ± 0.0018	0.9988 ± 0.0014
R %	84.3 – 112.1	80.4 – 108.8
RSD %	3.5 - 13.4	4.9 – 12.0

	Faeces	
	Bicalutamide	Andarine
a	0.0548	0.0076
b	1.2819	0.1190
r^2	0.9914 ± 0.0083	0.9993 ± 0.0008
R %	86.7 - 110.8	82.1 - 108.1
RSD %	5.3 - 12.1	5.6 - 10.7

	Serum	
	Bicalutamide	Andarine
a	0.0022	0.0016
b	-0.0037	0.0278
r^2	0.9994 ± 0.0023	0.9986 ± 0.0013
R %	82.7 - 110.5	86.5 - 109.4
RSD %	5.3 - 11.9	7.7 - 11.3

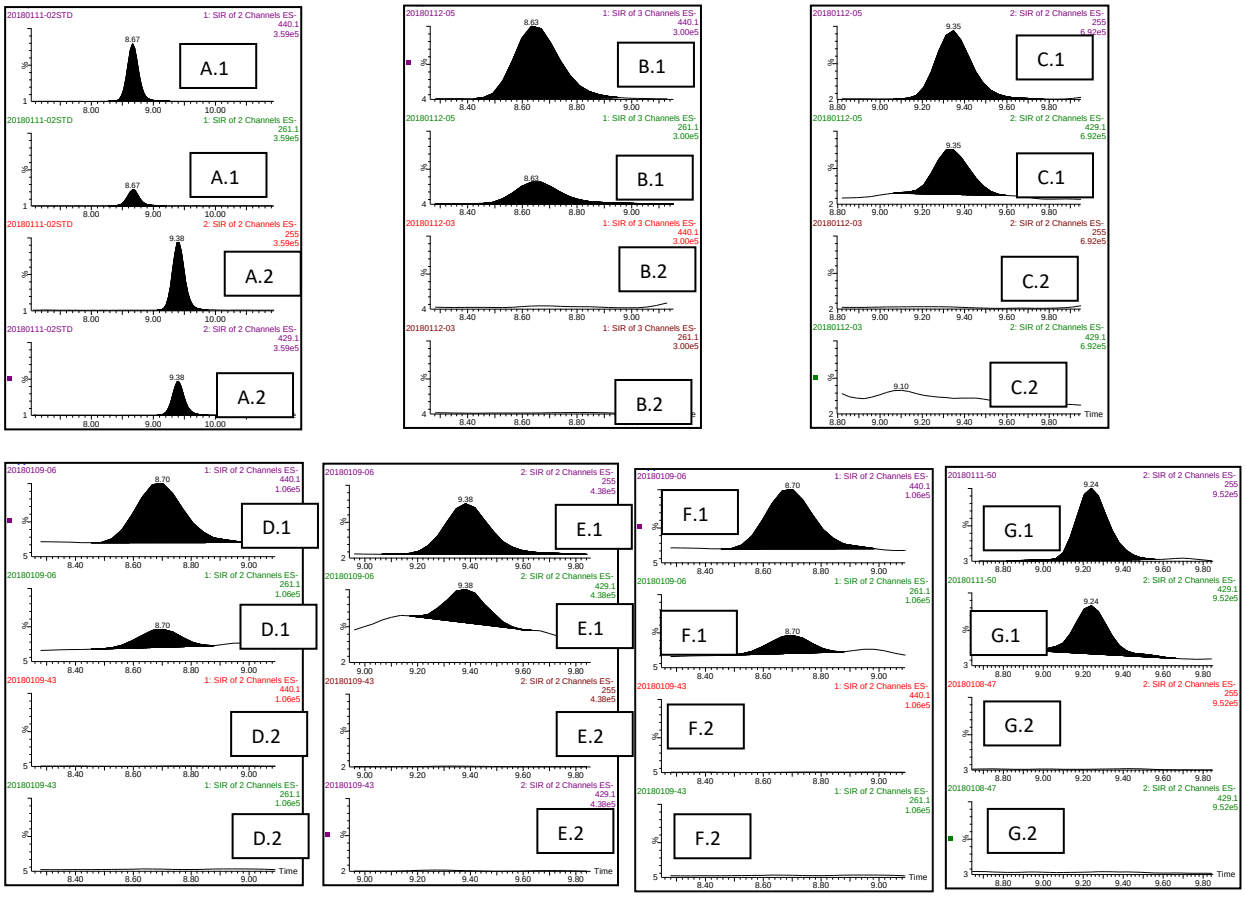


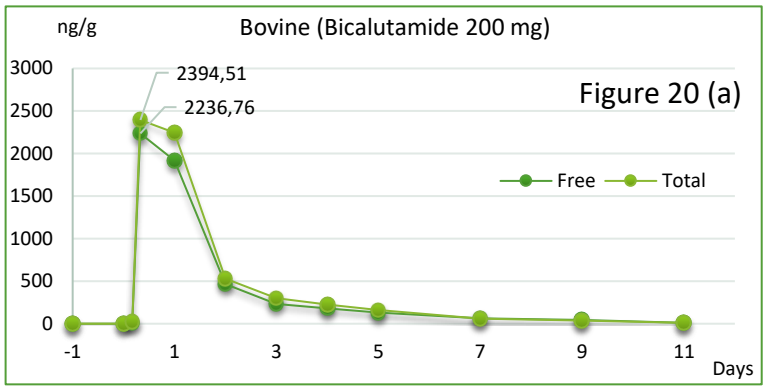
Figure 19. SIM Chromatograms: Standard solutions, blank samples and spiked samples.

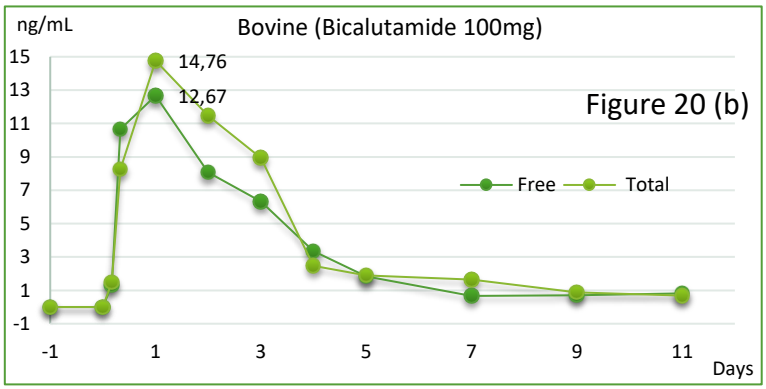
(A.1) Andarine, (A.2) Bicalutamide; Blank Urine (B.2) and Spiked Urine with Andarine (B.1); Blank Urine (C.2) and Spiked Urine with Bicalutamide (C.1); Blank Faeces (D.2) and Spiked Faeces with Andarine (D.1); Blank Faeces (E.2) and Spiked Faeces with Bicalutamide (E.1); Blank Serum (F.2) and Spiked Serum with Andarine (F.1); Blank Serum (G.2) and Spiked Serum with Bicalutamide (G.1).

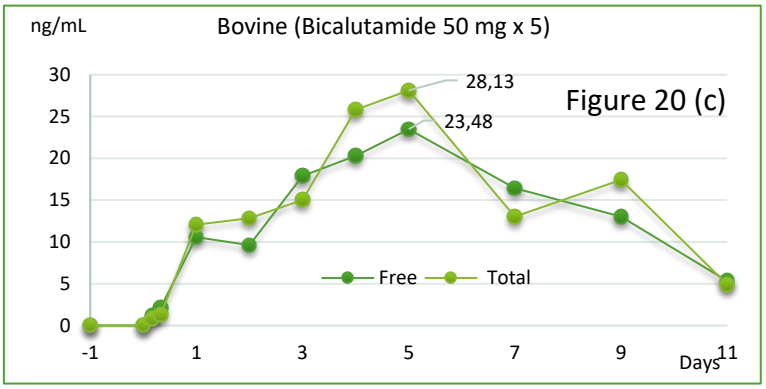
Excretion of Bicalutamide and Andarine

The results for free and total Bicalutamide in urine are presented in the Figure 20; in the faeces Figure 21 ; in the serum Figure 22 and the results of Andarine in the treated porcine are presented Figure 23.

Figure 20. Concentrations of Bicalutamide in Urine.







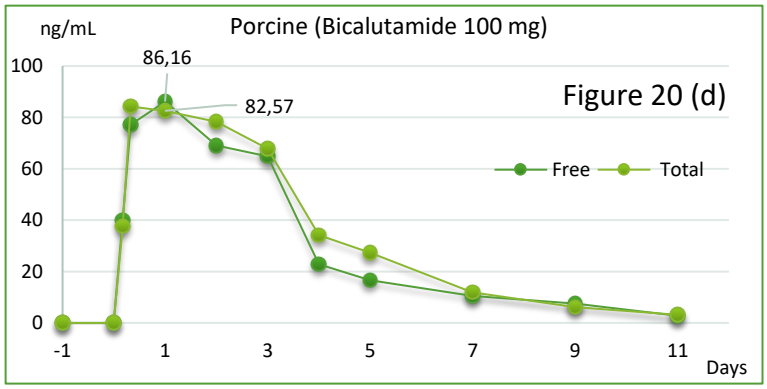
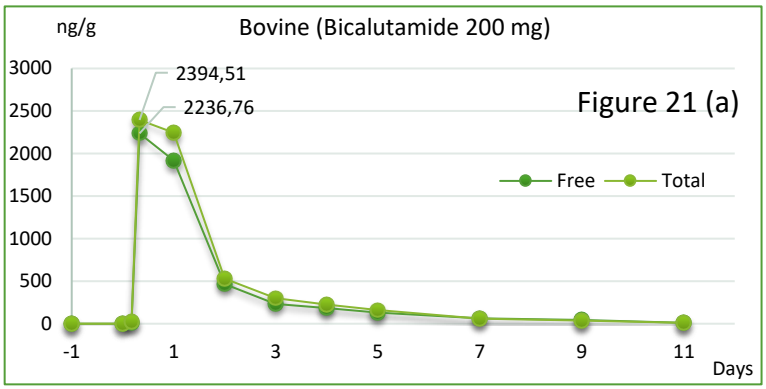
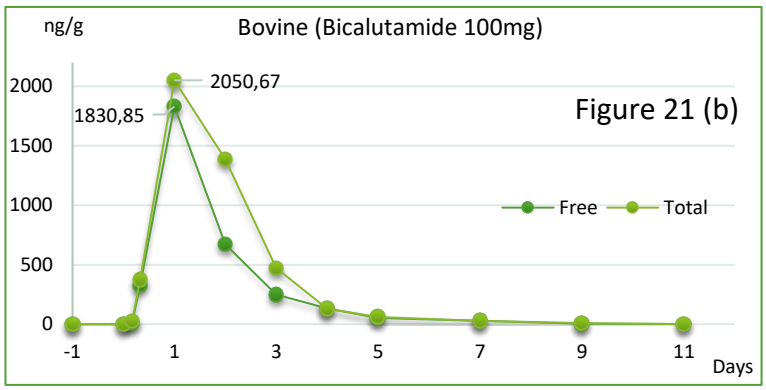
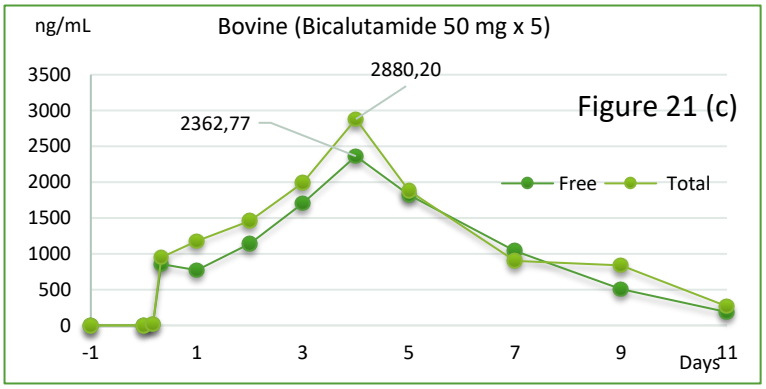


Figure 21. Concentrations of Bicalutamide in faeces.







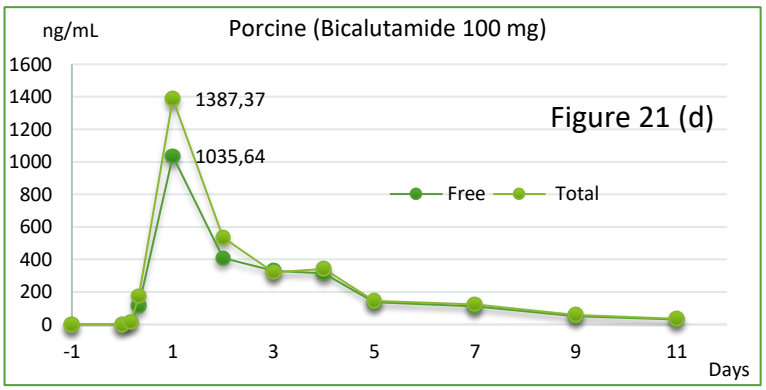
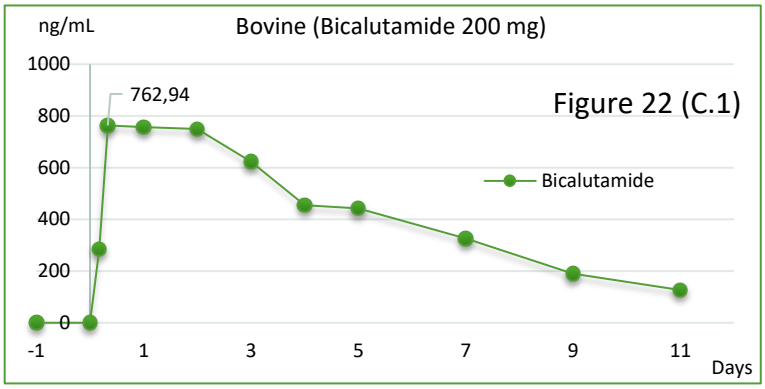
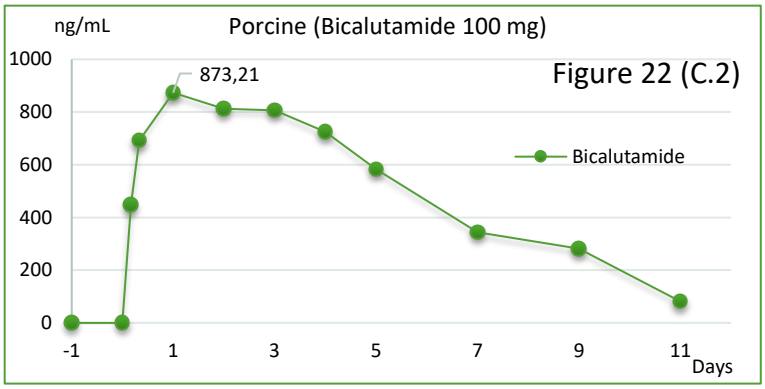
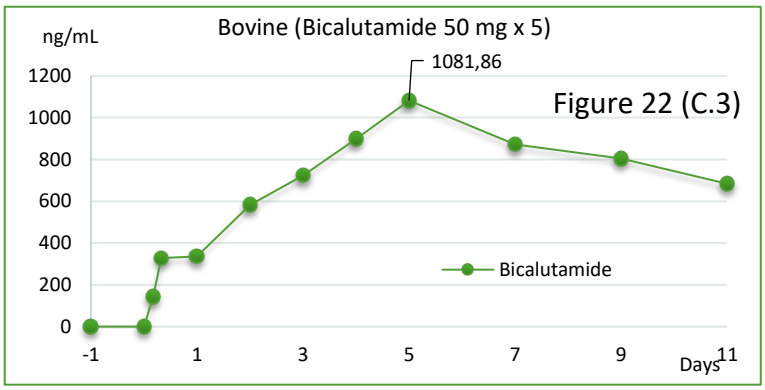


Figure 22. Serum concentrations of Bicalutamide.







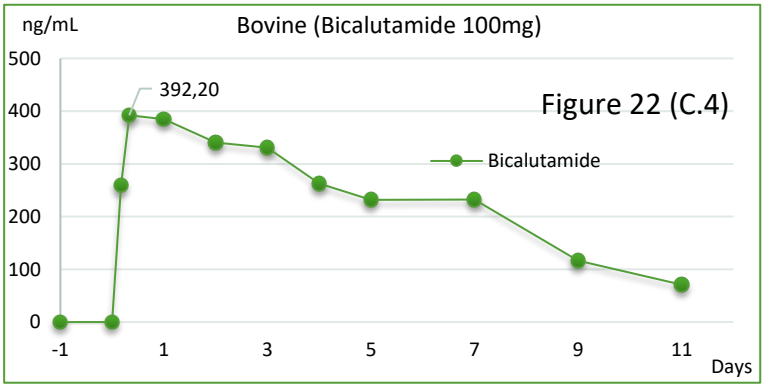
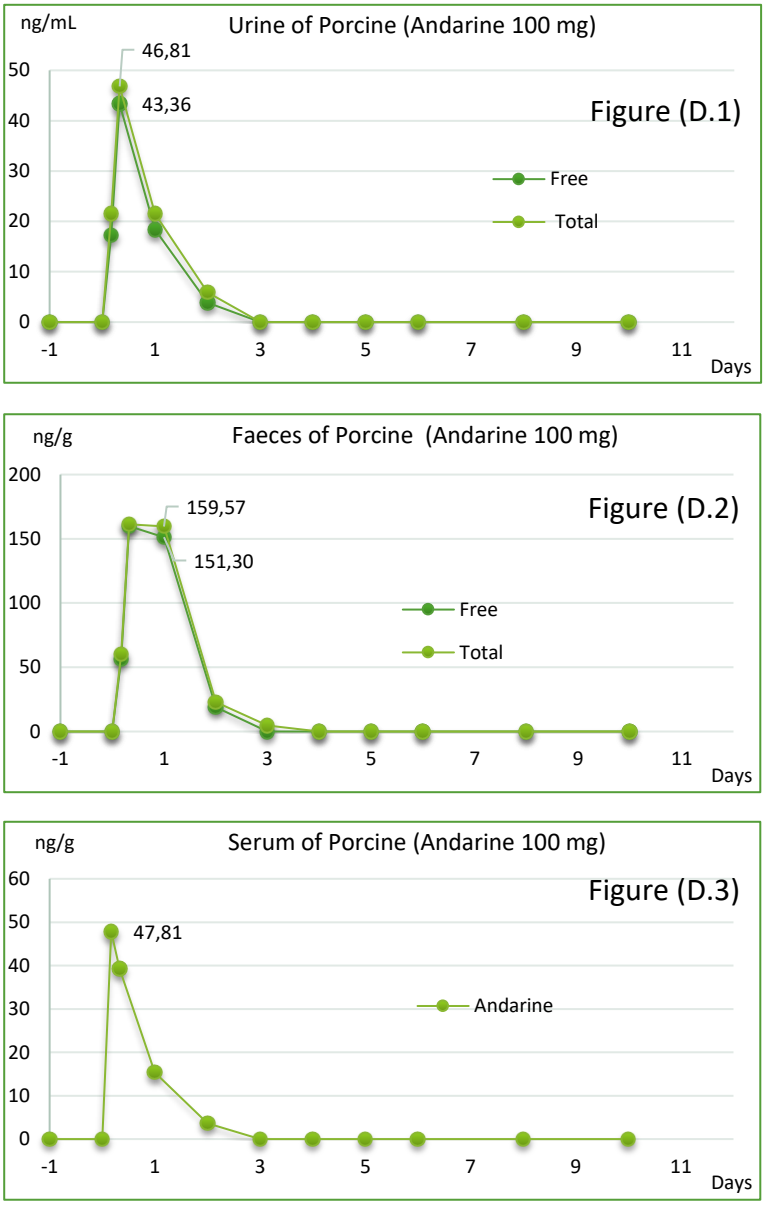


Figure 23. Concentrations of Andarine in Urine, Faeces and Serum of the treated porcine.



As expected, before administration no SARMs were detected in any of the studied matrices. Analysing the obtained concentration values, the lyophilized faeces showed highest concentrations for both species, both drugs and the different doses administered. This could be partly attributed to the lyophilisation process in which up to 80 % of the faecal weight is reduced by removing water and thus generating the concentration of non-volatile solutes such as SARMs and its metabolites.

Comparing the results obtained in treated animals with a single dose of Bicalutamide, a rapid increase of concentration can be observed (4 hs) which indicates a good absorption as in other animal species[18]. Excretion reaches its maximum by day 1, except in faecal matter with doses of 200 mg which had its maximum before. After maximum concentration in each matrix decreases slowly, describing asymmetric curves similar to those found in the first animal trial (page 49). These results in pigs and cattle, will support studies made by other authors in laboratory animals, where the almost total recovery of a single dose by collecting urine and faeces is described, finding less than 1 % in exhaled air. [18, 19].

Serum concentration curves of Bicalutamide in both species studied indicate a rapid absorption and a long period of permanence in blood, as in humans would probably be transported along with proteins mainly in albumin [27].

About the cumulative effect of daily doses of 50 mg, Figure 22 (C.3) shows the increase in serum concentration over the first 5 days of trials follows by similarly increases on the excretion (in urine and faecal matter). Clinical studies conducted in human volunteers with a daily administration of 50 mg describe an increase in serum concentration of Bicalutamide of up to 10 times [27]. The results obtained in our work for cattle, would be explained by the existence of biliary excretion of conjugated Bicalutamide and its reabsorption based on the described entero-hepatic circulation which would cause the accumulation effect [27, 52, 63].

The results obtained for Andarine presented a maximum fast concentration (4 hs) in all matrices and a rapid decrease, being not detectable at 3 days (in urine and serum). This behaviour is similar to previous studies carried out on other animals with a rapid appearance in the blood, indicating a fast digestive absorption [45, 52, 65].

Comparing the concentrations of Bicalutamide and Andarine in pigs, it is observed that Bicalutamide has a greater permanence in the animals, being detected until the last day of sampling in all matrices. On the other hand, Andarine is quickly eliminated, also the absorption is fast and for this reason

they present "sharped peaks" in serum, urine and faeces. The results presented in this work, referring to rapid absorption and distinctive permanence behaviour for Bicalutamide and Andarine, are similar to those described in previous work done by other authors [8].

Observing the Figures constructed with the concentrations of Andarine in the different matrices, it can be pointed out a first inconvenience for the control laboratories due to their short period of detection. These analytical drawbacks were mentioned in previous work carried out by other authors with laboratory animals and sports competition animals [45, 65, 66]. Andarine metabolisms are described as so intense that at low doses it is not possible to find the parent drug in urine [52]. In our case, it could be assumed that the dose given would be high enough to exceed/saturate the capacity of the metabolic pathways of pig removal and for this reason we found Andarine in urine and faeces.

Metabolites Study

In the case of animals treated with Casodex ® the work of finding metabolites was simplified by having characteristic fragments (Annex V; page 122). In the case of the pig treated with Andarine the characteristic fragments were first established (Annex VIII; page 134). Then analyses in SCAN mode and SRM mode were performed.

Bicalutamide metabolites

Table 17 shows the relevant ions found in SCAN mode and with an asterisk are marked the confirmed reactions in SRM mode. The information in this table shows that the metabolites found are the same as those previously found, as described in the Table 13 (page 50).

Table 17. Bicalutamide metabolites found in the treated steer.

	M1	M2	M3	M4	M5
[M-H$^-$]	605	621	445	509	525
Related Ions (m/z)	587	603	427	411	507
	543	445	185*	173*	445*
	429	427	255*	185*	427
	411*	185*	189*	255*	255*
	193*	255*			185*
	185	175			175
	255	271			
	159*	173			
		159			

Figure 24 shows a composite chromatogram constructed with the results obtained from the bovine treated with 200 mg Casodex ®. In time order it can be seen 2 peaks corresponding to M2, the largest at 3.64 min.; a first peak from M1 to 4.04 min.; then a peak of M5 (4.11 min.); another peak of M1 (4.19 min.); the peaks of M3 and M4 leave together at 4.29 min. and finally Bicalutamide (4.71 min).

By comparing the composite chromatograms in Figure 12 (page 52) with Figure 24, differences in all peak retention times can be observed, this is because the chromatographic system used in INTA – Concordia was different from that used in LabERCA (Nantes).

Another difference is the absence of metabolites M2c and M2d in the chromatogram of Figure 24, an explanation could be that these animals do not produce absent M2 metabolites, which could be due to inter-species differences due to the existence of different isoforms of some enzymes involved in the metabolism of SARMs [64]. Another explanation could be, as mentioned above, when changing the chromatographic system we can think that our method/instruments applied in this test are not able to detect them at a low concentration at which they were produced. Another explanation would be that the substances administered in both cases (Casodex®) were commercial products and could have some minimal differences in their composition, since they were produced by the same manufacturer, were acquired in different

countries and years. The lack or minimal production of M2c or M2d in treated animals could be the most likely cause because the tests carried out were subject to great biological variability (race, physiological status, age, etc.). Finally, the combination of all the above causes cannot be ruled out as another explanation either.

The details of the metabolites, ions and SRMs used for identification in the Annex IX (page 135).

Table 18 presents the ions found in the porcine samples after the mass spectrometry analysis. Comparing with the previous results from bovine samples (Table 17 y Table 13), it can be observed M1, M2, M3 and M4 but there was not found M5.

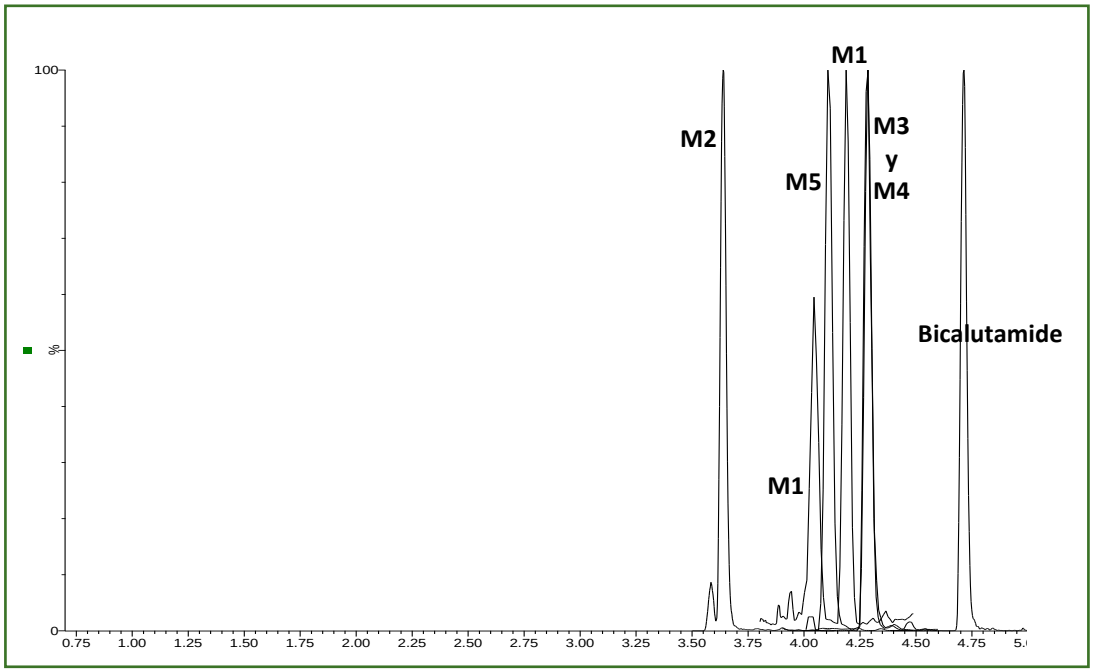


Figure 24. Composite chromatogram for the treated steer with 200 mg Casodex.

SRM: M1: 605>411; M2: 621>255; M3: 445> 255; M4: 509>255; M5: 525>255
Bicalutamide: 429.1>255.1

Table 18. Bicalutamide metabolites found in the treated porcine.

	M1	M2	M3	M4
[M-H$^-$]	605	621	445	509
Related Ions (m/z)	587	603	427	411
	561	445	409	185*
	543	365	185*	255*
	429	185*	255*	
	411*	255*	189*	
	185	175		
	255	189		
		271		
		173		
		159		

Figure 25 presents a composite chromatogram corresponding to urine samples and faeces of the pig treated with Casodex ®. This figure was constructed with SRM that confirm the presence of metabolites M1, M2, M3, M4 and Bicalutamide. As expected this chromatogram shows retention times similar to the metabolites in cattle treated with Casodex ®. In order of appearance can be observed: M2 with two peaks and the larger one at 3.65 min.; two peaks corresponding to M1 (4.04 min. and 4.19 min.); again M3 and M4 have almost simultaneous elution (4.3 min.). Further details of the metabolites, ions and SRMs used for identification in the Annex IX (page 135).

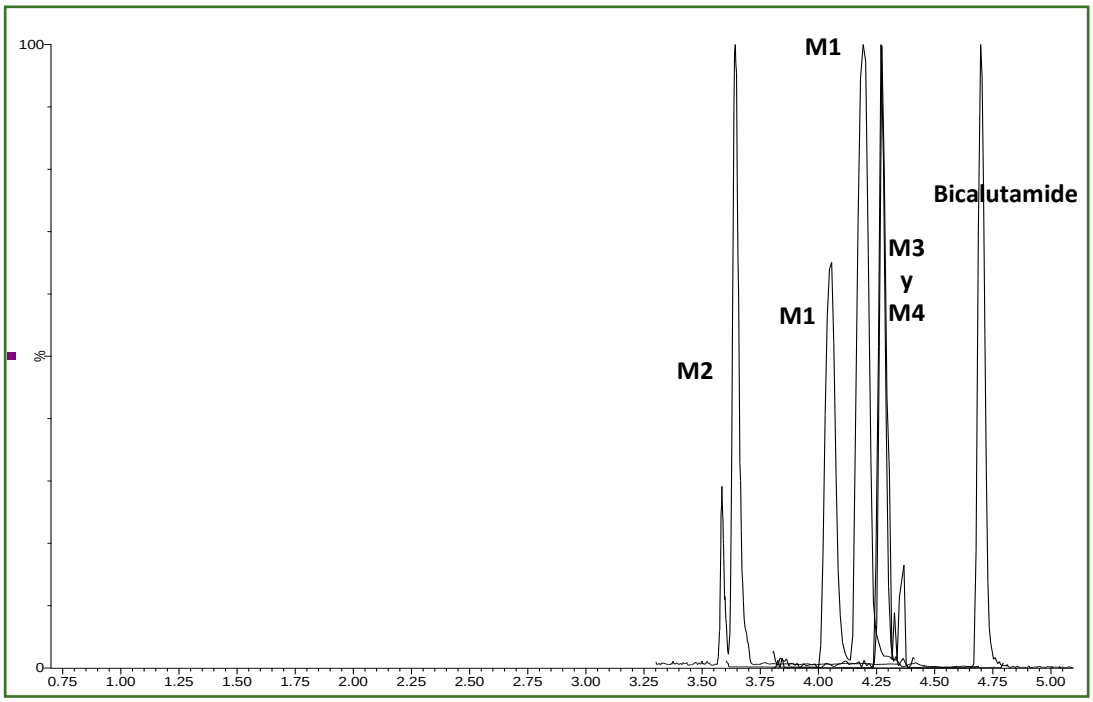


Figure 25. Composite chromatogram of the porcine after Casodex ® administration.

(M1: 605>411; M2: 621>255; M3: 445> 255; M4: 509>255 y Bicalutamide: 429>255).

Andarine Metabolites

Table 19 shows the ions used for the identification of each metabolite found and the ions confirmed by SRM and MRM are marked with asterisks.

Table 19. Andarine metabolites found in the treated porcine.

	DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8	DM9	DM10	DM11	DM12
M-H	205	221	301	307	397	398	410	426	590	483	616	494
Ions (m/z)	175*	190*	221*	205*	221*	261*	150*	261*	572*	205*	465*	414*
	159*	175*	190*	234*	171*	205*	175	289*	261*	277*	205	289*
	155*	171*	171*	175*	205	175*	221	205	175	205	150*	261*
			155*	155		171		175	205	171	440*	205*
				277							261	175
											289	221

Table 19 presents 12 metabolites of Andarine, marking a big difference with Bicalutamide that presented only 4 metabolites in pigs (

Table 18). This result would indicate the intense metabolism of this drug in pigs as described in previous works by other authors and it is remarkable the short period of detection (48 hs) found as we will see [52] [65].

Figure 26 presents a composite chromatogram corresponding to samples of the treated pig. The Figure is constructed with SRM of the 12 metabolites identified as "DM" (DM1 to DM12). In order, it can be observed in minute 2.42 peak DM9; DM5 at 3.05 min.; in 3.37 min simultaneously three peaks corresponding to DM7, DM3 and DM2; peak DM6 in 3.56 min. and peak DM10 in 3.58 min; peaks DM1 and DM4 are together in 3.84 min.; together also peaks DM12 and DM11 in 3.94 min.; Andarine is in minute 4.50 and very close is DM8 in 4.51 min. Annex IX presents other graphs showing in detail the order of elution and the SRM used for the identification of the metabolites found. The presence of the metabolites found were previously described in works carried out by other authors in studies *in-vitro* and *in-vivo* [36, 37, 41, 45, 67].

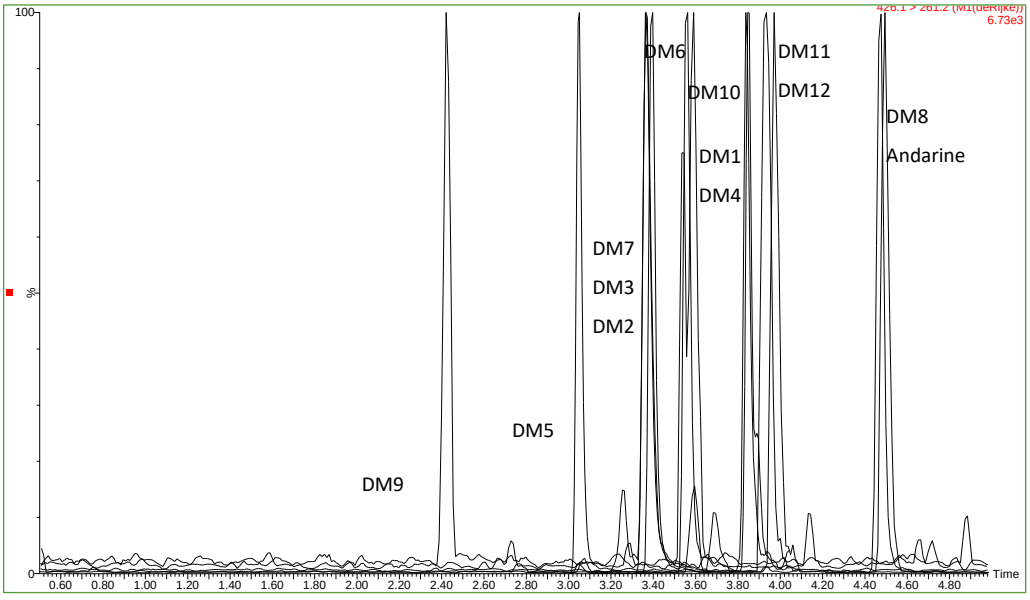


Figure 26. Composite chromatogram of the porcine treated with Andarine.

(DM1: 205>175; DM2: 221>175; DM3: 301>221; DM4: 307>205; DM5: 397>221; DM6: 398.1>205; DM7: 410.1>150; DM8: 426.1>261.2; DM9: 590>261; DM10: 483.1>205; DM11: 616.1>440.1; DM12: 494>414.1 y Andarine: 440.1>261).

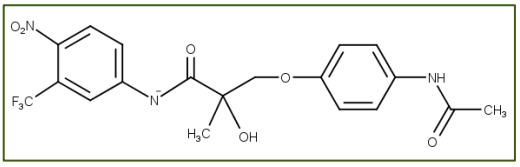


Figure 27. Chemical structure of Andarine (m/z = 440).

DM1: This metabolite would correspond to the deprotonated fragment coming from the rupture of the amide bond of the chain between rings of Andarine (Figure 27). Figure 28 shows the proposed chemical structure, consisting of prior studies [45] and according with m/z = 175 by loss of NO; m/z = 159 by loss of NO_2 and m/z = 155 by loss of HNOF. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143).

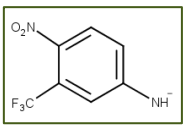


Figure 28. Chemical structure proposed for DM1 (m/z = 205).

DM2: This metabolite would correspond to DM1 hydroxylated. Figure 29 shows the proposed chemical structure that would explain the fragments found m/z = 190 by loss of HNO; m/z = 175 by loss of NO y m/z =171 by loss of HNOF. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143). This metabolite was previously reported in urine from horses treated with Andarine [45].

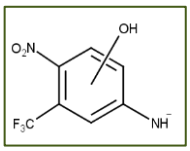


Figure 29. Chemical structure proposed for DM2 (m/z = 221).

DM3: This metabolite would correspond to the DM1 conjugated sulphate. Figure 30 shows the proposed chemical structure consisting with the fragment found m/z = 221 by loss of SO_3; m/z = 190 by loss of HNO and SO_3; m/z = 171 by loss of HNOF and SO_3; m/z = 155 by loss of HNO_2F and SO_3. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143). This metabolite was previously reported in urine from horses treated with Andarine [45].

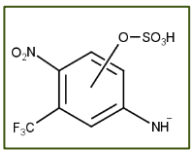


Figure 30. Chemical structure proposed for DM3 (m/z = 301).

DM4: The retention time and fragments found of this metabolite are consistent with a metabolite described in previous work [36-38, 41, 67]. As in previous works, it cannot propose a structure because of fragments size (m/z = 307) it could be the dephenylated Andarine, but the fragments (m/z = 234; m/z = 205; m/z = 175) observed with mass spectrometry do not match the structure of dephenylated Andarine.

DM5: This metabolite would correspond to the DM1 conjugated glucuronate. Figure 31 shows the proposed chemical structure consisting with the fragment found m/z = 221 by loss of glucuronic acid leaving the hydroxylated derivative and m/z = 171 by loss of HNOF. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143). This metabolite was previously reported in urine from horses treated with Andarine [45].

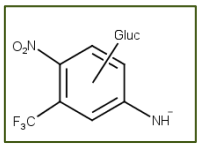


Figure 31. Chemical structure proposed for DM5 (m/z: 397).

DM6: This metabolite would correspond to Andarine deacetylated and deprotonated. Figure 32 shows the proposed chemical structure consisting with the fragment found, m/z = 261 is a characteristic fragment of Andarine that is generated by the loss of the amino-phenyl ring with a subsequent rearrangement [41]; m/z = 205 produced by rupture of the amide bond linking both rings, then m/z = 175 generated from m/z = 205 by loss of NO. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143).

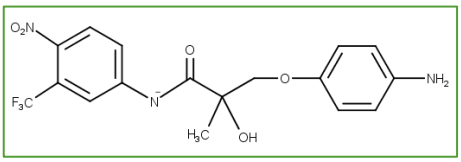


Figure 32. Chemical structure proposed for DM6 (m/z: 398).

DM7: This metabolite would correspond to deprotonated form generated from nitro-reduction of Andarine (Figure 27) with a reduction of 30 Da.

Figure 33 shows the proposed chemical structure consisting with m/z = 150 corresponding to phenyl-acetamide coming from rupture of ether bound. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143). This metabolite was previously reported in urine from horses treated with Andarine [45].

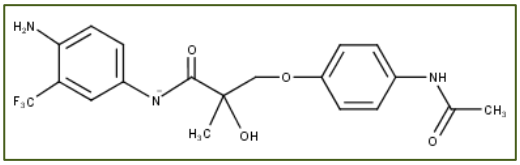


Figure 33. Chemical structure proposed for DM7 (m/z: 410).

DM8: This metabolite would correspond to hydroxylated DM7. There are several places where oxydril can enter in both rings. The presence of m/z = 261 (as describe for DM6) and m/z = 289 by loss of phenyl-acetamide group, indicates that the substituted trifluoromethyl ring does not receive oxydril (Figure 34). This metabolite was reported during an *in-vitro* trial with Andarine [38]. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143).

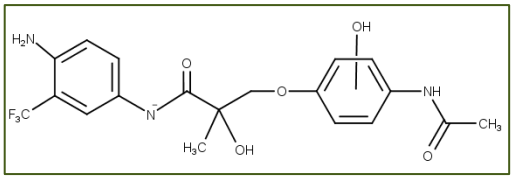


Figure 34. Chemical structure proposed for DM8 (m/z: 426).

DM9: It would correspond to the deprotonated form of Andarine and conjugated with glucuronic acid. The fragment m/z = 261 would indicate that the trifluoromethyl ring remains unchanged and the fragment m/z = 572 would

indicate loss of H_2O (Figure 35). This metabolite was reported during an *in-vitro* trial with Andarine [41]. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143).

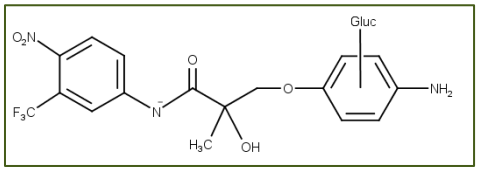


Figure 35. Chemical structure proposed for DM9 (m/z: 590).

DM10: The proposed chemical structure for this metabolite is shown in Figure 36. It would correspond to the conjugated derivative with glucuronic acid coming from the rupture of the ether bond in the chain that bound both rings. Fragment m/z = 205 would indicate absence of changes in the trifluoromethyl ring and fragment m/z = 277 is consistent with DM10 that loses glucuronic acid and a methyl group. This metabolite was reported during an *in-vitro* trial with Andarine [41]. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143).

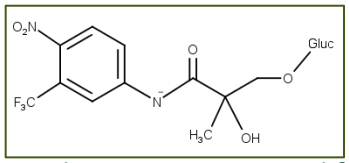


Figure 36. Chemical structure proposed for DM10 (m/z: 483).

DM11: This metabolite would correspond to Andarine conjugated with glucuronic acid in a deprotonated form. The fragments found would be consistent with the proposed structure (Figure 37) m/z = 440 come from the result of the loss of conjugated acid; m/z = 465 would show the result of removing the phenyl-acetamide ring (as a netral loss) and m/z = 150 woul be the deprotonated the phenyl-acetamide ring. This metabolite was reported during an *in-vitro* trial with Andarine [41]. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143).

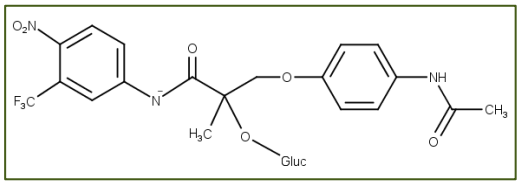


Figure 37. Chemical structure proposed for DM11 (m/z: 616).

DM12: This metabolite would correspond to Anadarine sulfate conjugated in a deprotonated form. Figure 38 shows the proposed chemical structure that would explain the fragments found, as described before m/z = 205 and m/z = 261 would indicate triflouromethylated ring unchanged; m/z = 414 by loss of SO3 and m/z = 289 by loss of phenyl-acetamide group. This metabolite was reported during an *in-vitro* trial with Andarine [41]. Table 19 presents the fragments found and Annex IX presents chromatographic details (page 143).

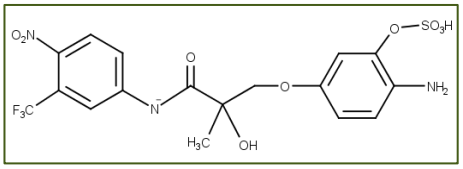


Figure 38. Chemical structure proposed for DM12 (m/z: 494).

Andarine and its metabolites in the excreta of the treated pig indicates its intestinal absorption. The appearance of 12 metabolites shows an intense metabolism in pigs as well as it was described by other authors for laboratory animals and in horses [45, 65].

Metabolites excretion

The study of the temporal window in the case of Bicalutamide was carried out with the transitions detailed in Table 14 (page 55) and in the case of Andarine metabolites are indicated in Table 20. The metabolite excretion profiles obtained from trial are presented below.

Table 20. Transitions MRM of Andarine metabolites.

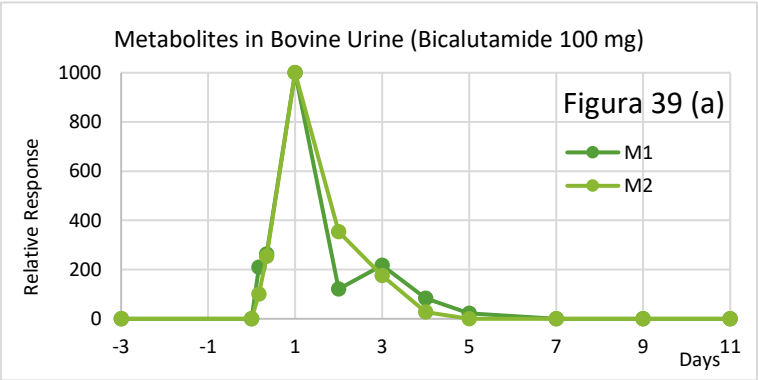
	[M-H$^-$]	MRM (m/z)		
M1	205	205>175	205>159	205>155
M2	221	221>190	221>175	221>171
M3	301	301>221	301>171	
M4	307	307>234	307>205	307>175
M5	397	397>221	397>171	
M6	398	398>175	398>205	398>261
M7	410	410>150		
M8	426	426>289		
M9	590	590>261		
M10	483	483>205		
M11	616	616>440	616>461	616>261
M12	494	494>414		

Figure 39 (a - p) show the results obtained for bovine (a - k) and porcine (m - p) treated with Casodex ® and Figure 40 (a - k) correspond to the pig treated with Andarine.

The figures are constructed on an arbitrary basis, where the signal of each Figure is relative to the maximum signal obtained for each metabolite. Since there are not standards of each metabolite, the signal could not be transformed into concentration units (at the moment of the trial).

However, these figures are very important for analytical control in livestock practices, since they indicate which metabolites are characteristic of each matrix and also how long they remain detectable. Annex IX (page 135) presents tables with the values used for the construction of these figures.

Figure 39. Excretion profile of Bicalutamide metabolites.



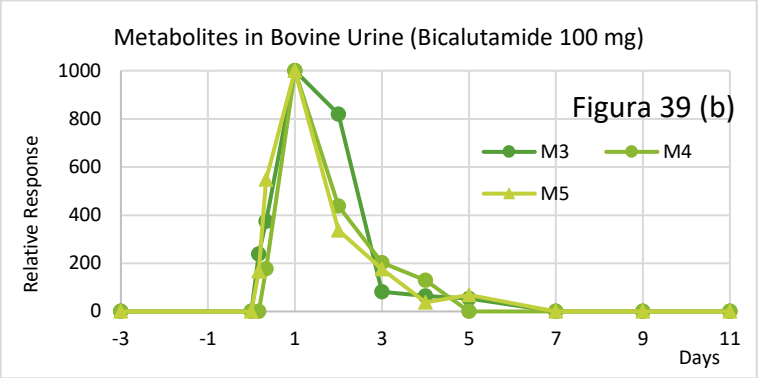
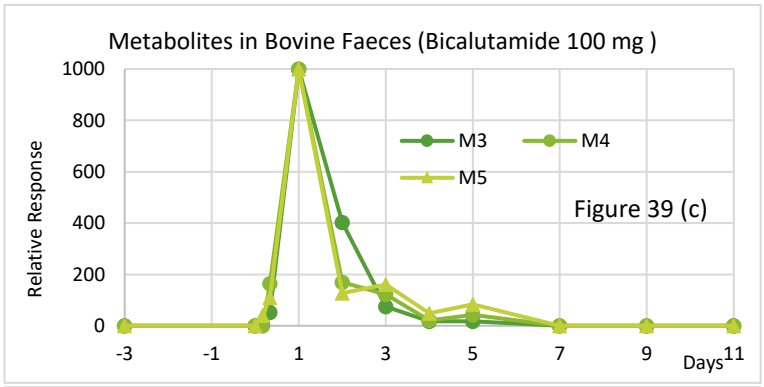
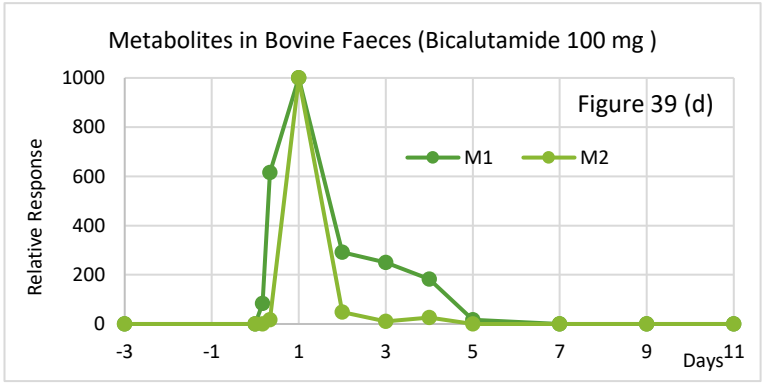


Figure 39 (a), (b), (d) and (c) show the elimination of Bicalutamide metabolites in urine and faeces of bovine animals treated with 100 mg Casodex®. It can be observed that the peak of the metabolites appears on day 1, there is a rapid increase and a slow decrease describing asymmetric curve.

Some metabolites were detected until day 5 (M1; M3 and M5) in urine and faeces, while the Bicalutamide unchanged was detected until day 11 in faeces and until day 7 in urine.





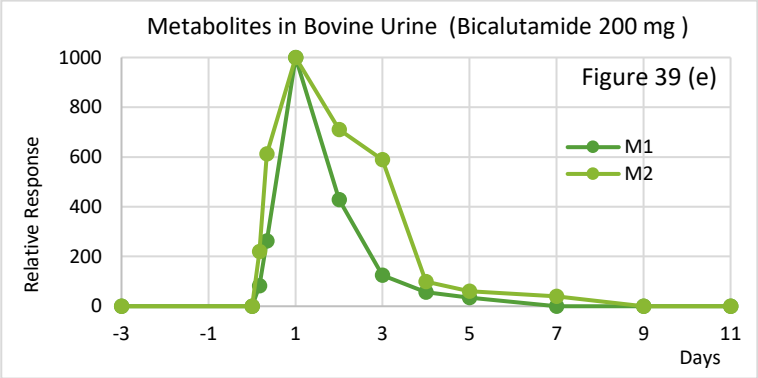
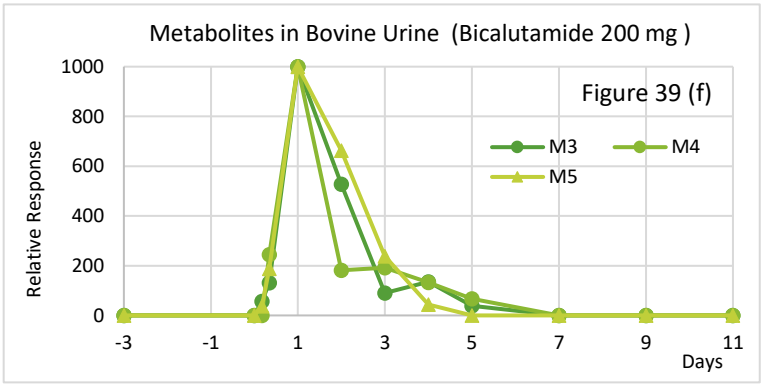
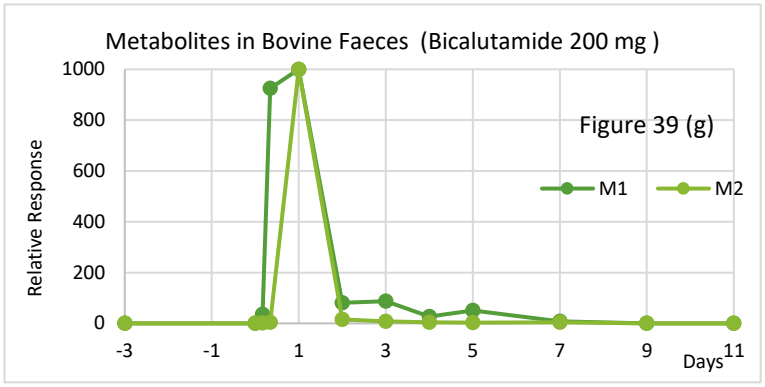


Figure 39 (e), (f), (g) and (h) show excretion of animals treated with 200 mg CDX. It can be observed that the concentrations of the metabolites increases rapidly at the beginning, until 8hs for M3 and day 1 and for the rest, then the decrease is slower, drawing asymmetric curves.

Metabolites that remain more detectable in faeces is M4 (until day 9) and in urine M2 (until day 7), while Bicalutamide unchanged remains detectable until day 11 in faeces and until day 9 in urine. This extension of the detection period of Bicalutamide compared to the bovine that received 100 mg could be related to the higher dose and due to the intestinal reabsorption of Bicalutamide (*entero-hepatic circulation*) [27]. On the other hand, the fact of detecting metabolites and Bicalutamide for longer periods of time in faeces than in urine could be due to the use of lyophilized faecal matter which has a concentration effect of non-volatile substances.





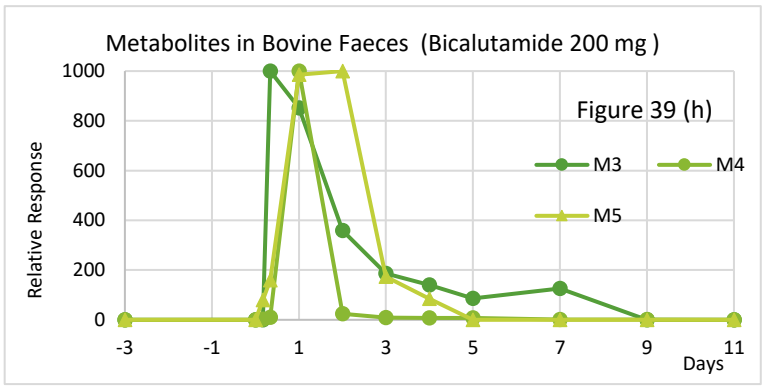
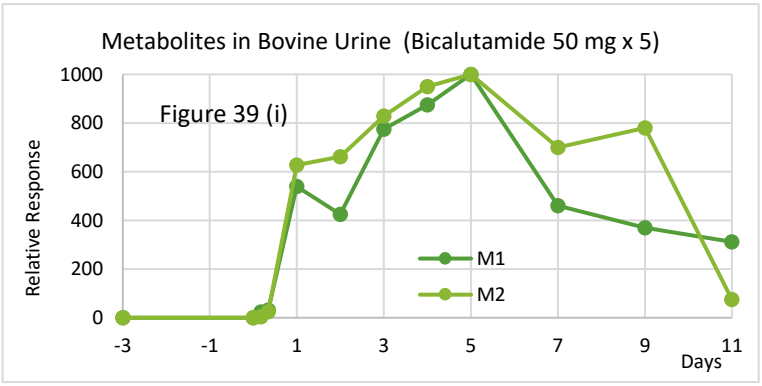
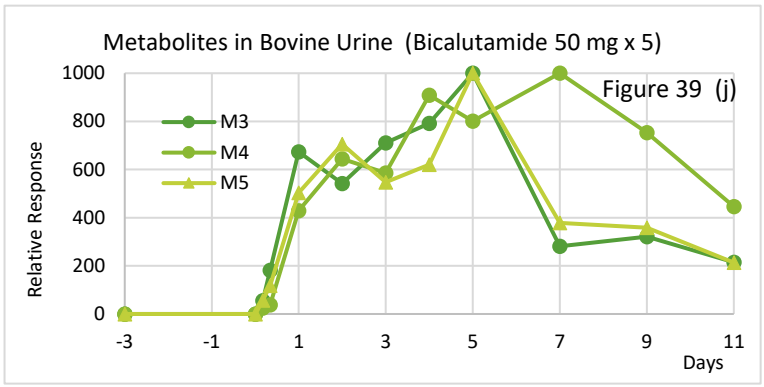
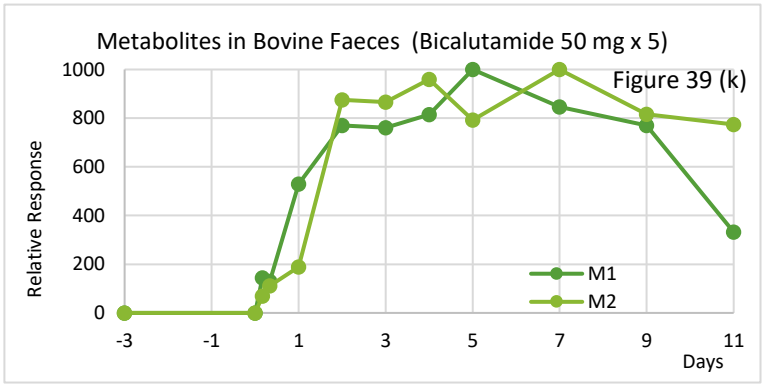
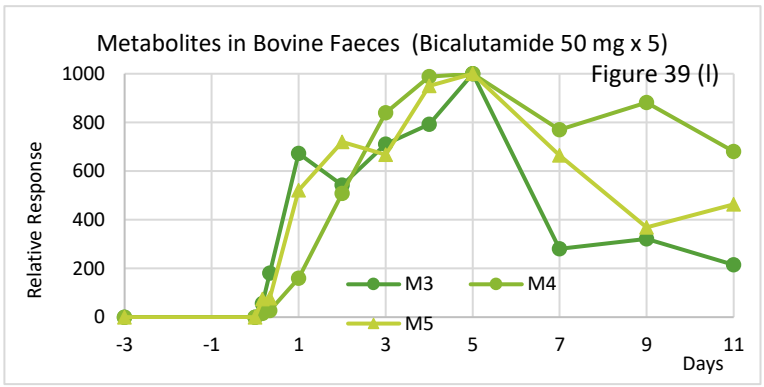


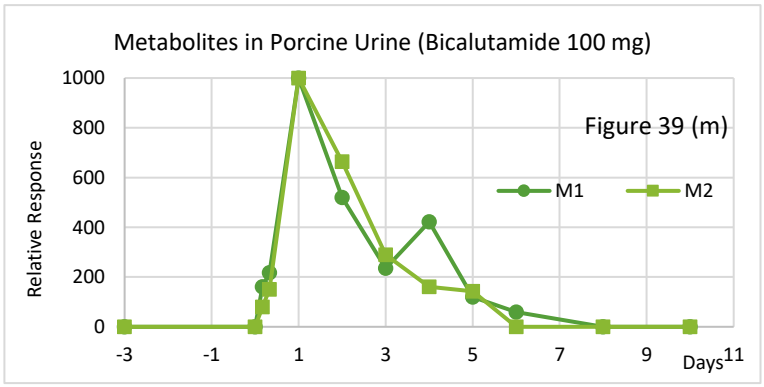
Figure 39 (i), (j), (k) and (l) show the variations in the relative signal of metabolites in urine and in faeces for the bovine that received the 5 daily doses of 50 mg. In urine, all metabolites can be seen to increase rapidly until day 1 and then a slowly increase; M4 continues to increase its signal until day 7 while the rest of the metabolites have their maximum on day 5; finally all metabolites signal decrease slowly and remain detectable until the end of the test. In faeces, the behaviour is very similar but in this matrix is M2 who increases its signal until day 7.




Metabolites in Bovine Urine (Bicalutamide 50 mg x 5)
Figure 39 (j)
Relative Response
1000
800
600
400
200
0
M3
M4
M5
-3
-1
1
3
5
7
9
11
Days


Metabolites in Bovine Faeces (Bicalutamide 50 mg x 5)
Figure 39 (k)
Relative Response
1000
800
600
400
200
0
M1
M2
-3
-1
1
3
5
7
9
11
Days


Metabolites in Bovine Faeces (Bicalutamide 50 mg x 5)
Figure 39 (l)
Relative Response
1000
800
600
400
200
0
M3
M4
M5
-3
-1
1
3
5
7
9
11
Days


Metabolites in Porcine Urine (Bicalutamide 100 mg)
Figure 39 (m)
Relative Response
1000
800
600
400
200
0
M1
M2
-3
-1
1
3
5
7
9
11
Days

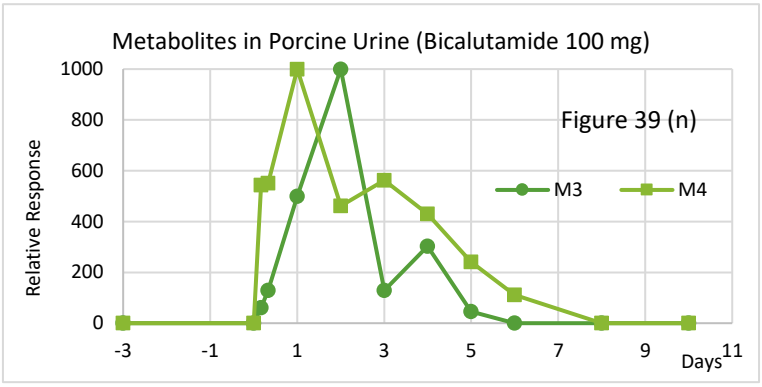
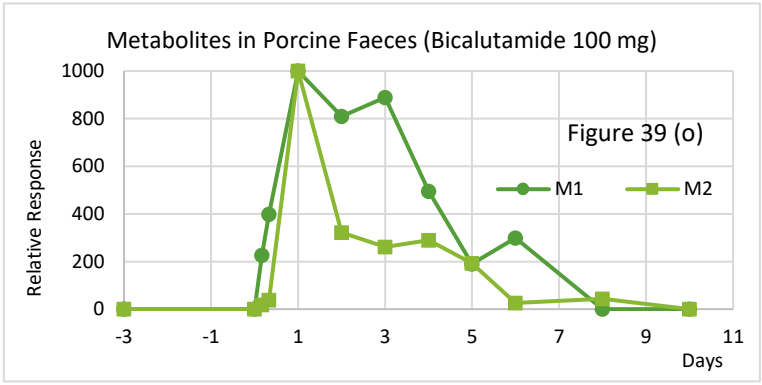


Figure 39 (m), (n), (o) and (p) show variations in the relative signals of metabolites found in urine and faeces of pigs treated with 100 mg CDX.

It is observed that after the administration of the drug (day 0) the increase of the signals is fast, showing the maximum on day 1 and only M3 in urine shows its maximum on day 2. Then the decrease is slow for all metabolites, describing asymmetrical curves. In urine, metabolites are detected until day 6 and in faeces until day 8, while Bicalutamide is detectable until the end of the test (day 10).



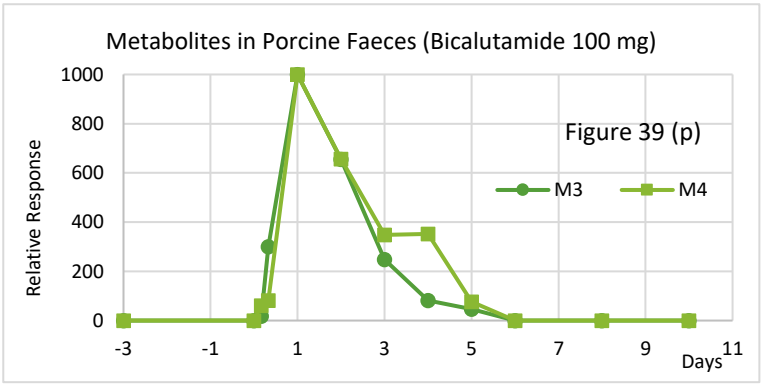


Figure 40. Excretion profile of Andarine metabolites.

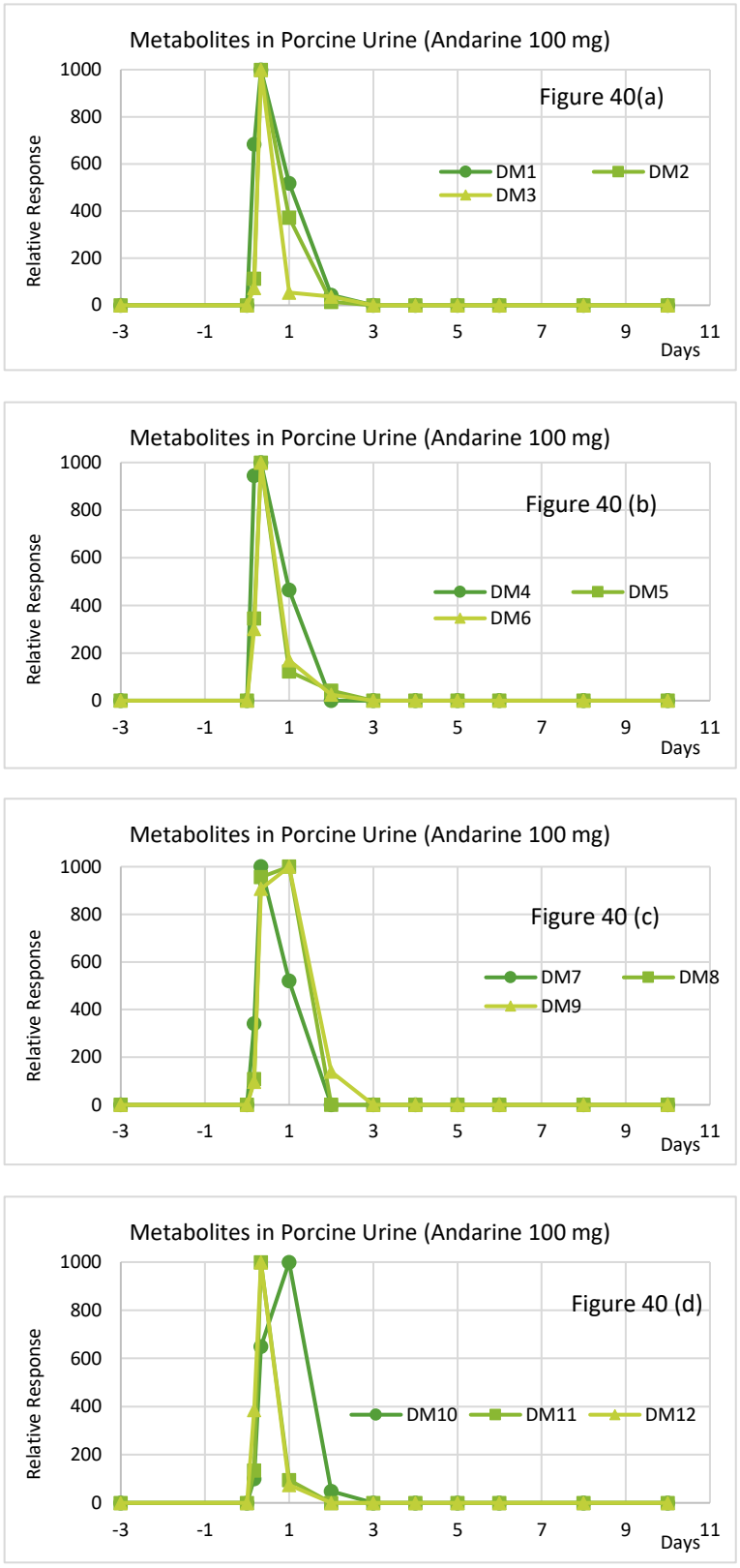
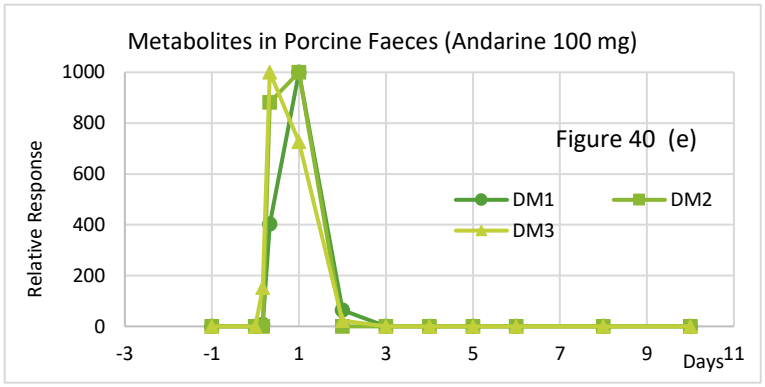
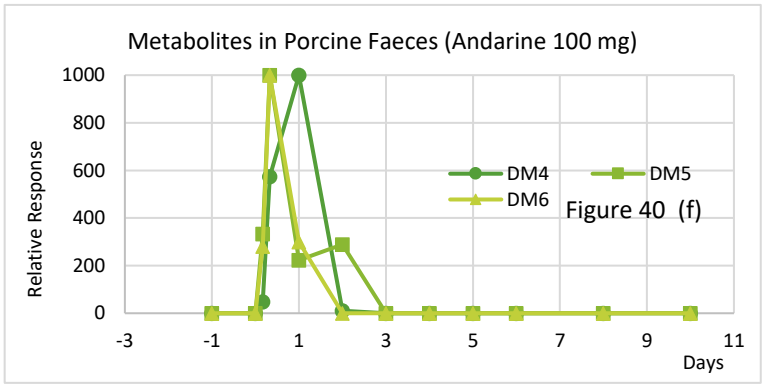


Figure 40 (a), (b), (c) and (d) show variations in the relative responses over time of metabolites found in urine of the porcine treated with Andarine. It can be seen that the increase of the signals is very fast as well as the decrease, it only takes a few hours to be undetectable. Only DM8, DM9 and DM10 have their maximum value on day 1, the other metabolites have their maximum at 8 hours post-administration. This fact could indicate that the maximum of Andarine and its metabolites would occur between 8 hs and 24 hs as it happens in other animals [45, 52].





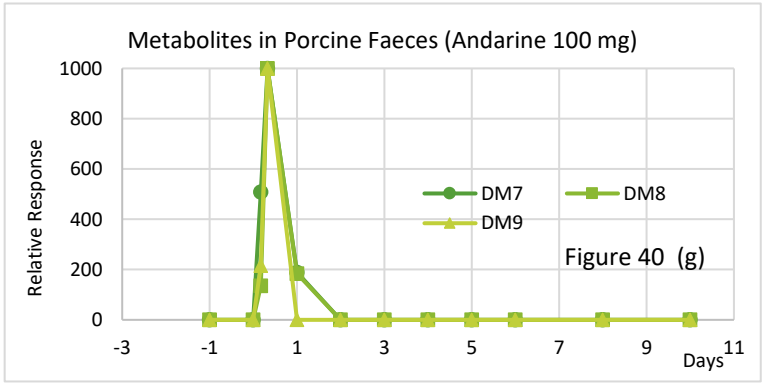
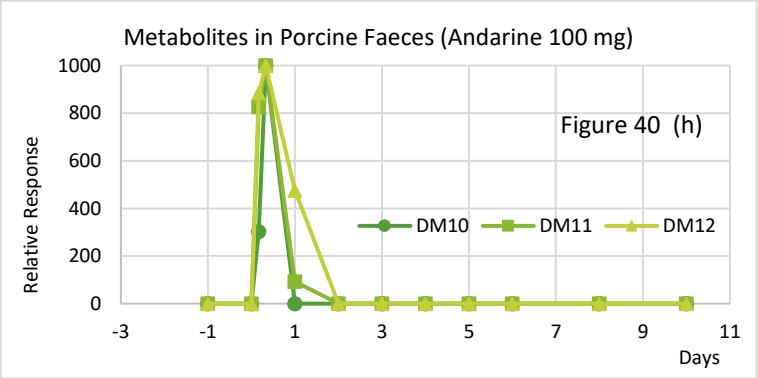


Figure 40 (e), (f), (g) y (h) present the variations of relative responses in the time of the metabolites found in faeces of the porcine treated with Andarine. In faeces the curves are similar to those described for metabolites in urine, you can see rapid increases and decreases that draw sharp peaks showing a rapid elimination of Andarine and its metabolites. Only DM1, DM2 and DM4 show their maximum signals at 24 hours post-administration and the rest of the metabolites show their maximum before (8 hs).



Partial Conclusions:

- A rapid method was developed for the determination of Bicalutamide and Andarine in serum, showing satisfactory results of Accuracy, Precision and linearity. The method allows great workflow and is ideal for routine analysis. Its limit of quantification was 2 ng/mL.
- The serum concentration of Bicalutamide was determined after the administration of an oral dose: presenting a maximum at 24 hours followed by a slow decrease with detectable concentrations until the last day of the test in pigs (day 10) and cattle (day 11).
- In the pig, Andarine has a maximum serum (478.1 ng/mL) between 4 to 8 hs and a rapid decrease that shows undetectable results on day 3.
- The faecal and urinary elimination profile was established for animals treated with Bicalutamide and Andarine. Phase I and Phase II metabolites from both drugs were found in the excreta of treated pigs and cattle. The information collected with chromatography and mass spectrometry detectors was used to

propose structures of the metabolites found for Bicalutamide and Andarine (except for DM 4).

- Bicalutamide metabolites described for Montbeliard bovine animals (M1-M5) are found in Angus bovine excreta. In the pig, the absence of metabolite M5 was observed.
- The pig treated with Casodex® presents detectable metabolites in urine until day 6 and in faeces until day 8.
- Cattle treated with Casodex® presents detectable metabolites with different periods of time: with 200 mg in urine until day 7 and in faeces until day 9; with 100 mg in urine until day 5 and in faeces until day 7 (for Angus breed); Montbeliar breed presents metabolites in both matrices until day 7; with 5 daily doses of 50 mg in urine and faeces until day 11. However, in all animals Bicalutamide's performance was better as a "marker analyte" of Casodex administration, in some animals staying longer and in others having a higher signal.
- In the porcine treated with Andarine, 12 metabolites were identified showing an intense metabolization. The urinary and faecal elimination profile was determined after oral administration of 100 mg.
- The metabolites of Andarine in the excreta, show a maximum before 24 hours follows by a fast decreasing, showing detectable concentrations in urine and faeces for 48 hours. This short stay poses a great analytical challenge for the control of this substance in livestock practices, at least for the studied matrices.

EVALUATION OF MARKERS METABOLITES FOR THE CONTROL OF LIVESTOCK PRACTICES

Analysing the metabolite excretion profiles previously discussed in Figure 18 (page 56), Figure 39 (page 82) and Figure 40 (page 88). The time window in which Andarine and Bicalutamide can be detected and identified metabolites can be determined. The absence of commercial standards is a limitation for the use of metabolites in the control of APA-SARMs. However, as current European and Argentine legislation prohibits the use of growth promoters in livestock practices, the presence of the drug or its metabolites indicate a violation of regulations.

In this work metabolites with different analytical behaviours (intensity and signal duration) have been found. For this reason and to evaluate the usefulness of each metabolite, comparative figures of *"relative abundances"* (chromatographic areas) were constructed for each metabolite excreted during all trials. (Figure 42 and Figure 43). These figures are intended to show the sensitivity of each metabolite by indicating the "intensity of the total chromatographic area" of each metabolite.

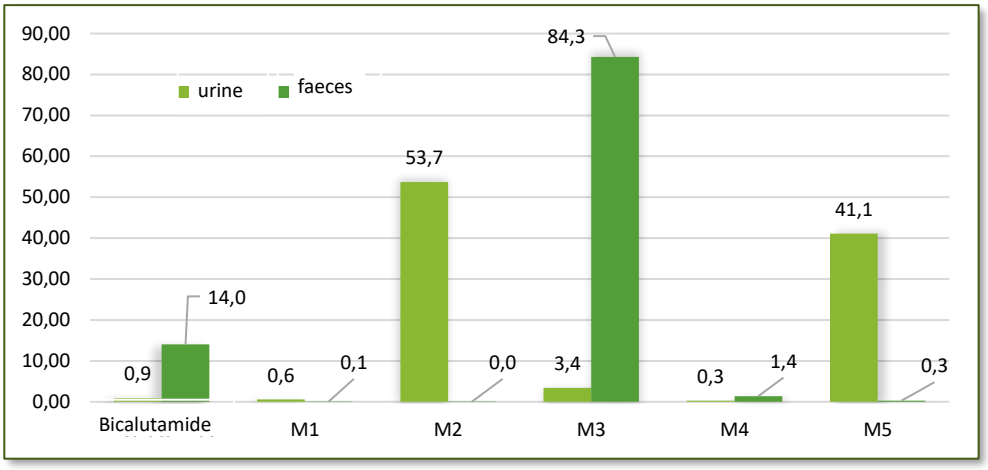


Figure 41. Relative abundances of Bicalutamide metabolites in excreta of bovine.

Figure 41 presents the *relative abundances* of Bicalutamide and its excreted metabolites (in faeces and urine) in cattle treated with different doses of Casodex ®. This figure shows a marked difference between the *abundance* of some metabolites in urine and faeces. This "*differential excretion*" of each metabolite would allow to concentrate the analytical efforts according to the matrix. It is important to note since the doses administered in this work are high and assuming that the abuse is performed with lower doses, only looking for "*metabolites more detectable*" could make possible to detect the case.

In the case of faecal matter, the percentages of Bicalutamide (14.0 %) and M3 (84.0 %) are higher. In urine, the situation is different, the most abundant were M2 (53.7 %) and M5 (41.1 %).

Figure 42 presents the "*relative abundances*" obtained for Bicalutamide and its metabolites in the excreta of pigs treated with Casodex®. Bicalutamide is found to be more abundant in faeces (81,7 %) than in urine (72,6 %). In addition, in faeces, M3 (17,4 %) has the highest abundance; in urine, M2 (16,4 %) and M1 (10,2 %) predominate. Finally it is noted that M4 has a low "*abundance*"

Figure 43 presents the "*relative abundances*" for Andarine and its 12 metabolites found in porcine excreta treated with Andarine.

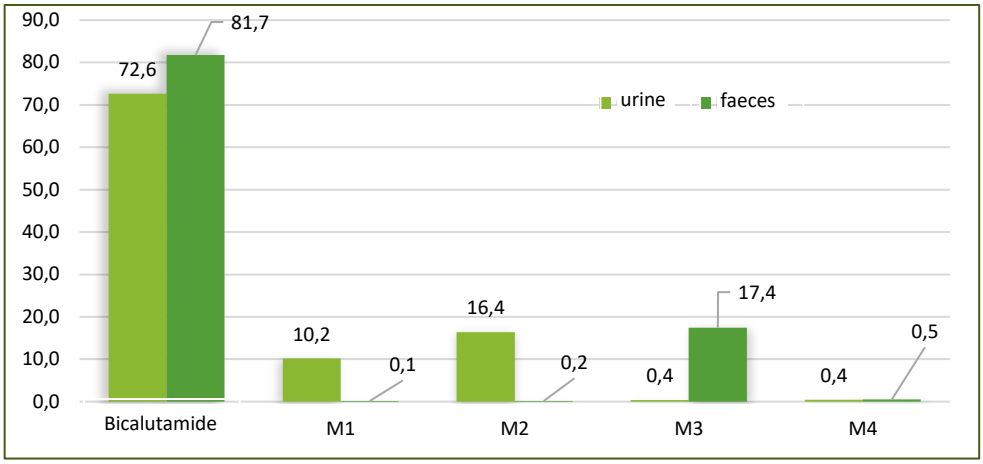


Figure 42. Relative abundances of Bicalutamide metabolites in excreta of porcine.

In faeces, Andarine (59.8 %) is the most *abundant* analyte follows by DM1 (7.8 %), DM2 (7.3 %), DM3 (7.7 %) and DM4 (6.0 %). In urine, Andarine (25.2 %) and DM3 (25.1 %) have the highest abundances; DM5 (13.4 %) and DM6 (10.5 %) and DM7 (6.7 %) have minor abundances.

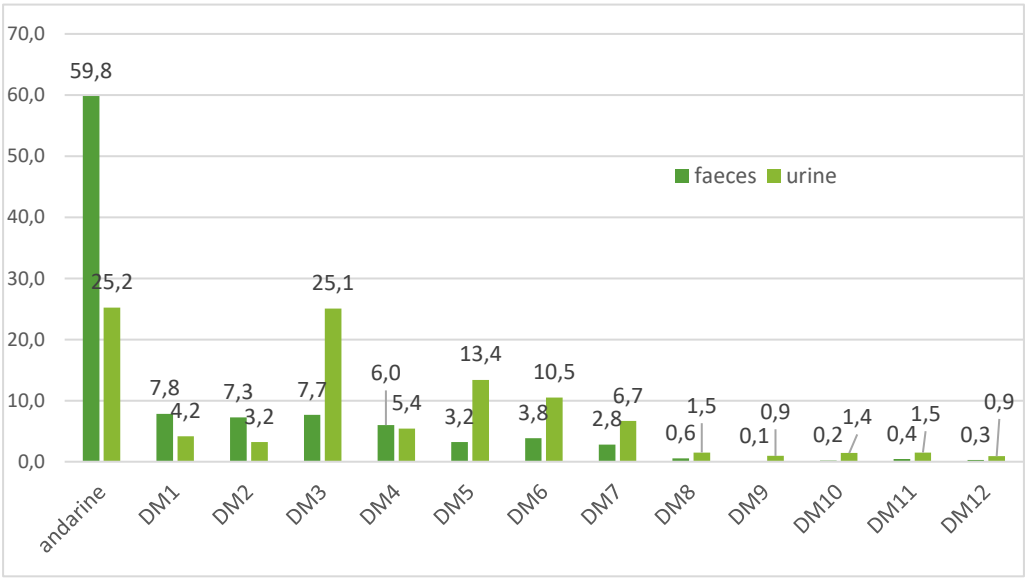


Figure 43. Relative abundances of Andarine metabolites in excreta of porcine.

Partial Conclusions

- In the case of cattle treated with Casodex®, M3 was the *"most detectable"* metabolites in faeces followed by Bicalutamide. Meanwhile, M2 and M5 in urine were more detectable metabolites.
- In the case of pigs treated with Casodex®, Bicalutamide is the *"most detectable"* in urine and faeces. In faeces, M3 could also be monitored, while in urine it could be monitored M1 and M2.
- In the case of pigs treated with Andarine, the drug unchanged is the *"most detectable"* in urine and faeces. Others abundant metabolites in faeces were DM1, DM2 and DM3. In urine, others abundant metabolites were DM3, DM5 and DM6.
- The lyophilized fecal matter showed its value as an analytical matrix for the control of administration of Bicalutamide and Andarine in livestock practices.

Conclusions

This work is the first study carried out with oral administration of Bicalutamide in food producing animals.

Analytical methods were developed and evaluated for the study of Enobosarm, Andarine, Bicalutamide and Hidroxyflutamide in urine and faeces. The methods showed the characteristics necessary for their routine use in control laboratories.

Analytical methods for the study of Andarine and Bicalutamide in serum were developed and evaluated. Achieving good analytical characteristics for the control of these drugs in pigs and cattle.

After oral administration of Casodex ®, Phase I and Phase II metabolites were detected in faeces and urine of four cattle and one pig. This is the first study in highlighting the usefulness of faecal matter as an important matrix for the control of Bicalutamide in livestock practices.

After oral administration of Andarine, metabolites of Phase I and Phase II were detected in faeces and urine of a pig.

Based on information obtained by liquid chromatography techniques coupled to mass spectrometry detectors chemical structures were proposed for 16 of the 17 metabolites corresponding to Bicalutamide and Andarine.

In the bovine excreta 5 metabolites of Bicalutamide were observed after Casodex® administration and 2 of them were reported for the first time in this work. The excretion profile of the 5 metabolites (faeces and urine) was established to obtain the respective temporary windows. Their intensity signal was evaluated to propose them as useful markers for the control in livestock practices. The selected ones were Bicalutamide unchanged and M3 in faeces; Bicalutamide unchanged, M2 and M5 in urine. In the case of Bicalutamide unchanged, its usefulness in urine is highlighted, despite the fact that it does not have a great signal but it is possible to quantify its concentration using a commercial pattern.

In porcine excreta treated with Casodex®, it was observed 4 of the 5 metabolites found in treated bovine. Then excretion profiles were determined

for each metabolite. In addition, the temporal window and the magnitude of each signal were evaluated to propose the following candidates: in faeces M3 and Bicalutamide and in urine M1, M2 and Bicalutamide unchanged.

Bicalutamide serum concentration was determined in animal trials (cattle and pig).

After Andarine administration, 12 metabolites were found in pig excreta. Andarine unchanged was established as the recommended marker considering the temporal windows and its intensity of the signal. In this trial, Andarine unchanged abundance in faeces were followed by DM1, DM2 and DM3; in urine they were DM3, DM5 and DM6.

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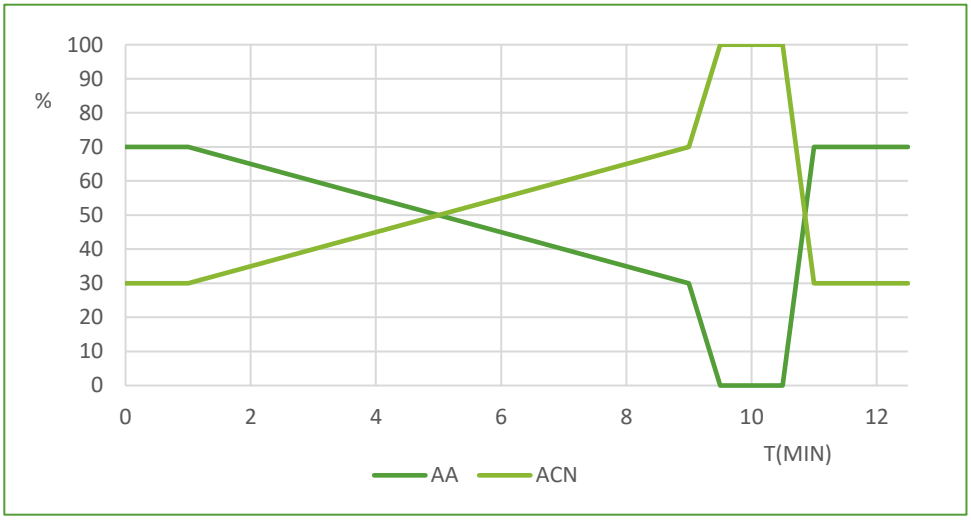
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Annex I

LABERCA CROMATOGRAPHIC SYSTEM

- Column ACQUITY UPLC BEH C18 (1.7 µm; 2.1 x 100 mm)
- Volume of Injection: 2 µL
- Mobil Phase: Acid Formic 0.1% (AA) / Acetonitrile (ACN)
- Flow: 0.6 mL/min



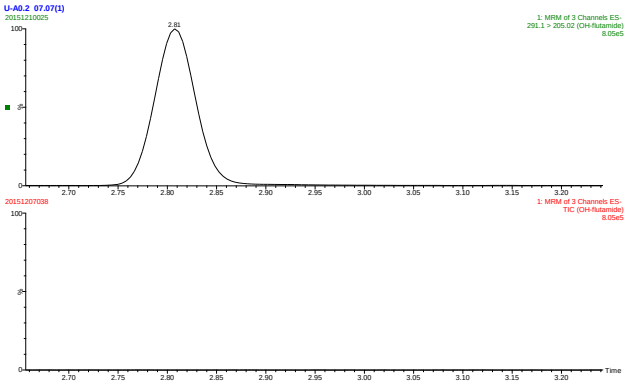
- Configuration of mass spectrometry detector

Parameter	
Ionization Electrospray	ESI -
Source Temperature	150 °C
Capillary voltage	- 2 kV
Source offset	30 V
Desolvation Temperature	450 °C
Cone Flow (nitrogen)	150 L/h
Desolvation Flow	650 L/h
Pressure de nebulization	7 Bar
Gas collision flow	0,15 L/min

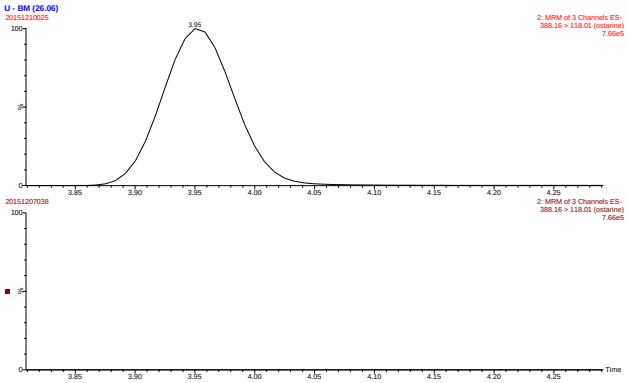
Chromatography

Chromatograms comparison are presented: extracted blank samples and spiked extract samples.

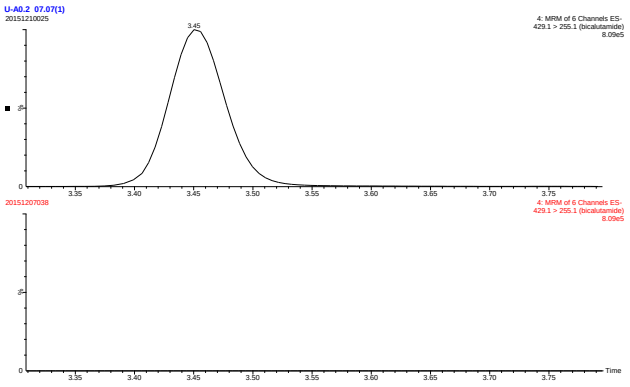
Chromatograms of 2 urine samples: below a Blank sample and above 0.2 ng/mL de **Hidroxyflutamide** (291.1>205.02).



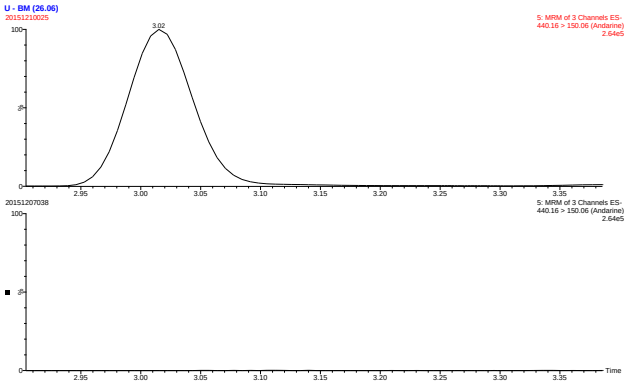
- **Enobosarm** (388.16>118.01)



- **Bicalutamide** (429,1>255,1)

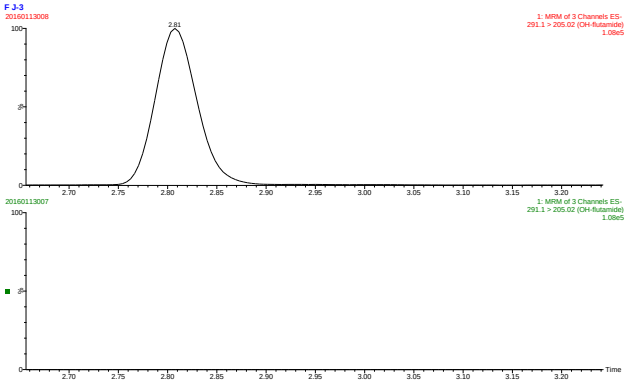


- **Andarine** (440.16>150.06)

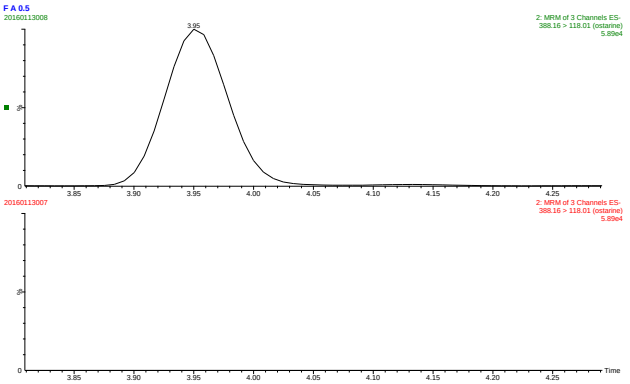


Chromatograms of faeces samples are presented below. The concentration used was 0.5 ng/g.

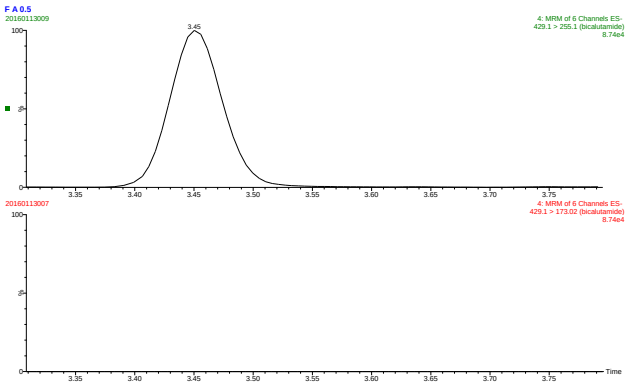
- **Hidroxyflutamide** (291.1>205.02)



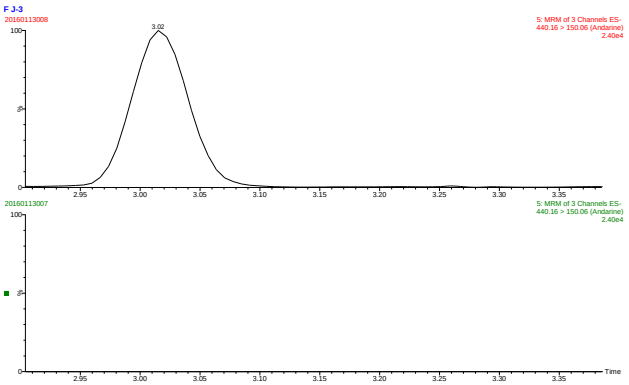
- **Enobosarm** (388.16>118.01)



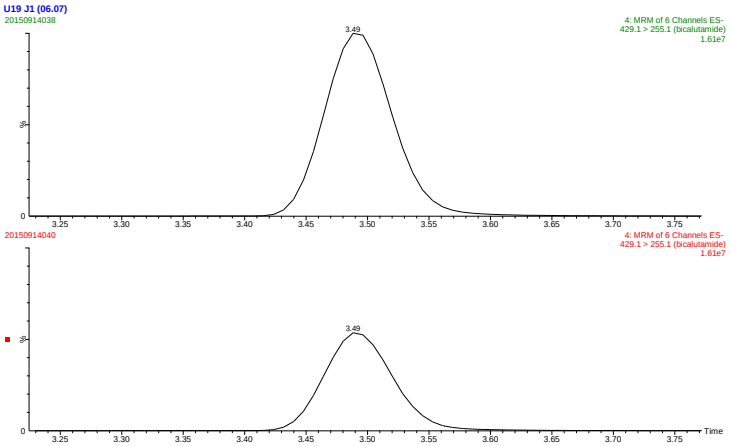
- **Bicalutamide** (429.1>255.1)



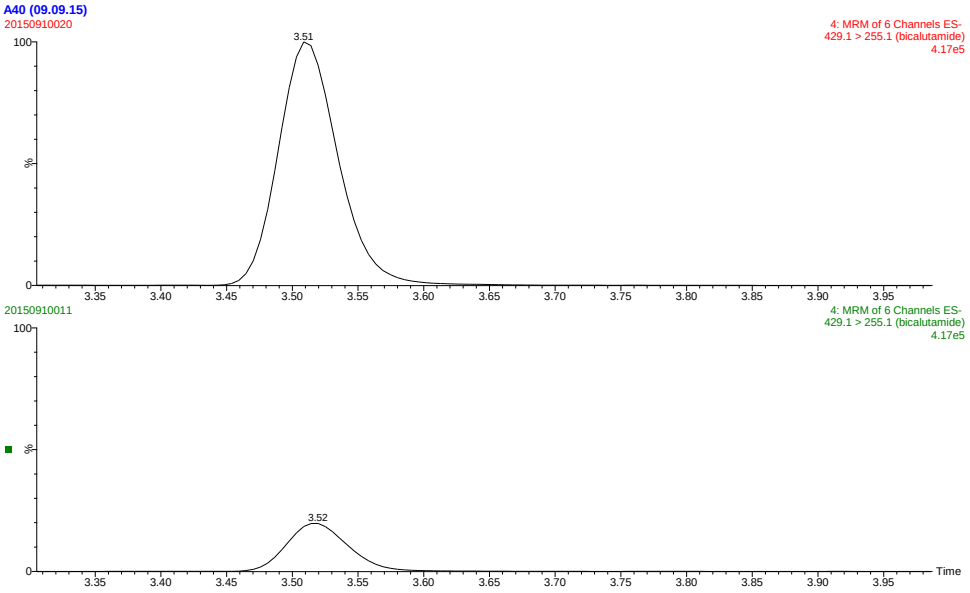
- **Andarine** (440.16>150.06)



Below chromatograms of a urine sample with the addition of Bicalutamide (above) and a urine sample of a treated animal (with 100 mg of Casodex ®).



Below chromatograms of a faeces sample with the addition of Bicalutamide (above) and a urine sample of a treated animal (with 100 mg of Casodex ®).



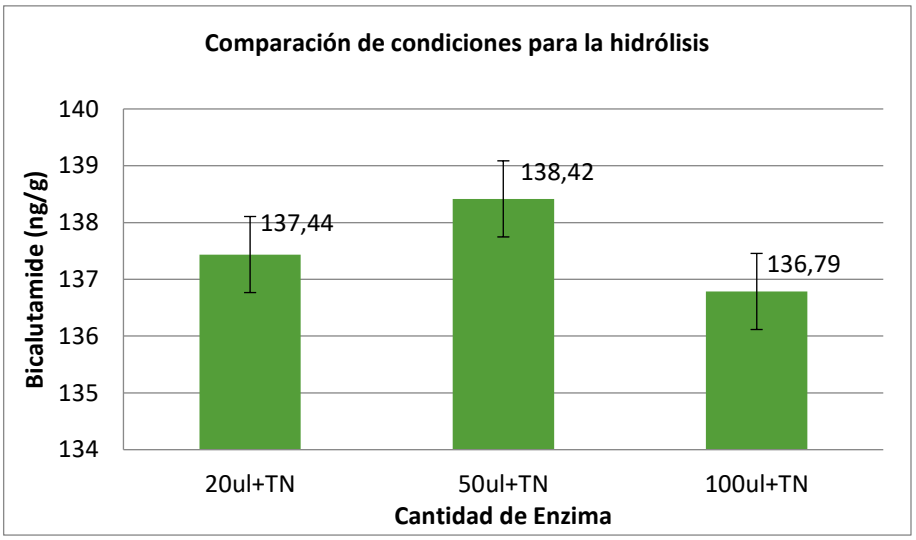
Annex II

HYDROLYSIS STUDIES AND STABILITY OF BICALUTAMIDE

Enzymatic Hydrolysis

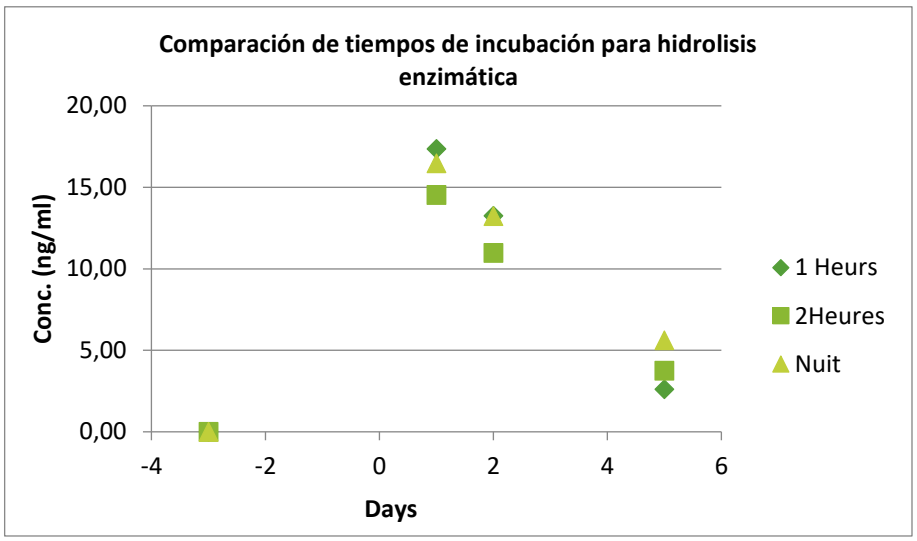
It was worked with urine samples and faeces of the treated animal. Different conditions (enzymes amount and incubation time) were tested to study the hydrolysis of conjugated Bicalutamide (Phase II metabolites).

The following Figure shows the results obtained using different amounts of enzymes (expressed in Volume: 20 µl; 50 µl; and 100 µl) for the analysis of the same faeces sample (incubating overnight at 50°C). The vertical lines of each bar represent the standard deviation found from the test (0.67 ng/g).



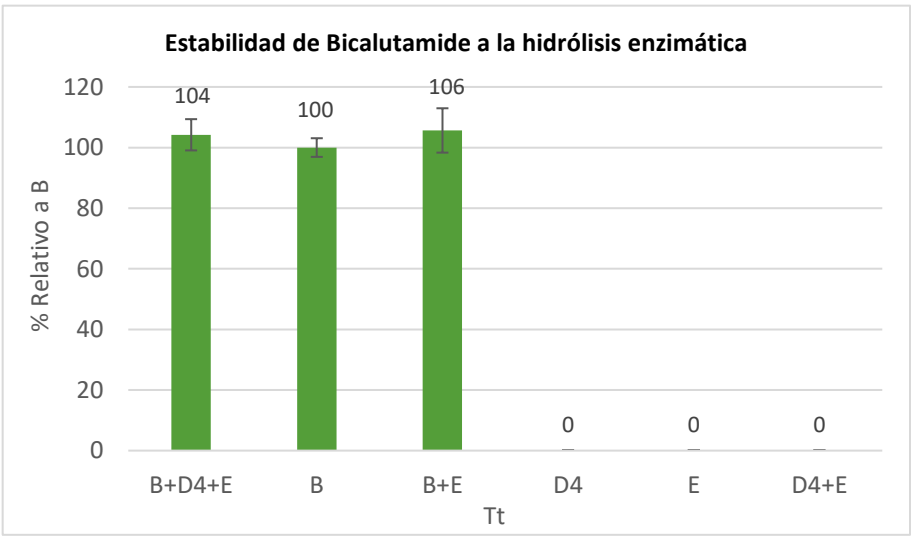
The results show that 20 µl of enzyme are equivalent to 50 µl and do not increase using 100 µl. There is no trend and the concentrations obtained with 20 µl or 50 µl do not show significant differences as the standard deviations overlap.

The following Figure shows the concentrations of Bicalutamide in urine from different days for different incubation times: one hour (1 Heures), two hours (2 Heures) and overnight (Nuit).



No marked differences or trends were observed between the trials in order to be able to select "the best condition" according to the concentration of Bicalutamide released. For operational reasons, overnight incubation was chosen (50°C).

Bicalutamide stability was tested for enzymatic action: it was studied to know if the enzymatic activity alters (increases/decreases) the Bicalutamide concentration. For this purpose, 5 tubes were incubated with the following mixtures: Bicalutamide + Bicalutamide-D4 + Enzyme (B+D4+E); Bicalutamide (B); Bicalutamide + Enzyme (B+E); Bicalutamide-D4 (D4) and Enzyme (E). After overnight incubation (10 hours) the concentrations of Bicalutamide were determined. Since the Bicalutamide amounts in each mixture were the same, the following figure was constructed by expressing Bicalutamide concentrations in relative percentages to tube B (100 %).



Little difference can be observed in the percentages (standard deviation) obtained between tubes with Bicalutamide and absence in tubes where it was not added. These results support the stability of Bicalutamide to enzymatic action.

Chemical Hydrolysis

The aim was to free Bicalutamide conjugated (Phase II metabolites) by testing 3 hydrolysis methods described in the literature consulted {Gomez, 2014 #91}{Kaabia, 2014 #138}.

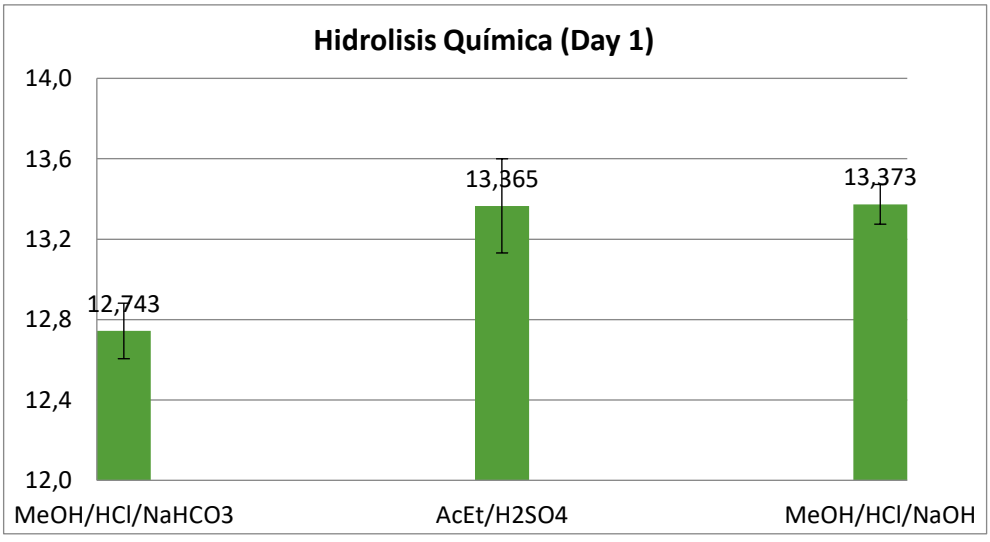
In this test it was worked with day 1 urine to obtain comparable results and each method was performed 2 to 5 times.

1) Method 1 (MeOH/HCl/NaOH): The urine sample was treated with the same SPE method (See Urines, page 41). The modification to perform the chemical hydrolysis was carried out after evaporating the 4 mL of methanol (elute), the residues were treated with hydrochloric acid (HCl) in methanol (MeOH), to stop the reaction sodium hydroxide (NaOH) was used.

2) Method 2 (MeOH/HCl/$NaHCO_3$): Method 1 was applied, but Sodium Hydrogen Carbonate (NaHCO3) was added to stop the reaction.

3) Method 3 (AcEt/H_2SO_4): for hydrolysis a mixture of ethyl acetate (Acet) with sulphuric acid (H2SO4) was used and stopped with NaOH.

Comparation de las concentracIons promedio de Bicalutamide determinadas en Urine del day 1.



Results obtained with chemical hydrolysis methods did not reach the values of the enzyme method (17.36 ng/mL).

In order to determine the absence of degradation of Bicalutamide/Bicalutamide-D4 (ISTD) to chemical agents, Bicalutamide and Bicalutamide-D4 were subjected to the conditions of chemical hydrolysis. Tubes with mixtures containing the same amount of Bicalutamide were prepared, similar to the Bicalutamide stability test for enzymatic activity. No alterations in the concentration of Bicalutamide (increase/decrease) were observed in any of the cases. Data from these trials are not included since the rest of the work was done by enzymatic hydrolysis.

The conclusions of these trials showed that Bicalutamide is resistant to the enzymatic action tested. It was established that enzymatic hydrolysis has a better performance, Volume of enzymatic extract and incubation time were set.

Annex III

METHOD EVALUATION

The following table show the signal (*S*) obtained after adding the analytes to *blank samples* (untreated animals). These data were used for the construction of *calibration curves*.

Urines

Calibration curves:

	Hidroxyflutamide	Enobosarm	Bicalutamide	Andarine
Concentration (ng/mL)	**S (A/IS)**	**S (A/IS)**	**S (A/IS)**	**S (A/IS)**
0.25	0.452	0.240	0.329	0.125
0.5	0.762	0.720	0.532	0.302
1	1.490	1.060	1.154	0.470
3	4.386	2.871	2.921	1.079
10	13.230	9.496	10.957	4.264
30	40.991	29.122	29.450	11.635

S (A/IS): is the relationship between the area of the analyte (A) and the area of the internal standard (IS).

	Hidroxyflutamide	Enobosarm	Bicalutamide	Andarine
Concentration (ng/mL)	**S (A/IS)**	**S (A/IS)**	**S (A/IS)**	**S (A/IS)**
0.25	0.521	0.331	0.316	0.198
1	0.826	0.546	0.515	0.265
0.5	1.530	0.916	1.077	0.292
3	4.120	3.476	2.540	0.830
10	13.923	11.028	10.499	3.399
30	48.213	28.156	28.824	10.058

	Hidroxyflutamide	Enobosarm	Bicalutamide	Andarine
Concentration (ng/mL)	**S (A/IS)**	**S (A/IS)**	**S (A/IS)**	**S (A/IS)**
0.25	0.673	0.392	0.360	0.138
0.5	0.784	0.484	0.688	0.299
1	1.835	1.176	1.150	0.369
3	5.051	2.720	3.296	1.284
10	15.794	9.298	8.721	3.281
30	38.928	31.276	28.351	10.796

Recovery test: were performed to calculate recovery (*accuracy*), variability (*Precision*) and limit of quantification (*sensitivity*).

Hidroxyflutamide			
Conc_Nom (ng/mL)	**C1**	**C2**	**C3**
0.25	0.252	0.293	0.255
0.5	0.540	0.538	0.554
1	1.151	1.015	1.041
3	3.555	2.953	2.899
10	9.711	9.810	9.811
30	34.567	29.629	29.182

Enobosarm			
Conc_Nom (ng/mL)	**C1**	**C2**	**C3**
0.25	0.194	0.183	0.194
0.5	0.539	0.581	0.541
1	1.016	1.081	1.115
3	3.199	3.177	3.059
10	11.840	10.677	10.494
30	29.352	33.623	30.420

Bicalutamide			
Conc_Nom (ng/mL)	**C1**	**C2**	**C3**
0.25	0.244	0.270	0.239
0.5	0.520	0.499	0.535
1	1.033	1.063	1.098
3	3.246	3.345	3.063
10	10.281	10.821	10.287
30	30.341	31.137	30.102

Andarine			
Conc_Nom (ng/mL)	**C1**	**C2**	**C3**
0.25	0.179	0.257	0.225
0.5	0.489	0.502	0.391
1	0.809	0.832	1.015
3	3.059	3.203	2.935
10	10.697	10.033	8.203
30	26.509	31.300	27.277

The results are summarised in the table Table 12 (page 46)

Faeces

Calibration curves: Since the concentrations found in faeces varied in a wide range, the slope of the curves changes and for this reason more than one curve was built for each analyte.

Hidroxyflutamide			
Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
0.5	0.803	0.843	0.883
1	1.580	1.772	1.413
3	4.697	5.032	5.242
10	16.214	16.454	16.734
30	45.851	48.598	45.527
50	75.525	71.865	77.501

Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
100	1.313	1.332	1.283
200	1.696	1.764	1.831
300	2.089	2.159	2.298
500	2.911	2.886	3.162
800	3.996	3.919	4.358
1000	5.185	5.361	5.096
1300	5.975	5.863	6.086
1600	7.006	7.208	7.410

Enobosarm			
Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
0.5	0.511	0.501	0.524
1	1.079	1.112	1.0116
3	3.012	3.171	3.047
10	14.201	14.113	13.919
30	37.728	37.477	37.594
50	56.210	57.299	58.666

Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
100	0.065	0.066	0.068
200	0.109	0.113	0.102
300	0.161	0.153	0.169
500	0.261	0.286	0.218
800	0.399	0.402	0.406
1000	0.484	0.464	0.475
1300	0.609	0.615	0.536
1600	0.787	0.691	0.765

Bicalutamide			
Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
0.5	0.6411	0.6357	0.6144
1	1.3367	1.3867	1.3292
3	4.0577	4.1266	4.1212
10	13.5737	14.2111	15.1293
30	34.7732	40.8141	38.7106
50	60.4741	63.3234	58.3000

Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
100	0.2426	0.2735	0.2513
200	0.4319	0.4596	0.4681
300	0.6530	0.6389	0.6451
500	0.9776	1.0022	0.9236
800	1.4252	1.3900	1.4965
1000	1.7101	1.8105	1.8021
1300	2.3373	2.3578	2.3474
1600	2.8973	2.7809	2.5733

Andarine			
Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
0.5	0.1317	0.1064	0.1472
1	0.3814	0.2882	0.3856
3	1.4317	1.4309	1.4170
10	5.2128	5.6285	5.3778
30	13.0808	13.1015	13.4488
50	21.0125	21.0398	21.0182

Concentration (ng/mL)	S1 (A/IS)	S2 (A/IS)	S3 (A/IS)
100	0.0656	0.0675	0.0586
200	0.1040	0.1040	0.0940
300	0.1421	0.1392	0.1243
500	0.2184	0.2047	0.1953
800	0.2760	0.3069	0.2521
1000	0.3286	0.3479	0.3231
1300	0.4458	0.4497	0.4181
1600	0.5500	0.5279	0.4885

Recovery Test:

Hidroxyflutamide			
Concentration (ng/mL)	**C1**	**C2**	**C3**
0.5	0.542	0.563	0.495
1	1.085	0.985	0.900
3	3.294	2.936	3.194
10	9.293	10.100	9.837
30	31.498	29.400	34.500
50	53.796	54.000	51.000
100	96.770	93.000	97.000
200	175.610	184.000	214.000
300	333.709	318.000	291.000
500	502.273	480.000	545.000
800	758.659	776.000	752.000
1000	1010.911	900.000	1120.000
1300	1281.476	1248.000	1287.000
1600	1597.087	1520.000	1456.000

Enobosarm			
Concentration (ng/mL)	**C1**	**C2**	**C3**
0.5	0.584	0.498	0.501
1	0.998	1.045	1.087
3	2.871	3.077	2.957
10	9.777	9.185	10.560
30	30.708	26.835	26.793
50	50.290	46.240	51.280
100	111.809	97.684	84.532
200	206.246	199.367	220.841
300	277.474	262.004	306.068
500	507.487	581.649	478.725
800	785.680	795.520	753.920
1000	903.100	983.100	1023.700
1300	1250.600	1369.940	1200.680
1600	1509.280	1685.760	1621.920

Bicalutamide			
Concentration (ng/mL)	**C1**	**C2**	**C3**
0.5	0.486	0.428	0.489
1	1.006	0.984	1.094
3	2.977	3.185	3.214
10	9.412	9.827	10.813
30	25.293	31.239	27.957
50	43.555	49.600	49.630
100	82.651	93.657	102.817
200	191.595	205.869	204.540
300	309.746	304.899	298.260
500	421.100	496.010	440.850
800	811.120	737.440	842.960
1000	932.600	913.300	941.900
1300	1227.590	1158.560	1201.460
1600	1429.760	1558.240	1653.440

Andarine			
Concentration (ng/mL)	**C1**	**C2**	**C3**
0.5	0.470	0.457	0.460
1	1.012	0.930	0.872
3	2.737	3.193	2.650
10	9.526	9.820	8.432
30	27.702	27.732	27.423
50	42.695	45.085	49.620
100	73.555	104.216	100.366
200	216.000	199.915	184.563
300	294.922	316.800	293.474
500	396.650	496.800	406.100
800	746.560	642.400	672.880
1000	941.700	904.100	802.200
1300	1110.070	1174.420	1044.290
1600	1426.240	1317.600	1362.720

Annex IV

FIRST ANIMAL TRIAL

Elimination profiles

The values of concentration, SD (standard deviation) and RSD were calculated by the determination per quintuplicate of each day.

Urines

Bicalutamide in urines (breed *Montbeliard*):

	Free		Total	
Day	***Conc(ng/mL)***	***SD (ng/mL)***	***Conc(ng/mL)***	***RSD %***
-3	ND	ND	ND	-
0	ND	ND	ND	-
0.17	2.82	0.06	2.84	2
0.33	5.50	0.65	8.79	12
1	13.15	0.42	17.36	3
2	9.62	1.56	13.25	16
3	6.95	0.25	11.06	4
4	1.74	0.08	4.55	5
5	2.32	0.17	2.61	7
6	0.95	0.01	1.72	2
7	0.72	0.02	1.24	3

Faeces

Bicalutamide in faeces (breed *Montbeliard*):

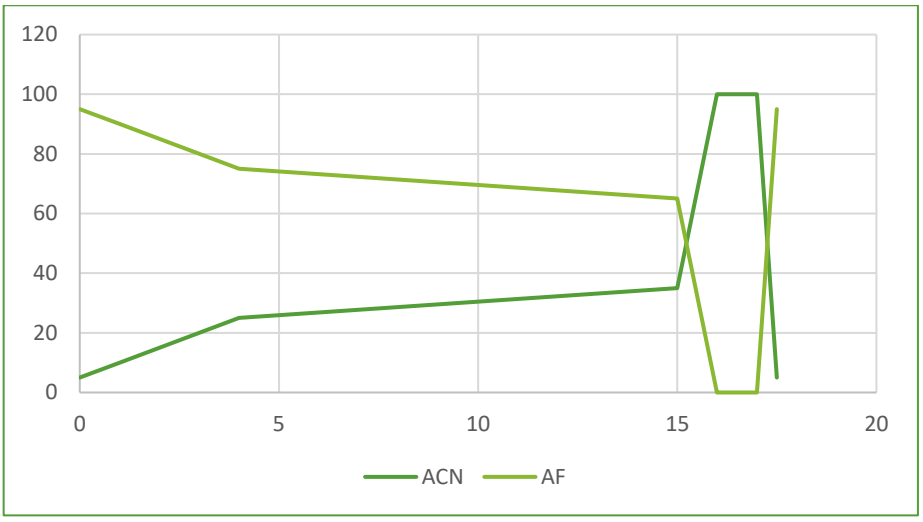
	Free		Total	
Day	**Conc (ng/mL)**	**SD (ng/mL)**	**Conc(ng/mL)**	**RSD %**
-3	ND	ND	ND	-
0	ND	ND	ND	-
0.17	7.10	0.33	2.83	5
0.34	119.07	3.08	130.33	3
1	1254.11	73.32	1346.66	6
2	776.58	10.12	966.56	1
3	471.25	40.29	491.76	9
4	213.52	22.24	247.92	10
5	112.32	2.55	117.69	2
6	82.00	3.59	60.70	4
7	52.90	1.43	37.35	3

Annex V

FIRST ANIMAL TRIAL: METABOLITES

Chromatographic System

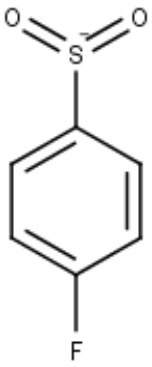
- Column ACQUITY UPLC BEH C18 (1.7 µm; 2.1 x 100 mm)
- Volume de Injection: 2 µL
- Mobil Phase: Acid Formic 0.1% (AF) / Acetonitrile (ACN)
- Flow: 0.4mL/min



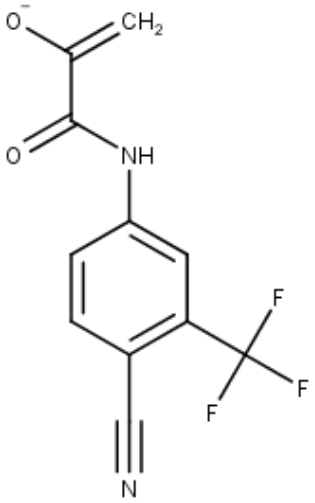
Characteristic fragments of Bicalutamide

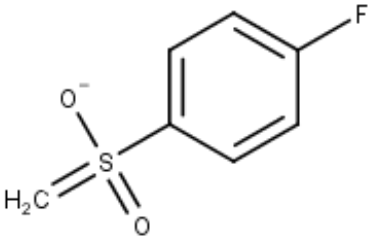
Based on previous studies of other authors it was possible identified the following fragments as characteristic for Bicalutamide [18, 19, 38, 63]:

m/z = 159

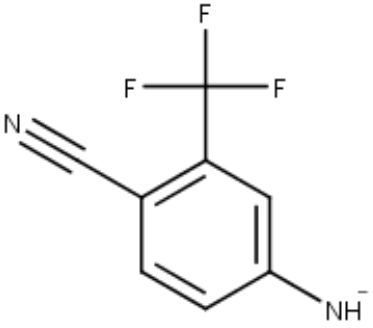


m/z = 255

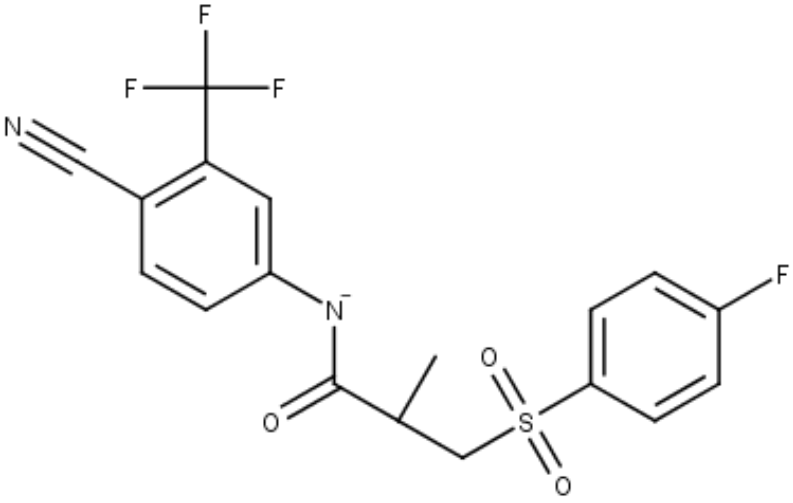




m/z = 173



m/z = 185



m/z = 411

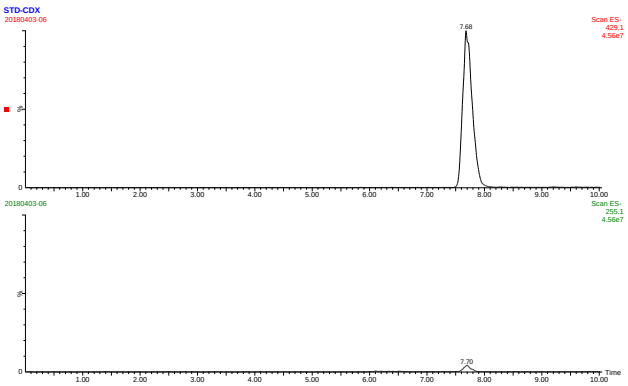
Annex VI

ADJUSTE OF CONE ENERGY

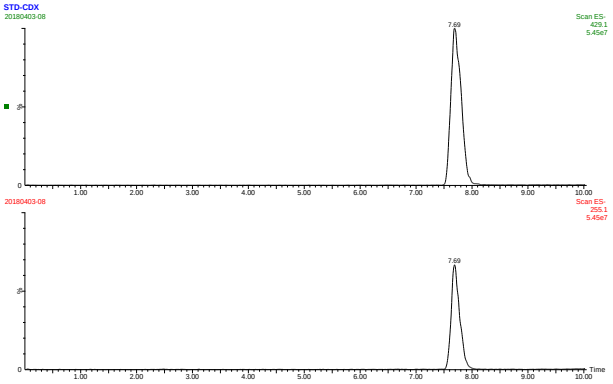
The following figures present the mass spectrometry responses in order to obtain the major response in single quadrupole mass spectrometry detector - Waters SQD (Laboratorio de contaminantes quimicos – INTA Castelar). It was used for the profile excretion of Bicalutamide and Andarine using Hidroxyflutamide as ISTD.

Bicalutamide (above m/z = 429 and below m/z = 255)

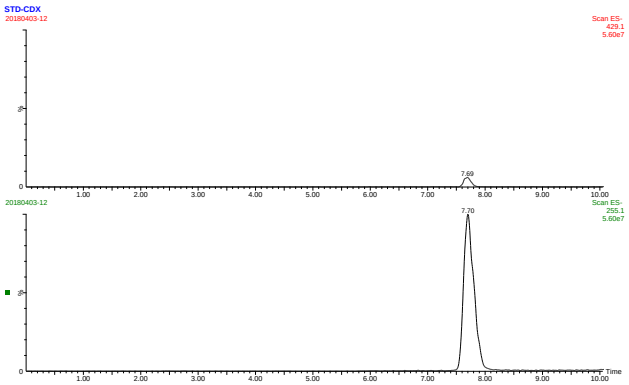
Cone energy: - 15 v



Cone energy: - 30 v

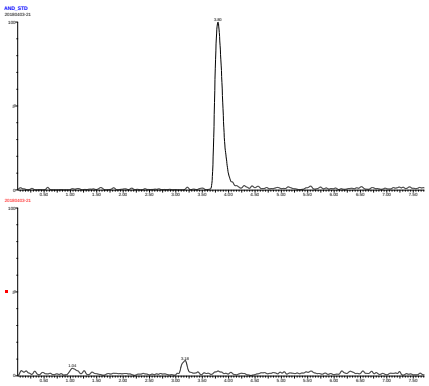


Cone energy: - 60 v

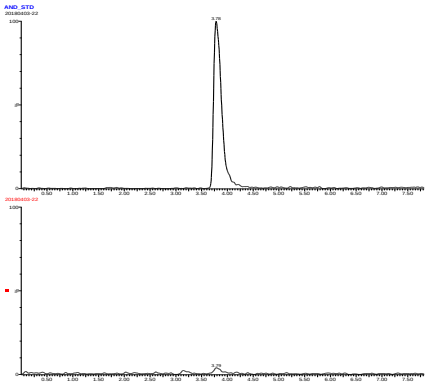


Andarine (above m/z = 440 and below m/z = 261)

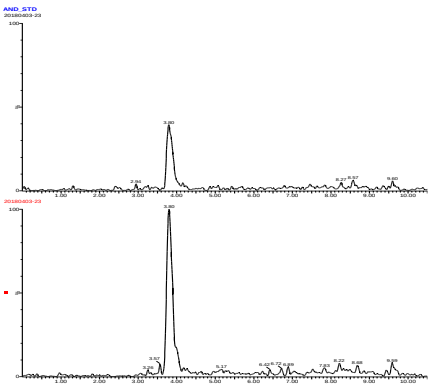
Cone energy: - 15 v



Cone energy: - 30 v

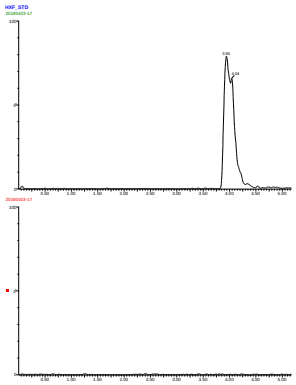


Cone energy: - 60 v

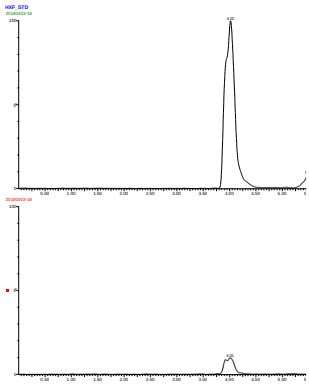


Hidroxyflutamide (above m/z = 291 and below m/z = 205)

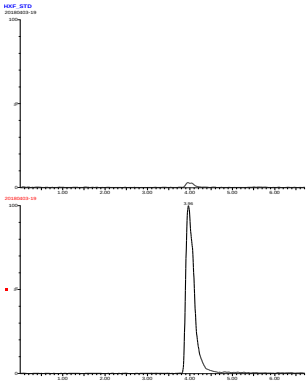
Cone energy: - 15 v



Cone energy: - 30 v



Cone energy: - 60 v



Based on this graphic information and others mass spectrometry tests in order to obtain the major signal for each compound the final cone energy established Bicalutamide 60 v; Andarine 40 v and Hidroxyflutamide 40 v (see Table 15, page 63).

Annex VII

METHOD EVALUATION

Calibration curves

It were used to determine Bicalutamide and Andarine concentrations and Hidroxyflutamide as ISTD.

The calibration curves were constructed several times (*S1*; *S2*; *S3*; etc.) because it were processed with samples. It were constructed to cover a wide range of concentrations.

Urines

	Bicalutamide				
Conc. (ng/ml)	***S1***	***S2***	***S3***	***S4***	***S5***
0.25	*0.181*	*0.159*	*0.152*	*0.178*	*0.107*
0.5	*0.341*	*0.312*	*0.313*	*0.307*	*0.289*
1	*0.691*	*0.585*	*0.574*	*0.629*	*0.622*
3	*1.954*	*2.007*	*1.718*	*1.754*	*1.623*
5	*3.318*	*3.189*	*3.134*	*3.363*	*3.645*
10	*6.755*	*6.068*	*6.186*	*6.331*	*7.897*
20	*13.515*	*13.543*	*11.899*	*11.660*	*13.244*
30	*17.519*	*18.410*	*18.459*		
50	*30.726*	*26.018*	*27.880*		
100	*56.158*	*48.922*	*49.338*		
120	*73.724*	*67.022*	*71.395*		
150	*85.006*	*72.998*	*77.441*		
200	*119.179*	*106.962*	*105.096*		

	Andarine				
Conc. (ng/ml)	**S1**	**S2**	**S3**	**S4**	**S5**
0.25	0.0697	0.0654	0.0619	0.0635	0.0672
0.5	0.1201	0.1245	0.1106	0.1143	0.0962
1	0.2652	0.2333	0.2070	0.2461	0.2244
3	0.7521	0.6504	0.6525	0.7164	0.6249
5	1.1807	1.0562	1.0925	1.1741	1.0743
10	2.3461	2.1699	2.0335	2.2069	2.3032
20	4.6167	4.5403	3.7836	4.0998	3.9200
30	6.4883	6.4516	6.452		
50	10.7944	10.4010	9.977		
100	20.9080	20.1136	18.171		
120	26.1530	22.3141	21.475		
150	32.0452	31.4926	30.084		
200	39.8037	36.3122	34.532		

Faeces

	Bicalutamide			
conc (ng/g)	**S1**	**S2**	**S3**	**S4**
1	0.425	0.484	0.469	0.380
2	0.861	0.777	0.792	0.849
5	2.088	2.363	2.009	1.961
10	4.083	4.427	4.735	3.871
15	6.115	6.087	6.483	5.337
20	8.392	9.799	9.484	7.974
50	20.592	23.001	20.060	
100	38.756	40.872	42.544	
150	59.950	70.665	58.626	
200	79.057	79.673	78.491	
300	120.845	128.518	113.353	
600	234.865	228.418	237.497	
1000	391.275	400.793	329.587	
1500	447.762	465.150	492.783	
2000	579.789	585.529	627.470	
2500	732.506	740.058	754.859	
3000	897.140	972.877	993.145	
3500	1004.574	1077.979	1201.122	

	Andarine			
conc (ng/g)	S1	S2	S3	S4
1	0.185	0.193	0.165	0.178
2	0.368	0.394	0.368	0.376
5	0.909	1.017	0.912	0.896
10	1.706	1.753	1.853	1.468
15	2.573	2.915	2.538	2.379
20	3.768	3.808	3.437	3.173
50	8.392	9.375	9.041	
100	17.641	17.924	18.427	
150	23.896	28.028	27.872	
200	37.510	36.323	35.795	
300	48.713	58.020	50.854	
600	101.355	102.557	91.779	
1000	171.281	174.899	145.516	
1500	186.179	203.450	213.314	
2000	259.771	270.944	278.896	
2500	333.849	340.841	344.391	
3000	413.268	434.793	434.699	
3500	442.740	470.897	524.969	

Serum

	Bicalutamide			Andarine		
conc (ng/ml)	S1	S2	S3	S1	S2	S3
1	0.247	0.239	0.235	0.0769	0.0800	0.0741
2	0.467	0.479	0.477	0.1394	0.1556	0.1481
5	1.192	1.262	1.157	0.3404	0.3432	0.3481
10	2.498	3.119	2.752	0.6839	0.7406	0.6649
15	3.636	4.107	3.803	1.0533	1.0836	1.0012
20	4.652	5.687	5.124	1.3051	1.3842	1.4038
50	9.431	8.663	8.582	3.0670	2.848	2.608
100	18.854	18.015	18.063	6.0707	6.052	5.494
150	28.580	26.642	26.634	9.1951	8.472	7.902
200	40.009	34.610	34.922	11.8530	10.152	9.467
300	58.209	49.865	49.413	16.8940	14.906	14.629
600	117.867	111.527	110.559	36.3668	33.846	29.781
1000	190.426	168.023	156.844	58.2987	54.834	47.741

Linearity

It were evaluated as *determination coefficient* (*r2*) from the calibration curves. The following tables present only the averages:

	Bicalutamide		
	Urine	***Faeces***	***Serum***
a	*0,617*	*0,392*	*0,229*
b	*0,125*	*4,231*	*0,313*
r2	*0,9951 ± 0,0051*	*0,9941 ± 0,0057*	*0,9971 ± 0,002*

	Andarine		
	Urine	***Faeces***	***Serum***
a	*0,205*	*0,165*	*0,053*
b	*0,382*	*0,162*	*0,053*
r2	*0,9951 ± 0,0058*	*0,9955 ± 0,0054*	*0,9982 ± 0,0006*

Accuracy

The accuracy was calculated as percentage recovery (R%) in the range of concentration found during the second animal trial.

Urine

	Bicalutamide					
conc (ng/ml)	***x1***	***x2***	***x3***	***R1***	***R2***	***R3***
0.5	*0.47*	*0.48*	*0.42*	*93.2*	*96.4*	*84.4*
1	*1.13*	*1.11*	*1.00*	*112.5*	*110.6*	*99.9*
5	*5.09*	*4.74*	*4.61*	*101.9*	*94.8*	*92.1*
10	*10.32*	*11.03*	*9.77*	*103.2*	*110.3*	*97.7*
50	*45.58*	*48.16*	*43.43*	*91.2*	*96.3*	*86.9*
100	*106.83*	*112.76*	*99.90*	*106.8*	*112.8*	*99.9*
200	*184.18*	*206.06*	*201.88*	*92.1*	*103.0*	*100.9*

	Andarine					
conc (ng/ml)	***x1***	***x2***	***x3***	***R1***	***R2***	***R3***
0.5	*0.42*	*0.49*	*0.42*	*84.0*	*97.3*	*84.9*
1	*1.08*	*1.04*	*0.97*	*108.1*	*104.4*	*97.0*
5	*4.54*	*4.03*	*4.11*	*90.8*	*80.6*	*82.2*
10	*9.27*	*8.62*	*8.13*	*92.7*	*86.2*	*81.3*
50	*53.06*	*49.70*	*51.47*	*106.1*	*99.4*	*102.9*
100	*106.17*	*98.34*	*101.14*	*106.2*	*98.3*	*101.1*
200	*185.07*	*210.30*	*195.67*	*92.5*	*105.2*	*97.8*

Faeces

	Bicalutamide					
conc (ng/g)	**x1**	**x2**	**x3**	**R1**	**R2**	**R3**
2	1.89	2.02	1.79	94.3	101.2	89.6
5	4.74	4.99	4.28	94.7	99.8	85.5
10	10.11	10.82	10.31	101.1	108.2	103.1
50	51.89	53.73	47.76	103.8	107.5	95.5
100	105.08	102.93	101.15	105.1	102.9	101.1
1000	1028.47	1107.65	1021.46	102.8	110.8	102.1
2500	2620.69	2271.53	2521.14	104.8	90.9	100.8
3500	3692.47	3382.18	3258.06	105.5	96.6	93.1

	Andarine					
conc (ng/g)	**x1**	**x2**	**x3**	**R1**	**R2**	**R3**
2	1.64	1.63	1.76	81.8	81.4	88.2
5	5.00	5.34	5.08	100.1	106.8	101.6
10	9.93	10.60	10.12	99.3	106.0	101.2
50	50.72	45.79	53.21	101.4	91.6	106.4
100	103.46	102.80	98.04	103.5	102.8	98.0
1000	1054.79	990.02	936.36	105.5	99.0	93.6
2500	2505.63	2331.33	2265.96	100.2	93.3	90.6
3500	3671.07	2844.28	3447.62	104.9	81.3	98.5

Serum

	Bicalutamide					
Conc_N (ng/ml)	**x1**	**x2**	**x3**	**R1**	**R2**	**R3**
2	2.17	1.98	1.96	108.3	98.9	97.8
5	5.42	5.28	4.91	108.4	105.6	98.1
10	9.26	8.24	8.62	92.6	82.4	86.2
50	48.47	51.50	43.71	96.9	103.0	87.4
100	110.57	92.37	93.57	110.6	92.4	93.6
200	218.94	209.70	194.31	109.5	104.8	97.2
1000	1060.06	907.34	970.22	106.0	90.7	97.0

	Andarine					
Conc_N (ng/ml)	**x1**	**x2**	**x3**	**R1**	**R2**	**R3**
2	1.99	2.26	2.01	99.6	112.9	100.6
5	4.22	4.74	4.02	84.4	94.7	80.5
10	10.46	10.43	9.87	104.6	104.3	98.7
50	53.79	45.66	48.82	107.6	91.3	97.6
100	106.79	92.37	100.40	106.8	92.4	100.4
200	213.90	200.60	216.06	106.9	100.3	108.0
1000	1041.04	912.06	1013.40	104.1	91.2	101.3

The following table presents R% and RSD% as ranges:

	Urine		***Faeces***		***Serum***	
	Bicalutamide	***Andarine***	***Bicalutamide***	***Andarine***	***Bicalutamide***	***Andarine***
R %	84.4 - 112.8	80.6 - 108.1	85.5 - 110.8	81.3 - 106.8	82.4 - 110.6	80.5 - 112.9
RSD %	5.2 - 6.8	3.3 - 8.3	1.9 - 7.7	2.9 - 12.9	5.1 - 10.3	3.2 - 8.5

Precision

The followings tables presents concentrations obtained for spiked samples. The analysis were made triplicated and at different concentration Urines: 0.25, 0.5, 1, 3 and 10 ng/mL; serum and faeces 1, 2, 5, 10 and 20 ppb (serum: ng/mL and faeces: ng/g).

Urines

	Bicalutamide (ng/mL)			
Conc_Nom	*C1*	*C2*	*C3*	*RSD%*
0.25	*0.20*	*0.23*	*0.27*	*13.4*
0.5	*0.46*	*0.51*	*0.53*	*6.6*
1	*0.95*	*0.98*	*1.04*	*4.6*
3	*2.71*	*2.94*	*3.26*	*9.3*
10	*9.73*	*9.76*	*10.34*	*3.5*

	Andarine (ng/mL)			
Conc_Nom	*C1*	*C2*	*C3*	*RSD%*
0.25	*0.23*	*0.24*	*0.28*	*12.0*
0.5	*0.45*	*0.49*	*0.54*	*8.5*
1	*0.93*	*0.99*	*1.07*	*7.1*
3	*2.79*	*3.01*	*3.18*	*6.5*
10	*9.54*	*10.32*	*10.47*	*4.9*

Faeces

	Bicalutamide (ng/g)			
Conc_Nom	*C1*	*C2*	*C3*	RSD%
1	*0.89*	*1.09*	*1.12*	*12.1*
2	*1.86*	*1.97*	*2.16*	*7.6*
5	*4.72*	*5.27*	*5.35*	*6.7*
10	*9.30*	*10.65*	*10.71*	*7.8*
20	*19.13*	*20.67*	*21.20*	*5.3*

	Andarine (ng/g)			
Conc_Nom	*C1*	*C2*	*C3*	RSD%
1	*0.79*	*0.86*	*0.97*	*10.7*
2	*1.79*	*1.96*	*2.14*	*8.9*
5	*4.68*	*4.85*	*5.26*	*6.0*
10	*9.31*	*9.89*	*10.42*	*5.6*
20	*18.72*	*19.65*	*21.22*	*6.4*

Serum

	Bicalutamide (ng/mL)			
Conc_Nom	*C1*	*C2*	*C3*	RSD%
1	*0.92*	*0.93*	*1.13*	*11.9*
2	*1.92*	*2.06*	*2.13*	*5.3*
5	*4.63*	*5.25*	*5.35*	*7.7*
10	*9.35*	*9.47*	*10.77*	*8.0*
20	*18.40*	*20.29*	*22.21*	*9.4*

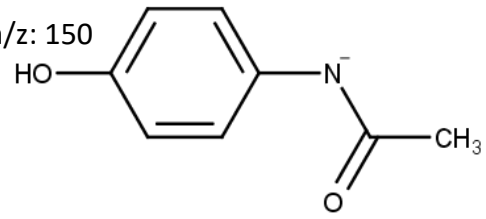
	Andarine (ng/mL)			
Conc_Nom	*C1*	*C2*	*C3*	RSD%
1	*0.92*	*0.94*	*1.12*	*11.3*
2	*1.86*	*2.04*	*2.17*	*7.7*
5	*4.35*	*4.56*	*5.35*	*11.1*
10	*9.32*	*10.68*	*10.71*	*7.8*
20	*18.22*	*20.64*	*21.40*	*8.3*

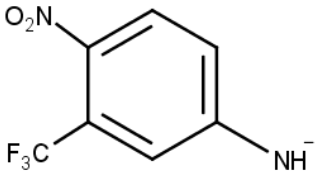
Annex VIII

CHARACTERISTIC FRAGMENTS OF ANDARINE

The following chemical structures are based on previous studies made by others authors [36, 38, 45, 52, 65].

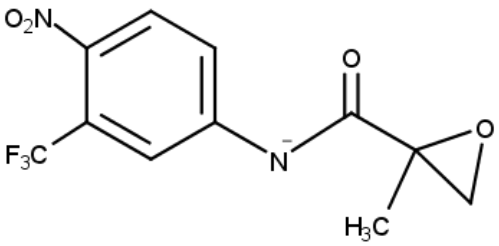
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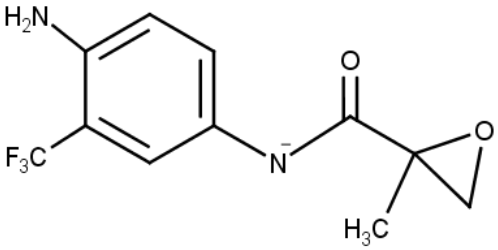




m/z: 205

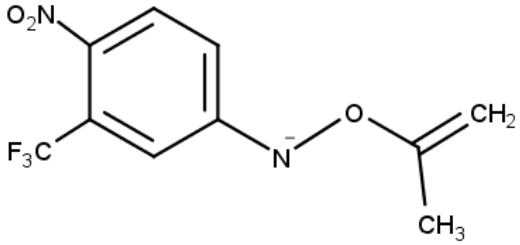
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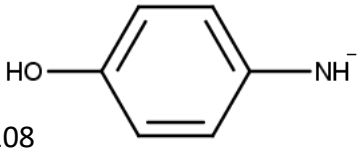




m/z: 259

m/z: 261





m/z: 108

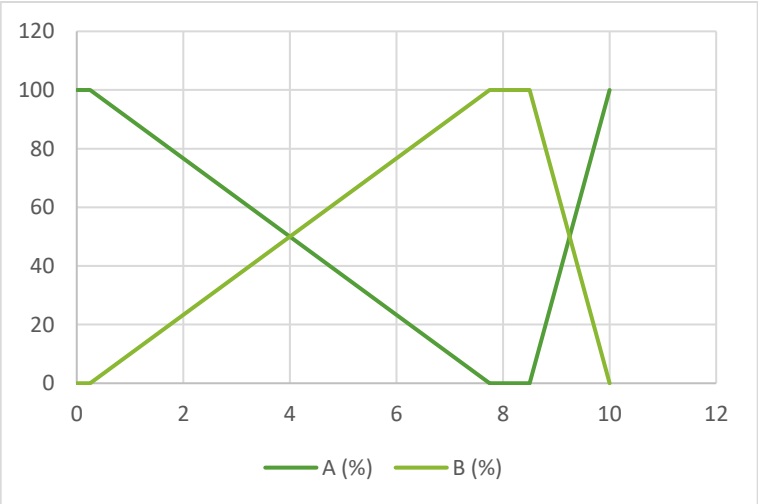
Annex IX

METABOLITES STUDY (2ND ANIMAL TRIAL)

Chromatographic System

- Column ACQUITY UPLC BEH C18 (1.7 µm; 2.1 x 100 mm)
- Volume de Injection: 2 µL
- Mobil Phase:
  - Solution A: Acid Formic 0.1% + Acetonitrile 10 %
  - Solution B: Acetonitrile
- Flow: 0.45 mL/min

T(min)	A (%)	B (%)
0	100	0
0,25	100	0
7,75	0	100
8,5	0	100
10	100	0



SARMS CONCENTRATIONS

	Steer N° 443-Casodex 100 mg				
	Bicalutamide Free	Bicalutamide Total	Bicalutamide Free	Bicalutamide Total	Bicalutamide
Day	Urine (ng/mL)	Urine (ng/mL)	Faeces (ng/g)	Faeces (ng/g)	Serum (ng/mL)
-1	*ND*	*ND*	*ND*	*ND*	*ND*
0	*ND*	*ND*	*ND*	*ND*	*ND*
0.17	*1.30*	*1.50*	*8.38*	*26.52*	*259.18*
0.33	*10.66*	*8.27*	*327.74*	*376.06*	*392.20*
1	*12.67*	*14.76*	*1830.85*	*2050.67*	*384.64*
2	*8.06*	*11.47*	*671.80*	*1387.17*	*340.22*
3	*6.32*	*8.93*	*248.89*	*471.09*	*330.94*
4	*3.36*	*2.47*	*132.17*	*131.07*	*262.67*
5	*1.83*	*1.90*	*52.22*	*61.26*	*231.77*
7	*0.67*	*1.65*	*28.61*	*27.30*	*232.63*
9	*0.70*	*0.87*	*3.04*	*8.84*	*116.66*
11	*0.81*	*0.68*	*2.25*	*2.60*	*71.12*

	Steer N° 421-Casodex 200 mg				
	Bicalutamide Free	Bicalutamide Total	Bicalutamide Free	Bicalutamide Total	Bicalutamide
Day	Urine (ng/mL)	Urine (ng/mL)	Faeces (ng/g)	Faeces (ng/g)	Serum (ng/mL)
-1	*ND*	*ND*	*ND*	*ND*	*ND*
0	*ND*	*ND*	*ND*	*ND*	*ND*
0.17	*2.52*	*2.54*	*9.48*	*22.68*	*284.95*
0.33	*4.80*	*5.85*	*2236.76*	*2394.51*	*762.94*
1	*17.74*	*24.48*	*1917.96*	*2244.41*	*757.13*
2	*9.79*	*16.49*	*466.39*	*528.35*	*748.95*
3	*7.05*	*7.66*	*232.63*	*299.16*	*623.15*
4	*5.77*	*8.59*	*181.44*	*224.11*	*455.21*
5	*2.38*	*3.94*	*129.09*	*160.48*	*442.18*
7	*1.35*	*2.16*	*61.44*	*59.45*	*325.53*
9	*0.64*	*1.01*	*43.75*	*37.10*	*189.87*
11	*0.63*	*0.72*	*11.07*	*11.48*	*126.68*

	Porcine N° 330-Andarine 100 mg				
	Andarine Free	Andarine Total	Andarine Free	Andarine Total	Andarine
Day	Urine (ng/mL)	Urine (ng/mL)	Faeces (ng/g)	Faeces (ng/g)	Serum (ng/mL)
-1	*ND*	*ND*	*ND*	*ND*	*ND*
0	*ND*	*ND*	*ND*	*ND*	*ND*
0.17	*17.26*	*21.51*	*563.65*	*602.26*	*47.81*
0.33	*43.36*	*46.81*	*1598.24*	*1612.92*	*39.27*
1	*18.35*	*21.47*	*1512.96*	*1595.68*	*15.39*
2	*3.86*	*5.91*	*189.14*	*228.15*	*3.61*
3	*ND*	*ND*	*ND*	*4.60*	*ND*
4	*ND*	*ND*	*ND*	*ND*	*ND*
5	*ND*	*ND*	*ND*	*ND*	*ND*
6	*ND*	*ND*	*ND*	*ND*	*ND*
8	*ND*	*ND*	*ND*	*ND*	*ND*
10	*ND*	*ND*	*ND*	*ND*	*ND*

	Steer N° 379-Casodex 50 mg during 5 days				
	Bicalutamide Free	Bicalutamide Total	Bicalutamide Free	Bicalutamide Total	Bicalutamide
Day	Urine (ng/mL)	Urine (ng/mL)	Faeces (ng/g)	Faeces (ng/g)	Serum (ng/mL)
-1	*ND*	*ND*	*ND*	*ND*	*ND*
0	*ND*	*ND*	*ND*	*ND*	*ND*
0.17	*1.13*	*0.82*	*14.98*	*13.67*	*143.48*
0.33	*2.13*	*1.31*	*856.68*	*947.68*	*327.42*
1	*10.59*	*12.05*	*770.52*	*1177.22*	*335.17*
2	*9.61*	*12.85*	*1142.03*	*1459.46*	*582.29*
3	*17.87*	*15.02*	*1703.29*	*1992.08*	*723.10*
4	*20.29*	*25.81*	*2362.77*	*2880.20*	*898.79*
5	*23.48*	*28.13*	*1820.46*	*1877.54*	*1081.86*
7	*16.43*	*13.04*	*1040.22*	*901.32*	*871.36*
9	*13.03*	*17.41*	*507.59*	*838.75*	*804.78*
11	*5.34*	*4.92*	*187.09*	*267.62*	*683.23*

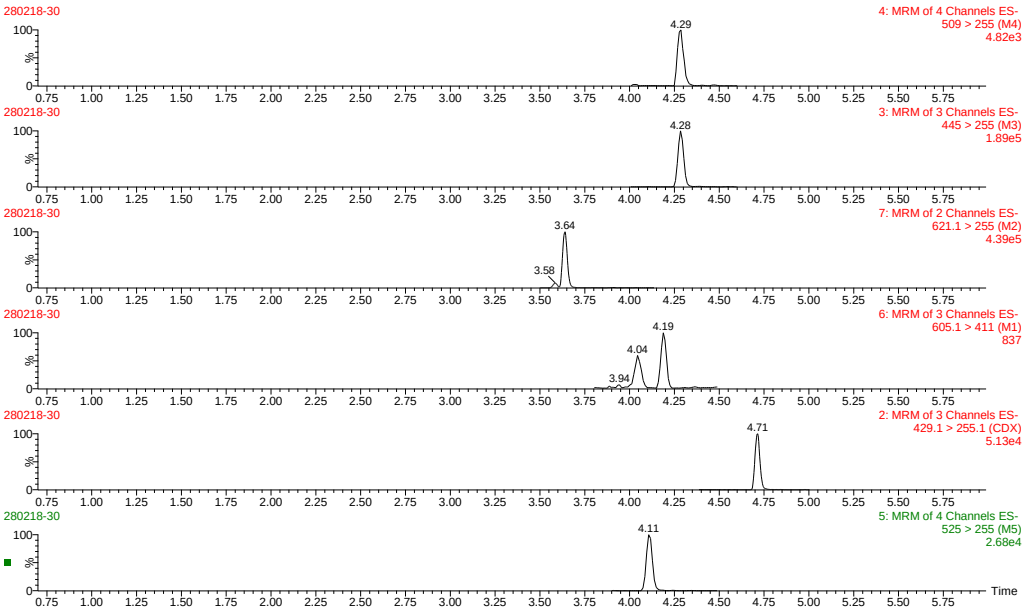
	Porcine N° 334 - Casodex 100 mg				
	Bicalutamide Free	Bicalutamide Total	Bicalutamide Free	Bicalutamide Total	Bicalutamide
Days	Urine (ng/mL)	Urine (ng/mL)	Faeces (ng/g)	Faeces (ng/g)	Serum (ng/mL)
-1	*ND*	*ND*	*ND*	*ND*	*ND*
0	*ND*	*ND*	*ND*	*ND*	*ND*
0.17	*40.04*	*37.52*	*13.91*	*14.06*	*448.16*
0.33	*77.17*	*84.24*	*115.33*	*174.92*	*691.99*
1	*86.16*	*82.57*	*1035.64*	*1387.37*	*873.21*
2	*69.09*	*78.29*	*409.10*	*533.86*	*812.59*
3	*64.84*	*67.87*	*329.85*	*320.95*	*806.49*
4	*22.80*	*34.25*	*313.34*	*342.09*	*724.56*
5	*16.64*	*27.34*	*137.07*	*144.66*	*583.04*
6	*10.45*	*11.84*	*111.10*	*123.32*	*344.19*
8	*7.54*	*6.21*	*49.51*	*57.99*	*282.05*
10	*2.73*	*3.16*	*29.85*	*35.57*	*81.34*

METABOLITES DE BICALUTAMIDE

This study was performed using a triple quadrupole mass detector XEVO TQ-D (Waters, Milford, MA, USA).

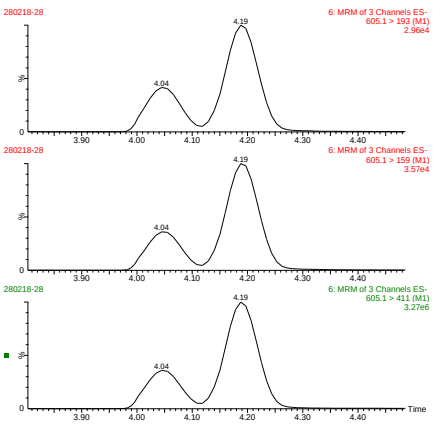
Bovines

The following chromatograms were obtained during the 2nd animal trial and are presented as an example. It shows the first metabolite is M2 follows by M3, M4, M5 and M1.

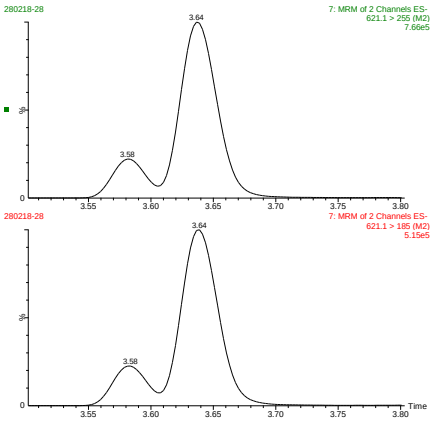


The following figures present the SRM used for the confirmation of each metabolites found:

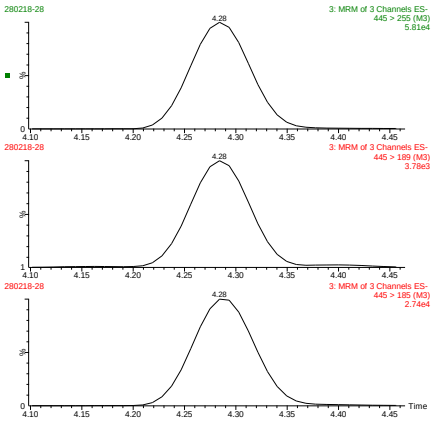
M1:



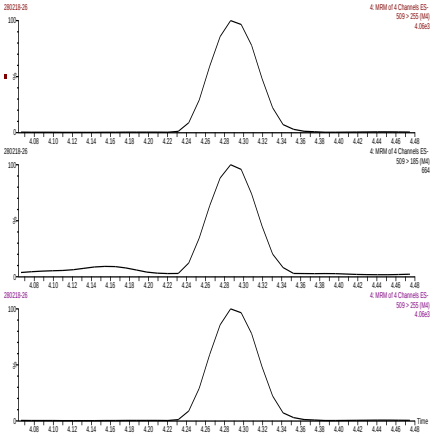
M2:



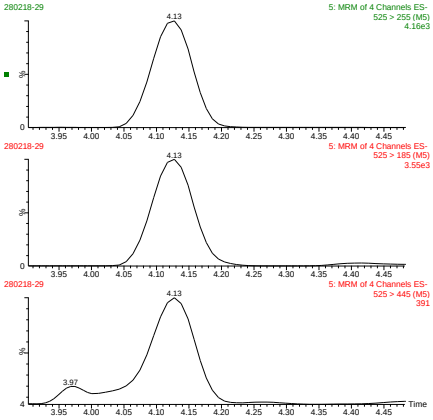
M3:



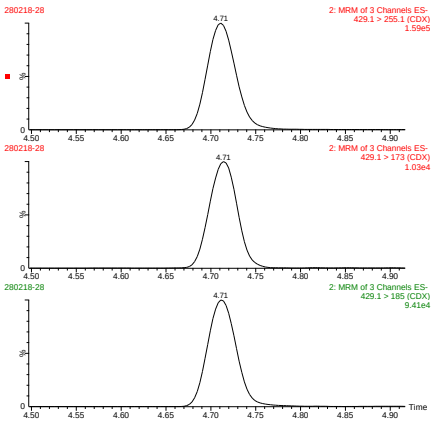
M4:



M5:

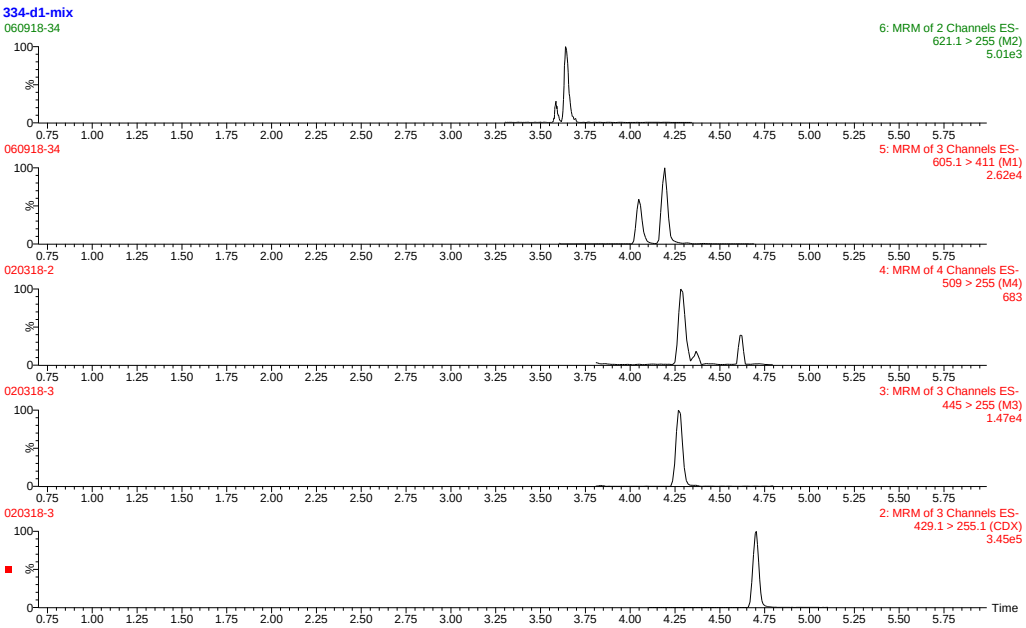


Bicalutamide



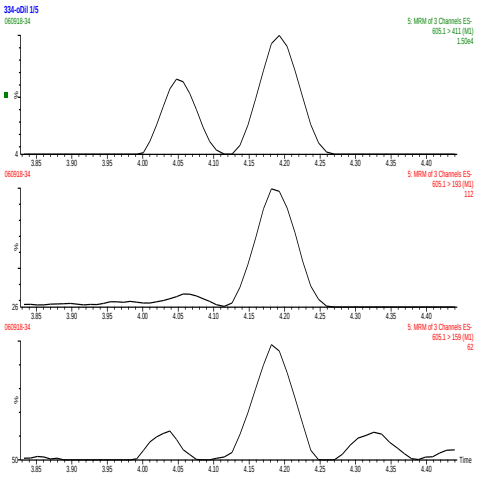
Porcine

The following chromatogram presents the metabolites found at the porcine treated with Casodex ®. It shows the metabolites found M1, M2, M3 y M4.

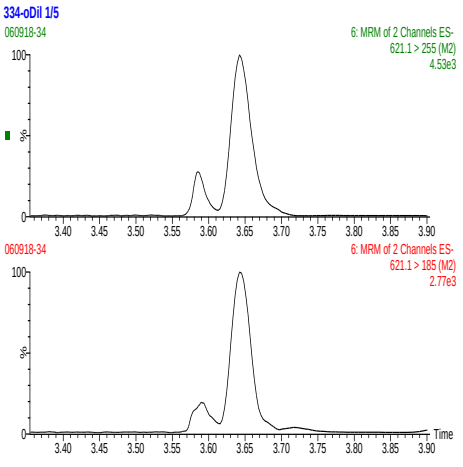


The following figures present the SRM used for the confirmation of each metabolites found:

M1:

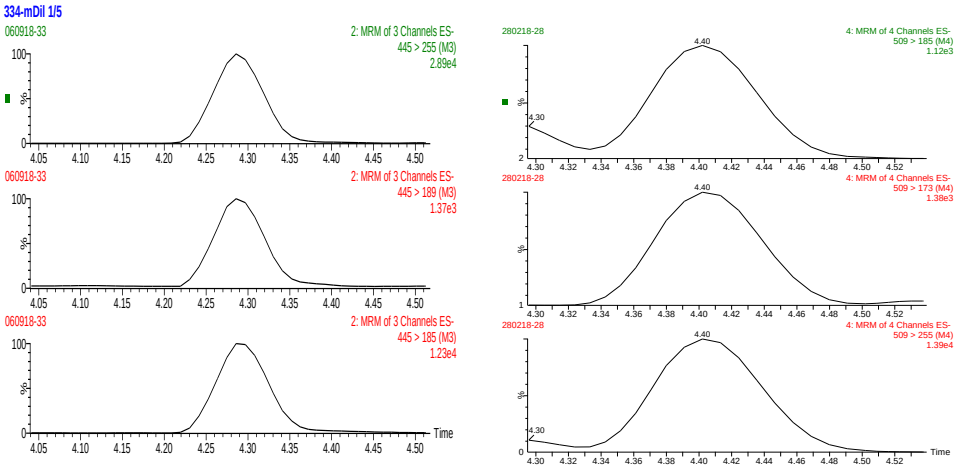


M2:

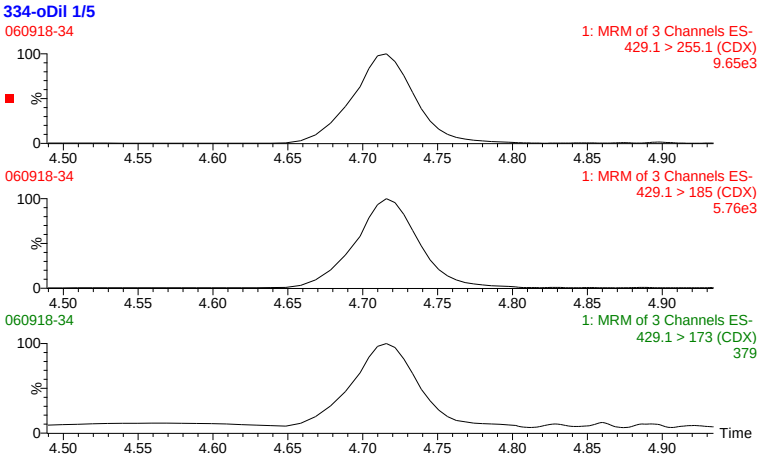


M3:

M4:

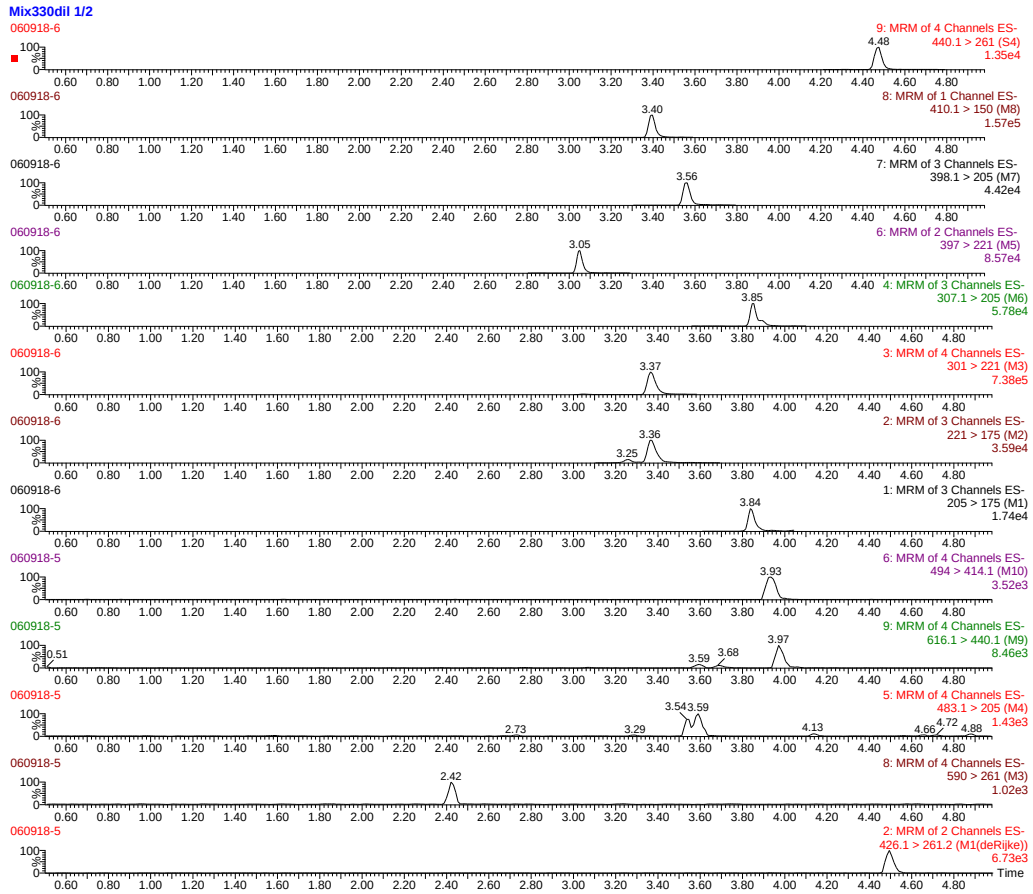


Bicalutamide



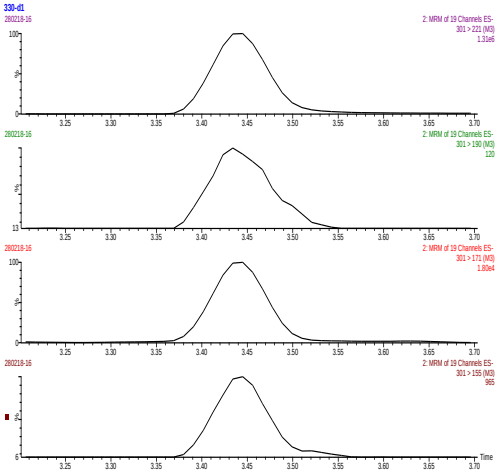
PORCINE METABOLITES OF ANDARINE

The following chromatogram presents the metabolites found at the porcine treated with Andarine. It shows the 12 metabolites found.

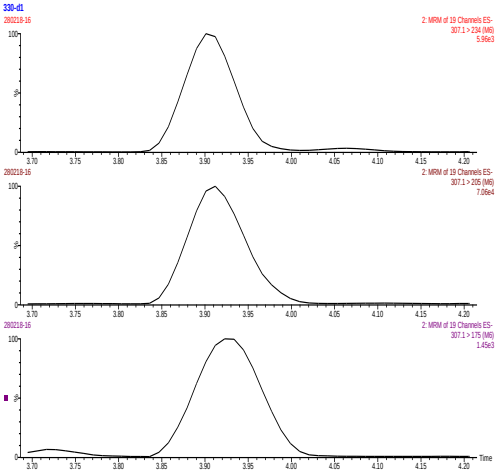


The following figures present the SRM used for the confirmation of each metabolites found:

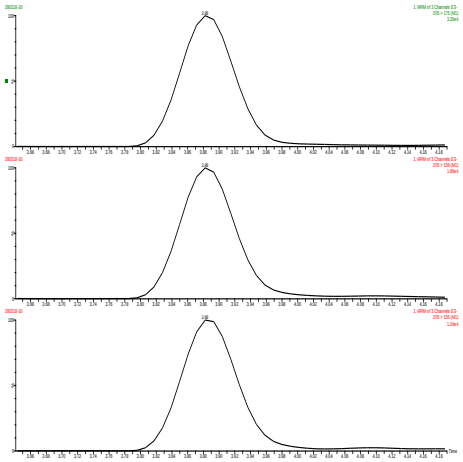
DM1



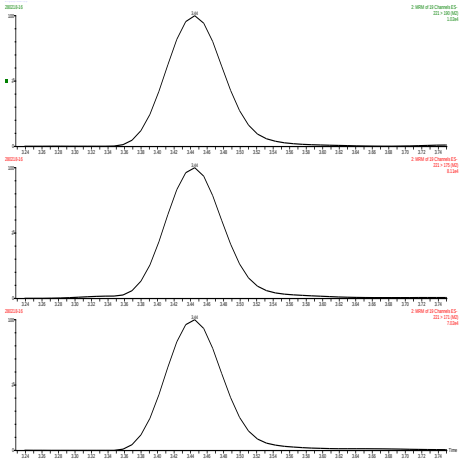
DM2



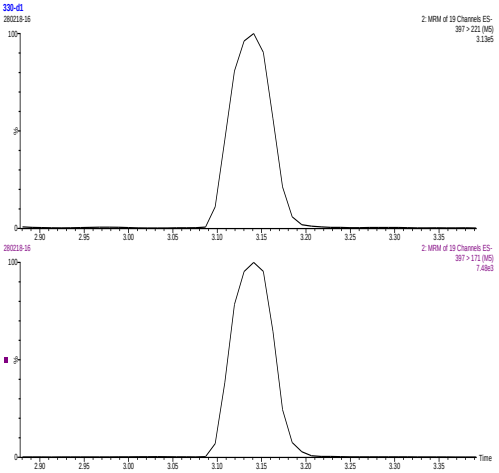
DM3



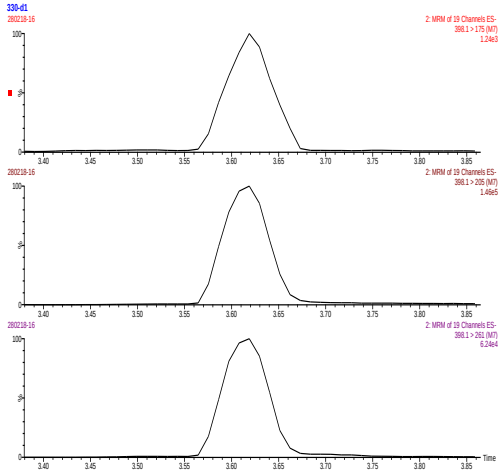
DM4



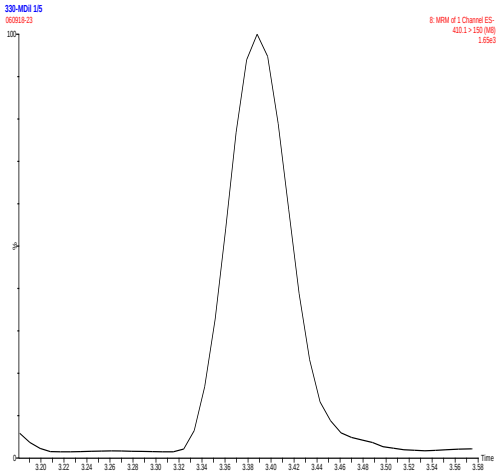
DM5



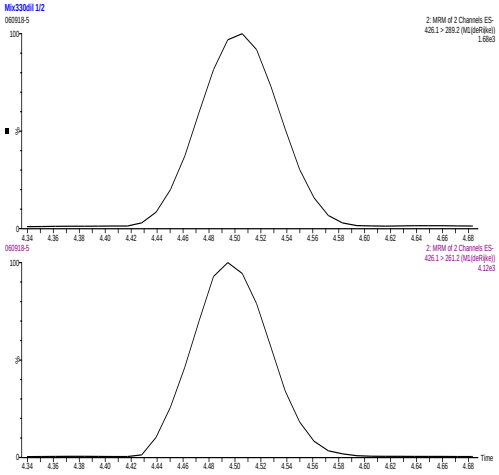
DM6



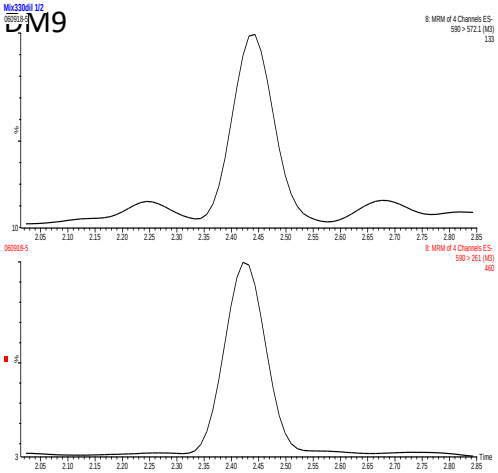
DM7



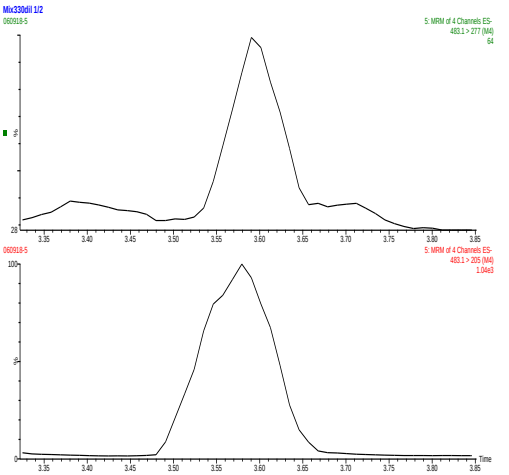
DM8



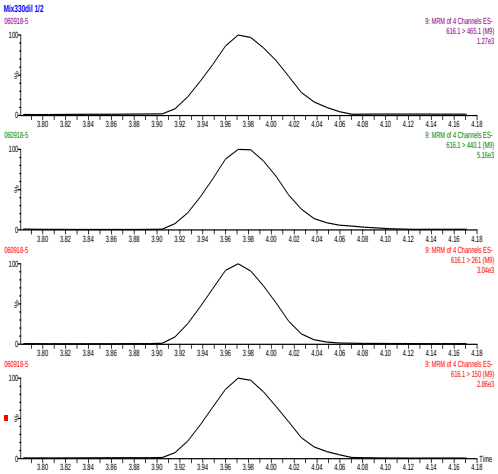
DM9



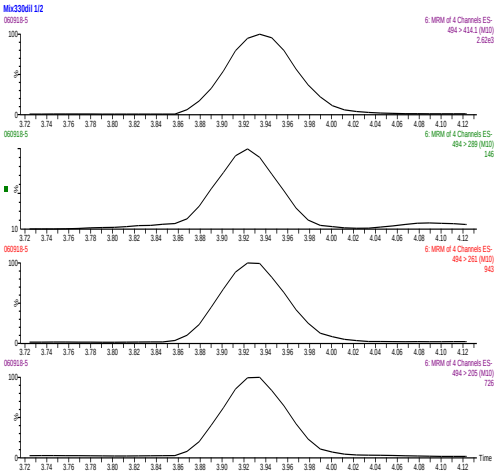
DM10



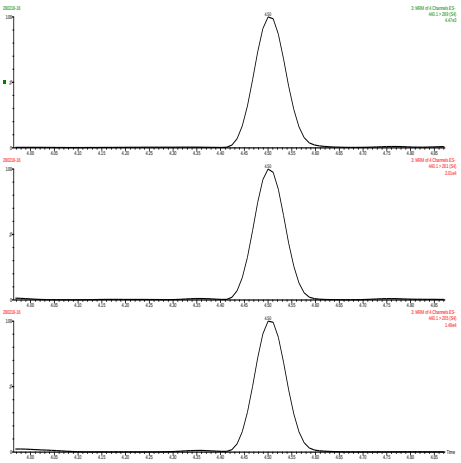
DM11



DM12



Andarine



DOCTORAT BIOLOGIE BRETAGNE SANTE LOIRE

Oniris
ÉCOLE NATIONALE
VÉTÉRINAIRE, AGROALIMENTAIRE ET DE L'ALIMENTATION
SANTÉ ET ALIMENTATION AU CŒUR DE LA VIE

Titre : Etude du métabolisme des SARMs de type Aryl-propionamide chez les animaux de production. Application à la recherche de biomarqueurs d'exposition et d'effets à des fins de contrôle.

Mots clés : SARMs ; métabolisme ; métabolites conjugués ; biomarqueurs ; LC-(HR)MS

Résumé : Les modulateurs spécifiques des récepteurs androgènes (SARMs), exercent leurs effets anabolisants exclusivement sur les muscles squelettiques et ne présentent pas d'activité androgénique. Sur la base de leurs mécanismes d'action et des résultats cliniques préliminaires, ces produits pourraient potentiellement être utilisés comme des substances anabolisantes. Dans ce contexte, l'objectif de la thèse, réalisée en co-tutelle internationale, est de développer des stratégies analytiques spécifiques et sensibles permettant de mettre en évidence leur utilisation frauduleuse chez l'animal de production (bovins et porcins). En s'appuyant sur des stratégies analytiques de couplage de chromatographie en phase liquide avec la spectrométrie de masse basse et haute résolution (LC-QqQ et HRMS), le métabolisme de quatre SARMs (Enobosarm, Andarine, Bicalutamide et Hydroxyflutamide) ainsi que leurs cinétiques d'élimination ont été étudiés chez deux espèces animales, après administration des composés par voie orale. Des métabolites biomarqueurs ont pu être identifiés dans les matrices urine, féces et sérum de bovins et porcins et des fenêtres de détection proposées. Ce travail de recherche contribuera à garantir au consommateur une sécurité chimique des denrées d'origine animale en assurant le suivi d'une nouvelle classe de composés anabolisants.

Title: Study of some aryl-propionamide SARMs metabolism in food producing animals. Highlighting biomarkers of exposure and effects for control purposes.

Keywords: SARMs, metabolism, phase II metabolites, biomarkers, LC-(HR)MS

Abstract: Compounds with anabolic properties have recently emerged: specific androgen receptor modulators (SARMs), currently being developed for the treatment of muscle-loss diseases. For example, commercially available Ostarine (MK-2866 / GTX-024 Enobosarm) exerts its anabolic effects exclusively on skeletal muscles and has no androgenic activity. Based on their mechanisms of action and preliminary clinical results, these products could potentially be used as anabolic substances. In this context, the aim of this international thesis is to develop analytical strategies to highlight their fraudulent use in production animal (cattle and pigs). Based on liquid-phase chromatography coupling techniques with low- and high-resolution mass spectrometry (LC-QqQ and HRMS), the study of the metabolism of SARMs (Enobosarm, Andarine, Bicalutamide and Hydroxyflutamide) after administration and identification of selective metabolites that can be used as biomarkers in animal serums, faeces and serums. This research will help to ensure the consumer's chemical safety of animal products by monitoring a new class of anabolic compounds.