Members of the jury:

Prof. Dr. ir. Stefaan De Smet (Chairman)

Department of Animal production, Faculty of Bioscience Engineering, Ghent University, Ghent

Prof. Dr. ir. Pascal Boeckx

Department of Applied analytical and physical chemistry, Faculty of Bioscience Engineering, Ghent University, Ghent

Prof. Dr. Hubert De Brabander

Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Merelbeke

Prof. Dr. ir. Peter Van Eenoo

Department of Clinical chemistry, microbiology and immunology, Faculty of Medicine and Health Sciences, Ghent University, Zwijnaarde

Dr. Philippe Delahaut

Health Department, CER Groupe, Marloie

Dr. Apr. Els Daeseleire

Technology and Food Science Unit - Food Safety, Institute for Agricultural and Fisheries Research, Melle

Prof. Dr. Bruno Le Bizec (Promoter)

Food contaminants and residue analysis laboratory (LABERCA), Nantes Atlantic College of Veterinary Medicine, Food Science and Engineering (Oniris), Nantes (Fr)

Prof. Dr. ir. Sven Mangelinckx (Promoter)

Department of Sustainable Organic Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent

Prof. Dr. ir. Norbert De Kimpe (Promoter)

Department of Sustainable Organic Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent

Promoters:	Prof. Dr. ir. Sven Mangelinckx and Prof. Dr. ir. Norbert De Kimpe
	Department of Sustainable Organic Chemistry and Technology
	Faculty of Bioscience Engineering, Ghent University
	Prof. Dr. Bruno Le Bizec
	Food contaminants and residue analysis laboratory (LABERCA), Nantes Atlantic College of Veterinary Medicine, Food Science and Engineering (Oniris), Nantes (Fr)
Dean of the Faculty:	Prof. Dr. ir. Marc Van Meirvenne
Rector of the University:	Prof. Dr. Anne De Paepe

UNIVERSITEIT GENT

Ir. Geert Janssens

Stable carbon isotope analyses of natural steroid hormones to determine their abuse in cattle

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Chemistry and Bioprocess Technology

Ghent, March 2016

The author: The promoters:

ir. Geert Janssens   Prof Dr. ir. Sven Mangelinckx

                                               Prof. Dr. ir. Norbert De Kimpe

                                               Prof. Dr. Bruno Le Bizec

Woord vooraf

Als u dit leest, betekent dit dat de eindmeet van dit project succesvol werd bereikt. Dat was onmogelijk geweest zonder de hulp van een heleboel mensen, en een aantal daarvan wil ik hierbij in het bijzonder vermelden.

Vooreerst hartelijk dank aan mijn promotoren aan de Vakgroep Duurzame Organische Chemie en Technologie, Prof. Dr. ir. Norbert De Kimpe en Prof. Dr. ir. Sven Mangelinckx, voor het mooie evenwicht tussen vrijheid laten en richting geven. Jullie stonden steeds onmiddellijk voor me klaar wanneer ik jullie nodig had, en dat werd enorm geapprecieerd.

To my promoter Prof. Dr. Bruno Le Bizec, and the entire staff at LABERCA, many thanks for your hospitality and for openly sharing your incredible expertise on this complex subject.

Walter Gillis, het was een hele eer dat je in mij de geschikte persoon zag. Bedankt dat je me voor dit onderzoek hebt voorgedragen. Geert De Poorter, bedankt voor het vertrouwen en me dit ook daadwerkelijk te laten uitvoeren. En Bert Matthijs, bedankt om me de kans en vrijheid te geven om het af te werken.

Dirk Courtheyn, zonder jouw onuitputtelijke kennis was deze hele onderneming onmogelijk geweest. Bedankt me steeds met raad en daad bij te staan.

Team ISORA, jullie inzet was enorm. Ingrid, Ingrid, Veronique en Eva, liefste trezen, bedankt voor alles.

Jona, bedankt voor je onvoorwaardelijke steun, je geduld en je liefde. Milo en Magnus, bedankt voor alle inspiratie, die ver buiten de grenzen van dit proefschrift mijn leven kleurt.

Tenslotte wil ik ook mijn familie en al mijn vrienden bedanken voor hun steun.

Geert

Table of contents

List of abbreviations

2-DE	Two dimentional electrophoresis
AAA-PD	5α-Pregnane-3α,20α-diol
AAB-PD	5α-Pregnane-3α,20β-diol
AAS	Anabolic-androgenic steroid
ABA-PD	5α-Pregnane-3β,20α-diol
ABB-PD	5α-Pregnane-3β,20β-diol
ACN	Acetonitrile
ADD	Androsta-1,4-diene-3,17-dione
AED	Androstenedione
AEdiol	5-Androstene-3β,17α-diol
AFFIRMS	Immunoaffinity combined with isotope ratio mass spectrometry
A-PDione	5α-Pregnane-3,20-dione
BAA-PD	5β-Pregnane-3α,20α-diol
BAB-PD	5β-Pregnane-3α,20β-diol
BBA-PD	5β-Pregnane-3β,20α-diol
BBB-PD	5β-Pregnane-3β,20β-diol
B-PDione	5β-Pregnane-3,20-dione
CAM	Crassulacean acid metabolism
CER	Centre d'Economie Rurale
C-IRMS	Combustion-isotope ratio mass spectrometry
CRL	Community Reference Laboratory
DES	Diethylstilbestrol
DHEA(-S)	Dehydroepiandrosterone (sulphate)
D/H	Deuterium/hydrogen
DNA	Deoxyribonucleic acid
E1	Estrone
ELISA	Enzyme-linked immunosorbent assay
ERC	Endogenous reference compound
Etio	Etiocholanolone
EU	European Union
EURL	European Union reference laboratory
FASFC	Federal Agency for the Safety of the Food Chain
FLSFCG	Federal laboratory for the safety of the food chain Gentbrugge
GC	Gas chromatography
GC-(MS/C-IRMS)	Gas chromatography coupled in parallel to both mass spectrometry and combustion-isotope ratio mass spectrometry
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HTC	High-temperature conversion
IAC	Immunoaffinity chromatography
IR	Infrared
ISO	International organization for standardization
IUPAC	International Union of Pure and Applied chemistry
LC	Liquid chromatography
LLE	Liquid liquid extraction
LOQ	Limit of quantification

MDGC	Multidimensional gas chromatography
MIPS	Molecularly imprinted polymers
MRL	Maximum residue limit
MS	Mass spectrometry
NIU	National Investigation Unit
NMR	Nuclear magnetic resonance
PD	Pregnanediol
PDB	Pee Dee Belemnite
PG	Progesterone
PTV	Programmed temperature vaporizer
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SFC	Supercritical fluid chromatography
SPE	Solid phase extraction
SPME	Solid phase micro extraction
T/E	Concentration ratio of testosterone over epitestosterone
TLC	Thin layer chromatography
UPLC	Ultra performance liquid chromatography
USA	United States of America
USSR	Union of Socialist Soviet Republics
VPDB	Vienna Pee Dee Belemnite
WADA	World Anti Doping Agency
αE2	17α-Estradiol
αT	17α-Testosterone
βE2	17β-Estradiol
βT	17β-Testosterone

1. General introduction

Nowadays, a great variety of substances described as hormones is known and can be defined in a number of ways, according to origin, functionality, chemical structure,… Following the classical approach, hormones are defined as chemical substances secreted into the bloodstream by endocrinal glands to fulfill their messenger function and trigger the demanded response in the targeted tissue.

Considering that not all hormones are produced by the body itself, but can be administered as well, distinction is made between endogenous and exogenous hormones. Endogenous hormones are assimilated by the body itself, whereas exogenous hormones enter the organism from outside.

Exogenous substances with hormonal activity can either be xenobiotic substances, which do not naturally occur in the organism itself, or homologues of endogenous hormones.

The best way to categorize hormones is by chemical structure and functionality. Distinction is made between amine-derived substances with hormonal activity (examples are β-agonists, thyroid hormones), peptide hormones (examples are insulin, growth hormone) and steroid hormones. Since the latter are the main subject of this thesis, further discussion will be limited to these compounds.

1.1. Definitions of steroid hormones

1.1.1 Steroid nomenclature

Steroids are characterized by a skeleton of perhydrocyclopentanophenanthrene, also referred to as sterane.[1] It is a cyclic structure consisting of three fused cyclohexane rings (ring A, B and C) and one cyclopentane ring (ring D), shown in figure 1.1 with position numbering, containing 17 carbon atoms in total. By addition of double bonds, bond scissions, ring expansions or contractions in the skeleton, and/or by the addition of different functional groups to this core structure, a wide variety of compounds can be obtained, for which a specialized nomenclature is used.[2] The objective of this paragraph is to provide the basic rules of this nomenclature, required to interpret correctly various terms in this thesis.
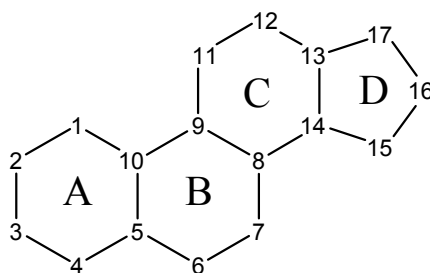
Figure 1.1: Structure of perhydrocyclopentanophenanthrene, including position and ring notations

When steroids are pictured as projections onto the plane of the paper, atoms or groups oriented downwards are noted as α, whereas substituents emerging from the plane are noted as β-oriented, indicated with a dashed or solid wedge, respectively. For substituents with an unknown orientation, or for mixtures of stereoisomers, a wavy line is used.[2] The sterane core possesses six asymmetric carbon atoms. The usual orientation at the bridgeheads is 8β, 9α, 10β, 13β and 14α, and therefore does not need to be specified in the names or figures unless it is different. The core structure with this configuration, and without alkyl groups at position 10, 13 and 17 is referred to as gonane. When a methyl group is present in position 13, the 18-carbon structure is referred to as estrane, and with methyl groups in position 10 and 13, it is called androstane, containing 19 carbon atoms. By addition of alkyl substituents in position 17 of the androstane nucleus, a number of additional hydrocarbon backbones can be obtained, also bearing specific stem names, of which the 21-carbon pregnane nucleus, and the 27-carbon cholestane nucleus need to be mentioned. The side chains in position 17 are in β-orientation, unless stated otherwise.[2] All these structures and the stem names are included in figure 1.2.

The configuration at bridgehead 5 needs to be specified if known, by addition of 5α or 5β in front of the stem name. When the orientation is not known, the notation ξ is used.[2,3]

Unsaturation of the steroid nucleus is indicated by changing –an(e) to –en(e), -adien(e), -yn(e),... anticipated by the position of the unsaturation(s).[2] Substituents of the steroid nucleus are noted with prefixes or suffixes, accompanied by the position and orientation. Alcohol groups are indicated as suffixes or prefixes to the stem name, -ol or hydroxy-, respectively. When multiple alcohol groups are present, this becomes –diol, -triol,... or dihydroxy-, trihydroxy-,... The same rules apply to ketones, where the prefix oxo- and the suffix –one are used. When both alcohol and ketone

groups are present, ketones take priority as suffixes.[2,3] Alkyl substituents, on the other hand, can only be described using the appropriate prefix (methyl-, ethyl-,...).[2] When both a substituent and a side chain are present at position 17, the α-orientation of the substituent does not need to be noted.
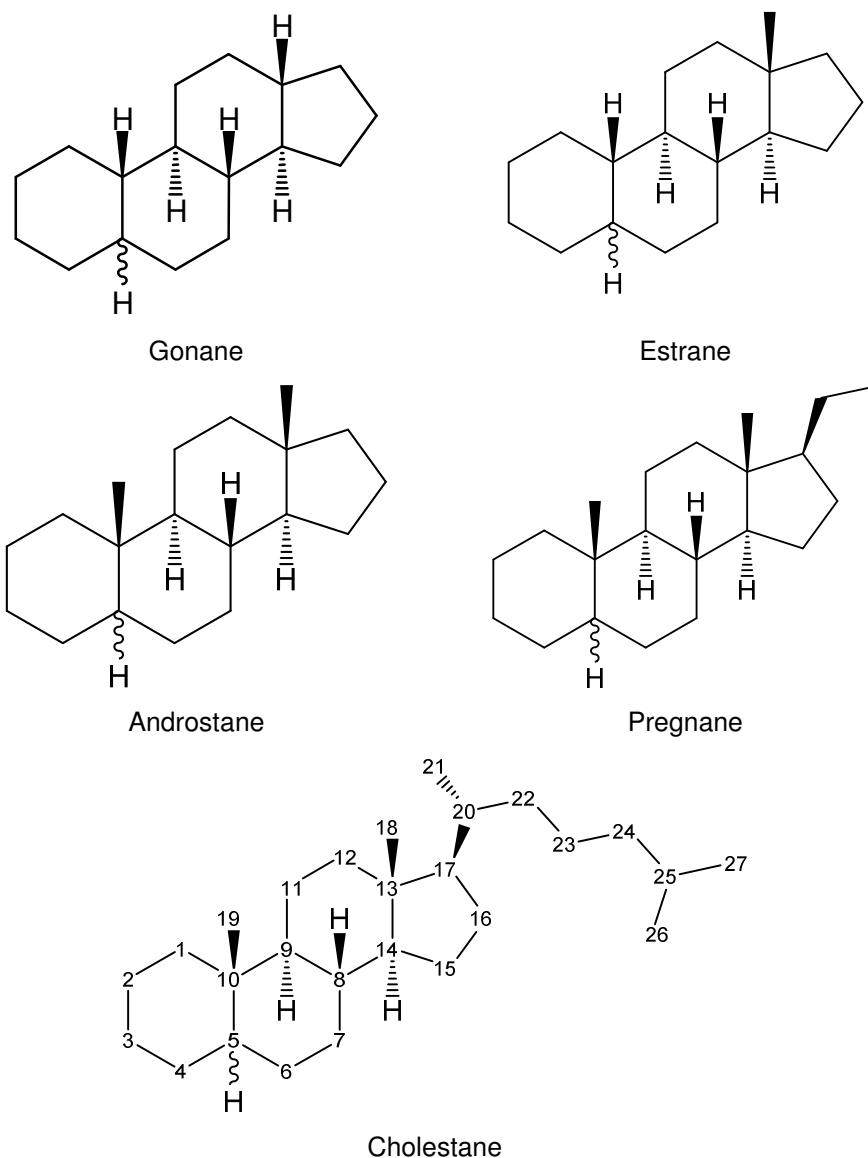
Figure 1.2: Structure of the different steroid nuclei and their stem names. Position numbering is only provided for cholestane.

Finally, beside the rules provided by the official International Union of Pure and Applied Chemistry (IUPAC) discussed above, a set of trivial names is often used to describe a number of important steroids and improve readability of a text. Some of these trivial names are IUPAC-approved, other are commonly used in the field. If these trivial names are used as a basis for naming derivatives or stereoisomers, the

derived trivial name must make the nature of the modification completely clear.[2,3] To illustrate the system of nomenclature, all steroids of importance for this manuscript are presented in table 1.1, including their full systematic name, structure and possible trivial name.

Table 1.1: Names, structure and used abbreviation of the steroids of importance for this manuscript

Trivial names *IUPAC name* Used abbreviation	Structure	Trivial names *IUPAC name* Used abbreviation	Structure
5-Androstene-3β,17α-diol *Androst-5-en-3β,17α-diol* AEdiol		*5β-Pregnane-3β,20β-diol* BBB-PD	
5α-Pregnane-3α,20α-diol AAA-PD		5β-Pregnanedione *5β-Pregnane-3,20-dione* B-PDione	
5α-Pregnane-3β,20α-diol ABA-PD		((17)β-)Estradiol *Estr-1,3,5-trien-3,17β-diol* βE2	
5α-Pregnane-3α,20β-diol AAB-PD		(17)α-Estradiol *Estr-1,3,5-trien-3,17α-diol* αE2	
5α-Pregnane-3β,20β-diol ABB-PD		((17)β-)Testosterone *17β-Hydroxyandrost-4-en-3-one* βT	

4

5α-Pregnanedione

5α-Pregnane-3,20-dione

A-PDione

(17)α-Testosterone;
Epitestosterone

17α-hydroxyandrost-4-en-3-one

αT

Pregnanediol

5β-Pregnane-3α,20α-diol

BAA-PD

Dehydroepiandrosterone

3β-Hydroxyandrost-5-en-17-one

DHEA

5β-Pregnane-3β,20α-diol
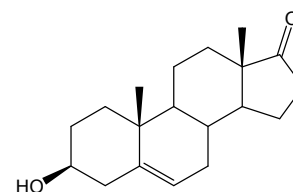
BBA-PD
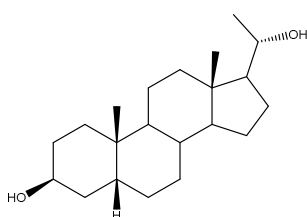
Etiocholanolone

3α-Hydroxy-5β-androstan-17-one

Etio

5β-Pregnane-3α,20β-diol
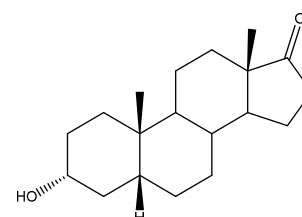
BAB-PD
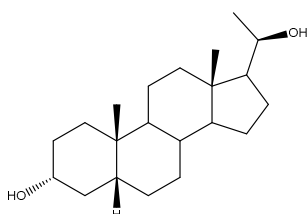
Progesterone

Pregn-4-en-3,20-dione

PG
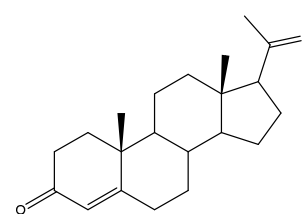
1.1.2 Classification and functionality of endogenous steroid hormones

Steroids encompass a large number of compounds, classified in different groups with varying functionality. Next to their endogenous functionality, a number of steroid hormones are well known for their growth promoting capabilities in farm animals, which will be discussed in the following paragraph.

Sex steroid hormones, or gonadal steroids, play an important role in the regulation of behavior, morphogenesis and functional differentiation of the reproductive system in vertebrates, and are divided into three subgroups.[4,5]

The first group are the male sex steroids, or androgens. Endogenous androgens are characterized by a 19-carbon, androstane steroid nucleus, with hydroxyl or carbonyl groups at position 3 and 17. They are primarily produced in the Leydig cells in the testes, and to a lesser extent in the ovary and the adrenal cortex, and are

responsible for the development of both primary and secondary sex characteristics of male animals.[4,6] Besides their androgenic effects, they are also wellknown for their anabolic qualities. Used as growth promoters, they achieve body weight gain by increased feed conversion and nitrogen retention in animals, resulting in increased protein deposition at the expense of body fat.[6] Although the balance between androgenic and anabolic characteristics is highly variable in different androgens, they always possess both properties, and therefore are often referred to as anabolic-androgenic steroids (AAS).[5] Testosterone is the most common example of endogenous androgens.

The second group are the female sex steroids, or estrogens. Endogenous estrogens are characterized by a 18-carbon, estrane steroid nucleus, in which the A-ring is converted to a phenol structure, and with an additional hydroxyl or carbonyl group at position 17.[6,7] They are produced in the ovaries and placenta in females, and in the testes in males. They are responsible for the development of primary and secondary female sex characteristics.[4,6] In ruminants, an increased overall nitrogen retention and utilization, improved growth rate and lean tissue accretion are obtained under the influence of estrogens. However, in many other mammalian species, amongst which humans, growth is inhibited by female sex steroids.[6] Estradiol is the best known example of endogenous estrogens.

The third group are the progestagens, also referred to as gestagens or hormones of pregnancy. Endogenous progestagens have a 21-carbon, pregnane skeleton, and are synthesized in the corpus luteum, the placenta and the adrenal cortex.[4,6] Progestagens are essential for the uterine development necessary for implantation, blastocyst development, and maintenance of the fetus and of uterine tone during pregnancy.[8] Although they posses anabolic qualities as well, these are less pronounced than for the androgens or estrogens, and progestagens are known to increase body fat deposition.[6] Progesterone is the most common endogenous progestagen.

The growth promoting results obtained through administration of synthetic analogues of endogenous sex steroid hormones are variable, dependent upon the characteristics of the animal.[6] The effects are most pronounced in ruminants, as opposed to pigs and poultry, where much more variable results were obtained. Also sex and age of the animal is of importance: androgens will be more effective when administered to heifers or steers as opposed to bulls, whereas estrogens generally

work better in the latter, and treatment of veal calves at a too early age can be detrimental for later growth.

Also, the nature of the treatment is a determining factor. The administered substances have an optimal dosage, below which the effects will be negliable, and above which they do not bring additional gain or might even be harmful.[6] Furthermore, combinations of two or more anabolic agents are often used. In this way, undesirable side effects of one agent can be mitigated by another, and a synergistic effect on growth or daily gain can be observed. Therefore, estradiol and testosterone are usually combined for the treatment of heifers, whereas a combination of estradiol and progesterone is more common for the treatment of steers, bulls and calves.[6,9-14] Because endogenous steroids are rapidly metabolized, they are usually administered in an esterified form. In the systemic circulation, the esters are hydrolyzed by blood esterases, resulting in a sustained release.[15] In countries where regulated use of synthetic analogues of endogenous sex steroid hormones for growth promoting purposes is permitted, treatment is usually done by implantation into the ear of the animal.[9,11] The implantation allows a more gradual release of the steroids into the bloodstream, prolonging the effects over time. Additionally, by removing the ear after slaughter, a source of high residual contamination is easily avoided, as opposed to injection sites when the animals are treated intramuscularly.

Although the focus of this study is on sex steroid hormones, a second important group of steroid hormones, corticosteroids, needs to be mentioned. Similar to progestagens, they have a pregnane nucleus. Additionally, they are characterized by a hydroxyl or carbonyl group at position 11, a carbonyl group at position 3 and position 20 and a hydroxyl group at position 21.[16] Most endogenous corticosteroids have a double bond between carbon 4 and 5. Corticosteroids are divided into two groups: glucocorticoids, for example cortisol, carry a hydroxyl group at position 17, whereas mineralocorticoids, for example aldosterone, do not. Both examples are shown in figure 1.3. In the body, mineralocorticoids play an important role in the regulation of electrolyte and water metabolism, whereas glucocorticoids are involved in gluconeogenesis, glycogen deposition and protein metabolism. Additionally, glucocorticoids exhibit a powerful anti-inflammatory activity, leading to its widespread medical and veterinary use.[5]

Originally, a negative correlation between corticosteroids and growth has been reported in cattle and sheep, causing fat deposition and reduced protein content.[6] However, administered in combination with β-agonists or anabolic steroids, positive growth promoting abilities were observed due to synergetic effects. Additionally, treatments with low concentrations of corticosteroids were reported to improve feed intake, increase weight gain and reduce feed conversion ratio.[16-18]
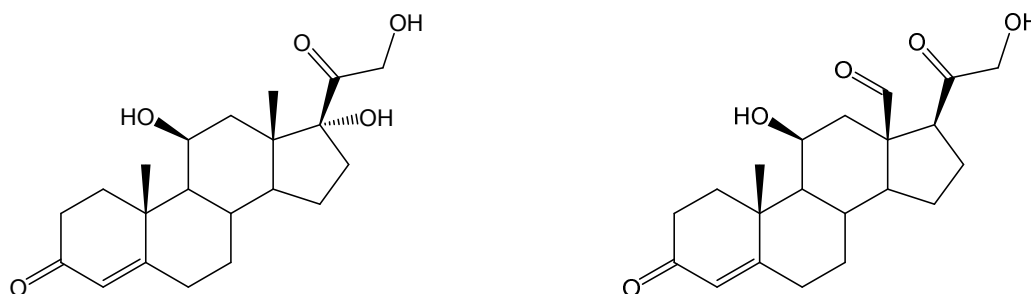
Figure 1.3: Structure of the glucocorticoid cortisol (11β,17,21-trihydroxypregn-4-en-3,20-dione) (left) and the mineralocorticoid aldosterone (11β,21-dihydroxypregn-4-en-3,18,20-trione) (right)

1.2. Legal framework

As a precautionary measure to protect consumers' health, the use of hormonal substances as growth promoters has been prohibited in the European Union (EU) since 1988.[19] Despite international pressure from countries in which restricted use of certain hormonal growth promoters is permitted, the EU maintained its decision based on risk evaluations,[20,21] resulting in the currently active legislative triptych.[22-24]

With regard to synthetic analogues of naturally occurring sex steroid hormones, their use is restricted as laid down in article 3a of Council directive 96/22/EC, amended twice,[20-21] prohibiting the administration of substances having an estrogenic, androgenic or gestagenic action to farm or aquaculture animals.[22] Exception is made for therapeutic veterinary use, which is strictly regulated in veterinary law.

A second Council directive, 96/23/EC, lays down the ground rules for inspection of unwanted residues of substances in living animals or products thereof.[23] Sex steroid hormones are included in annex I of this directive as substances having anabolic effect and unauthorized substances, group A3: steroids. The member states are

responsible for the implementation of these guidelines into annual national residue plans.

Finally, Commission decision 2002/657/EC specifies the analytical criteria required for the implementation of the national residue plans.[24] Group A substances can be monitored with qualitative methods, since no maximum residue limits (MRLs) have been set. Authorized confirmation methods for this group require a liquid or gas chromatographic separation (LC or GC), combined with mass spectrometric or infrared spectrometric detection (MS or IR). The identification criteria are based upon the comparison between the sample and a calibration standard, an external standard included in the same measurement series. Regarding the chromatographic separation, the relative retention time needs to correspond within an interval of ± 0.5% for GC, and ± 2.5% for LC. Mass spectrometric identification is based upon the correspondence of the relative ion intensities, for which a specific point system is used based upon the exact application. For a successful identification of a group A substance, four identification points need to be earned.

With exception of Italy and the Netherlands, which regard them as group A, most member states classify corticosteroids as group B substances, or veterinary drugs, within the subgroup 2f: other pharmacologically active substances (including unlicensed substances which could be used for veterinary purposes). For these substances, a wider range of authorized confirmation methods is described, and when mass spectrometry is used, three earned identification points is sufficient.[24] For the licensed corticosteroids, prednisolone, methylprednisolone and dexamethasone, MRLs are also defined in various matrices.[25,26]

In the Belgian national legislation, restrictions on the use of hormonal substances, and the consequences of illegal use, are laid down in the law of 15 July 1985.[27] Sex steroid hormones are comprised under article 3§2, prohibiting the prescription and administration of substances with estrogenic, androgenic or gestagenic action to farm or aquaculture animals, with exception of legitimate veterinary treatment; the same prohibition is in force for corticosteroids, included in substances with hormonal action, described in article 3§3. The law is accompanied by a series of executive decisions (royal and ministerial decrees), assigning the competent authority for inspection and

designating suitable laboratories for the required analyses, amongst other practical aspects.[28]

When evaluating the current legislation, an obvious void becomes apparent with regard to the inspection on illegal use of synthetic analogues of endogenous steroid hormones. Since the concentrations of these substances are highly variable according to species, sex, age and physiological state of the animal, no decision level for their concentration in matrices from animal origin have been set.[16,25] Since the official analytical methods are based upon identification using MS or IR detection, and because these substances can also occur in the animals naturally, it is impossible to draw conclusions regarding abuse based on these results.[16] As advised by the European Union Reference Laboratory (EURL), analytical approaches based on gas chromatography coupled to combustion-isotope ratio mass spectrometry (GC-C-IRMS) can be used as a confirmation method to elucidate the origin of steroid hormones in samples from animal origin,[29] which will be discussed in detail in the following chapters. However, these techniques are not included into Commission decision 2002/657/EC. It has been underlined that this decision requires an update to allow a harmonized approach for IRMS-based methods, amongst other reasons.[30]

1.3. Screening strategies for endogenous sex steroid abuse

The preferred analytical methods for the detection of abuse of hormonal substances in cattle have changed regularly throughout the years, mainly as a consequence of instrumental improvements and availability, combined with evolutions in sample preparation. At the early stage in the 1960s and 70s, thin layer chromatography (TLC) was the standard method for the detection of group A substances. During the 1980s and 1990s, immunoassay techniques, such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), became popular and offered the means for control on a larger scale, but were later on largely replaced by the more definitive mass spectrometric-based techniques. Indeed, with the instruments becoming more robust and affordable, a shift towards GC-MS methods occurred during the 1980s. By the end of the 90s, LC-MS based methods became common good, evolving into ultra performance liquid chromatography (UPLC) more recently, used for screening methods including a large number of compounds. However, for

the confirmatory analysis after a non-compliant screening result for steroid hormones, GC-MS methods are still commonly applied.[17] Whereas a shift back towards GC-MS is observed in the field of human anti-doping, this trend is not visible for the detection of hormone abuse in cattle yet. As stated in the previous paragraph, traditional mass spectrometry does not allow differentiation between endogenous sex steroids and synthetic analogues thereof. However, it can still be a valuable tool to screen for suspicious samples.

1.3.1. Steroid concentrations

Establishing threshold concentration values for endogenous sex steroids in matrices of animal origin is a three phase process. First, results must be obtained from a non-treated population. Second, a statistical analysis of these 'normal' concentrations is required to propose a level above which the concentration of the substance can be considered as 'abnormal'. Finally, samples from treated animals are required to evaluate the determined threshold. The greatest difficulties in this process lie within the fact that a large control population is required to provide statistically significant results, and that an acceptable balance is required between false positive and false negative outcomes.[29] In the past, recommended threshold concentrations have been formulated by the Community Reference Laboratory (CRL) regarding 17β-testosterone in serum samples, and 17β-estradiol in serum and muscle samples, as described in table 1.2.[31]

Table 1.2: Recommended concentrations for non-compliance by the CRL

Substances	Matrix	Recommended concentration
17β-Testosterone	Serum	Male < 6 months: 10 ppb
		Male 6 - 18months: 30 ppb
		Female < 18 months: 0.5 ppb
17β-Estradiol	Serum	0.1 ppb
	Muscle	1 ppb

Although large scale population studies resulted in suggested thresholds levels for a number of endogenous steroids in bovine urine,[32,33] it became clear that the discriminating power of single steroid concentrations is limited. In sports doping

control, the relationship between the levels of multiple steroids, or steroid profiling, is used as a screening strategy for anabolic abuse, which started with the ratio of testosterone over epitestosterone (T/E), and eventually evolved into 'the athlete biological passport', a personalized set of biomarker levels obtained through longitudinal study of the individual athlete.[34] Unfortunately, a personalized approach such as 'the animal biological passport' is impossible due to the magnitude of the livestock population and the limited lifespan of the animals, and also the T/E ratio was proven to be an inadequate marker in bovines.[35] However, steroid profiling based measurements of multiple end products of the bovine phase II metabolism, which will be discussed in chapter 2, proved to be a promising approach for future application.[36-38] Steroid profiling is not exclusive to urine analysis: different matrices can be evaluated using GC-MS and LC-MS. This can be helpful in analysis where no urine is available, for example imported meat from countries allowing the use of natural steroids.[39]

Finally, metabolomic approaches to steroid urine profiling have been described.[40-42] This emerging field of 'omics' research focuses on large scale and high-throughput measurements, in an untargeted mode, of small molecules in biological matrices. Traditionally, nuclear magnetic resonance (NMR) spectrometry was the technique of preference for this type of research, but due to recent technological advances, high resolution mass spectrometry (HRMS) is rapidly gaining in popularity because of its sensitivity and capability in structural elucidation.[43] In the presented studies, urine samples from untreated and bovines treated with synthetic analogues of endogenous steroids, are analyzed with GC-MS,[40] or LC-HRMS,[41,42] after a minimal sample preparation. Afterwards, the generated urine profiles are statistically analyzed to reveal significant differences between the control group and the treated group. The presented methods allowed the successful differentiation between both groups, and can therefore be used as adequate screening techniques by themselves. Moreover, a large number of potential biomarkers, most likely steroid metabolites based on their mass spectra, were present. After identification, these might be implemented for steroid profiling purposes.[40-42]

1.3.2. Indirect screening approaches

The above described metabolomic approach is by nature untargeted, and therefore not necessarily linked to steroid concentrations. Although originally mostly applied for medicinal and pharmaceutical research, metabolomic research has been frequently adopted for the investigation of steroid hormone abuse over the past decade, using either NMR spectrometry,[44] or HRMS, for the untargeted analysis of urine,[41-45] and serum samples.[46] Additionally, holistic approaches to screen for steroid abuse are not limited to the metabolome, but have been extended to the proteome and transcriptome as well.[47-50] For the first, protein fingerprinting from liver and plasma samples was accomplished by combining two dimensional electrophoresis (2-DE) protein separation with mass spectroscopic and western blotting detection, and allowed to effectively differentiate between treated and untreated bovines.[47,48] For the second, untargeted gene expression analysis was performed using either complement DNA (cDNA) bioarray or RNA-sequencing techniques on various bovine tissue samples.[49,50] Besides successful differentiation between treated and untreated animals, the studies revealed over expressed genes which are not unique to bovines, indicating applicability of the technique for other species and sports doping control.

From the above, it is clear that administration of steroid hormones affects the levels of a large variety of non-steroidal molecules inside the treated animal significantly. These indirect biomarkers can be used to construct high-throughput analytical strategies, which are more suitable for screening large numbers of samples as opposed to the discussed untargeted approaches. For this reason, research on indirect biomarker approaches of all kinds (immunological parameters, blood chemistry parameters, peptides, proteins,…) to screen for steroid abuse gained a lot of interest over the past years, most of which was blood sample based.[43,51-55] However, it became clear that indirect biomarker screening faces similar challenges as steroid based screening: multi-biomarker profiling is required because of the limited discriminating power of a single compound threshold, and extended data from a control population and animals treated with various combinations of prohibited substances are needed to provide sufficiently validated methods capable of detecting varying hormonal abuse.[43] Therefore, the bridge between controlled experiments and application into the field remains largely uncrossed.

Finally, one of the oldest screening methods for hormone abuse is histological survey. It has been reported that administration of steroid hormones can induce macroscopic and microscopic changes in specific organs, mainly in genital tract organs and sex accessory glands, but also in the thymus, thyroid and liver.[15,56,57] Although it is hard to convert histological observations into a practical screening tool, this knowledge can be useful for official veterinarians performing control upon slaughter when selecting samples.

1.4. Outline of the current study

As described, a lot of interesting different approaches remain to be further explored to construct screening methods to detect abuse of synthetic analogues of endogenous steroid hormones in cattle. However, without adequate confirmatory techniques, screening methods serve no purpose. As for these confirmation methods, far less optional routes are available. Almost two decades ago, the capacity of GC-C-IRMS to differentiate between treated and untreated animals was illustrated. Steroid preparations were shown to have different $^{13}C/^{12}C$ ratios than steroids produced by the animals. Therefore, the correct measurement of this carbon isotope ratio of steroids in urine samples, using IRMS, allows to elucidate their endogenous or exogenous origin. However, its application into the field for real life control purposes remained extremely limited. The general aim of the current research was to provide fully validated IRMS-based methods for the detection of abuse of synthetic analogues of endogenous sex steroids, which could be implemented as such for official control purposes. This was accomplished in a four step approach.

First, an extensive literature research was performed, which is presented in **chapter II**. In this review, all relevant theoretical principles are provided, required to understand why it is possible to detect abuse of synthetic analogues of endogenous steroids based on $^{13}C/^{12}C$ ratio measurements, and which factors will influence the outcome of such an analysis. Afterwards, the required instrumental setup and necessary sample preparation techniques are discussed. Also, an overview of every published method regarding this application is provided. Finally, the link is made to doping control, where this type of analysis is more commonly applied, which proved to be a valuable source of information.

Second, based on the acquired knowledge from literature, a confirmation method to detect abuse of synthetic analogues of endogenous estrogens in cattle using gas chromatography coupled to both mass spectrometry and combustion-isotope ratio mass spectrometry in parallel (abbreviated as GC-(MS/C-IRMS) in this document) was developed, which is described in **chapter III**. Special attention was given to the method validation, and to the impact of the parallel coupled MS in the setup, which had not previously been described for the analysis of steroid hormones.

Third, the developed method was extended to include the detection of abuse of synthetic analogues of androgens, as described in **chapter IV**. After thorough validation, the detection method was tested on two bovines treated with esters of 17β-estradiol and 17β-testosterone. Finally, the detailed information from this administration study allowed a direct comparison with alternative confirmatory approaches.

Fourth, the method was adapted for the detection of synthetic analogues of the third group of sex steroid hormones, progestagens, which is described in **chapter V**. After selection of suitable target analytes for this analysis, a number of slightly different analytical approaches were evaluated against each other. The most promising approach was validated and successfully tried on samples from bovines treated with progesterone.

Finally, chapter VI provides the general discussion of the accomplishments, the global conclusions and suggestions for future research.

2. Use of isotope ratio mass spectrometry to differentiate between endogenous and synthetic homologues in cattle: a review

2.1. Abstract

Although substantial technical advances have been achieved during the past decades to extend and facilitate the analysis of growth promoters in cattle, the detection of abuse of synthetic analogs of naturally occurring hormones has remained a challenging issue. When it became clear that the exogenous origin of steroid hormones could be traced based on the $^{13}C/^{12}C$ isotope ratio of the substances, GC-C-IRMS has been successfully implemented to this aim since the end of the past century. However, due to the costly character of the instrumental setup, the susceptibility of the equipment to break-down and the complex and time consuming sample preparation, this method is up until now only applied by a limited number of laboratories. In this review, the general principles as well as the practical application of GC-C-IRMS to differentiate between endogenous steroids and exogenously synthesized homologous compounds in cattle will be discussed in detail, and will be placed next to other existing and to be developed methods based on isotope ratio mass spectrometry. Finally, the link will be made with the field of sports doping, where GC-C-IRMS has been established within the World Anti-Doping Agency (WADA) approved methods as the official technique to differentiate between exogenous and endogenous steroids over the past few years.

2.2. Introduction

Since the 1930s, the positive effects of testosterone on muscle building have been well-known. Throughout the years, growth-promoting qualities of a great variety of hormonal compounds were discovered. Anabolic steroids were introduced into the world of sports during the 1940s and 1950s, which led to widespread abuse by the time detection methods became more available in the early 1970s.[58,59] By that time, the use of steroid hormones and thyreostatics had become common practice in stock farming.[60] However, it became clear that next to the beneficial effects of steroid hormones, a great number of adverse effects could be attributed to these substances, in particular disruption of the reproductive system.[61,62] In 1981, the European Commission issued a first directive prohibiting the use of a number of hormonal substances, namely stilbenes (e.g. diethylstilbestrol (DES)) and thyreostatics, in stock farming.[63] After careful consideration, the European Commission later expanded its decision, resulting in the current legislation which bans the use of all hormonal substances for growth promoting reasons.[21,22] As other countries (e.g. USA, Canada, New-Zealand, Australia), allow the use of synthetic forms of six natural steroid hormones (estradiol, progesterone, testosterone, melengestrol acetate, trenbolone acetate and zeranol) as growth promoters, the use of these substances is fiercely controversial.

Over the past decade, great advances have been achieved in residue detection with gas and liquid chromatography coupled to mass spectrometry, which allows the accurate and simultaneous detection of small quantities of a large number of prohibited substances in different matrices of animal origin.[64,65] But the discordance between the decreasing number of positive results in samples of animal origin and the analysis of confiscated illegal preparations containing synthetic homologous compounds of natural steroid hormones reveals the shortcomings of the used analytical techniques when it comes to the detection of abuse of this type of substances.[16] More specifically, the classical mass spectrometric techniques are unable to make the distinction between endogenous (produced by the animal itself) and exogenous (administered) or synthetic forms of natural steroid hormones.

It was clarified during the 1990s that a differentiation between endogenous natural anabolic steroids and exogenous homologues could be made by measurement of $^{13}C/^{12}C$ isotope ratios of these substances, both in humans and bovines.[66,67] At this

point, gas chromatography linked to combustion-isotope ratio mass spectrometry (GC-C-IRMS) emerged as a promising technique to demonstrate the abuse of exogenous homologues of natural steroid hormones in cattle.

The objective of this review is threefold. First, the underlying principles of the isotopic difference between endogenous and exogenous steroids will be explained, and how this can result in an applicable parameter. Next, the practical implementation of GC-C-IRMS as an analytical technique will be reviewed, focusing on the sample preparation, which is the bottleneck of GC-C-IRMS analysis. Finally, the use of GC-C-IRMS to differentiate between exogenous and endogenous steroids in cattle will be positioned in the broader picture of analysis of natural steroid hormones, linking it to other techniques and the other field of application, which is sports doping control.

2.3. Underlying principles of $\Delta^{13}C$ values

To understand the difference between endogenous and exogenous homologues or synthetic forms of natural steroids, the difference between steroids biosynthesized in the animal or synthesized in laboratory/industrial conditions needs to be clarified. Since androgens and estrogens are the steroid hormones most commonly used as growth promoters, the synthesis of these compounds will be explained, given its relevance for IRMS.

2.3.1. Bovine steroid biosynthesis

In mammals, all steroid hormones are derived from cholesterol. Cholesterol can be taken up from the feed directly, or can be biosynthesized from acetyl co-enzyme A. By a multi-step pathway, cholesterol is converted into steroid hormones, amongst which androgens and estrogens, as displayed in scheme 2.1. All reactions involved in the biosynthesis of steroid hormones are enzyme-regulated and most of them are reversible. The enzymes are listed in table 2.1. The first reaction in steroid anabolism is the cleavage of the side chain from cholesterol, thus forming pregnenolone.

During androgen biosynthesis, two pathways can be followed, starting from pregnenolone. They are referred to as the 4-ene and the 5-ene pathway. In the 4-ene pathway, the intermediates are characterized by a double bond between the carbons

at position 4 and 5, whereas the 5-ene pathway intermediates have a double bond between the carbons at position 5 and 6.

In meat producing animals, 5-ene precursors of testosterone are more likely to be found than 4-ene precursors, with an exception for cervid species.[68]
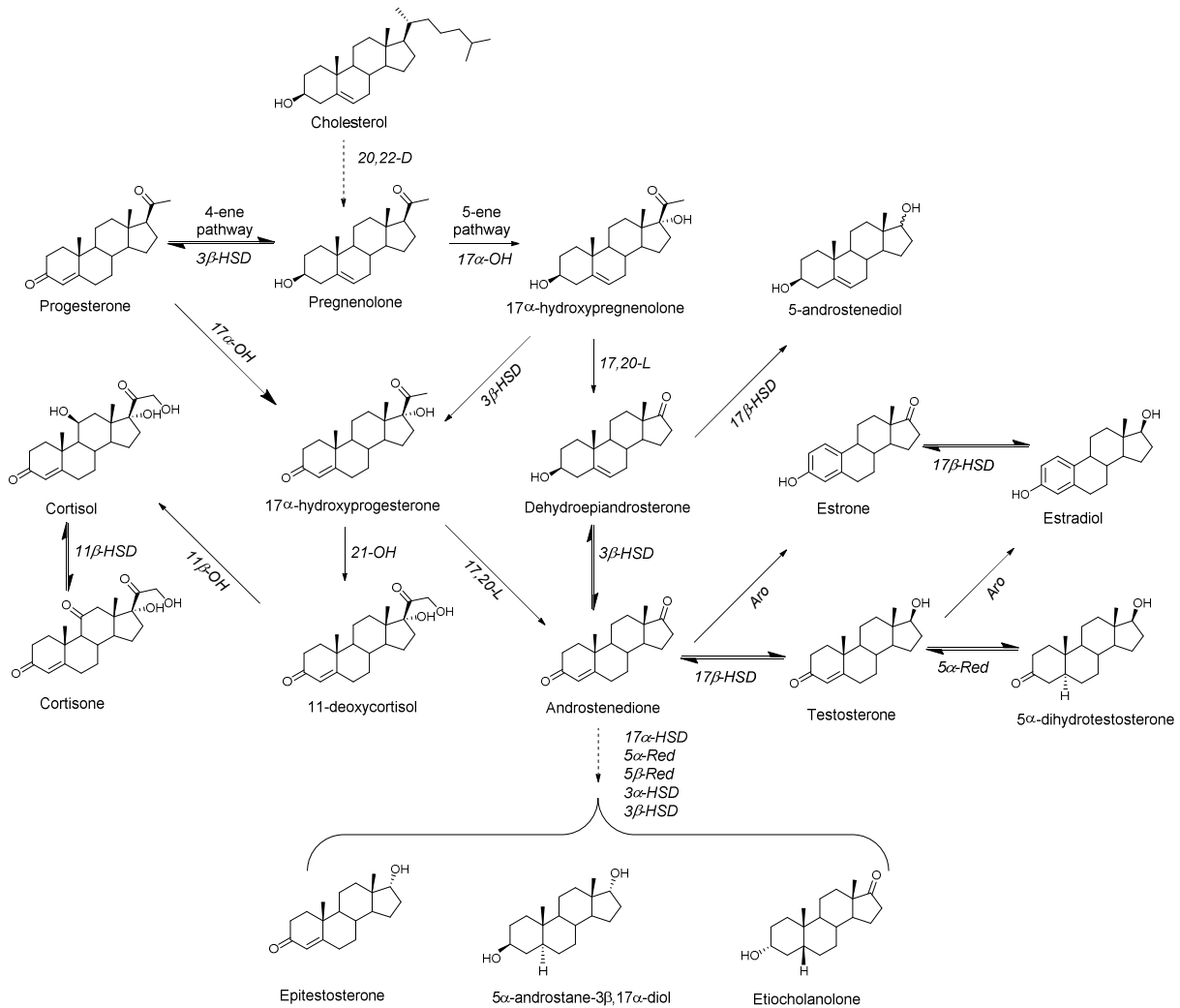
Scheme 2.1: Representation of the biosynthetic pathways of endogenous steroids in bovines.[36,68,69] The dotted arrow indicates a multistep reaction involving different enzymes. Enzymes are printed in italic, the abbreviations are explained in table 2.1

In the body, the adrenal cortex produces large amounts of dehydroepiandrosterone (DHEA). This excess DHEA is conjugated to a sulphate group, forming dehydroepiandrosterone sulphate (DHEA-S). The sulpho-conjugated form of DHEA is significantly less bioactive and so, DHEA-S can be considered as the body's steroid reserve. When unconjugated, DHEA is transformed into the more active 4-

androstenedione, which could in turn lead to the formation of testosterone after reduction of the 17-keto group.[70,71] Estrogens are the products of the aromatization of androgens. One important route of estrogen production is the transformation of androstenedione (AED) into estrone (E1) and by further reduction of the 17-keto group to 17β-estradiol which is amongst the most bioactive compounds of the C18-group.

Table 2.1: Overview of the enzymes regulating the steroid pathways[70]

Abbreviation	Enzyme
20,22-D	20,22-Desmolase
17α-OH	17α-Hydroxylase
3β-HSD	3β-Hydroxysteroid dehydrogenase,$\Delta^{5,4}$ isomerase
17,20-L	$C_{17,20}$-Lyase
17β-HSD	17β-Hydroxysteroid dehydrogenase
17α-HSD	17α-Hydroxysteroid dehydrogenase
3α -HSD	3α-Hydroxysteroid dehydrogenase
Aro	Aromatase
5β-Red	5β-Reductase
5α-Red	5α-Reductase
21-OH	21-Hydroxylase
11β-OH	11β-Hydroxylase
11β-HSD	11β-Hydroxysteroid dehydrogenase

For most of them, steroid hormones circulate in the blood stream under their free form, with the exception of estrone sulphate and the above mentioned DHEA sulphate. High levels of steroids (after an administration for instance) suppress the release of the corresponding releasing hormone (e.g. GnRH), providing negative-feedback control of hormone levels. The levels of the different steroid hormones in serum and plasma are highly dependent upon the age and gender of the animals. In plasma from untreated bulls, measured concentrations of 17β-testosterone ranging from below the detection limit up to almost 6 ng mL^{-1} were reported,[72] whereas the level was approximately tenfold lower in mature females.[68] The reported 17β-estradiol levels are significantly lower, ranging from below the detection limit up to approximately 50 pg mL^{-1} in plasma from untreated bulls, and peaking at approximately 80 pg mL^{-1} in serum of untreated heifers.[73] In younger animals (< six

months), the presence of steroids is generally lower, resulting in different compliance thresholds for the presence of 17β-testosterone and 17β-estradiol in serum, as suggested by the Community Reference Laboratory, given in table 1.2.

It was reported that plasma levels of estrogens and gestagens are highly variable in heifers throughout the estrus cycle.[74,75] Progesterone concentrations raised gradually up to approximately 10 ng mL^{-1} as the corpus luteum developed, whereas the concentration of 17β-estradiol was more variable, peaking approximately one day before estrus. 17β-Testosterone concentrations appeared to be unchanged during the estrus cycle.[76]

After further metabolisation, steroid metabolites are excreted in urine mainly conjugated. This results in the presence of both sulpho- and glucuro-conjugated phase II steroid metabolites in urine and feces. The latter glucuro-conjugated steroids are the products of conjugation to glucuronic acid. Average concentrations of 17β-testosterone and 17β-estradiol of 3.5 ng mL^{-1} and 0.21 ng mL^{-1}, respectively, were reported in urine of male animals. For female animals, this was 0.58 ng mL^{-1} and 0.63 ng mL^{-1}, respectively.[37] Concentrations in younger animals (< seven months) were significantly lower.[77] However, the 17β-form of steroid hormones is usually metabolized to less active 17α-isomers, which will therefore be more abundant in urine. Indeed, in the same study, the reported average concentrations of 17α-testosterone were 8.13 ng mL^{-1} and 14.30 ng mL^{-1}, and of 17α-estradiol 79.07 ng mL^{-1} and 5.54 ng mL^{-1}, for heifers and bulls, respectively. However, average concentrations of 17α-estradiol, reported from a population study in the United Kingdom, were tenfold lower.[32]

Regarding progesterone, most of the measured urine samples were below the detection limit for both male and female animals.[77]

Although the biosynthesis of the more active steroid hormones is similar in all mammalian species, important differences in the catabolic end products of the steroid metabolism between species are known.[68] Also between bovines and humans, the steroid profile found in urine differs significantly. This results in a number of characteristic changes in steroid analysis of bovine urine compared to human urine, which will be discussed later in the text.

2.3.2. Semisynthesis of steroid hormones

Pharmaceutical companies mostly use semisynthetic methods for steroids, starting from sterols present in plant material, also called phytosterols. Soy is most frequently used as source of plant sterols, next to *Dioscorea* species, succulent plants also known as yams.[78,79] Commonly used phytosterols are stigmasterol and diosgenin, originating from soy and succulent plants, respectively.[80] Through a one step microbial degradation, androsta-1,4-diene-3,17-dione (ADD) is formed from soy sterols, from which conversion to other steroid hormones is possible.[81] Semisynthetic methods are the most profitable and by far the most commonly used, as the source material is available in large quantities and the production process is fairly easy. However, semisynthesis is not the only available process for the production of steroid preparations. Total synthesis of steroids will be discussed in section 2.4.2.

2.3.3. Origin of isotopic difference, C_3 and C_4 plants

$^{13}C/^{12}C$ Ratios of exogenous steroid hormones are significantly different from those of endogenous steroids. The reason for this difference lies within the origin of the molecule. As discussed above, steroid hormone preparations are produced from phytosterols available from plant material. Plants use atmospheric carbon under the form of carbon dioxide (CO_2) for the biosynthesis of their compounds, amongst which the plant sterols. The integration of CO_2 is achieved through photosynthesis. There exist three types of photosynthetic pathways.

C_3 Plants incorporate CO_2 by using a natural process which is named the Calvin cycle. In this pathway, CO_2 is incorporated by being attached onto a five carbon counting sugar molecule which is subsequently split, leading to the formation of intermediates composed of three carbon atoms. This cycle is regulated by a number of enzymes. The enzyme ribulose-1,5-diphosphate carboxylase is responsible for the fixation of CO_2. Since this enzyme has a greater binding capacity for $^{12}CO_2$ over $^{13}CO_2$, it discriminates against incorporation of the heavy carbon isotope. This way, plant material of C_3 plants becomes enriched in ^{12}C. Typical C_3 plants are wheat and soy.

The incorporation of CO_2 by C_4 plants is done by a process called the Hatch-Slack-cycle. In this pathway, CO_2 is attached to phosphoenolpyruvate leading to intermediates composed of four carbon atoms. The enzyme responsible for the

fixation of CO_2 is phosphoenolpyruvate carboxylase, which incorporates $^{13}CO_2$ and $^{12}CO_2$ without substantial preference. The actual substrate for the enzyme is not CO_2, but hydrogen carbonate (HCO_3^-) formed through dissolution of CO_2 in a slightly basic medium. Thus, in this pathway, the incorporation of CO_2 is mainly limited by its diffusion into the leaf and its dissolution in water. This process has a far less pronounced effect on the $^{13}C/^{12}C$ ratio compared to the pathway involving ribulose-1,5-diphosphate carboxylase. This results in a lower discrimination against the heavy carbon isotope, leading to organic compounds with a higher $^{13}C/^{12}C$ ratio in comparison to C_3 plants. Maize is a typical C_4 plant.

A third type of plants uses the Crassulacean Acid Metabolism (CAM). This mechanism uses a combination of both the Calvin- and the Hatch-Slack-cycle, resulting in plant material with a $^{13}C/^{12}C$ ratio which is usually, but not always, higher than those of C_3 plants. The pineapple is a typical CAM plant.[82]

The plants which are used for the production of exogenous steroid hormones are typically C_3 plants, while food and feed, out of which endogenous steroids are produced, typically are composed of a mixture of C_3 and C_4 plants. Since the exogenous steroids are formed out of a single carbon source, being a C_3 plant, it can be understood that the exogenous steroids will be more enriched in ^{12}C than endogenous steroids.[83,84]

After administration of exogenously produced gonadic steroids, the measured $^{13}C/^{12}C$ isotope ratio of the corresponding steroid metabolites will be the consequence of the $^{13}C/^{12}C$ values of both the endogenously produced and the administered steroids, which is represented in scheme 2.2. As mentioned before, the intake of exogenous steroids has a negative effect on the endogenous steroid production. Since the exogenously produced steroids are enriched in ^{12}C, compared to endogenously produced homologous compounds, and because of hindered endogenous production, the measured $^{13}C/^{12}C$ ratio will be significantly lower in case of administration of exogenous steroids compared to when no steroids were administered and the $^{13}C/^{12}C$ isotope ratio of the metabolite is solely dependent upon the endogenous production. This forms the basic principle to differentiate between endogenous steroids and exogenously synthesized homologous compounds using IRMS. However, despite the negative feedback mechanism, significant endogenous

production can still occur after administration. The higher the endogenous production of the steroid hormone, the less pronounced the effect of the administration will be on the measured $^{13}C/^{12}C$ isotope ratio. This process, also referred to as endogenous dilution, can be a seriously limiting factor for the detectability of treatment with synthetic homologues of endogenous steroids.
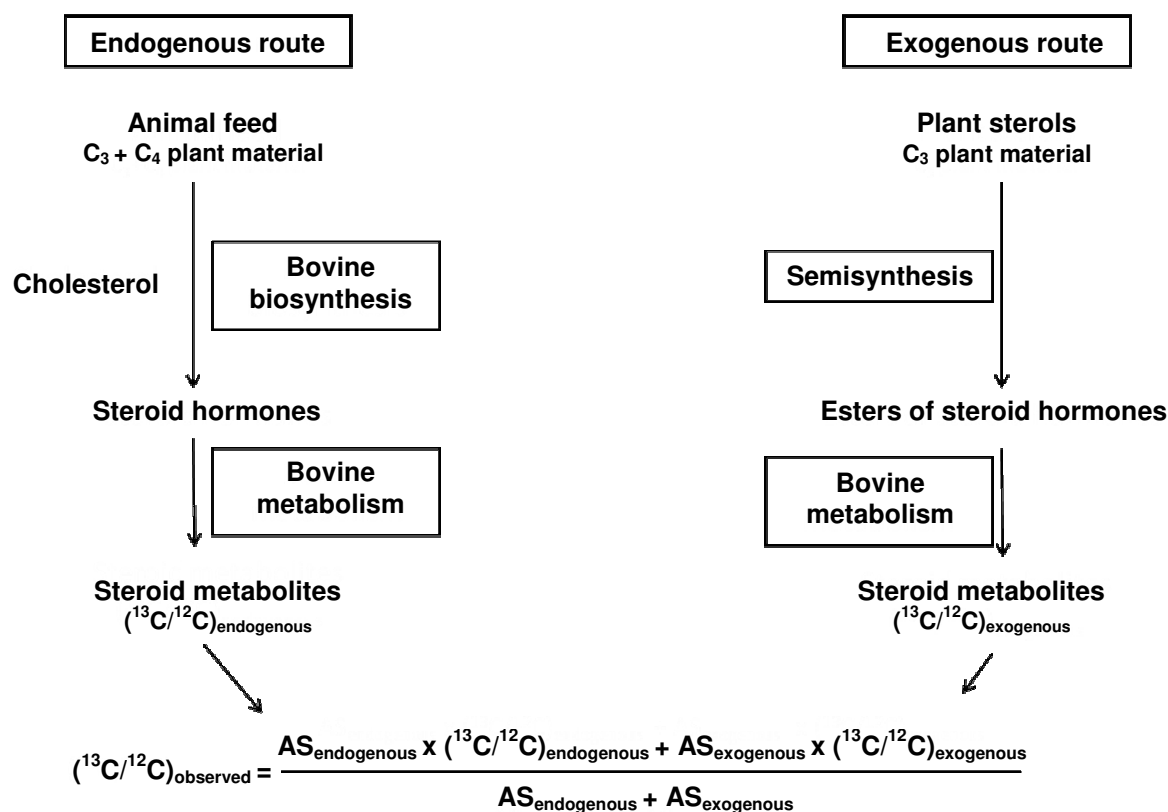
Scheme 2.2: Representation of the observed carbon isotope ratios and the relation to endogenous steroids and exogenous homologues. AS stands for "Amount of the steroid"

2.3.4. δ^{13}C values

2.3.4.1. Definition

The $^{13}C/^{12}C$ (= R) isotope ratios are expressed in regard to a reference standard as δ^{13}C values per mille, according to the following formula:

$$\delta^{13}C[‰] = (((^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{std})/ (^{13}C/^{12}C)_{std}) \times 10^3 = ((R_{sample} - R_{std})/R_{std}) \times 10^3$$

The reference standard for carbon is a calcium carbonate,[85] also referred to as Pee Dee Belemnite (PDB). The PDB formations are skeletal remains of mollusks that are

deposited in South Carolina (USA), and that have an R value or R_{ref} of 0.011237199.[86] Since stocks of PDB are not available anymore, a new standard, called Vienna Pee Dee Belemnite (VPDB), was suggested, having a $\delta^{13}C$ value of +1.95‰ relative to PDB.[82] In the meantime VPDB is generally accepted as a standard, and as such, the currently used formula expressing $\delta^{13}C$ values in promille with respect to VPDB becomes:

$$\delta^{13}C_{VPDB} = ((R_{sample} - R_{VPDB})/R_{VPDB})) \times 10^3 = (R_{Sample}/R_{VPDB} - 1) \times 10^3$$

2.3.4.2. Factors influencing exogenous $\delta^{13}C$ values

The possible methodologies to differentiate between endogenous natural steroids and exogenously synthesized preparations of homologous compounds are based on the source material of the preparations, a single C_3 plant source, which results in substances relatively enriched in ^{12}C in comparison to their endogenously produced counterparts. This hypothesis is supported by results of analysis of preparations and bulk materials in which $\delta^{13}C$ values ranging from -25.9 ‰ down to -32.8 ‰ were measured.[67,87] However, in a more recent study on testosterone products, 9% of the analysed preparations possessed $\delta^{13}C$ values smaller than -25.9 ‰, which are within the endogenous range.[88] All of these samples were illegal steroid preparations, confiscated in 2009 at border-level seizures. These results suggest that another source material for the production of steroid preparations might be available.

Although, at the moment, semisynthesis from plant material is the production method of choice, it is not the only possibility. In 1959, the total enantioselective synthesis of steroids was first brought to industrial production. The reaction of 6-methoxy-1-tetralone with vinylmagnesium bromide and 2-methylcyclopentane-1,3-dione to form an intermediate with a sterane core formed the basic structure of all steroid molecules, which eventually led to the production of estrone.[89] The reaction was referred to as the Torgov reaction, after its discoverer. The Torgov reaction was responsible for large-scale industrial production of steroid hormones in the USSR. and Germany, and still provides the foundation of the reaction currently used for the production of pharmaceutical steroid hormones at factories of Schering in Berlin.[90] Unfortunately, no information on the carbon isotopic content of these substances is available to our knowledge, but this process might be responsible for the deviating $\delta^{13}C$ value of -22.7 ‰ measured in an old preparation of boldenone.[91]

Therefore, continuous attention must be paid to the influence of production methods on carbon isotope ratios of preparations. Rapidly evolving knowledge on the biosynthesis of cholesterols and other sterols in prokaryotic and eukaryotic organisms might lead to the availability of new sources for steroid hormones.[92]

2.3.4.3. Factors influencing endogenous $\delta^{13}C$ values

When evaluating the $\delta^{13}C$ values of endogenous steroid hormones in different animals and humans, significant individual differences are observed. Factors known to cause these differences are described below.

The most important cause, both for humans and bovines,[84,93,94] is the diet, in correspondence with the principle "you are what you eat". The larger the portion of C_4 plants in the diet, the more the endogenous steroids will be enriched in ^{13}C. Both studies on bovines provided $\delta^{13}C$ values of the urinary steroids within a range of -19‰ to -23‰ in case of a C_4 plant diet, and -24‰ to -32‰ in case of a C_3 plant diet. For humans, regional differences in $\delta^{13}C$ values are observed as well, but these can be explained by the corresponding differences in diet.[95,96] Logically, the range of the $\delta^{13}C$ values of the endogenous steroids in case of a C_3 plant diet, and of the steroidal preparations is largely the same. This poses a critical problem, since it makes distinction between endogenous steroids and administered synthetic homologues extremely difficult.

Neither the breed of animal, nor the age, was found to have an influence on the endogenous $\delta^{13}C$ values. However, it was noticed that the age does play a role in the detection of steroid abuse. Younger animals have a lower endogenous production of steroid hormones than older ones. When preparations are administered, the measured $\delta^{13}C$ values are determined by both the $\delta^{13}C$ values of the exogenous and endogenous steroids and in relation to the amount in which they are present. Thus, when the endogenous production is low, exogenous steroids make up a larger portion of the total steroid content, and their influence on the measured $\delta^{13}C$ values will be more pronounced. This facilitates detection of abuse.[94]

In humans, a difference in $\delta^{13}C$ values between males and females in an experimental group was observed, with the steroid hormones in females being more enriched in ^{12}C. This was probably caused by the extended use of oral contraceptives, which are in fact exogenous steroid hormones, in the females subgroup.[95]

Since a number of bacteria are capable of the bioconversion of sterols and steroids,[97] concerns have been raised about phytosterols, present in animal feed, which could be converted into steroids in the intestinal track. The direct absorption of these steroids might have a direct influence on the measured $\delta^{13}C$ values.[86,91] However, until now there is no evidence of such an event altering the measured values. Additionally, microbial contamination after sampling can impact the measured $\delta^{13}C$ values, which is discussed in detail in paragraph 2.4.2.1.

2.3.5. Endogenous reference compounds and $\Delta^{13}C$ values

Due to the mentioned variability in individual $\delta^{13}C$ values, it is hard to set a threshold $\delta^{13}C$ value for the differentiation between endogenous steroids and synthetic derivatives. This problem is solved by measuring $\delta^{13}C$ values of both metabolites and precursors of steroid hormones in urine. The $\delta^{13}C$ values of the chosen precursor molecules should not be influenced by the administered exogenous compound. These components are called Endogenous Reference Compounds (ERCs). In theory, the $\delta^{13}C$ value of the ERC is not significantly different from the $\delta^{13}C$ value of endogenous steroids.[98] The difference between the $\delta^{13}C$ values of the ERC and the metabolite is defined as the $\Delta^{13}C$ value. The $\Delta^{13}C$ value is an unambiguous parameter for the presence of exogenous steroids. When no steroids are administered, $\delta^{13}C$ values of metabolites and precursors will be close to each other, which results in a low $\Delta^{13}C$ value. When synthetic steroid hormones are administered, $\Delta^{13}C$ values will be higher. This difference is represented in figure 2.1. In the past, for sports doping control, a value of more than 3‰ was considered as the demonstration of the presence of exogenous steroids.[99] For cattle, however, an official $\Delta^{13}C$ threshold value has not been designated. Still, research data suggests that the 3‰ threshold value could also be implemented for bovine urine samples.[100,101] $\Delta^{13}C$ values of an untreated reference population do not exceed a value of 2‰, and measured $\Delta^{13}C$ values of positive samples significantly exceed values of 10‰. However, it needs to be noted that a universal 3‰ compliance threshold is no longer applied by the WADA. More details on recent changes in the WADA technical documents are provided in paragraph 2.4.3.
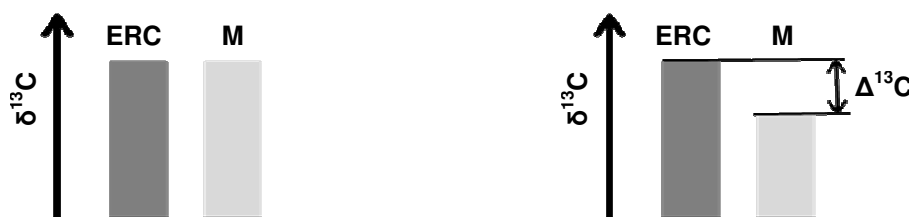
Figure 2.1: Visualization of $\Delta^{13}C$ value and the effect of (a) non-administration, (b) administration of exogenous steroid hormones. ERC stands for Endogenous Reference Compound, M for metabolite

Sufficient knowledge of the metabolic pathways of steroid hormones, described in section 2.3.1, is necessary for the adequate selection of ERCs, as well as the information on their natural abundance. DHEA and androst-5-ene-3β,17α-diol are tested and approved as ERCs for the detection of estradiol and testosterone abuse in cattle. The most often used testosterone metabolites in bovine species are etiocholanolone, epitestosterone and 5α-androstane-3β,17α-diol.[100] 17α-Estradiol is the target metabolite for 17β-estradiol (and esters) administration.[101]

Even though there is no direct evidence, it is suspected that DHEA might be used as a growth promoter in cattle.[102] In this case, an additional ERC to the detection of DHEA abuse has to be selected. Because a number of steps in the steroid pathways are hypothetical and not always fully understood,[103] it is important to verify always adequacy of an ERC – metabolite combination with analytical data of an administration study.

One method on human urine was published in literature, in which no ERC was used.[104] The aim was to simplify the analysis to provide a rapid screening method, in which $\delta^{13}C$ values of approximately -28‰ and lower are considered suspicious.

After the residues of the administered compounds are excreted from the body, or in case of significant endogenous dilution, as discussed in paragraph 2.3.3, the detection of the administration will no longer be possible. The detectability, as well as the time frame for detection, are largely dependent upon the exact nature and quantity of the administered substance, as well as the method of administration. Data on this subject in bovines are relatively scarce. However, the detectability of a single intramuscular administration of 250 mg of testosterone enanthate was evaluated.[67] By means of the simultaneous analysis of two different testosterone metabolites, epitestosterone and etiocholanolone, the administration could be detected up to three

weeks after injection. Treatment of bovines with a single intramuscular injection of estradial valerate, dosed at 0.05 mg/kg, was detectable up to two weeks after administration.[101] However, information is lacking in this study to further evaluate in detail the evolution of the $\Delta^{13}C$ values in time, and no urine samples taken later than two weeks after administration were analysed.

Finally, it needs to be stressed that, although exceeding a $\Delta^{13}C$ based threshold is an unambiguous confirmation of non-compliance, this is not necessarily true the other way around. As the portion of C_4 plant material in the feed decreases, the $\delta^{13}C$ value of the ERCs decreases as well. When C_4 plant material is completely removed from the feeding regime, the $\delta^{13}C$ values of both the ERCs and metabolites will be close together even in case of treatment, thus severely compromising the discriminating power of the method and enlarging the possibility of false negative results.

2.4. Analytical implementation of GC-C-IRMS for the differentiation between endogenous steroid hormones and synthetic homologues

Although the theory is straightforward, the practical application of carbon isotope measurement of steroids in bovine urine is a demanding task. In this chapter, first the instrumental setup for carbon isotope ratio measurement will be presented. Next, sample preparation, which is the main bottleneck of the analysis, will be discussed. Finally, a number of quality control measures will be listed.

2.4.1. IRMS setup for measurement of steroid hormones, GC-C-IRMS

In the instrumental development, two major breakthroughs were necessary to provide a system allowing the carbon isotopic measurement of steroids in samples. The first was the direct coupling of a gas chromatograph (GC) to a combustion furnace.[105,106] The second was the development of a multicollector detection system, allowing the simultaneous detection of the same compound bearing different isotopes. The coupling of a multicollector isotope ratio mass spectrometer (IRMS) to a GC via an online combustion system allowed the continuous measurement of carbon isotope

ratios, which was first accomplished by Barrie et al. using a dual collector type IRMS.[107]

After preparation, the sample first enters this system by injection onto the GC. The function of the GC is to obtain separation of analytes from each other and from matrix impurities. The analytes need to be free from any co-elutions, since they can alter the $\delta^{13}C$ values. Next, the output of the GC is directly linked to a combustion oven, since the IRMS measures the isotopic content in the form of CO_2 gas. In the combustion oven, the steroids are converted to CO_2 and H_2O. It is essential that all of the analyte is converted and sent to the IRMS, as incomplete combustion can result in incorrect measurements. To ensure complete combustion, the reaction is performed at very high temperatures, near 1000 °C. Then, the combustion oven is linked to a water trap, to ensure that only carbon dioxide continues to the IRMS. When water enters the IRMS, it could result in the formation of HCO_2^+ by protonation of carbon dioxide, and thus interfere with the isotopic measurement. This water trap can be a membrane based filter with a Nafion membrane or a cryogenic trap.[70] After the water trap, the CO_2 gas is directed to the IRMS through the gas inlet, into the ion source. In the ion source, $CO_2^{+\bullet}$ ions are generated in a high vacuum by the impact of electrons. Finally, these ions are transmitted to the detector.

The IRMS of nowadays is a magnetic sector instrument, which contains three Faraday cups as detector. This will allow the simultaneous measurements of three types of $CO_2^{+\bullet}$ ions, with m/z 44, 45 and 46. There is only one type of ion present with m/z 44, $^{12}C^{16}O_2^{+\bullet}$. Corresponding to m/z 45, three different ion isotopomers will be collected: $^{13}C^{16}O^{16}O^{+\bullet}$, $^{12}C^{17}O^{16}O^{+\bullet}$ and $^{12}C^{16}O^{17}O^{+\bullet}$. In the third Faraday cup, five isotopomers with m/z 46 will be collected: $^{12}C^{18}O^{16}O^{+\bullet}$, $^{12}C^{16}O^{18}O^{+\bullet}$, $^{12}C^{17}O^{17}O^{+\bullet}$, $^{13}C^{17}O^{16}O^{+\bullet}$ and $^{13}C^{16}O^{17}O^{+\bullet}$. According to the relative abundance, the ^{17}O isotopomers at m/z 46 are considered negligible. Thus, the signal at the Faraday cup for m/z 46 measures $^{12}C^{18}O^{16}O^{+\bullet}$ and $^{12}C^{16}O^{18}O^{+\bullet}$. Next, the ^{18}O abundance, determined from the ratio of m/z 46/44, combined with an assumed relationship between the ^{17}O and ^{18}O isotopic abundances, allows to calculate the contribution of $^{12}C^{17}O^{16}O^{+\bullet}$ and $^{12}C^{16}O^{17}O^{+\bullet}$ to the signal at m/z 45.[98] This procedure is referred to as the ^{17}O correction. The first to apply an ^{17}O correction was Craig, using the following formula for the relation between the quantities of the different oxygen isomers: $^{17}O/^{16}O = K(^{18}O/^{16}O)^a$ with a = 0.516, and K = 0.0099235.[108] Nowadays,

alternative [17]O corrections are available, leading to slightly different results over a range of less than 0.1‰.[109]

Since the steroids are completely converted into CO_2 and H_2O during combustion, GC-C-IRMS provides no information on the identity of the measured compound. Therefore, an additional GC-MS analysis is in order. However, it has been argued that this is an insufficient measure to assure identification of the combusted compound.[110] A setup has been developed, in which the sample is split after GC and send to MS and C-IRMS simultaneously, which allows identification of the analyte and measurement of its $\delta^{13}C$ values at the same time (figure 2.2).[111,112] There is currently no official abbreviation to describe this setup, and therefore, an unofficial abbreviation, GC-(MS/C-IRMS), will be used in this document to describe this coupling.
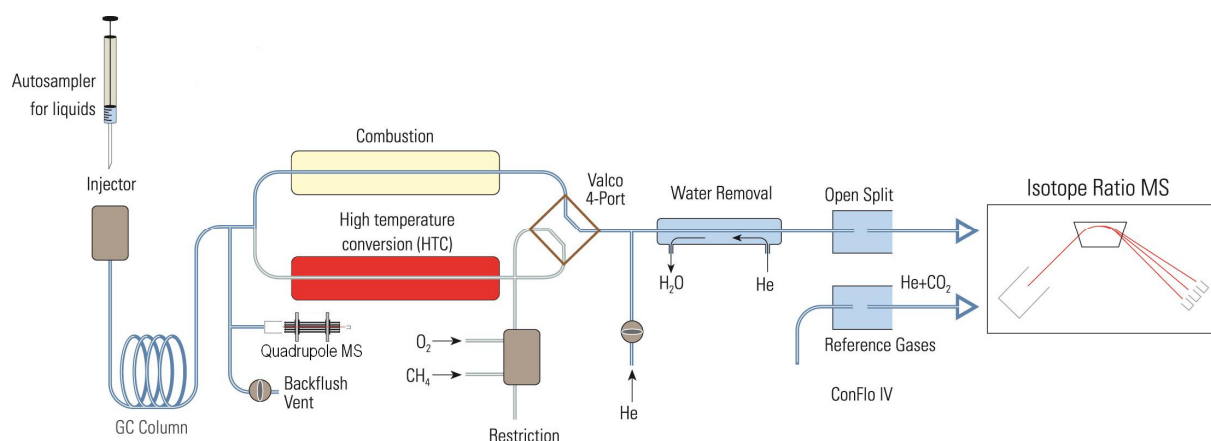
Figure 2.2: Schematic picture of GC-(MS/C-IRMS) instrumental setup (©Thermo Fisher Scientific)

2.4.2. Sample preparation

Nowadays, the greatest difficulty in isotope analysis is to obtain purified samples with few interferences. The analyte peaks in GC should be free of any co-elution.

In this chapter, the different techniques used in the cleanup procedure prior to GC-C-IRMS will be overviewed and discussed. Next, the published strategies employed for the analysis of bovine urine will be presented.

2.4.2.1. Evaluation of employed preparation techniques

A number of analytical techniques are used in every method for steroid sample preparation prior to GC-C-IRMS. These techniques are in analogy with the methods used in sample preparation for regular GC-MS. However, it is of utmost importance that the $\delta^{13}C$ values of the analytes are not influenced by a natural phenomenon due to the mode of sample preparation, which can sometimes lead to kinetic or isotopic fractionation.

Samples

The first part of every analysis is sampling. Low concentration of target analytes in urine and successive purification steps require large sample volumes, sized up to 50 mL. Only one method using another matrix, namely bile, is described in the literature.[113]

Proper storage of urine samples is required. When urine samples are not stored properly, chemical, enzymatic and microbial degradations take place. In human urine, it has been observed that microbial contamination will induce hydrolysis of glucuronide and sulphate conjugates, followed by modifications of the steroid structure by oxidoreduction.[114,115] It has been proved that long term improper storage of human urine samples led to changing $\delta^{13}C$ values.[116] To this aim, samples were stored at room temperature for several weeks. Then, the samples were inoculated with old urine samples which already showed severe symptoms of degradation. Prior to analysis, the samples were once again stored at elevated temperatures for a week. Afterwards, significant changes in $\delta^{13}C$ values were observed. These changes were mainly noticed in free and sulphoconjugated steroids. Therefore, it is advised to use glucuroconjugated steroids for isotopic measurement when samples show signs of degradation.[116] The reasons for the fractionation could not be identified, and since proper storage is a key issue, further study on this topic is needed. Cooled storage of the urine samples was adviced, however, more detailed information on optimal preservation of the samples was not reported.

Hydrolysis

Only a small fraction of the steroids is eliminated unconjugated in urine. The majority is either glucuroconjugated or sulphoconjugated and requires deconjugation during the analysis.

Deconjugation of the glucuroconjugates can be done enzymatically, and is often performed using β-glucuronidase from *E. coli* in a phosphate buffer, maintaining a stable pH. Enzymes from *Helix pomatia*,[67,117] and Abalone entrails,[94] can be used as well to perform this hydrolysis, but this can degrade to some extent the steroid, leading to a different structure. Results with a higher reproducibility are observed with *E. coli* glucuronidase.[118] Also, the use of β-glucuronidase from *P. canaliculata* was reported for the hydrolysis of steroids in horse urine.[119]

Glucuronidase from *Helix pomatia* is also capable of hydrolysing sulphoconjugated steroids. However, this capacity is limited and most frequently, a chemical approach is used to this aim. The cleavage of the sulphate by chemical solvolysis is usually obtained by the addition of concentrated sulphuric acid in ethyl acetate, both for bovine and human samples.[84,101,120] No isotope fractionation during hydrolysis has been reported so far.

Contrary to the described sample preparation of bovine urine for GC-C-IRMS, solid phase extraction is performed prior to hydrolysis in a significant number of methods reported for the preparation of human urine.[118,121-127] This is a common practice for the sample preparation prior to regular GC-MS analysis, since matrix interferences might limit the activity of the used enzymes. A partial deconjugation may lead to an underestimation of the concentration of the steroids, which can lead to quantification errors.[128] The $^{13}C/^{12}C$ value may be affected as well.

Solid phase extraction (SPE)

Often, a combination of SPEs is used during sample preparation. Most frequently, C_{18} columns are utilised, sometimes in combination with silica columns. Use of an OASIS HLB stationary phase has been reported as well.[129] An advantage of SPE is its practical applicability in every laboratory since it requires limited space, no additional equipment and is easy to handle. Although no influence on $\delta^{13}C$ values by SPE treatment has been reported for steroids,[130] fractionation has been reported for other applications.[131,132] For the treatment of human urine, C_{18} columns are used as well, with only a few publications employing another stationary phase, namely Sephadex LH20.[126,133]

Next to the use of SPE to eliminate matrix impurities, it has been used as a tool to split the steroids in human urine by gradual elution from the SPE column.[80,121] The

separation of analytes into different subsamples can facilitate the GC procedure before IRMS measurement.

Liquid-liquid extraction (LLE)

Liquid-liquid extraction (LLE) is commonly performed to extract the steroids from an aqueous solution. The use of successive LLEs under different pH conditions has been reported as a means to separate androgens from estrogens.[100,101] Because of their phenolic steroid structure, estrogens are deprotonated at a high pH and reside as soluble phenolate in the aqueous phase, allowing the selective extraction of unconjugated androgens using an organic extraction solvent. After extraction, the pH of the aqueous phase is adjusted, allowing the extraction of unconjugated estrogens.
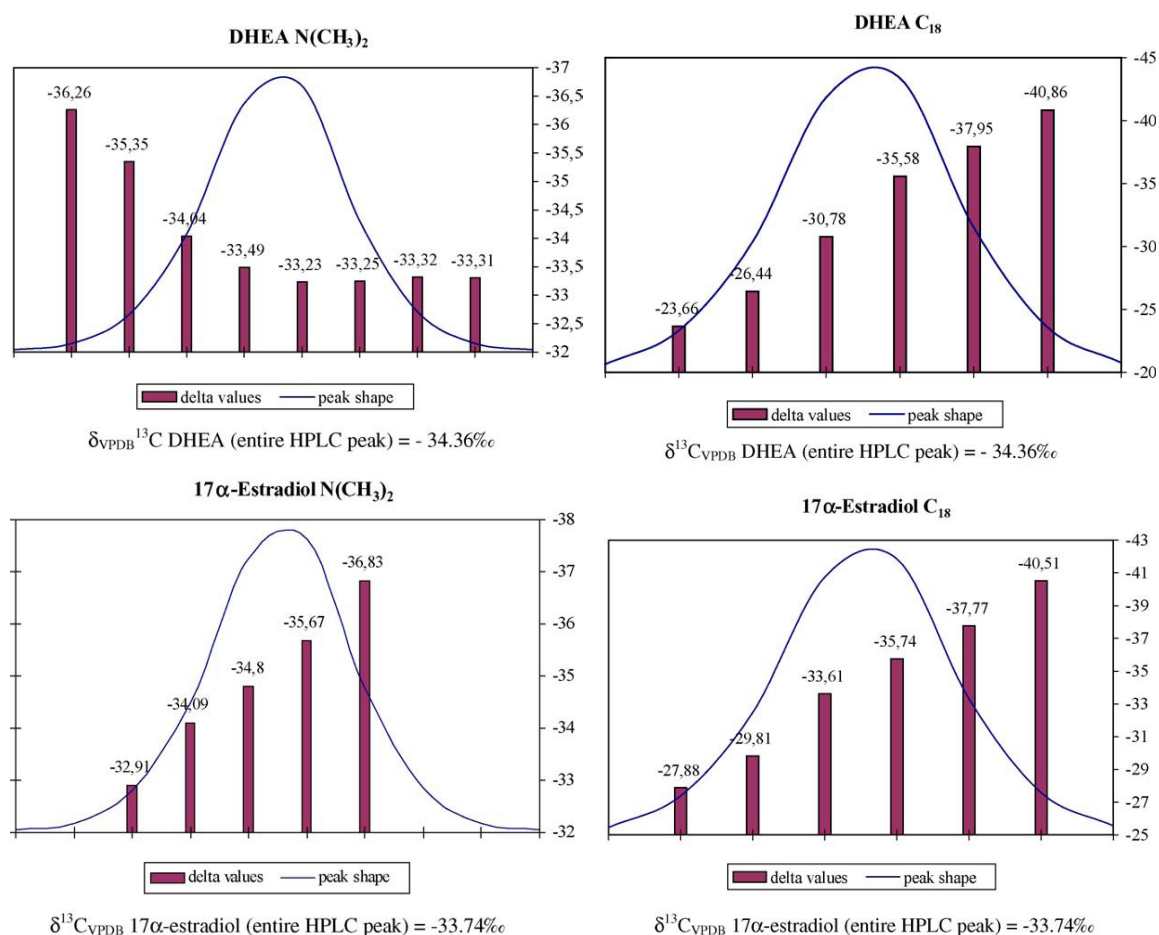
Figure 2.3: Graphic representation of the distribution of $\delta^{13}C$ values of steroids in a HPLC-peak. On the left side, the distribution of DHEA and 17α-estradiol on a $N(CH_3)_2$ stationary phase are displayed; on the right side, the distribution of DHEA-acetate and 17α-estradiol acetate on a C_{18} stationary phase are displayed[101]

High Performance Liquid Chromatography (HPLC)

When available, high performance liquid chromatography or HPLC is the sample preparation method of preference. It allows specific separation of analytes by selection of a target fractionation window. Generally, reversed phase HPLC with a C_{18} column is employed, but use of straight phase HPLC with an aminopropyl column,[67] and a 3-(dimethylamino)propyl column has been reported as well.[100,101,129] However, there is a downside to the application of semi-preparative HPLC since it has been demonstrated that it could lead to isotopic fractionation.[134,135] The heavier isotopomers display a different retention behaviour on column than the lighter isotopomers, resulting in varying elution times in accordance with the $^{13}C/^{12}C$ ratio of the molecules. This is illustrated in figure 2.3. Thus, when an analyte is only partially collected due to a too narrow fractionating window, isotopic fractionation occurs. Therefore, the retention times have to be verified with each HPLC injection series by standard injection of the target analytes.[101] Additionally, the retention times can be evaluated in every sample by the addition of an internal standard, as described in the following chapters. For the observed difference in behavior of DHEA and 17α-estradiol on the $N(CH_3)_2$ stationary phase, visible in figure 2.3, there is currently no explanation.

Immunoaffinity chromatography (IAC)

Immunochemical methods are frequently utilised in sample preparation for steroid analysis other than GC-C-IRMS.[85,133,136,137] In immunoaffinity chromatography (IAC), antibodies are immobilized on a stationary phase and target analytes are retained on column by specific antibody recognition. It was hoped that due to its great analyte specificity, a one step IAC cleanup method would replace existing multi step cleanup procedures prior to GC-C-IRMS, thus significantly simplifying the analysis. The applicability was investigated in both animal bile samples and human urine samples.[113,138] An IAC cleanup proved to be insufficient for the bile samples, with too many impurities still present during GC. Even though no isotopic fractionation was observed in the human urine samples, LC sample preparation was preferred over IAC because with LC, multiple analytes can be separated at the same time, in contrast to IAC where a different stationary phase is required for the specific isolation of each analyte.

Derivatization

Steroids are usually derivatized prior to GC-analysis. During this process, hydroxyl groups are esterified to decrease the polarity of the molecule and increase the volatility. Derivatization significantly improves the peak shape during GC separation. In contrast to regular GC-MS analysis,[139,140] the number of derivatization options is limited in combination with GC-C-IRMS for several reasons.

First of all, the number of carbon atoms added to the molecule by derivatization needs to be minimized, since they affect the isotopic measurement. At the moment, acetylation is the derivatization method of preference, adding an acetyl group to each derivatization site of the steroid (hydroxyl functions). Commonly,[100,101] acetylation is performed by the addition of pyridine and acetic anhydride, but a number of publications reported acetylation without pyridine.[67,84]

The influence of the acetate moieties on the measured $\delta^{13}C$ value of the acetylated steroids is corrected as follows:

$$D_{OH} = D_{OAc} + 2m (D_{OAc} - D_{Ac})/n$$

D_{OH} is the $\delta^{13}C$ value of the underivatized steroids, D_{OAc} is the $\delta^{13}C$ value of the acetylated steroids, D_{Ac} is the $\delta^{13}C$ value of the acetylating reagent, n the number of carbon atoms in a molecule and m is the number of hydroxyl groups to be acetylated.[120,121,123,130] To facilitate the calculation of $\Delta^{13}C$ values, it is advised to compare precursors and metabolites with an equal number of carbon atoms and derivatization sites, which eliminates the need for this correction.

The use of O-trimethylsilylation to replace acetylation was investigated. The advantage of trimethylsilylation over acetylation is an even better behavior of the compounds in GC, and a more controllable derivatization reaction. The disadvantage is the addition of three carbon atoms per derivatization site, thus affecting the isotopic measurement more than acetylation.[117] It has also been reported that silicon deposits originating from the silylation reagent might deactivate the combustion oven.[141] Derivatization reagents containing fluorine atoms are unsuited for use in combination with C-IRMS, since fluorides released upon combustion might compromise the efficiency of the combustion furnace as well.[122,142]

In some methods used on human urine, steroids are analyzed without derivatization,[143,144] but generally, the chromatographic behaviour of steroids containing more than one hydroxyl group is insufficient to meet the chromatographic criteria required for IRMS analysis.

The use of hydropyrolysis - pyrolysis at elevated temperatures in the presence of hydrogen gas and a platinum or molybdenum catalyst - before GC-C-IRMS measurement has been suggested.[139] During hydropyrolysis, all functional groups are stripped from the steroid, but the carbon skeleton is left intact. This technique would eliminate the need for derivatization and its associated problems. Hydropyrolysis on steroids has been studied, but no easily applicable method is currently available.[145,146] Still, hydropyrolysis might be a useful technique for future application.

The effects of an adaptation of the GC-C-IRMS system, replacing the traditional back flush system after GC by a precolumn solvent removal, has been investigated. Such a system would improve chromatography drastically, eliminating the need to derivatize the steroids.[147]

Gas Chromatography coupled to Mass Spectrometry (GC-MS)

Analysis using GC-MS needs to be performed before GC-C-IRMS analysis for three reasons.

First of all, it is used for the identification of the compounds. As mentioned before, GC-C-IRMS only provides information on the isotope ratio of the measured compound. The identification is done according to the legal criteria.[24] Therefore, an internal standard is added before injection to assess the relative retention times of the analytes. Suggested internal standards are 19-nortestosterone and ethynyltestosterone in bovine urine,[67,117] and 5α-androstane-3β-ol in human urine.[125]

Secondly, the quality of the prepared sample is observed. It needs to be ensured that the peaks are free of any impurity or co-elution before GC-C-IRMS measurement. The mass spectrum of the analytes is compared to a standard solution and/or a spiked extracted aqueous sample, injected in the same series.[67,82,84]

Finally, GC-MS is used to determine the concentration of the targeted steroid precursors and metabolites. The concentration is estimated by comparison with an internal standard.[67] An estimation of the concentration is required to either concentrate or dilute the sample to match the IRMS linear range operational area of the employed IRMS device.

For these reasons, it is understandable that the GC parameters, as well as the used column, of the GC-MS measurement need to be identical to those used in the GC-C-IRMS system. As for the performance of the GC-analysis, there are a few precautions in regard to regular steroid GC-MS analysis.

As mentioned above, baseline separation of the analytes is the objective of the GC step. This is the determining factor when setting the GC oven temperature program and deciding the dimensions of the column. Temperature gradient or carrier gas flow rate do not attribute to isotopic fractionation effects.[148]

When measuring analytes with a low concentration, the impact of column bleeding on the $\delta^{13}C$ values may become significant and the automatic correction performed by the IRMS's software may become inadequate. Therefore, not all columns are suitable for GC-C-IRMS analysis and the use of columns with low-bleed specification is advised.

Split injection has to be avoided as differences in boiling point might induce isotopic fractionation.[82] Also, the splitless time needs to be evaluated for each analyte and deactivation of the liner is essential to prevent memory effects.[94] The number of publications in which programmed temperature vaporizer (PTV) large volume injection is used is limited, but isotopic fractionation caused by large volume injection has not been reported so far.[84,113] Use of PTV injection might hold some advantages. The fact that the solvent is not brought onto the column improves the chromatography, which reduces the need for derivatization.[113] Also, the injection of larger volumes allows the reduction of the required sample volume.[84]

With all the above kept in mind, it needs to be said that GC in itself does influence the $^{13}C/^{12}C$ ratio in a very specific way. It is observed that ^{13}C containing molecules migrate slightly more rapidly than U-^{12}C species on the GC-column.[149] This effect is known as the chromatographic isotope effect, which is caused by interactions between solute and stationary phase, dominated by van der Waals dispersion

forces.[150] Therefore, different $^{13}C/^{12}C$ ratios will be measured at the beginning and at the end of the peak. Thus, a correct and complete integration over the peak in its entirety is essential for correct IRMS measurements. In order to achieve this, the peak needs to be perfectly baseline separated first. It has to be noted that when baseline separation is only just reached in GC-MS, this might not be sufficient for IRMS. In GC-C-IRMS, the sample passes through a number of capillaries and the combustion furnace after being eluted from GC. This causes band broadening which may cause barely separated peaks to remerge. Second, since the peak at m/z 45 appears slightly before the one at m/z 44, both signals need to be integrated separately. Third, in combination with defining the peak, the background needs to be defined in a consequent way, so that all measurements can be corrected accordingly. Finally, it is of utmost importance that the peak integration is performed in a consequent and identical way for every analyte measured in a sample. Since $\delta^{13}C$ values of different ERCs and metabolites are compared, inconsistent measurements of an ERC and a metabolite might have a serious influence on the calculated $\Delta^{13}C$ value. Therefore, the use of software is preferred over manual peak integrations, since this eliminates a human bias factor.[110,151]

2.4.2.2. Integrated analytical sample preparation strategies for the differentiation between endogenous steroids and synthetic homologues in bovine urine

Now that the different steps of sample preparation prior to GC-C-IRMS have been presented, the art is to combine the right elements into an efficient and effective analytical strategy. In the following part, a brief summary of the different published analytical methods for the differentiation between endogenous steroids and synthetic homologues in bovine urine is given. An overview providing more detail on the methods is represented in table 2.2.

The first analytical procedure for the differentiation between endogenous steroids and synthetic homologues in bovine urine was published in 1998 by Ferchaud et al.,[67] and consisted of an enzymatic hydrolysis, two SPEs, two subsequent liquid-liquid extractions (LLEs) and one HPLC cleanup. A different derivatization method was used for the GC-MS analysis and the GC-C-IRMS analysis. The measured analytes were DHEA as ERC, and etiocholanolone and epitestosterone as metabolites of testosterone. In the following two publications, the method was fine

tuned with a number of minor adaptations. The hydrolysis method was adapted,[94] and subsequently, a new derivatization method was introduced, using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)/NH_4I/dithiothreitol (DTT)/CH_3CN for both GC-MS and GC-C-IRMS.[117] In this report, the use of androst-5-ene-3β,17α-diol as an additional ERC and epiandrosterone and 5α-androstane-3β,17α-diol as additional metabolites of testosterone was suggested.

Another method, published by Balizs et al., contains a significant number of differences.[84] In a first step, glucuronides were hydrolyzed with β-glucuronidase from *E. coli*. Afterwards, sulphuric acid and ethyl acetate were used to deconjugate the sulphated compounds. Additionally, a combination of two semi-preparative HPLC fractionations was introduced. Finally, DHEA, epitestosterone, 17β-testosterone and etiocholanolone were measured.

In 2005, a method for the detection of the administration of exogenous estrogens was presented,[101] containing a complex and very thorough analytical strategy making use of multiple LLEs, SPEs and HPLC purifications. After hydrolysis using β-glucuronidase from *E. coli* and a SPE with a C_{18} cartridge, the sample was divided into three fractions using two successive LLEs. In an alkaline environment, the deconjugated androgens reside in the organic phase, being extracted as "fraction A". "Fraction E" containing the estrogens was isolated at a lower pH. The sulphated compounds remain in the aqueous phase, called the sulphate fraction or "fraction S". Afterwards, each of the fractions was further purified separately. Another novelty in this procedure was the use of an additional HPLC fractionation after the derivatization of the steroids when the GC-MS analysis pointed out that co-elution of the target peaks was still occurring or when a sufficient level of purity was not reached. Androst-5-ene-3β,17α-diol served as the endogenous reference compound (ERC), while 17α-estradiol was the measured analyte for estrogen administration.

Later, additional measurement of 5α-androstane-3β,17α-diol and etiocholanolone as testosterone metabolites in the same method resulted in a complete strategy to detect both androgen and estrogen abuse in cattle.[100]

Finally, it has to be mentioned that, even though corticosteroids have only received little attention in regard to IRMS-analysis, a method to detect administration of glucocorticoids in cattle has been developed,[129] with DHEA as ERC and cortisol as metabolite.

Table 2.2: Overview of the main characteristics of the different sample preparation methods for the differentiation between endogenous and exogenous steroids in bovine urine, in chronological order according to the date of publication

reference	sample volume	hydrolysis	solid phase extraction (SPE)	liquid-liquid extraction (LLE)	high performance liquid chromatography (HPLC)	derivatization	sample preparation, sequence of preparative techniques in the described method	
[67]	50 mL	*Helix pomatia* juice	1: C$_{18}$ stationary phase 2: silica stationary phase	two subsequent LLEs with a sodium hydroxide solution	aminopropyl stationary phase	(for GC-MS): MSTFA-TMIS (for GC-C-IRMS): acetic anhydride	Hydrolysis - SPE1 - LLE - SPE2 - HPLC - Derivatization	
[94]	50 mL	*Abalone* entrails	1: C$_{18}$ stationary phase 2: silica stationary phase	two subsequent LLE with a sodium hydroxide solution	aminopropyl stationary phase	(for GC-MS): MSTFA-TMIS (for GC-C-IRMS): acetic anhydride	Hydrolysis - SPE1 - LLE - SPE2 - HPLC - Derivatization	
[117]	50 mL	*Helix pomatia* juice	1: C$_{18}$ stationary phase 2: silica stationary phase	two subsequent LLE with a sodium hydroxide solution	aminopropyl stationary phase	MSTFA/NH$_4$I/DTT/CH$_3$CN (1000/5/0.25/100, v/w/w/v)	Hydrolysis - SPE1 - LLE - SPE2 - HPLC - Derivatization	
[84]	20 mL	1: β-glucuronidase *E. coli* 2: sulphuric acid and ethyl acetate	C$_{18}$ stationary phase		1: C$_{18}$ stationary phase 2: C$_{18}$ stationary phase (different gradient mobile phase)	acetic anhydride	Hydrolysis1 - SPE – Hydrolysis2 - HPLC1 - HPLC2 - Derivatization	
[101]	20 mL	1: β-glucuronidase *E. coli* 2: sulphuric acid and ethyl acetate	1: C$_{18}$ stationary phase 2: silica stationary phase	two subsequent LLE resulting in three fractions (fraction A, fraction E and fraction S)	1: 3-(dimethylamino)propyl stationary phase 2: C$_{18}$ stationary phase	pyridine and acetic anhydride	Hydrolysis1 - SPE1 - LLE	fraction A and E: SPE2 - HPLC1 - Derivatization - HPLC2 fraction S: Hydrolysis2 - SPE1 - SPE2 - HPLC1 - Derivatization - HPLC2
[100]	20 mL	1: β-glucuronidase *E. coli* 2: sulphuric acid and ethyl acetate	1: C$_{18}$ stationary phase 2: silica stationary phase	two subsequent LLE resulting in three fractions (fraction A, fraction E and fraction S)	1: 3-(dimethylamino)propyl stationary phase 2: C$_{18}$ stationary phase	pyridine and acetic anhydride	Hydrolysis1 - SPE1 - LLE	fraction A and E: SPE2 - HPLC1 - Derivatization - HPLC2 fraction S: SPE1 - Hydrolysis2 - SPE2 - HPLC1 - Derivatization - HPLC2
[129]	1: 20 mL	1: *Helix pomatia* juice	1: OASIS	two subsequent LLE resulting in three fractions (fraction A, fraction E and fraction S). Only fraction S is analysed further	1: 3-(dimethylamino)propyl stationary phase	1: oxidation with potassium dichromate and acetonitrile	1: Hydrolysis1 - SPE1 - Derivatization1 - HPLC2	
	2: 20 mL	2: β-glucuronidase *E. coli* 3: sulphuric acid and ethyl acetate	2: C$_{18}$ stationary phase 3: silica stationary phase		2: C$_{18}$ stationary phase	2: pyridine and acetic anhydride	2: Hydrolysis2 - SPE2 - LLE - SPE2 - Hydrolysis3 - SPE3 - HPLC1 – Derivatization2 - HPLC2	

2.4.3. Control measures

As the performance criteria of GC-C-IRMS analysis are not uniformly dictated, and because of the complexity of the sample preparation methods, a number of measures, which are not necessary for validation of GC-MS methods, are in order to verify the functionality of the method and to assure the correctness of the results.[30] It is not within the objective of this review to provide detailed information and guidelines on the validation of GC-C-IRMS methods, because different approaches to this issue are possible. However, in the following chapter a number of issues will be addressed which should be integrated in every validation or quality control system regarding steroid analysis using GC-C-IRMS. In the past few years, the WADA revised their guidelines with regard to doping control analyses using GC-C-IRMS.[152,153] As discussed in paragraph 2.5.2, there are many similarities between the use of IRMS for doping control in athletes and for growth promoter control in cattle, and therefore, these documents are a very valuable source of information when designing quality control and validation procedures for this application.

Instrumental performance

The IRMS instrument measures the $\delta^{13}C$ values by comparison to the reference CO_2 gas, present in the system. In order to obtain correct values, this reference gas needs to be calibrated. This was usually performed using a certified alkane mixture.[80,121] Nowadays, however, it is advised to perform calibration using certified material that resembles the target analytes as much as possible.[82] Since steroids behave rather differently during gas chromatography than alkanes, this might lead to an erroneous calibration.[129] Additionally, in the most recent version of the WADA technical document, in force since the beginning of 2016, it is recommended to use a mixture of steroids covering the range of $\delta^{13}C$ values normally found in urine samples.[153]

Prior to analysis, a number of measures to ensure an adequate operation status of the IRMS system are required. Stability of the instrument is checked by introducing a number of CO_2 pulses of equal quantity and verifying if the measured deviation of $\delta^{13}C$ values is within range.

The IRMS instrument has a linear range of measurement which is defined by the manufacturer. For a classical type instrument, $\delta^{13}C$ value measurements with signal intensities between 1 and 10 nA at m/z 44 are considered reliable,[112,154,155] or values

above 0.8 mV depending on the type of instrument.[156] Before every injection series, the linear performance of the IRMS apparatus is verified by introducing a series of CO_2 pulses with rising intensities. The correlation coefficient of the measured concentrations is the indication for the linearity.[121,129]

The system needs to be checked for leaks. This can be accomplished by performing a scan to detect background gases, for example m/z 40 for argon or m/z 18 for water.[82] It needs to be added that an unexplained drift in $\delta^{13}C$ values over a longer period of time has been reported.[157]

Compound specific linear range and limit of quantification

It has been reported that GC-C-IRMS linearity is compound-dependent, meaning that for each substance, correct measurements can only be obtained within a limited and specific concentration range. Therefore, it is advisable to verify the range for each analyte by a series of standard injections at various concentrations.[154,156] As a criterion, the most recent WADA guidelines state that the measured $\delta^{13}C$ values may not deviate more than 0.5‰ of the mean value within the linear range.[153]

In relation to this, the same document describes that the limit of quantification (LOQ), described as the lowest concentration in urine which still provides a measurable signal in the linear range with a standard deviation (SD) smaller than 1‰, needs to be determined for all measured analytes.

Measurement uncertainty

Since 2014, the WADA guidelines dictate that the estimated combined standard measurement uncertainty must be below 1‰ for every analyte, but it does not describe in detail how this should be calculated. Next to the within lab reproducibility, the method and system bias are the most important factors contributing to the measurement uncertainty. The method bias, or the isotopic fractionation caused by the sample preparation, is usually evaluated by comparing the $\delta^{13}C$ values of steroid standards of the target analytes and extracted spiked aqueous samples. In case of absence of isotopic fractionation, the values need to be the same within a statistical variation.[82] The system or calibration bias, also referred to as trueness, is evaluated through the difference between the measured and certified values of steroid standards with known and traceable $\delta^{13}C_{VPDB}$ values. Ideally, both method and

system bias can be evaluated simultaneously, through the measurement of urine samples containing steroids with known $\delta^{13}C_{VPDB}$ values.

Compliance criteria (threshold Δ^{13}C values)

In recent years, the WADA abandoned a universal 3‰ criterion for all ERC-metabolite couples to declare a sample non-compliant, and moved to a more specific set of non-compliance criteria, as illustrated in table 2.3. Since no official criteria are currently available for the evaluation of bovine urine samples, threshold $\Delta^{13}C$ values for every ERC-metabolite couple need to be determined during the method validation. To this aim, a sufficient amount of samples from a non-treated control population need to be analyzed. The compliance threshold can be calculated as the mean $\Delta^{13}C$ value plus three times the standard deviation (μ + 3 x SD).[152] Whereas the 2014 WADA guidelines included a control population based threshold value as an additional non-compliance criterion, this was changed to a laboratory performance criterion in the new version.

Table 2.3: IRMS thresholds according to WADA requirements 2016[153]

					$\Delta^{13}C_{ERC\text{-}Metabolite}$	
Metabolite:	**βT**	**αT**	**Androsterone**	**Etio**	**5α-androstane-3α,17β-diol and/or 5β-androstane-3α,17β-diol**	**Boldenone (metabolites) and formestane**
Case 1	>3‰				>3‰ (either diol)	
Case 2					>3‰ (both diols)	
Case 3		>4‰				
Case 4			>3‰			
Case 5				>4‰		
Case 6			2-3‰		>3‰ (either diol)	
Case 7				3-4‰	>3‰ (either diol)	
Case 8					>4‰ and δ≤-27‰ (first diol)	
Case 9						>4‰

Routine control measures

A number of measures were already mentioned above, such as injection of standard solutions to verify peak purity and the use of internal standards to assess stability. Additionally, it is suggested to inject a solvent blank in the beginning of a sequence, to check absence of contamination. The injection of an extracted water-blank sample

is suggested to help identifying the presence of impurities. Finally, it is advised to include positive and negative control samples to verify the aptitude of the method.[82]

2.5. Detection of abuse of synthetic homologues of endogenous steroids, the greater picture

Research into methods for the differentiation between endogenous steroids and synthetic homologues stretches beyond the carbon isotope ratio analysis of bovine urine. The aim of this chapter is to briefly present other applications, allowing positioning of the information into a broader frame. First, other isotope based techniques to differentiate between endogenous steroids and exogenous homologues in cattle will be described. Second, the detection of abuse of synthetic forms of endogenous steroids in men will be presented by highlighting the similarities and differences between sports doping control and detection of growth promoter abuse, both using GC-C-IRMS.

2.5.1. Other isotope ratio mass spectrometric methods for the differentiation between endogenous steroids and synthetic homologues

Next to carbon isotope ratio measurement, GC coupled to IRMS can also be used to determine the deuterium/hydrogen (D/H) ratio of steroids. During the development of a setup to perform this analysis, a number of problems were encountered. The quantitative conversion of the analytes to hydrogen gas (H_2) proved to be difficult, but was eventually resolved by the use of high-temperature conversion (TC), during which the analytes are pyrolysed at temperatures of approximately 1400 °C. Also, the low abundance of deuterium in combination with the presence of helium (He) as the carrier gas made precise measurements of the ions with an m/z ratio of 3 nearly impossible. This problem was resolved by augmenting the analyte concentration and by adding lenses in the detector to filter out He.[158]

Although gas chromatography-high-temperature conversion-isotope ratio mass spectrometry (GC-TC-IRMS) has not yet been used to detect D/H ratios of steroids in bovine urine, a method has already been developed for the measurement of human urine samples.[159] A number of significant differences between D/H and $^{13}C/^{12}C$ ratios

need to be kept in mind. First of all, the carbon isotope ratio of endogenous steroids depends on the diet, whereas the D/H ratio is largely influenced by the drinking water. Also, constant conditions of sample preparation are important, since hydrogen atoms of hydroxyl groups can equilibrate with the ambient atmosphere. It has been suggested that D/H ratio measurement might provide a powerful tool to be used together with $^{13}C/^{12}C$ ratio measurement, especially in cases where the $\Delta^{13}C$ value is near the threshold value. Due to the large relative mass difference between D and H in comparison with ^{13}C and ^{12}C, more pronounced isotopic effects are expected, which could result in significant δD signatures.[88,159] Piper et al. calculated the first reference-population based threshold values and were able to successfully differentiate between compliant and non-compliant samples, even in a case where this was not possible based on the $\delta^{13}C$ values.[160]

Although GC-C-IRMS is the most widespread method for carbon isotope measurement, it is not the only option available. Initially, the coupling of LC to IRMS proved to be more difficult than that of GC to IRMS. However, an interface to achieve this coupling, based on a wet chemical oxidation of organic compounds to CO_2, has been commercially available since 2004. But still, LC-IRMS encountered a number of limitations, which hindered the practical application. The main restriction is the impossibility to apply organic mobile phases. These mobile phases contribute to the formation of CO_2, detected simultaneously with the analytes. Using pure water as the mobile phase, the required baseline separation of analytes was difficult to achieve. This problem was recently overcome by the development of temperature-programmed high-performance liquid chromatography.[161] Using temperature gradients and HPLC at elevated temperatures, analyte separation can be significantly improved. Since the isotopic accuracy of temperature-programmed HPLC-IRMS seems to be less affected by errors compared to GC-C-IRMS,[162] this technique could be promising for the future carbon isotope measurement of steroids.

Finally, multidimensional gas chromatography coupled to combustion isotope ratio mass spectrometry (MDGC-C-IRMS) for the detection of exogenous steroids in urine has been suggested. The use of multiple GC columns in series greatly improves the separation capacity, therefore eliminating the need for an extended sample

preparation. To this aim, a prototype instrumental setup was designed to perform comprehensive two-dimensional gas chromatography combustion isotope ratio mass spectrometry (GCxGC-C-IRMS).[163] For the coupling of the two GC columns, a longitudinally modulated cryogenic system (LMCS) was used. This allows the components eluting from the first column to be cryogenically trapped and continuously transferred as a narrow band onto the second column in two to ten seconds intervals, resulting in a secondary chromatogram every two to ten seconds. However, extensive adaptations to the combustion reactor, back flush system, water trap and capillaries were required in order to minimize peak broadening and make the setup functional. Also, no software for the interpretation of steroid peaks which were sliced by the LMCS was available and needed to be written.[164] Still, promising results were obtained by testing the setup with steroid standards. Afterwards, the system was further refined, and human urine samples were successfully analyzed using a sample preparation method from which HPLC purification could be eliminated. Still, due to the complex nature of the setup and its lacking commercial availability, GCxGC-C-IRMS is not easily applicable.

2.5.2. Differentiation between endogenous steroids and synthetic derivatives in men

Synthetic forms of endogenous steroids are not only abused as growth promoters in cattle, but as horseracing and sports doping agent as well. In the field of sports doping, GC-C-IRMS is also used as the preferred method for the unambiguous differentiation between endogenous anabolic steroids and synthetic homologues. Since the same doping regulations are applied worldwide, and since the World Anti-Doping Agency (WADA) recently obliged all of its laboratories to be able to perform GC-C-IRMS analysis, this application is more investigated and documented than the growth promoter abuse, which is mainly a European issue. Because of the large similarities between both applications, this research contains a valuable source of information when designing new strategies for cleaning up bovine urine samples, as illustrated upon discussing sample preparation techniques.

However, awareness of the differences is in order. While DHEA is considered an ERC in bovine urine, its abuse as doping product is known and as such it is not suited as a reference. For doping control, 17α-testosterone, pregnanediol, 11β-

hydroxyandrosterone, 5α-androst-16-en-3β-ol and 11-ketoetiocholanolone are commonly utilized as the ERCs, androsterone, etiocholanolone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol function as metabolites.[123,130] This shows that even though the steroid metabolism is similar in all mammalian species, important differences between humans and bovines need to be kept in mind, especially when it comes to metabolites of testosterone. Since there is no abuse of estrogens as doping, its metabolites are not analyzed.

Furthermore, bovine urine is considered a more difficult matrix than human urine, and therefore demands more extensive sample preparation,[84] although the amount of urine available is usually higher than in human sports doping testing.

Since doping control research has greatly expanded during recent years, it holds a number of applications not yet available to routine growth promoter control.

While WADA provides a clear list of criteria with regard to GC-C-IRMS analysis and results, these are completely lacking in current European legislation, causing unclarity and legal disputes.[30]

While rapid screening criteria for suspicious bovine urine samples are still being investigated, steroid profiling in athletes is far more developed and actively applied.[165,166] In the past, samples with a ratio of testosterone over epitestosterone (T/E) larger than 4 were considered atypical,[99] and threshold values for DHEA, testosterone and epitestosterone were set as well. More recent, 'the athlete biological passport', a personalized set of biomarker levels obtained through longitudinal study of the individual athlete, was developed to identify atypical samples.[34]

The availability of adequate reference material facilitates method development and interlaboratory comparison of results.[167] In the field of sports doping, reference standards are now available,[168] and a protocol for calibration of the reference gas is present.[169] Next to that, matrix-based reference material is being evaluated at the moment.[170] Currently, the number of laboratories performing routine GC-C-IRMS analysis on bovine urine is very limited, but as the number might increase in the future, such developments will be required.

2.6. Conclusions

Gas chromatography coupled to combustion-isotope ratio mass spectrometry offers a unique method for the unambiguous differentiation between natural hormones and homologous synthetic compounds in cattle. The advantage of being able to detect exogenous steroids in an undisputable way gravely outweighs the difficulties of the complex sample preparation methods. Still, extensive study will be required in facilitating the implementation and broadening the application of this technique.

Recent evolutions in high throughput sample preparation methods, such as solid phase micro extraction (SPME), supported and immobilized liquid extraction and robotic liquid handlers, can provide powerful means to simplify and speed up the analysis, which remains up until now laborious and time consuming.

Next to evolutions in sample preparation, technical improvements in IRMS technology holds promises for future applications. Enhancement of the sensitivity of carbon isotopic measurement would greatly improve the detection of steroid abuse and could allow smaller sample volumes. Also, recent developments in liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) could provide new strategies for the future. Finally, the use of hydrogen IRMS for the differentiation between endogenous steroids and exogenous homologues shows promising first results, although further elaboration is required.

It can be hoped that the increased attention and recent evolutions concerning GC-C-IRMS in doping control will boost the development in the field of growth promoter detection.

It is clear, however, that parallel to the further development of GC-C-IRMS as a highly specialized confirmation method, the development of targeted steroid profiling to provide applicable screening strategies is necessary in order to isolate suspicious samples for isotope ratio analysis.

3. Application of gas chromatography-(mass spectrometry/combustion-isotope ratio mass spectrometry) (GC-(MS/C-IRMS)) to detect the abuse of 17β-estradiol in cattle

3.1. Abstract

Although the ability to differentiate between endogenous steroids and synthetic homologues based on their $^{13}C/^{12}C$ isotopic ratio has been known for over a decade, this technique has been scarcely implemented for food safety purposes. In this study, a method was developed using gas chromatography-(mass spectrometry/combustion-isotope ratio mass spectrometry) (GC-(MS/C-IRMS)) to demonstrate the abuse of 17β-estradiol in cattle, by comparison of the $^{13}C/^{12}C$ ratios of the main metabolite 17α-estradiol and an endogenous reference compound (ERC), 5-androstene-3β,17α-diol, in bovine urine. The intermediate precision was determined as 0.46‰ and 0.26‰ for 5-androstene-3β,17α-diol and 17α-estradiol, respectively. To the best of our knowledge, this is the first reported use of GC-(MS/C-IRMS) for the analysis of steroid compounds for food safety issues.

3.2. Introduction

The influence of steroid hormones on muscle/meat building has been known for over 70 years, which led to widespread use in both sports and stock farming, respectively. While their immediate effect on animals from the farmer's point of view is clear, risk assessment was subjected to debates because of divergent opinions at the international levels, e.g. Codex Committee on Residue of Veterinary Drugs in Foods (CCRVDF) and Scientific Committee on Veterinary Measures relating to Public Health (SCVPH). Whereas hormones are licensed in various countries worldwide, they are banned for use as growth promoters in the European Union since 1988.[19] As a result, monitoring the abuse of steroid hormones in large scale surveillance

programs for food safety reasons is mandatory for all member states.[23] When looking at the results of these monitoring plans from 2000 up to 2010,[171] an annual average of approximately 8% of the non-compliances for steroids (group A3) are attributable to 17β-estradiol. However, these monitoring programs are still based on the classical approach using either GC-MS or LC-MS, which are unable to provide unambiguous results when it comes to the detection of synthetic analogues of naturally occurring steroid hormones in urine. In case of estradiol, a population study on the presence of natural steroids in bovine urine in the UK showed that when setting a concentration threshold value for 17α-estradiol in bovine urine to indicate 17β-estradiol abuse, a confirmatory analysis is required since there is an overlap in the concentration of 17α-estradiol between treated and non-treated animals.[32] Since it became clear during the late 1990s that a distinction could be made between endogenous steroids and exogenous homologues based on their carbon isotopic composition ($^{13}C/^{12}C$) – reported as $\delta^{13}C$ values (‰) – analyses using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) have been adopted in the field of sports doping control and food safety.[67,84,94,100,101,113,117,129,130,172] Exogenous steroids which are synthesized from plant material are enriched in ^{12}C compared to endogenously produced steroids because the source material originates from plant species which are naturally low in ^{13}C content. The $\delta^{13}C$ values of exogenous steroids are usually lower than -30‰, whereas $\delta^{13}C$ values of endogenous steroids reflect the diet and are usually above -28‰. Because of the individual variability of the $\delta^{13}C$ values, mostly caused by differences in diet, both precursors, also called endogenous reference compounds (ERCs), and metabolites of the targeted steroid hormone are measured. Only the $\delta^{13}C$ values of the metabolites are influenced by the administration of the exogenous steroid and therefore, the difference between the $\delta^{13}C$ value of the ERC and the metabolite, expressed as $\Delta^{13}C$ (‰), provides proof of administration. Still, in the field of food safety, the use of GC-C-IRMS to differentiate between endogenous steroid hormones and synthetic homologues in cattle has only been applied scarcely until now[172], and the number of published methods is slowly growing. [67,84,94,100,101,113,117,129]

In this chapter, a method for the detection of 17β-estradiol administration to cattle is presented using gas chromatography-(mass spectrometry/combustion-isotope ratio mass spectrometry) (GC-(MS/C-IRMS)) for the measurement of 5-androstene-3β,17α-diol as ERC and 17α-estradiol as metabolite in urine. Sample preparation was based on the previously published method,[101] with minor adaptations to adjust to the current laboratory situation and to allow further automation.

3.3. Material and methods

3.3.1. Urine samples

Non-compliant samples

One male and one female bovine, aged between 18 and 24 months and weighing approximately 400 kg, were treated with a single intramuscular injection containing 1 mg/kg body weight testosterone (administered as 1.194 mg/kg testosterone propionate) and 0.2 mg/kg body weight estradiol (administered as 0.276 mg/kg 17β-estradiol-3-benzoate). Urine samples were collected before and during the first 27 days after administration and stored frozen at -21°C. Afterwards, the samples were stored at -85°C until assay.

Compliant samples

Twenty-nine urine samples of pregnant cows were collected at different farms to be used as reference population samples. The samples were stored at -85°C until assay.

Spiked samples

Routine samples in which no 17α-estradiol could be detected and with concentrations of 5-androstene-3β,17α-diol below 5 ppb, were used for the preparation of samples spiked with 17α-estradiol and 5-androstene-3β,17α-diol, to be used for validation and quality control.

3.3.2. Reagents and chemicals

All reagents and solvents were of analytical grade and were provided by Sigma-Aldrich (Bornem, Belgium). The solvents for liquid chromatography were of LC- and

HPLC-grade from Biosolve (Valkenswaard, the Netherlands). 17β-Testosterone acetate was supplied by Sigma-Aldrich (Bornem, Belgium). Other steroids were obtained from Steraloids (Wilton, NY, USA). SPE C_{18} cartridges were obtained from Achrom (Zulte, Belgium). β-Glucuronidase was from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany). Pyridine and acetic anhydride used in derivatization reactions were from Sigma-Aldrich (Bornem, Belgium).

3.3.3. Instrumentation

HPLC-UV

During sample preparation, two subsequent HPLC purifications were performed. The first system used was a Waters Alliance 2690 equipped with a UV-detector (diode array detector, DAD), operated between 205 and 235 nm, and an automated fraction collector. The system was set up with a C_{18} functionalized precolumn (Grace Alltima C_{18}; 7.5 mm x 3 mm; 5 μm) and a C_{18} functionalized column (Grace Alltima C_{18}; 250 mm x 3 mm; 5 μm). An isocratic method was used with a rinsing phase at the end of the run. A mobile phase made of H_2O/MeOH (95/5; v/v) (solvent A) and MeOH (solvent B) was used, held at a constant composition (A:B; v/v) of 37:63. The flow rate was set at 0.6 mL/min, column temperature at 40°C and the injected volume was 100 μL. The second Waters Alliance 2690 system was equipped with two diol functionalized columns (LiChrospher Diol; 250 mm x 4 mm; 5 μm) in series. An isocratic method was used with a mobile phase of isooctane/isopropanol (85/15; v/v) and a rinsing phase at the end of the run. The flow rate was set at 1 mL/min, column temperature at 40°C and the injected volume was 100 μL.

GC-(MS/C-IRMS)

The samples were analyzed with a Thermo Trace GC Ultra gas chromatograph, equipped with a RXI 5 SIL MS column (Restek – 30 m; 0.25 mm ID; 0.25 μm df) and a Thermo Scientific TriPlus autosampler. After GC, the sample was split by means of a T-piece, which was coupled to a Thermo DSQ II single quadrupole mass spectrometer at one end, and to a Thermo MAT 253 isotope ratio mass spectrometer, via the Thermo Scientific GC Isolink, at the other end. Gas flows were regulated using the Thermo Scientific Conflo IV interface. Eight μL was injected at 20 μL/sec into the injector in programmed temperature vaporizer mode. The initial

injector temperature was 100°C, which was held for 0.05 min with a vent flow of 20 mL/min. The temperature was raised at 8°C/min and held at 280°C for 2 min during sample transfer on column. The initial GC oven temperature was 110°C, which was held for 1.5 min. The temperature was then raised to 280°C at 8°C/min and held for 2 min. Finally, the temperature was raised to 320°C at 50°C/min and held for 3 minutes. The carrier gas was helium at a constant flow rate of 1.5 mL/min. The temperature of the transfer line was set at 300°C. The steroids were detected on MS using full scan mode (m/z 50 to 400). The combustion furnace was set at 950°C and was oxidized prior to each series of analyses for 1h. The combustion gases were passed through a Nafion membrane filter for water removal. After ionization, the ions with m/z 44, 45 and 46 were magnetically separated and simultaneously measured in three Faraday collectors. The CO_2 reference gas was calibrated with a mixture of steroids with certified and traceable $\delta^{13}C_{VPDB}$ values, measured with an elemental analyzer (5-androstene-3β,17α-diol monoacetate (-27.0‰), β-testosterone acetate (-30.5‰) and dehydroepiandrosterone (DHEA) acetate (-32.1‰)). Carbon isotope ratios of the compounds were expressed relative to Vienna Pee Dee Belemnite (VPDB). The shift of the $\delta^{13}C_{VPDB}$ value due to the formation of acetates is corrected as follows:

$$D_{OH} = D_{OAc} + 2m (D_{OAc} - D_{Ac})/n$$

D_{OH} is the $\delta^{13}C_{VPDB}$ value of the underivatized steroids, D_{OAc} the $\delta^{13}C_{VPDB}$ value of the acetylated steroids, D_{Ac} the $\delta^{13}C_{VPDB}$ value of the acetylating reagent, n the number of carbon atoms in a molecule and m is the number of hydroxyl groups to be acetylated[100].

3.3.4. Sample preparation

A schematic overview of the analytical strategy is presented in scheme 3.1. The urine samples were centrifuged (15 min, 3113 g) prior to analysis, to avoid obstruction of the solid phase extraction (SPE) cartridges in a later stage. To 10 mL of the centrifuged urine sample, 3 mL phosphate buffer 0.8 M (pH 6.8) and 50 μL glucuronidase from *E. coli* were added. Hydrolysis was performed for 15h at 37°C. Then, the sample was brought onto a C_{18} SPE column, which was first conditioned with 6 mL of methanol and 6 mL of H_2O. The column was subsequently washed with 6 mL H_2O and 5 mL H_2O/acetonitrile (ACN) (80/20; v/v), and eluted using 8 mL

H$_2$O/ACN (10/90; v/v). The eluted sample was evaporated to approximately 300 µL under a nitrogen stream at 40℃, and 0.5 mL of 1 M sodium hydroxide was added. Next, a liquid-liquid extraction (LLE) was performed using 4 mL of n-pentane/diethyl ether (92.5/7.5; v/v). The organic layer was kept and labeled "fraction A", containing androgenic steroids, amongst which the targeted ERC 5-androstene-3β,17α-diol. Next, the pH of the aqueous layer was adjusted using glacial acetic acid and a second liquid-liquid extraction using 4 mL of n-pentane/diethyl ether (92.5/7.5; v/v) was performed. The organic layer was kept and labeled "fraction E", containing estrogenic steroids, amongst which the target metabolite 17α-estradiol. Both fractions were evaporated under a nitrogen stream at 37℃ after the addition of 100 µL of glycerol solution (10% in methanol) to serve as a keeper solution, and mixed with 70 µL of water and 40 µL of fluoxymesterone in methanol (50 ng/µL) as internal standard. Prior to injection onto the first HPLC-system, the fractionation windows for the ERC and metabolite were determined through the threefold injection of a standard solution containing fluoxymesterone, 17α-estradiol, 5-androstene-3β,17α-diol and 17β-testosterone. The collected fractions "A" and "E", containing 5-androstene-3β,17α-diol and 17α-estradiol, respectively, were evaporated under a nitrogen stream at 37℃ and dissolved in 120 µL of isopropanol/isooctane (10/90; v/v). An aliquot of 20 µL of a medroxyprogesterone standard solution (100 ng/µL) was added as internal standard, used for both verification of the retention time and estimation of the analyte concentration during the second HPLC purification. The samples were injected after calculating the fractionation windows with the threefold injection of a standard solution containing medroxyprogesterone, 17α-estradiol, 5-androstene-3β,17α-diol and 17β-testosterone. The collected fractions were evaporated to dryness under a nitrogen stream at 40℃ and 25 µL of both acetic anhydride and pyridine were added. The derivatization was done overnight at room temperature in a closed vial. Afterwards, the sample was evaporated to dryness under a gentle nitrogen stream at 40℃ and the residue was dissolved in the appropriate volume of isooctane to provide measurement within the linear range of the IRMS apparatus. Finally, after the addition of noretiocholanolone acetate (4 ng/µL) as external standard, the two fractions were injected onto GC-(MS/C-IRMS) for further characterization of δ^{13}C$_{VPDB}$ of 5-androstene-3β,17α-diol diacetate and 17α-estradiol diacetate.
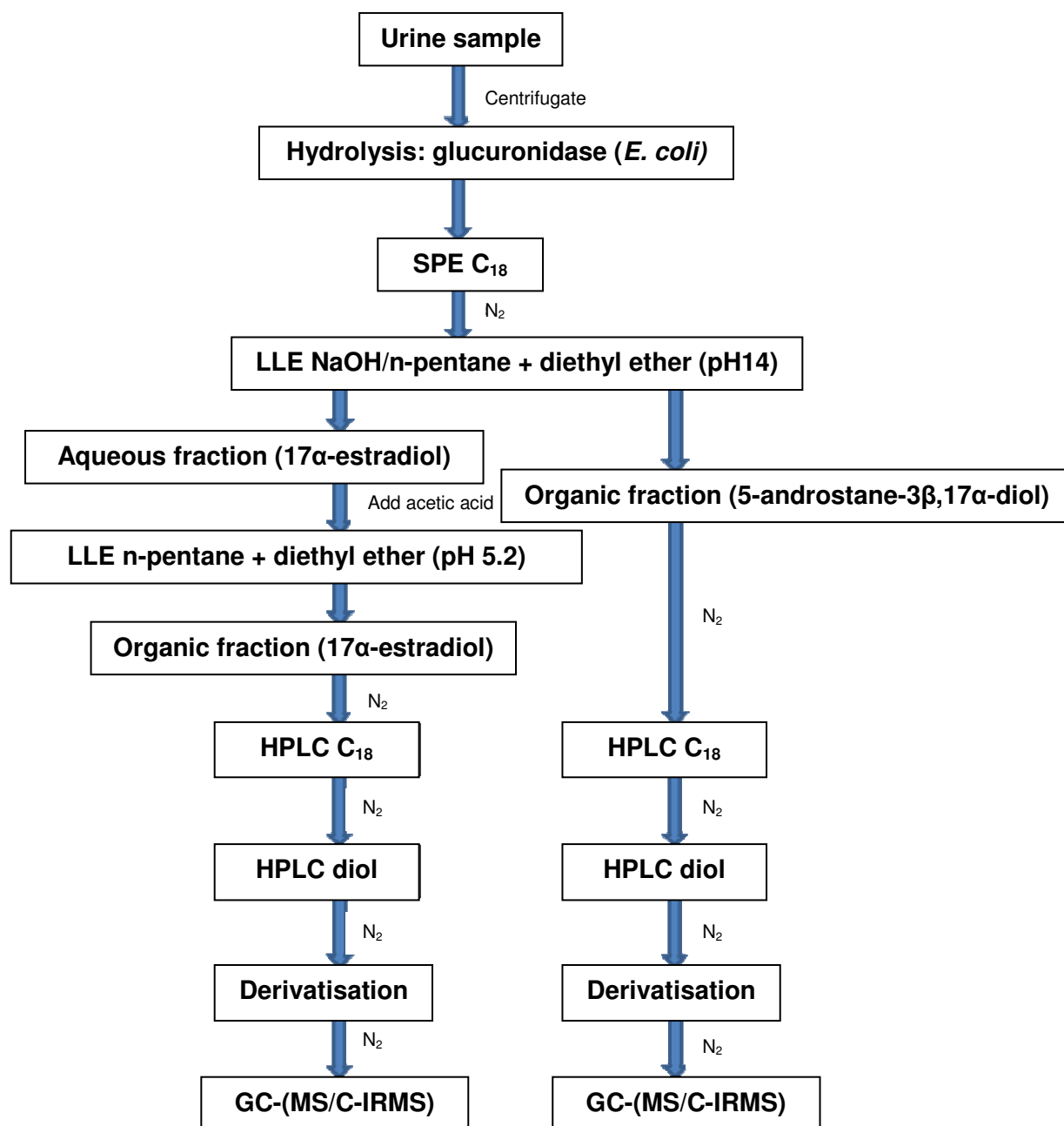
Scheme 3.1. Analytical strategy for the extraction and purification of 17α-estradiol and the endogenous reference compound 5-androstene-3β,17α-diol. LLE stands for liquid-liquid extraction

3.4. Results and discussion

3.4.1. Sample preparation and analysis

The presented sample preparation method is based on the previously published method by Buisson et al.,[101] with a number of adaptations to speed up the process or to allow further automation in the future. First, only 5-androstene-3β,17α-diol was

measured as ERC. By not measuring dehydroepiandrosterone (DHEA), the necessity for the time consuming analysis of the sulphoconjugated steroids was eliminated.

Second, one SPE purification step and the two preparative HPLC steps were replaced by just two subsequent HPLC purifications prior to derivatization. Because the use of a 3-(dimethylamino)propyl-functionalized silica gel column resulted in unstable retention times – after a sudden shift, the retention times of the analytes differed significantly – a diol functionalized stationary phase was selected for the second HPLC purification. Even though a cleanup procedure without the straight phase HPLC purification provided accurate results, the addition of the second HPLC preparation step was preferred to further reduce the risk of impurities coeluting with the analytes. The additional effect of this HPLC step on the sample cleanup is illustrated in figure 3.1.
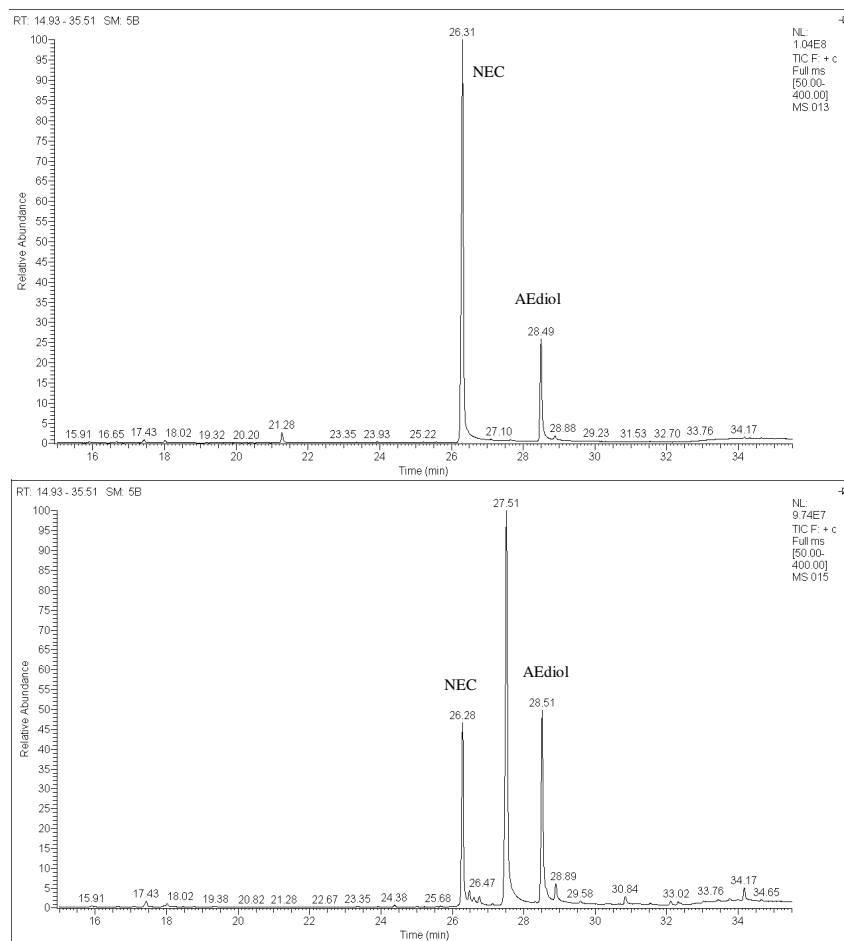

Figure 3.1. MS chromatograms of the androgen fraction of a urine sample of a pregnant cow after the complete sample preparation procedure (above) and after sample preparation without straight phase HPLC purification (below), showing the internal standard noretiocholanolone acetate (NEC) and 5-androstene-3β,17α-diol (AEdiol). The data were produced in full scan mode (m/z 50 – 400).
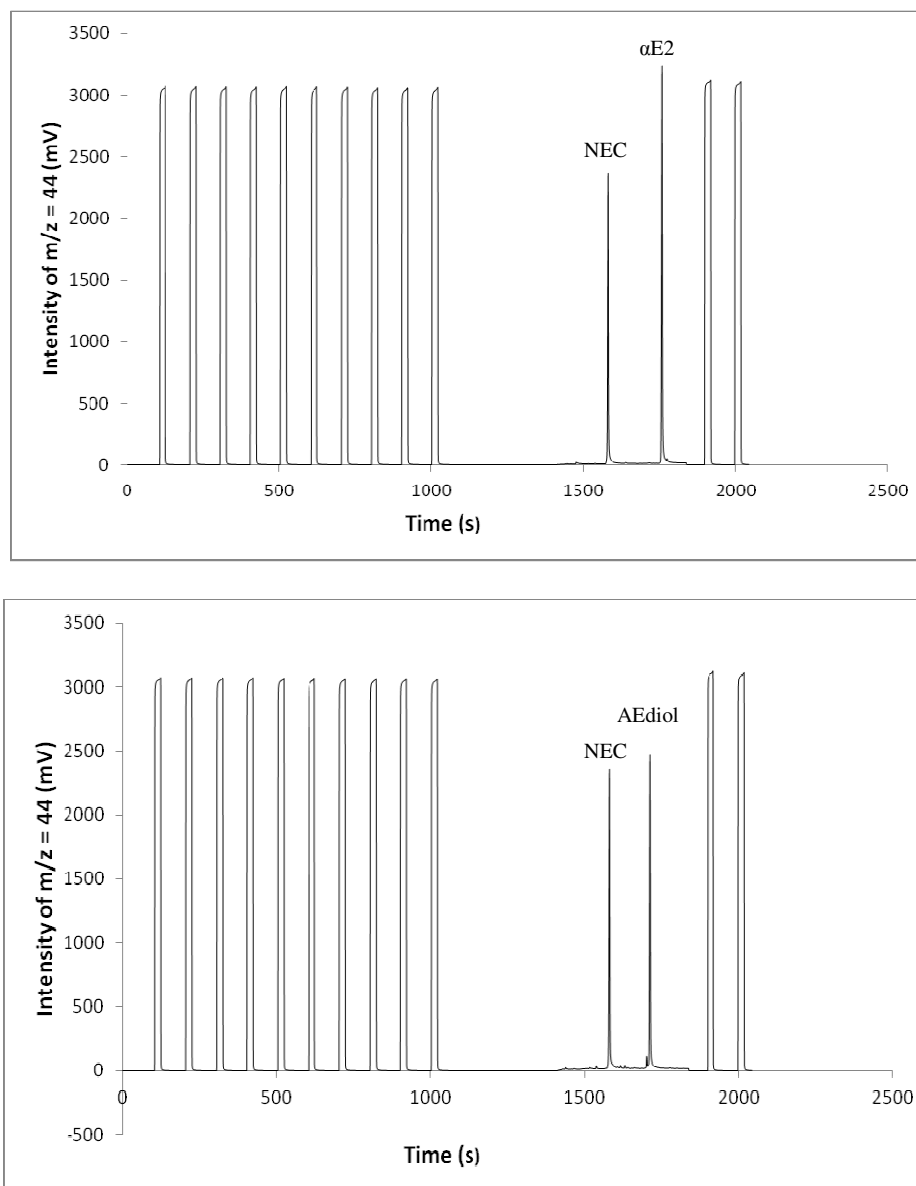
Figure 3.2. IRMS chromatograms (m/z = 44) of a positive bovine urine sample, showing the internal standard noretiocholanolone acetate (NEC), the metabolite 17α-estradiol diacetate (αE2) (above) and the ERC 5-androstene-3β,17α-diol (AEdiol) (below).

Third, by replacing the previously used splitless injection by programmed temperature vaporization (PTV)-injection, the required sample volume was successfully reduced from 20 mL to 10 mL. Finally, the separate GC-MS analysis, which was until now required prior to each GC-C-IRMS analysis of steroids, could be eliminated. By estimating the concentration of the analytes by means of UV-detection during the final HPLC step, the dilution factor of the sample could be successfully determined to obtain measurements well within the linear working range of the C-

59

IRMS-apparatus. Additionally, by means of the parallel coupled MS in the GC-(MS/C-IRMS) setup, the analytes could be successfully identified and controlled for purity simultaneous with the isotope ratio measurement, thus avoiding possible criticism on GC-C-IRMS that identification is not done during the same injection as isotope ratio measurement.[110] The resulting IRMS chromatograms of a urine sample of a treated animal after sample preparation are shown in figure 3.2. The rectangular peaks at the beginning and end of the chromatogram are pulses of the used CO_2 reference gas. The fifth CO_2 pulse is used by the software for the normalization of the measurements. The resulting chromatograms are clean and the analyte peaks are baseline separated and free of any coelutions, demonstrating the performance of the purification method. However, due to the necessary extensive sample preparation, the yield of the analytes is highly variable. The recovery of the sample preparation was calculated under reproducibility conditions at different concentrations, and ranged from 40% to 80% for 5-androstene-3β,17α-diol and from 40% to 76% for 17α-estradiol.

3.4.2. Method validation

GC-C-IRMS has been accepted as the confirmation method for the differentiation between endogenous steroid hormones and synthetic homologues. Still, official guidelines for the validation of IRMS analysis are currently lacking. However, the sample cleanup procedures remain complex, with many different purification steps involved. Since every cleanup step introduces a risk on isotopic fractionation, the robustness assessment was mandatory.

Linear working range

The range in which the isotope ratio mass spectrometer produces accurate measurements of $\delta^{13}C_{VPDB}$ values was determined by a series of injections of 17α–estradiol diacetate and 5-androstene-3β,17α-diol diacetate in increasing amounts, from 2.5 ng up to 80 ng on column, in sixfold at each level. To determine the linear range, a combination of three criteria was applied. First, the standard deviation (SD) of the repeated injection at each individual concentration level needs to be smaller than 0.5‰. Second, the difference between the lowest and highest measured

$\delta^{13}C_{VPDB}$ value needs to be smaller than 1.25‰. Third, the standard deviation over the entire range must be below 0.75‰ as well.

The injections of 5 ng, corresponding with peak intensities just below 500 mV, clearly show deviating $\delta^{13}C_{VPDB}$ values and a larger spreading of the results, hence the lower limit of the linear range lies between 500 mV and 1000 mV, corresponding with approximately 7 ng of the steroids on column. For the injection of 80 ng of 5-androstene-3β,17α-diol diacetate, lower $\delta^{13}C_{VPDB}$ values are observed, which might be due to peak fronting. The injection of 2.5 ng did not yield measurable results. Using the mentioned criteria, the compound specific linearity ranged from approximately 7 ng to 60 ng on column for AEdiol, and 7 ng to 80 ng on column for αE2, as can be seen in figure 3.3. The measured standard deviation within the linear domain were 0.23‰ and 0.13‰ for 5-androstene-3β,17α-diol diacetate and 17α–estradiol diacetate, respectively.

Intermediate precision or within lab reproducibility

To determine the intermediate precision, a blank urine sample was spiked with 5-androstene-3β,17α-diol and 17α-estradiol at 200 µg L^{-1}. The sample was divided into 12 subsamples which were analyzed by three different operators on three different dates, over a time span of two months. The first series consisted of 6 subsamples, the second series of 2 subsamples and the third series of 4 subsamples. The results are presented in table 3.1. The sample standard deviation (S.D.) (n=12) of the $\delta^{13}C_{VPDB}$ were 0.46‰ and 0.26‰ for 5-androstene-3β,17α-diol and 17α–estradiol, respectively. Standard deviations beneath 0.5‰ are considered acceptable.[117,123,172]

Isotope fractionation or method bias

The $\delta^{13}C_{VPDB}$ values after sample preparation of six water samples spiked at 200 µg L^{-1} with 5-androstene-3β,17α-diol and 17α–estradiol were compared with those of four standard injections, not subjected to sample preparation, to assess the isotope fractionation occurring during sample preparation. The mean difference between the $\delta^{13}C_{VPDB}$ values was 0.33‰ for 5-androstene-3β,17α-diol and 0.04‰ for 17α–estradiol. In comparison with results from the literature for a similar experiment with other steroids,[84] these differences are significantly lower than previously reported

values, leading to the conclusion that the isotope fractionation – if any – is acceptable.

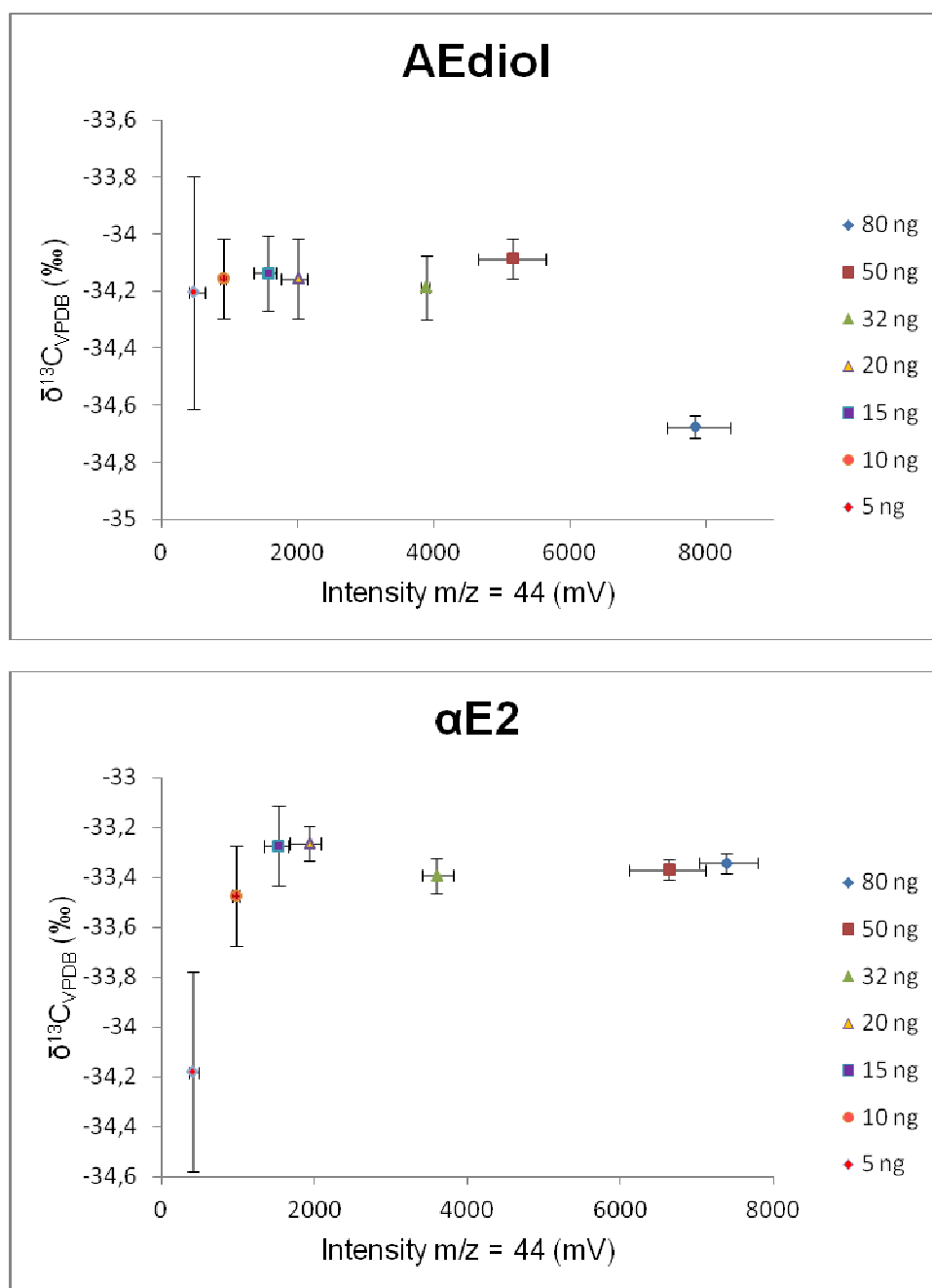**Figure 3.3.** $\delta^{13}C_{VPDB}$ values (expressed in ‰) of 17α-estradiol diacetate (αE2) (lower) and 5-androstene-3β,17α-diol diacetate (AEdiol) (upper) and the corresponding peak intensities (expressed in mV) for the injected amounts of 5 ng, 10 ng, 15 ng, 20 ng, 32 ng, 50 ng and 80 ng on column (six injections for each amount). Vertical error bars represent the SD (‰), horizontal error bars represent the range (mV).

Table 3.2. Intermediate precision of the $\delta^{13}C_{VPDB}$ values (expressed in ‰, not corrected for the acetate moiety) of a spiked urine sample [200 ppb of 17α-estradiol (αE2) and 5-androstene-3β,17α-diol (AEdiol)], analyzed on three different days**.**

	$\delta^{13}C_{VPDB}$ AEdiol (mean ± S.D.)(‰)	$\delta^{13}C_{VPDB}$ αE2 (mean ± S.D.)(‰)
Series 1 (n=6)	-32.86 ± 0.27	-31.53 ± 0.15
Series 2 (n=2)	-33.16 ± 0.01	-32.02 ± 0.24
Series 3 (n=4)	-33.75 ± 0.18	-31.92 ± 0.07
Mean (n=12)	-33.26	-31.82
S.D. (n=12)	0.46	0.26

Trueness or system bias

The trueness, or system bias, was evaluated by the injection of standards of dehydroepiandrosterone acetate (-31.9‰) and 17β-testosterone acetate (-30.3‰) with traceable and certified $\delta^{13}C_{VPDB}$ values, obtained through EA-IRMS analysis by an accredited laboratory. These standards were included into six separate series of measurement, and allowed to evaluate the system bias. The mean difference between the certified and the measured $\delta^{13}C_{VPDB}$ values was 0.88‰ and 0.25‰ for dehydroepiandrosterone acetate and 17β-testosterone acetate, respectively, resulting in an average system bias of 0.57‰.

Specificity

Ten reference standards, ten spiked water samples, sixteen spiked urine samples and fifteen urine samples collected from pregnant cows were analyzed according to the described method. For all samples, the identification of 5-androstene-3β,17α-diol and 17α–estradiol was done according to the legal criteria,[24] by comparison of retention time and the abundance of six fragment ion ratios of the analytes with those of a standard injected in the same series. This way, the four identification points required to identify Group A substances using mass spectrometry, as described in paragraph 1.2, are obtained. The typical MS-spectra of 5-androstene-3β,17α-diol and

17α–estradiol are depicted in figure 3.4. For all the samples, the analytes could be correctly identified and no impurities or coelutions were observed.

Complementary, the MS-data were evaluated using AMDIS-software. This program employs specific algorithms on the MS-data to detect interfering peaks hidden beneath others. Additionally, AMDIS compared the MS-data of the analytes with that of a standard injection in the same series, to provide a "Net Match"-factor and a "Purity"-factor for all the samples. The objective was to study if these "factors" could be used as a criterion for the evaluation of both the identity and the purity of the analytes. In the AMDIS-data, the "Net Match"-factor ranged from 92 to 100 and the "Purity"-factor from 88 to 97. For future analysis, the use of fragment ion abundance ratios will only be required if the "Net Match"-factor is below a threshold value of 92 and if the "Purity"-factor is below a threshold value of 88.
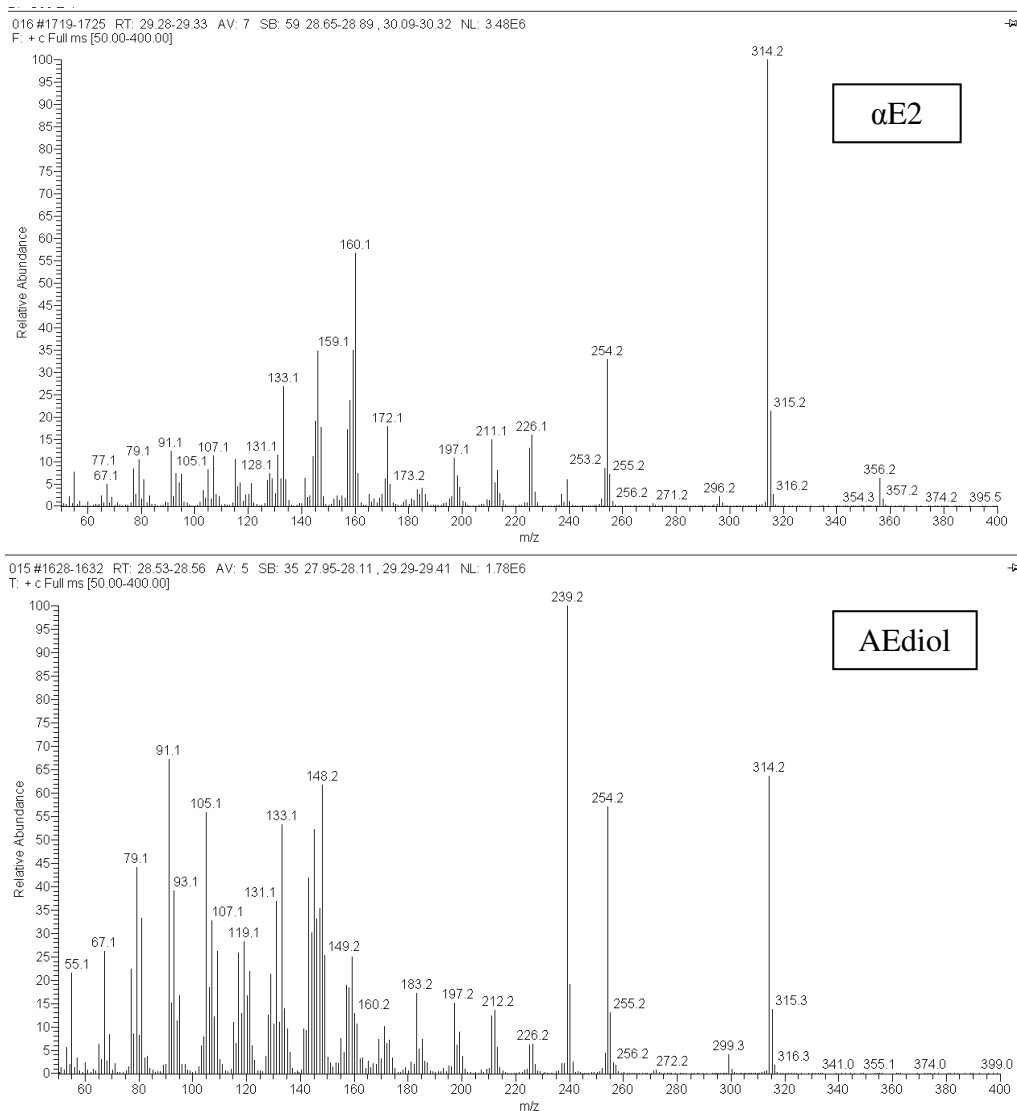
Figure 3.4. MS-spectra of 17α-estradiol diacetate (αE2) (above) and 5-androstene-3β,17α-diol (AEdiol) (below). Fragmentation was done by electron ionization with an ion source temperature of 250 °C. The data were produced in full scan mode (m/z 50 – 400).

Non-compliant threshold value

In the past, for doping control purposes, the World Anti Doping Agency (WADA) used a threshold value of $\Delta^{13}C$ >3‰ for non-compliant samples, but moved recently to a more detailed decision system, for specific ERC-metabolite couples, as discussed in chapter 2. However, 17α-estradiol is not of interest for anti-doping analyses, and for bovine samples, no official compliance criteria currently exist. To determine a suited threshold value for this specific application, $\Delta^{13}C_{VPDB}$ values were determined in a compliant control population of 29 pregnant cows. Pregnant cows' urine is preferred in this case, since the concentrations of 17α–estradiol in regular samples are often

too low for reliable measurement with C-IRMS. The data obtained from the samples of the compliant control population allowed to calculate a $\Delta^{13}C_{VPDB}$ threshold of 2.32‰ as the mean value plus three times the standard deviation (μ + 3 x SD). To add an additional safety margin, the applied threshold value above which samples are evaluated as non-compliant, was set at 3‰.

To verify the adequacy of the developed method and the determined threshold value, six urine samples collected from treated animals were analyzed according to the described procedure. The resulting $\delta^{13}C_{VPDB}$ values are presented in table 3.2. All $\Delta^{13}C$ values are above 14‰, clearly illustrating the potential of the method to detect positive samples, as well as the validity of the used threshold value.

Table 3.3. $\delta^{13}C_{VPDB}$ values (expressed in ‰) of 17α-estradiol and 5-androstene-3β,17α-diol in six non-compliant urine samples of bovines treated with 17β-estradiol.

Sample N°	$\delta^{13}C_{VPDB}$ AEdiol (‰)	$\delta^{13}C_{VPDB}$ αE2 (‰)	$\Delta^{13}C_{VPDB}$ (AEdiol- αE2) (‰)
1	-15.57	-30.23	14.66
2	-15.64	-30.15	14.51
3	-15.26	-29.99	14.73
4	-15.45	-30.24	14.79
5	-15.17	-30.11	14.94
6	-15.38	-30.08	14.70

3.4.3. MS detector influence on isotope ratio measurement

To evaluate the influence of the parallel coupled MS on the isotope ratio mass spectrometers' measurements, the experiment performed to determine the linear range, as described above, was repeated twice, once after uncoupling the mass spectrometer and again after recoupling. The results for 17α–estradiol diacetate and 5-androstene-3β,17α-diol diacetate of the three carbon isotope ratio measurement series are presented in figure 3.5. No differences in $\delta^{13}C_{VPDB}$ values between the three series, other than those caused by natural spreading of the results, could be observed. The repeatability of the standard injections at different concentrations without and with the MS coupled in the setup revealed neither significant differences in precision nor accuracy.
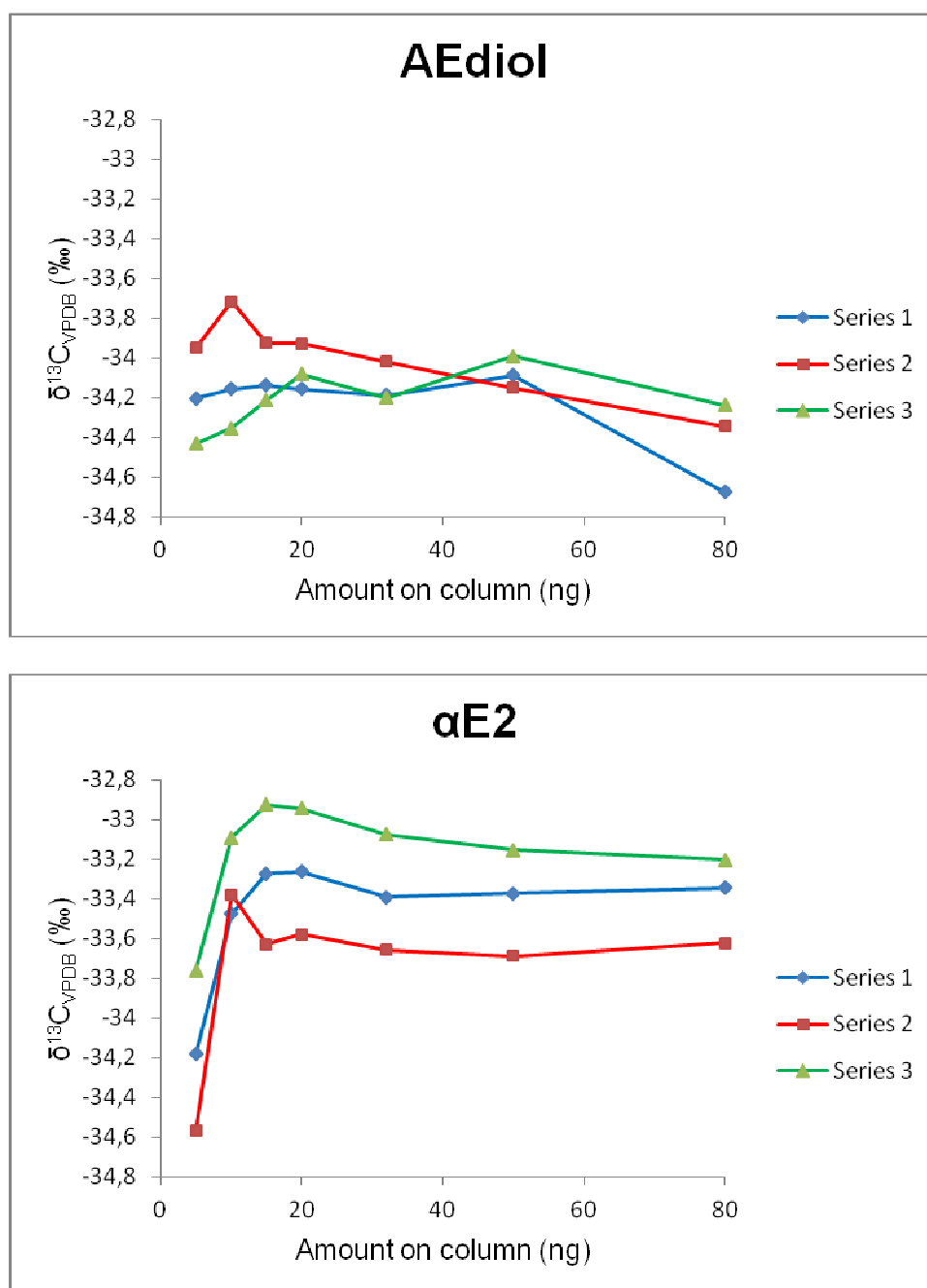
Figure 3.5. $\delta^{13}C_{VPDB}$ values (expressed in ‰) of 17α-estradiol diacetate (αE2) (lower) and 5-androstene-3β,17α-diol diacetate (AEdiol) (upper) and the corresponding amounts (expressed in ng) for the injected amounts of 5 ng, 10 ng, 15 ng, 20 ng, 32 ng, 50 ng and 80 ng on column (mean values of six injections for each amount). Series 1 and series 3 were performed with a GC-(MS/C-IRMS) setup, series 2 with a GC-C-IRMS setup.

Since the same sample amount is split between the MS and the IRMS in the GC-(MS/C-IRMS) setup, the measured peak intensities for the GC-C-IRMS setup generally were slightly higher. However, the difference is limited and causes no

significant disadvantages within the regular range of the measurements, between 10 ng and 40 ng on column, as illustrated in figure 3.6.
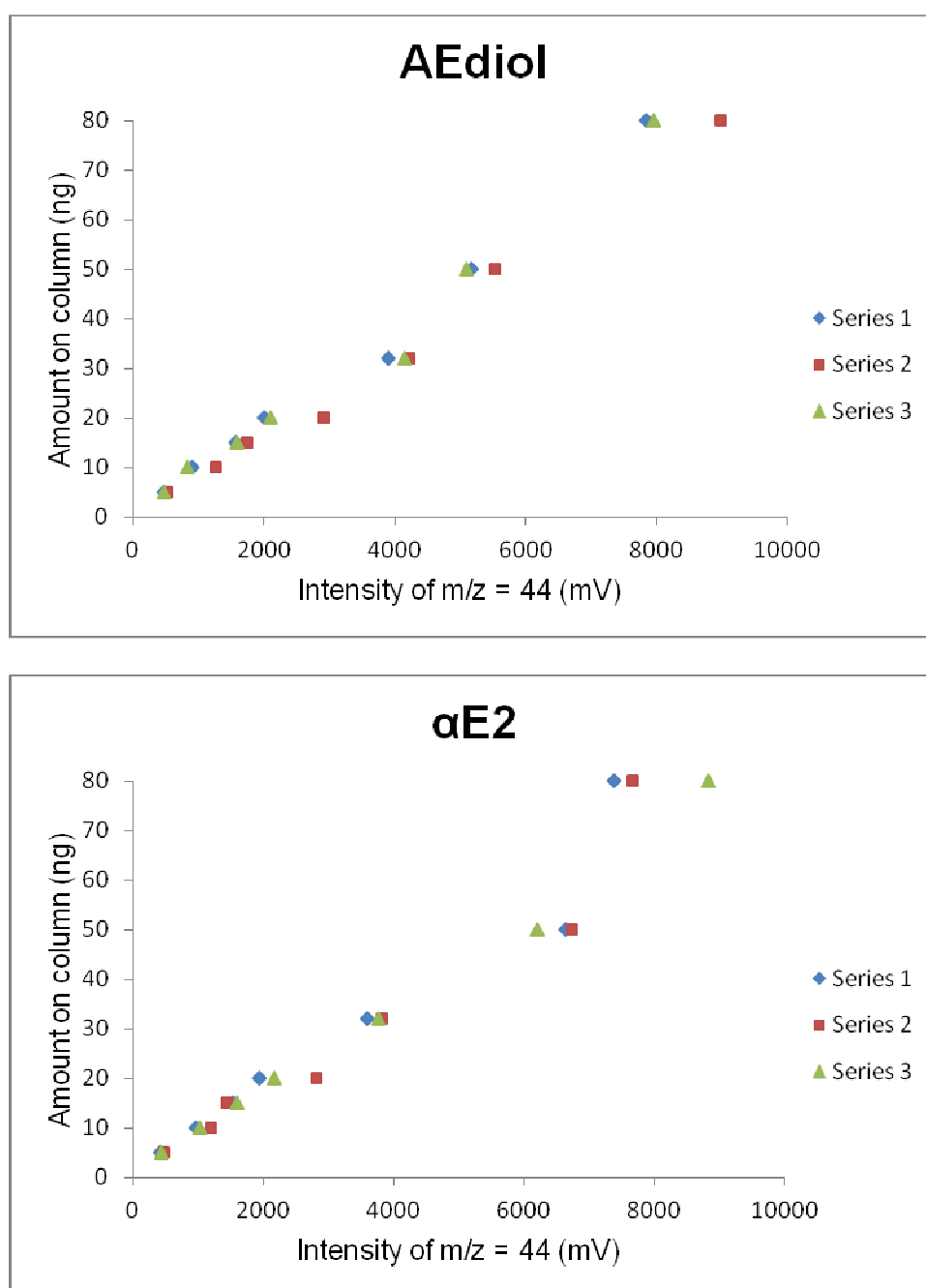
Figure 3.6. Average peak intensities (expressed in mV) and the corresponding amounts on column (expressed in ng) of 17α-estradiol diacetate (lower) (on the right) and 5-androstene-3β,17α-diol diacetate (AEdiol) (upper). Series 1 and series 3 were performed with a GC-(MS/C-IRMS) setup, series 2 with a GC-C-IRMS setup.

3.5. Conclusions

GC-(MS/C-IRMS) offers a powerful tool for the detection of steroid abuse. It is clear, however, that the described procedure needs to be extended in the future to include a number of additional metabolites and ERCs to allow simultaneous detection of abuse of a broader range of steroids. Still, analysis with GC-(MS/C-IRMS) is relatively complex, the sensibility of the apparatus is limited and the sample preparation remains laborious and time consuming, making the application for routine analyses limited to experienced laboratories. Therefore, adequate screening procedures need to be worked out to complement the C-IRMS confirmatory analyses. Publications on using profiles of direct metabolites of steroids for screening of steroid abuse indicate that applicable strategies will be available soon,[36,174,175] and research into the use of indirect biomarkers holds interesting promises for future application.[40,42,46,174-178] GC-MS and LC-MS analyses based on both targeted and untargeted profiling would provide rapid and powerful screening methods, to be used in combination with GC-(MS/C-IRMS) confirmation methods.

4. Simultaneous detection of androgen and estrogen abuse in bovines by gas chromatography-(mass spectrometry/combustion-isotope ratio mass spectrometry) (GC-(MS/C-IRMS)) evaluated against alternative methods

Adapted from Janssens G.; Mangelinckx S.; Courtheyn D.; De Kimpe N.; Matthijs B.; Le Bizec B. *J. Agric. Food Chem.* **2015**, *63*, 7574-7581.

4.1. Abstract

The administration of synthetic homologues of naturally occurring steroids can be demonstrated by measuring $^{13}C/^{12}C$ isotopic ratios of their urinary metabolites. Gas chromatography-(mass spectrometry/combustion-isotope ratio mass spectrometry) (GC-(MS/C-IRMS)) was used in this study to appraise in a global approach isotopic deviations of two 17β-testosterone metabolites (17α-testosterone and etiocholanolone) and one 17β-estradiol metabolite (17α-estradiol) together with those of 5-androstene-3β,17α-diol as endogenous reference compound (ERC). Intermediate precisions of 0.35‰, 1.05‰, 0.35‰ and 0.21‰, respectively, were observed (n=8). To assess the performance of the analytical method, a bull and a heifer were treated with 17β-testosterone propionate and 17β-estradiol-3-benzoate. The method permitted the demonstration of 17β-estradiol treatment up to 24 days. For 17β-testosterone treatment, the detection windows were 3 days and 24 days for the bull and the heifer, respectively. The potential of GC-(MS/C-IRMS) to demonstrate natural steroid abuse for urinary steroids was eventually compared to those of mass spectrometry (LC-MS/MS) when measuring intact steroid esters in blood and hair.

4.2. Introduction

The positive effects of steroids on weight gain and feed conversion are well known since the middle of the past century, after which they were readily used in stock

farming for fattening purposes and in sports for muscle building. Eventually, the use of these substances was restricted, and until today, remains prohibited as a growth promoter in the European Union.[22] Therefore, the abuse of steroid hormones is monitored in mandatory surveillance programs for food safety in all the EU member states.[23] For the official control laboratories, analytical guidelines and performance criteria are available, demanding that a positive screening result is followed by a confirmatory analysis using gas or liquid chromatography coupled to mass spectrometry (GC-MS or LC-MS).[24] However, using GC or LC-MS, the distinction between endogenous steroids, produced by the animals themselves, or synthetic homologues derived from them, which can be administered, is commonly difficult because they will be detected as structurally identical compounds. Also, an unambiguous assessment of the origin of the urinary metabolites based on their concentrations is hard to accomplish because of the large variations between individual animals.[16] Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was developed in the late 90's to offer an alternative to historical quantitative approaches.[64,67,101] Very recently, the European Union Reference Laboratory (EURL) advised to make use of GC-C-IRMS to demonstrate the origin of steroids in urine samples.[29]

Steroid preparations are usually synthesized from phytosterols originating from plant sources which are relatively enriched in ^{12}C, with carbon isotope ratios, expressed relative to Vienna Pee Dee Belemnite (VPDB) as $\delta^{13}C_{VPDB}$ values, usually lower than -30‰. In comparison, endogenously produced steroids have a $^{13}C/^{12}C$ ratio which reflects that of the mixture of plant materials in the feed, with $\delta^{13}C_{VPDB}$ values usually above -28‰. Because of the fact that it is difficult to set non-compliance $\delta^{13}C_{VPDB}$ threshold values due to large individual differences between animals, both endogenous reference compounds (ERCs) and metabolites of the targeted steroid hormones are measured. Since the ERC is not impacted by the steroid treatment, a substantial $\delta^{13}C$ difference between ERC and metabolite may be observed for a certain period of time post administration. The $\Delta^{13}C_{VPDB}$ (‰) is the cornerstone of the abuse demonstration. While this technique is nowadays mandatory for accredited WADA laboratories,[130,153] it is applied in the food safety arena to a minor extent.[172]

The first aim of the presented research was to develop a common analytical backbone able to provide carbon isotopic measurements for main metabolites of 17β-

testosterone (βT), namely etiocholanolone (Etio) and 17α-testosterone (αT), and of 17β-estradiol (βE2), namely 17α-estradiol (αE2), thus allowing the simultaneous detection of abuse of synthetic homologues of androgenic and estrogenic steroid hormones. The second aim was to apply the validated method for an experimental study involving treatment of a bull and a heifer, determining the detection window capability of the analytical approach and allowing a direct comparison with other strategies including the monitoring of steroid esters in blood.

4.3. Material and methods

4.3.1. Chemicals

All reagents and solvents were of analytical grade and were provided by Sigma-Aldrich (Bornem, Belgium). The solvents for liquid chromatography were of LC- and HPLC-grade from Biosolve (Valkenswaard, the Netherlands). 17β-Testosterone acetate was supplied by Sigma-Aldrich (Bornem, Belgium). Other steroids were obtained from Steraloids (Wilton, NY, USA). SPE C_{18} cartridges were provided by Achrom (Zulte, Belgium). *Escherichia coli* β-glucuronidase was from Roche Diagnostics GmbH (Mannheim, Germany). Dry pyridine and acetic anhydride were from Sigma-Aldrich (Bornem, Belgium).

4.3.2. Sample description

Animal experiment

One male and one female bovine (Belgian white-and-red breed), aged 22 and 19 months and weighing 310 kg and 269 kg, respectively, were used for an administration study. After arrival at the farm until the end of the experiment, they were fed with a typical feed concentrate (2.0 kg daily), which contained mainly C_3 plant material with added vitamins and minerals, and had access to dried grass, chopped corn and water *ad libitum*. It needs to be noted that the amount of chopped corn, consumed by the animals, was significantly larger than that of the feed concentrate and the dried grass. After 21 days, they were treated with a single intramuscular injection into the dorsal neck muscles, containing 1 mg kg^{-1} bw testosterone (administered as 1.194 mg kg^{-1} 17β-testosterone propionate (βTP)) and 0.2 mg kg^{-1} bw estradiol (administered as 0.276 mg kg^{-1} 17β-estradiol-3-benzoate

(βE2B)) as an oil based preparation. Urine samples were collected before and during the first 27 days after administration, and stored frozen at -21 ℃. When all the samples were collected, they were stored at -85 ℃ in a monitored temperature environment until assay.

The animal experiment was performed at the Faculty of Veterinary Medicine of Ghent University (B), in line with the guidelines of the ethical committee, with approval code EC2011/163.

Compliant samples

Eleven urine samples of non-treated pregnant cows were collected at different farms. Additionally, twelve urine samples from animals, *a priori* non-treated according to routine analyses, were added to obtain a sufficiently large reference population. The samples were stored at -85 ℃ in a monitored temperature environment until assay.

Spiked samples

A urine sample which contained low concentrations of AEdiol (12.4 µg L^{-1}), Etio (3.5 µg L^{-1}), αE2 (not present) and αT (0.45 µg L^{-1}), was spiked with these substances at 100 µg L^{-1} and divided into eight subsamples to be used during the method validation.

4.3.3. GC-(MS/C-IRMS) sample preparation and analysis

To provide adequate GC-(MS/C-IRMS) data, an extensive sample preparation is necessary to definitely avoid signal interferences that may generate inaccuracy in the ^{13}C/^{12}C measurement. After hydrolysis for 15 h with β-glucuronidase at 37 ℃ and pH 6.8, the urine samples, with a volume of 10 mL, are brought onto a C$_{18}$ solid phase extraction (SPE) column, conditioned with 6 mL of methanol and 6 mL of H$_2$O. Next, the column was washed with 6 mL H$_2$O and 5 mL H$_2$O/acetonitrile (ACN) (80/20; v/v), and finally the sample was eluted with 8 mL H$_2$O/ACN (10/90; v/v). Then, two subsequent liquid-liquid extractions (LLEs) with 4 mL n-pentane/diethyl ether (92.5:7.5; v/v) at different pH (pH 14 and pH 5.2) are performed to separate estrogenic and androgenic steroids. The extracts are further purified using a reversed phase HPLC-fractionation followed by a normal phase for particular fractions containing target compounds. The four obtained fractions containing the isolated

targeted analytes (αE2, AEdiol, Etio and αT) are eventually acetylated overnight at room temperature with pyridine and acetic anhydride before separate injection into the GC-(MS/C-IRMS), with noretiocholanolone being added as internal standard. Details of the sample preparation procedure and instrumental setup are described in a previous published work.[179] However, a limited number of adjustments, described below, have been introduced in the original analytical backbone to include a wider range of steroid metabolites, including those of 17β-testosterone.

Sample preparation

The volume of the glycerol solution, used as a keeper after the LLE steps, was reduced from 100 µL to 30 µL. Medroxyprogesterone replaced fluoxymesterone as the internal standard in the estrogen fraction during reversed phase HPLC. Finally, progesterone was used as internal standard during the normal phase HPLC step instead of medroxyprogesterone. Because of the weak UV-detector response for Etio, a progesterone standard was used instead to determine the adequate fractionation windows during the HPLC steps.

HPLC-UV

For the reversed phase HPLC, the C_{18} functionalized pre-column and analytical column were replaced by a Kinetex (Security Guard Ultra C18 for 2.1 mm ID) and a Kinetex C_{18} column (XB-C18; 250 mm x 4.6 mm; 5 µm), respectively. The chromatographic conditions were slightly altered: column temperature was changed from 40 ℃ to 50 ℃, flow rate from 0.6 mL min^{-1} to 0.8 mL min^{-1} and mobile phase composition from 37:63 to 35:65 (A:B; v/v), with H_2O/MeOH (95:5; v/v) as solvent A and MeOH as solvent B.

The normal phase HPLC-setup, using two diol functionalized columns in series (LiChrospher Diol; 250 mm × 4 mm; 5 µm) and a constant mobile phase of isooctane/isopropanol (85:15; v/v), was left unchanged.

GC-(MS/C-IRMS)

The T-pieces, used to split the sample after the GC-column to the mass spectrometer and isotope ratio mass spectrometer for simultaneous detection, were replaced by SilFlows ([TM] SGE Analytical Science). The RXI 5 SIL MS column (Restek −30 m; 0.25

mm i.d.; 0.25 µm df) GC-column was replaced by an Optima 17MS column (Machery Nagel – 30 m; 0.25 mm i.d.; 0.25 µm df). The injection and GC program were adapted accordingly. The initial injector temperature was set at 100 °C, which was held for 0.10 min with a vent flow of 20 mL min^{-1}. The temperature was raised at 8 °C min^{-1} and held at 320 °C for 2 min during sample transfer on column. The initial GC oven temperature was 110 °C, which was held for 1.5 min. The temperature was then subsequently raised to 220 °C at 30 °C min^{-1}, to 270 °C at 6 °C min^{-1} and to 300 °C at 2 °C min^{-1}. Finally, the temperature was raised to 330 °C at 50 °C min^{-1} and held for 3 min. The carrier gas was helium at a constant flow rate of 1.2 mL min^{-1}. The injection volume of 8 µL was left unchanged.

4.3.4. LC-MS/MS sample preparation and analysis

LC-MS/MS analysis

The quantitative analyses performed in this study were carried out on a Thermo Accela HPLC system coupled to a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer.

The LC was equipped with a C$_{18}$ column (Kinetex C18; 2.1 mm x 150 mm; 1.7 µm) at a temperature of 70 °C. Solvent A was prepared by adding 400 µL of acetic acid to 1600 g of MeOH. For the preparation of solvent B, 1600 g of H$_2$O was mixed with 320 g of MeOH and 400 µL of acetic acid. Initially, the mobile phase composition was set at 35:65 (A:B; v/v). Between 1 and 4 min, the composition was linearly increased to 50:50 (A:B; v/v). During the following minute, the composition changed to 75:25 (A:B; v/v). After a final rapid increase in 10 s, the mobile phase was held at 95:5 (A:B; v/v).

The triple quadrupole was operated in the electrospray ionization mode, and the signals were recorded in the selected reaction monitoring mode (SRM).

Prior to sample analysis, a solvent blank and two standard solutions containing 20 ng mL^{-1} of the analytes (αE2, αT, AEdiol and Etio) in H$_2$O/MeOH (56:44; v/v) were injected, all with added internal standard (1 ng mL^{-1} of 17β-testosterone-d$_2$ (Ds at position 16) and 40 ng mL^{-1} of 17β-estradiol-d$_3$ (Ds at position 16 and 17). This protocol was performed in order to calculate the one-point calibration curve covering 0 to 40 ng mL^{-1}.

Sample preparation

The sample preparation protocol used for this analysis, was very similar to the protocol used prior to GC-(MS/C-IRMS) described above, but adapted to a sample volume of 5 mL and omitting a number of steps. Since there was no need to isolate the different analytes for quantification, the alkaline LLE and the two HPLC-purifications were unnecessary. Also, there was no need to derivatize the compounds. Thus, the sample preparation consists of a hydrolysis with β-glucuronidase, followed by one SPE and one LLE purification step. Finally, the extract was reconstituted in 125 µL of MeOH/H_2O (80:20; v/v) of which 10 µL was injected into the LC-MS/MS. Samples were spiked at 2 ng mL^{-1} with 17β-testosterone-d_2 and at 80 ng mL^{-1} with 17β-estradiol-d_3 to serve as internal standard for the quantification of the androgens and estrogens, respectively.

4.3.5. Analysis of the preparation

The administered βE2B and βTP were dissolved separately in methanol to prepare standard solutions of 10 ng µL^{-1}. Next, 100 µL of each solution were hydrolyzed using 2 mL of 1 M NaOH in MeOH. After 15 min at 65 °C, 8 mL of 2 M formic acid in H_2O were added to stop the hydrolysis. Then, the samples were brought onto a SPE C_{18} column, washed with 6 mL of H_2O and eluted two times with 4 mL of MeOH. The eluate was evaporated to dryness under a nitrogen stream at 37 °C and reconstituted into 40 µL of MeOH and 70 µL of H_2O. Afterwards, a reversed phase HPLC fractionation and an acetylation step were performed identical to those used during the preparation of the urine samples. Finally, the samples were reconstituted into 80 µL of isooctane and analyzed with GC-(MS/C-IRMS).

4.4. Results and discussion

4.4.1. Analytical strategies used

GC-(MS/C-IRMS) method

The primary objective of the current research was to develop a method allowing the detection of βT abuse. Based on the literature,[35,67,100] Etio and αT were retained as diagnostic markers of βT administration. During the two HPLC steps, separate fractions for both metabolites were successfully collected, resulting in very pure extracts suitable for IRMS analysis, as illustrated in figure 4.1.

Finally, in combination with the already included fractions from a previous study,[179] the method allows for the simultaneous detection of estrogen and androgen abuse, with αE2 as metabolite of βE2 and AEdiol as endogenous reference compound (ERC) for both βE2 and βT. The extraction yield for αT and Etio was between 36 and 69%, and between 31 and 71%, respectively, which is very similar to the yield of AEdiol and αE2, previously published.[179]
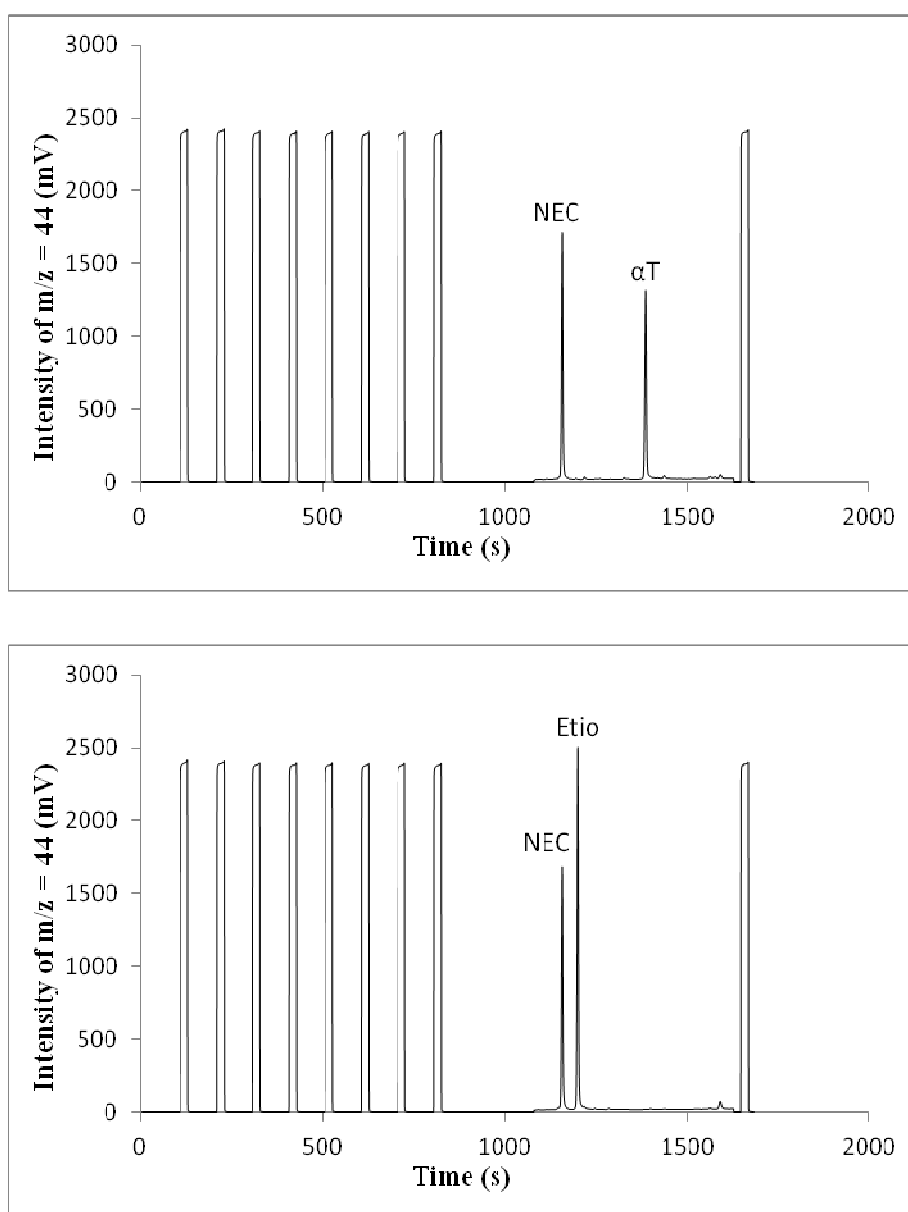

Figure 4.1. IRMS chromatograms (m/z = 44) of a compliant bovine urine sample, showing the internal standard noretiocholanolone acetate (NEC), 17α-testosterone (αT) (above) and etiocholanolone (Etio) (below).

HPLC-MS/MS method

While performing the analysis for the routine detection of estrogen abuse in 2013, it became clear that the majority of the received urine samples did not contain a sufficient quantity of αE2 to provide an IRMS measurement within the linear range. This is in line with findings from the literature, placing an average concentration of αE2 at around 6000 pg mL^{-1} for heifers, and less than 500 pg mL^{-1} for bulls.[32,73] Therefore, a quantitative screening method with a similar but severely simplified sample preparation was initially developed to assess which samples contained more than 20 ng mL^{-1}, necessary to provide IRMS-analysis within the linear range, filtering out large numbers of samples and avoiding unnecessary work.

Afterwards, when including the testosterone metabolites into the method, the quantitative screening gained a second functionality. Because of the low UV-absorption of Etio, it was not possible to determine the adequate dilution of this fraction for IRMS analysis based on the UV-detection during the straight phase HPLC fractionation, as it is done for the other analytes. However, it was possible to determine the dilution and provide measurement within the linear range based on the LC-MS/MS output.

Finally, it must be pointed out that, although this method provides accurate and reproducible results in the lower concentration range, a linear calibration curve only provided a perfect match ($R^2 > 0.99$) up to 50 ng mL^{-1}. Concentrations between 50 and 100 ng mL^{-1}, mentioned with regard to the animal experiment, should be considered as an approximation, whereas even higher concentrations must be interpreted as "elevated". Still, these data were found valuable to include.

Hydrolysis of the steroid preparations

The existence of steroid preparations having $\delta^{13}C_{VPDB}$ values similar to endogenous steroids has recently been reported for sports doping products.[88,180-182] To assure that the $\delta^{13}C_{VPDB}$ values of the preparations used during the animal experiment were not within the endogenous range, the preparations were analyzed prior to treatment. An alkaline hydrolysis protocol for preparations, based on present experience at the laboratory and similar to published methods,[180,182] was used. To confirm complete hydrolysis of the preparations and avoid any potential influence on the $\delta^{13}C_{VPDB}$ values, the absence of intact steroid esters was verified by UV-detection during the

HPLC-separation. The measured $\delta^{13}C_{VPDB}$ values, corrected for the acetate moieties, after hydrolysis were -32.13‰ and -31.70‰ for βE2 and βT, respectively, which is significantly different from endogenous values and should allow the detection of the administration.

4.4.2. Method validation

The method validation was performed similar to the previous study,[179] as described in chapter III, and the most important results are summarized in table 4.1 and figure 4.2.

Table 4.1. Summary of the method validation results of 5-androstene-3β,17α-diol (AEdiol), 17α-estradiol (αE2), 17α-testosterone (αT) and etiocholanolone (Etio)

Validation parameter	AEdiol (ERC)	αE2	αT	Etio
AMDIS 'Net Match' factor (specificity)	94	90	94	99
AMDIS 'Purity' factor	91	88	81	87
Linear range (ng of analyte on column) (min – max)	7.5 - 50	15 - 80	10 - 80	10 - 80
Intermediate precision (‰) (sample standard deviation (n=8))	0.21	0.35	0.35	1.05
Mean difference between spiked water samples and standards (method bias) (‰) (n=8)	0.18	0.66	0.33	0.05
Trueness (average system bias) (‰)	0.57	0.57	0.57	0.57
Combined measurement uncertainty (%)	0.70	0.99	0.80	1.23

The validation results for AEdiol and αE2 were comparable to those obtained during the previous study, and for αT, similar results were obtained. For Etio, however, two results stand out. First, the intermediate precision was noticeably higher than for the other compounds. Still, similar and even higher values have been considered acceptable both in published methods for sports doping control,[123,125] and lifestock control,[84,113] although reported for other steroids or matrices. For the determination of the intermediate precision during the initial validation, the eight spiked urine subsamples were analyzed two by two in four separate measurement series, performed by three different analysts over a time span of three months. To reevaluate the result for Etio afterwards, the measurements of the compliant control

80

sample for routine analyses, analyzed in 14 different measurement series over a time span of eight months, were assessed. The calculated intermediate precision based on these data (n=14) was 0.76‰, which is significantly better.

Second, the non-compliance $\Delta^{13}C_{VPDB}$ threshold value, based on 21 measurements from untreated cows, executed in five series of measurement over a time span of four months, and calculated as the mean value plus three times the standard deviation (µ + 3 x SD), as described in chapter III, was 3.47‰. This is above the 3‰ threshold value applied for doping control in the past, as is displayed in figure 4.2. Remarkably, similar observations have been made with regard to sports doping control, where a number of reference population studies indicated that a 3‰ threshold is too low in certain cases.[123,125] Because of these observations, the new guidelines of the World Anti Doping Agency (WADA) describe a more detailed list of compliance criteria, with different combinations of threshold values for specific ERC-metabolite combinations including an elevated threshold for the metabolite Etio. Additionally, each laboratory for sports doping control must perform a reference population study to determine if their performance is good enough to apply these criteria.[153] The presented findings suggest similar precaution must be used for the analysis of bovine urine. Therefore, the applied threshold for routine analysis was fixed at 3‰ for the couples αE2-AEdiol and αT-AEdiol, and at 4‰ for Etio-AEdiol.
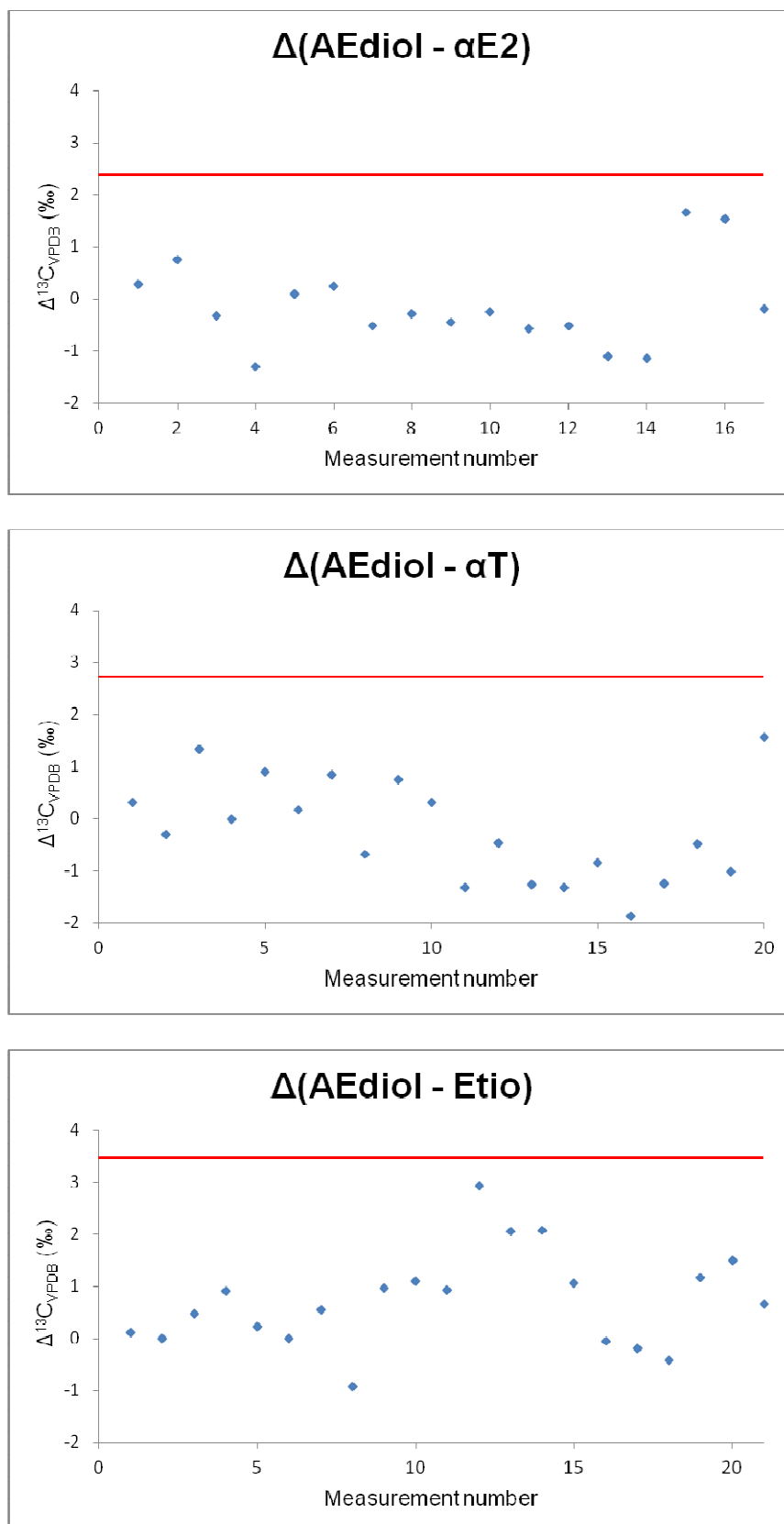
Figure 4.2. $\Delta^{13}C_{VPDB}$ values (expressed in ‰) of 17α-estradiol (αE2) (upper), 17α-testosterone (αT) (middle) and etiocholanolone (Etio) (lower), with 5-androstene-3β,17α-diol (AEdiol) as endogenous reference compound, from a compliant control population

Specificity of the method was verified according to legal criteria,[24] identical as described in paragraph 3.4.2, and the absence of impurities and coelutions was verified by inspection of the MS data. Additionally, AMDIS, specialized software to evaluate peak purity and identification of the analyte peaks, was used for this purpose.

No calculations of measurement uncertainty could be found in publications regarding the use of GC-C-IRMS to detect steroid abuse in cattle. Although information on this subject is scarce in publications regarding sports doping control as well, the work of Polet et al. pays detailed attention to the subject.[183] One of the suggested approaches is to estimate the combined measurement uncertainty as the square root of the sum of the squares of the different uncertainty components attributing to the measurement uncertainty, according to the following formula:

$$\sqrt{u(\text{calibration bias})^2 + u(\text{reference value})^2 + u(\text{method precision})^2}$$

This approach was used to estimate the combined measurement uncertainty for each analyte, of which the results are included in table 4.1. U(calibration bias) corresponds with the measured system bias, as calculated in chapter III, and u(method precision) corresponds with the intermediate precision, both also included in table 4.1. U(reference value) is the SD on the certified steroids used to perform the calibration, specified as 0.3‰ by the supplier on the certificate. Finally, since the method bias also contributes to the measurement uncertainty, it was included in the calculation as well.

4.4.3. Results of the animal experiment

An administration study was required to assess the potential of the developed method to detect steroid abuse in cattle, and to adequately evaluate the pharmacokinetics and the detection window after administration. Trying to observe gender differences, a male and female animal were treated. Prior to treatment, the diet of both animals was kept constant for three weeks, to assure stable $\delta^{13}C_{VPDB}$ values for the ERC during the experiment. Finally, the animals were treated with 400 mg of βT and 80 mg of βE2, injected as a propionate and benzoate ester,

respectively. While a typical approved dosage in the USA, administered by implant, is 20-45 mg per animal for βE2, and 140-200 mg for βT,[11] laboratory experience with the analysis of injection sites indicated dosages sometimes up to several hundred mg more. The selected treatment holds the middle ground between both scenarios. As other studies reported detection windows up to three weeks, as discussed in chapter II, samples were collected until 27 days after administration to be able to provide detailed pharmacokinetic data on the treatment.

Of course, the low number of treated animals is a seriously limiting factor. It is impossible to draw final conclusions regarding the influence of gender, based on measurements of only one male and one female animal. The same can be said regarding the determined detection windows: a much larger number of treated animals, and a variation of different treatments, are required to obtain statistically relevant data. Therefore, these results should be regarded as preliminary. However, they did allow to take an interesting glance at the examined parameters, and add important additional information on a subject for which only a limited number of animal studies have been published so far.

Effect of the treatment on measured $\delta^{13}C_{VPDB}$ values

The $\delta^{13}C_{VPDB}$ values of the four measured analytes in the urine samples after treatment are displayed in figure 4.3.

The measured $\delta^{13}C_{VPDB}$ values of AEdiol remain stable after treatment at -15.49 ± 0.49‰ and -14.80 ± 0.57‰ in the male and female, respectively, confirming the functionality as a reference compound for both androgens and estrogens, as already described elsewhere.[100,101] This enriched level for endogenous values fits well with the reported measurements from cattle fed with a diet containing a high percentage of maize.[100,101] As expected, the results suggest that gender does not influence the endogenous $^{13}C/^{12}C$ ratio of the ERC significantly.

Already the first day after treatment, the $\delta^{13}C_{VPDB}$ of the metabolites of both βE2 and βT approached their lowest values, contrary to previous testosterone administration studies, in which a more gradual decline over a time span of several days was described.[67,94,117] This difference can be contributed to the currently used preparation, 17β-testosterone propionate, which is known to have a more rapid and shorter lasting effect because of the short carbon chain length of the ester, as

opposed to 17β-testosterone enanthate, although influence of other factors such as age, weight or type of feed of the animals cannot be excluded.
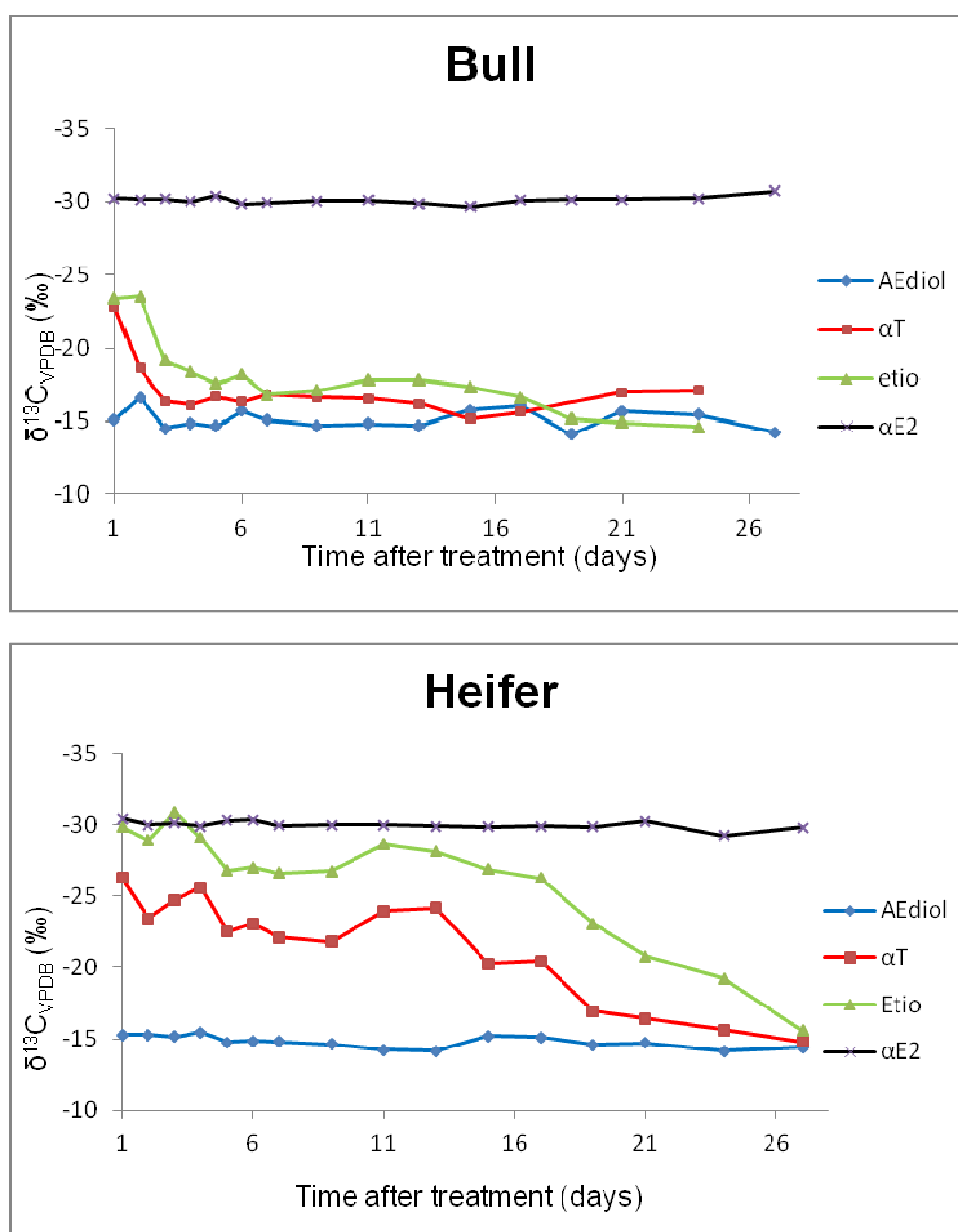
Figure 4.3. δ13C$_{VPDB}$ values (expressed in ‰) of 5-androstene-3β,17α-diol (AEdiol), 17α-estradiol (αE2), 17α-testosterone (αT) and etiocholanolone (Etio) in the urine samples from a bull (upper) and a heifer (lower) after treatment with 17β-testosterone propionate and 17β-estradiol benzoate.

The δ13C$_{VPDB}$ values of αE2 remain stable after injection, at -30.13 ± 0.24‰ and -29.90 ± 0.70‰ for the male and female, respectively. The small difference in value between the used preparation and the urine samples indicate a very low endogenous

production of estrogens and therefore, the treatment remained visible until the end of the experiment, 27 days after administration.

However, regarding the androgen metabolites, differences between both animals are more pronounced. The difference between the injected testosterone $\delta^{13}C_{VPDB}$ value and the more depleted $\delta^{13}C_{VPDB}$ value in the various female urine samples is close to zero for both Etio and αT. It would indicate that the contribution of the endogenous steroids in the female is insignificant versus the concentration of exogenous residues. For the male, the relative concentration contribution of the endogenous steroids is rather high as attested by the difference observed between $\delta^{13}C_{VPDB}$ values of the injected testosterone and the most depleted level of Etio found in the urine samples after treatment (6‰).

As a consequence, while βTP treatment could be proven up to 24 days after injection in the female, this was limited to three days for the male. The very small detection window for 17β-testosterone treatment forms a serious limitation when applying it to detect such abuse for official control purposes. However, since βE2 was proven to be a more powerful growth promoter in male animals than βT, both substances can be expected to be administered together to enhance the effects.[184] Still, the detection of βE2 administration has its boundaries as well. While endogenous dilution determines the detection window for the androgens, it is limited for the estrogens by their abundance in the urine samples (figure 4.4). While the results 27 days after administrations still indicate the administration in both animals, the measurements are just below the linear range of the IRMS and would have been discarded during routine analysis.

When evaluating the results for both testosterone metabolites, the carbon enrichment was prolonged in Etio, compared to αT for which the $^{13}C/^{12}C$ ratio returned to the endogenous value within two days after treatment in the bull, making it a less suitable metabolite in male animals.[100] In the heifer, however, the $\Delta^{13}C_{VPDB}$ value for αT remained above the threshold for up to 17 days, thus proving its usefulness.
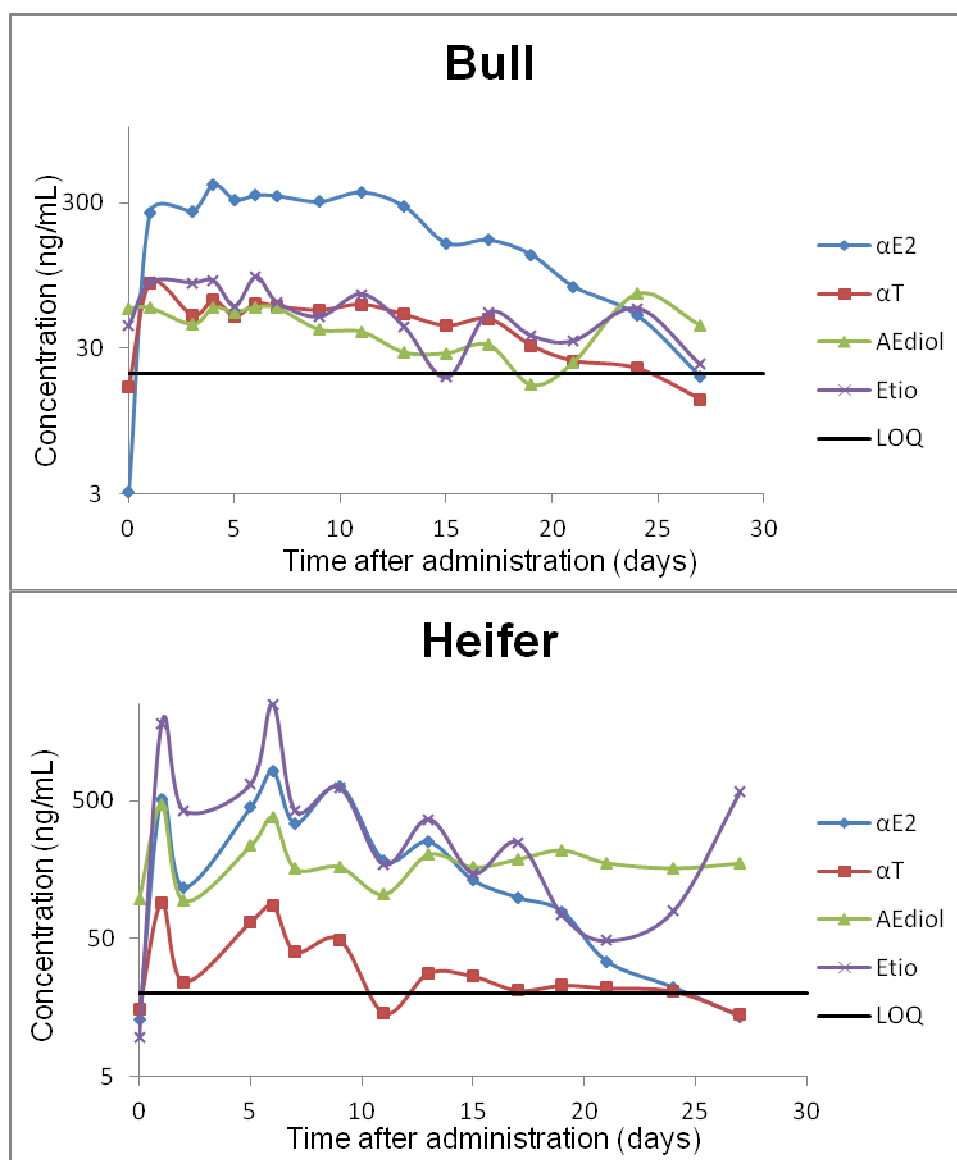
Figure 4.4. Concentrations (expressed in ng mL^{-1}), measured with LC-MS/MS, of 5-androstene-3β,17α-diol (AEdiol), 17α-estradiol (αE2), 17α-testosterone (αT) and etiocholanolone (Etio) in the urine samples from a bull (upper) and a heifer (lower) after treatment with 17β-testosterone propionate and 17β-estradiol benzoate.

Effect of the feeding on measured δ^{13}C$_{VPDB}$ values

After the animals were obtained, their feeding regime was kept constant during the entire experiment. The treatment was only performed after 21 days, to assure that the measurements of the ERC after treatment were stable and reflected the current diet. Urine samples were collected during this adaptation period as well, of which the results are displayed in figure 4.5. In the samples from the bull, an upward trend in measured δ^{13}C$_{VPDB}$ values is visible, which corresponds with the described effect of an enlarged portion of maize in the feed.[84,94] The values of both the ERC and the

87

metabolites shift simultaneously, with the $\Delta^{13}C_{VPDB}$ values never exceeding 0.89‰. However, this trend is not very pronounced, since apparently the previous diet already contained a significant portion of C_4 plant material, and for the heifer, this trend was only visible for AEdiol. Unfortunately, the four target analytes could not be measured simultaneously in any of the samples before treatment, and for αE2, none of the samples contained a sufficient amount to provide an adequate result.

It is clear that the feeding regime in this experiment created an ideal situation for the treatment to be detectable. As discussed in chapter II, as the portion of C_4 plant material in the feed decreases, the $\delta^{13}C_{VPDB}$ values of the ERC, AEdiol in this case, will decrease as well. Eventually, the difference in $\delta^{13}C_{VPDB}$ values between AEdiol and the administered substances will become so small, that it will no longer be possible to establish the treatment through the measured $\Delta^{13}C_{VPDB}$ values.

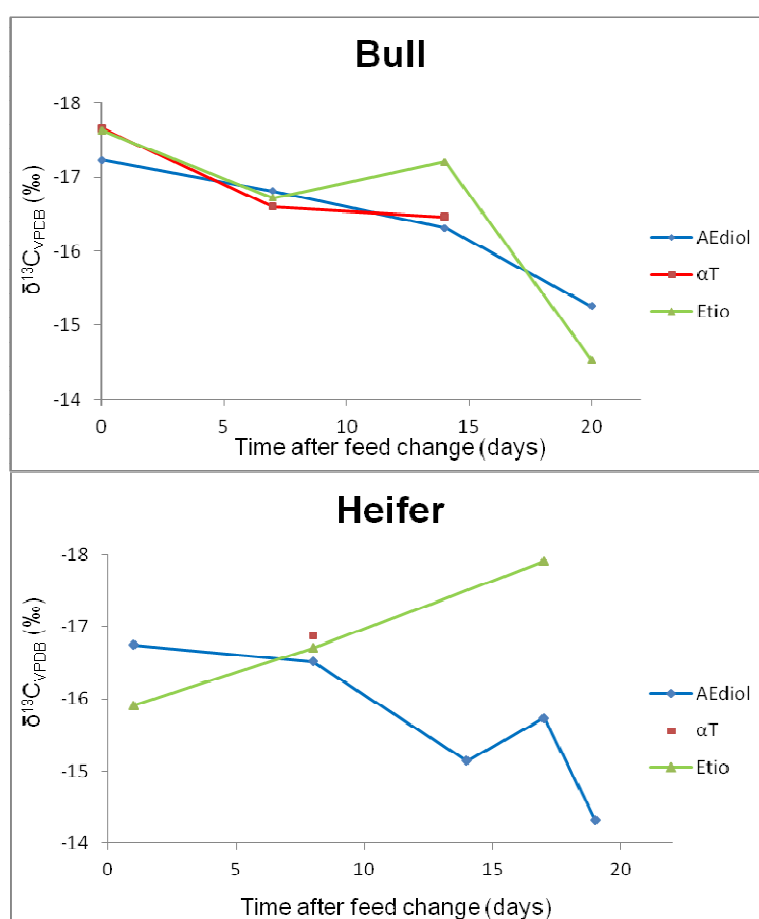**Figure 4.5**. $\delta^{13}C_{VPDB}$ values (expressed in ‰) of 5-androstene-3β,17α-diol (AEdiol), 17α-testosterone (αT) and etiocholanolone (Etio) in the urine samples from a bull (left) and a heifer (right) after changing the feed composition.

Comparison to other detection methods

As an alternative to the analysis of urine samples with IRMS, abuse of synthetic homologues of naturally occurring steroid hormones in cattle can also be proven by detection of the intact steroid esters, used for the treatment, in hair or blood samples, using other mass spectrometric techniques.[185] During the administration study, serum samples were collected at different times from both animals for the detection and quantification of βTP and βE2B using ultra high performance liquid chromatography-tandem mass spectrometry. The used method, as well as the obtained results from these and other treated animals, were discussed by Kaabia et al.[186] The amount of βE2B in the serum samples increases rapidly already one day after injection, and only starts decreasing between 9 and 13 days after injection in both animals. A good correspondence can be observed between the kinetic curve of βE2B in serum and the concentration of αE2 in the urine samples of both animals (figure 4.6). βE2B remained detectible up to 17 days after administration. Unfortunately, no βTP was detected in the samples from the bull. However, in samples from three other heifers which received a similar treatment, βTP remained detectable up to 2 days, indicating a high esterase activity.

Thus, the detection window for the administration of both substances was wider when using the GC-(MS/C-IRMS) approach, while the difference between both analyses was most pronounced when looking at testosterone abuse in the heifer, which could be detected up to 17 days longer compared to the analysis of the serum samples. However, it is expected that the IRMS detection windows will become narrower as the relative portion of C_3 plant material in the feed increases, whereas a dietary change would not affect the presence of the administered steroid esters in serum. Also, the analytical method for the analysis of the plasma samples is significantly less complex and time consuming than the sample preparation prior to IRMS analysis.
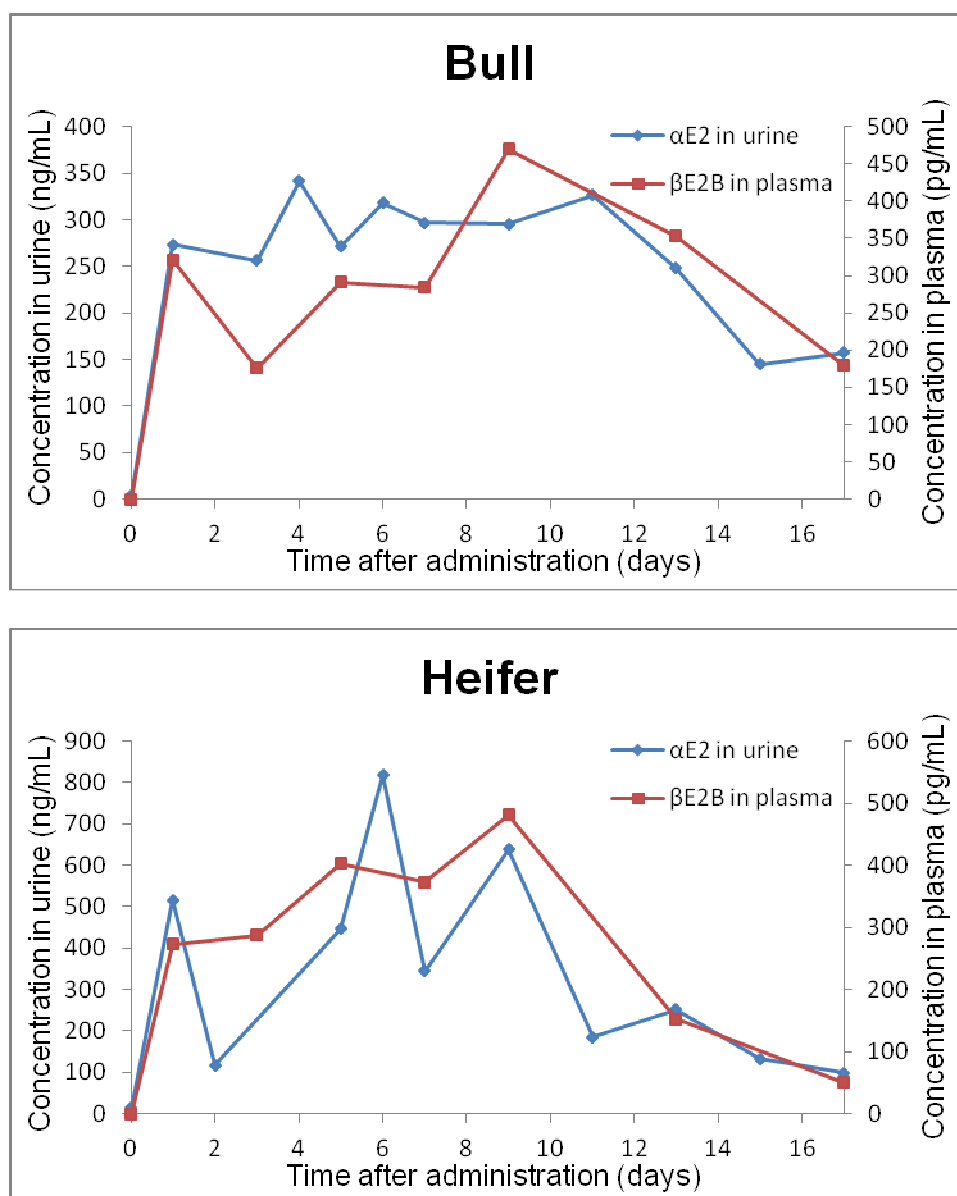
Figure 4.6. Concentrations of 17β-estradiol benzoate (βE2B) in plasma (expressed in pg mL^{-1}) and 17α-estradiol (αE2) in urine (expressed in ng mL^{-1}) from a bull (upper) and a heifer (lower) after treatment with 17β-testosterone propionate and 17β-estradiol-3-benzoate. Adapted from Kaabia et al.[186]

As mentioned above, a third way to undisputedly demonstrate the abuse of synthetic homologues of naturally occurring steroid hormones, is by detection of the intact steroid esters in hair. Unfortunately, no hair samples were analyzed during the current study. However, detection methods using GC-MS/MS and LC-MS/MS, and results from administration studies with esters of βT and βE2 are available.[185,187-189]

Regarding a single intramuscular βE2B administration, detection windows from two weeks up to 70 days have been reported.[185,187] Regarding βTP, no data could be produced on the successful detection of its incorporation into bovine hair after treatment, although this could have been due to the low dosage used in the study.[189] Nevertheless, in studies using other esters of testosterone, detection windows similar to those reported above for βE2B were observed, up to 70 days after treatment.[185,189] Even though this method offers great perspectives due to its large detection windows, the result is highly variable and influenced by additional factors such as pigmentation of the hair and sampling distance from the injection site. It is clear that GC-(MS/C-IRMS) is an excellent technique for the detection of abuse of homologues of naturally occurring steroids, and that the method presented is well functioning. However, the inclusion of additional ERCs would provide an even more powerful result, and since different steroids are often abused together, the addition of metabolites of other substances to the analysis would broaden the scope and detection possibilities. Still, as illustrated by this study, the technique is limited by both the abundance of steroids in urine samples and the required complex sample preparation. Therefore, this technique is complementary to the detection of steroid esters in blood, for which a far less laborious and time consuming sample preparation can be used, and in hair, which could offer a larger timeframe for detection.

5. The use of gas chromatography-(mass spectrometry/combustion-isotope ratio mass spectrometry) (GC-(MS/C-IRMS)) to demonstrate progesterone treatment in bovines

Adapted from Janssens G.; Mangelinckx S.; Courtheyn D.; De Kimpe N.; Matthijs B.; Le Bizec B. **2015**, submitted to *J. Chromatogr. A*.

5.1. Abstract

Currently, no analytical method is available to demonstrate progesterone administration in biological samples collected from rearing animals, and therefore, tracking the abuse of this popular growth promoter is arduous. In this study, a method is presented to reveal progesterone (PG) treatment on the basis of carbon isotope measurement of 5β-pregnane-3α,20α-diol (BAA-PD), a major PG metabolite excreted in bovine urine, by gas chromatography-(mass spectrometry/combustion-isotope ratio mass spectrometry) (GC-(MS/C-IRMS)). 5-Androstene-3β,17α-diol (AEdiol) is used as endogenous reference compound. Intermediate precisions (n=11) of 0.56‰ and 0.68‰ have been determined for AEdiol and BAA-PD, respectively. The analytical method was used for the very first time to successfully differentiate urine samples collected from treated and untreated animals. Unexpectedly, characterization of urine samples collected from animals treated with 17β-testosterone and 17β-estradiol indicated that the $^{13}C/^{12}C$ ratio of BAA-PD was affected by the treatment.

5.2. Introduction

As a measure to meet consumer's demands, the use of hormonal substances as growth promoter in stock farming is prohibited within the European Union.[22] Abuse of these substances is actively monitored,[23] for which specific analytical guidelines are in place.[24] While these surveillance programs are adequate for the detection of xenobiotic residues, they fall short when it comes to tracing administration of sexual
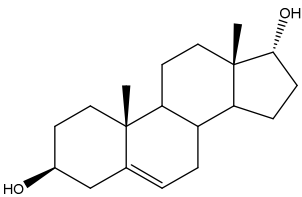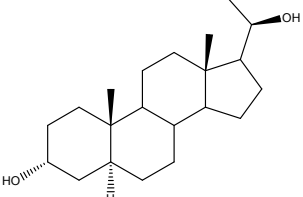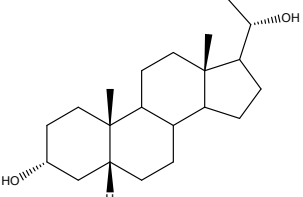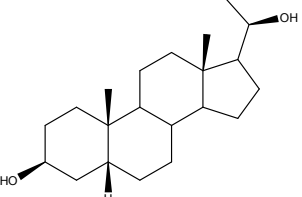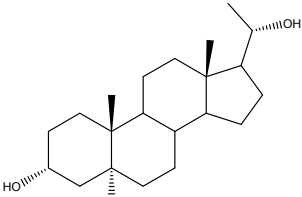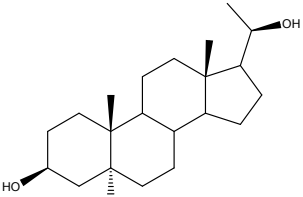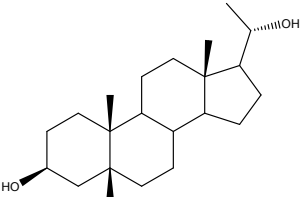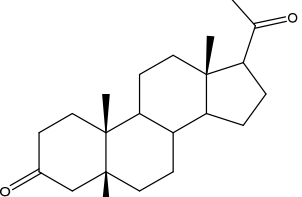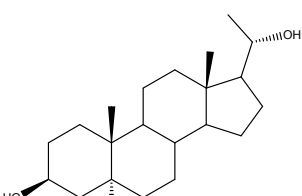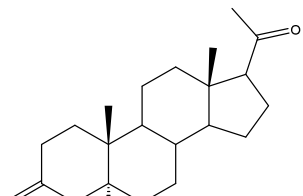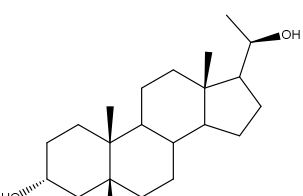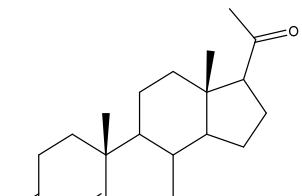
hormones in cattle. For the latter, two main analytical options are currently implemented by European Member States. First, residues of the administered steroid esters, which are considered xenobiotic, can be detected in blood, hair and injection sites for a certain period of time after administration using GC-MS or LC-MS based methods.[185-190] Second, because the $^{13}C/^{12}C$ isotope ratio, expressed relative to Vienna Pee Dee Belemnite (VPDB) as $\delta^{13}C_{VPDB}$ values, of steroid preparations is usually different from that of endogenously produced steroids, abuse can be detected in bovine urine samples using gas chromatography coupled to combustion-isotope ratio mass spectrometry (GC-C-IRMS). Because it is difficult to set threshold values which indicate the treatment, at least two analytes are measured with IRMS: an endogenous reference compound (ERC), of which the $\delta^{13}C_{VPDB}$ value is not affected by the steroid treatment, and a metabolite of the administered substance. When the difference between the $\delta^{13}C_{VPDB}$ values of the ERC and the metabolite, the $\Delta^{13}C_{VPDB}$ value, exceeds a threshold determined through the statistical evaluation of results of samples from a compliant population, the treatment is analytically and administratively demonstrated.[67,100,101,172,179,191]

Progesterone, however, remains largely overlooked by current analytical methods, despite indications of its abuse through results from material samples and preparations.[9,192] In 15% of the preparations, confiscated by the Belgian authorities at farms from 2010 until 2015 and found positive for hormonal substances, progesterone was found. In veterinary practice, progestagens are commonly used for reproductive performance in cows, regulating estrus and thus facilitating insemination at the farm. In itself, progesterone is a less potent growth promoter than androgens or estrogens, but still, in heifers, it can be used to eliminate heat to obtain better growth and improved feed conversion.[10] But more importantly, because of its anti-estrogenic activity, it is often administered together with estradiol, canceling out some of the unwanted side-effects from the latter.[10] In the USA, where regulated administration of certain growth promoters is permitted, both substances are combined in implants at 10 mg estradiol and 100 mg progesterone for calves, and twice this dosage for steers and bulls,[193] providing good growth promoting results.[12-14]

The aim of this study is to provide an original analytical method for the unambiguous demonstration of progesterone abuse in samples from animal origin. A

GC-(MS/C-IRMS) approach was developed on bovine urine according to a three-stage process. Suitable candidate target analytes were investigated; an overview is given in table 5.1 together with their chemical structures and abbreviations. Afterwards, two analytical protocols were designed and evaluated against each other. Eventually, the capacity of the most efficient protocol was validated before use on incurred urine samples collected from treated and untreated cattle.

Table 5.1. Overview of the steroids of interest in this chapter, the used abbreviations and the structures.

Structure Name Abbreviation	Structure Name Abbreviation	Structure Name Abbreviation	Structure Name Abbreviation
5-Androstene-3β,17α-diol AEdiol	5α-Pregnane-3α,20β-diol AAB-PD	5β-Pregnane-3α,20α-diol BAA-PD	5β-Pregnane-3β,20β-diol BBB-PD
5α-Pregnane-3α,20α-diol AAA-PD	5α-Pregnane-3β,20β-diol ABB-PD	5β-Pregnane-3β,20α-diol BBA-PD	5β-Pregnane-3,20-dione B-PDione
5α-Pregnane-3β,20α-diol ABA-PD	5α-Pregnane-3,20-dione A-PDione	5β-Pregnane-3α,20β-diol BAB-PD	Progesterone PG

5.3. Material and methods

5.3.1. Reagents and chemicals

All reagents and solvents were of analytical grade and were supplied by Sigma-Aldrich (Bornem, Belgium). Liquid chromatography solvents were of LC- and HPLC-grade from Biosolve (Valkenswaard, the Netherlands). 17β-Testosterone acetate was obtained from Sigma-Aldrich (Bornem, Belgium). Other used steroids were purchased from Steraloids (Wilton, NY, USA). SPE C_{18} cartridges were from Achrom (Zulte, Belgium). *Escherichia coli* K12 β-glucuronidase was provided by Roche Diagnostics GmbH (Mannheim, Germany). Dry pyridine and acetic anhydride were obtained from Sigma-Aldrich (Bornem, Belgium).

5.3.2. Samples

Non-compliant samples

To provide non-compliant samples, three female bovines were treated with progesterone (PG) at the Centre d'Economie Rurale (CER groupe) (Marloie, Belgium). The approval code from the ethical commission for the experiment was CE/Santé/ET/004. Cow A and B were of the Holstein breed, aged four years and weighing 565 kg and 529 kg, respectively. They received daily 2 kg of a typical feeding concentrate (corn (18.5%), dried sugar beet pulp (11.6%), soy meal (9.3%), palm-kernel expeller (9.2%), wheat meal (8.3%), colza meal (8.3%), alfalfa (7.6%), spelt wheat (7.0%), gluten feed from corn (5.0%), sunflower meal (4.0%), gluten feed from wheat (3.0%), barley (2.5%), vinasse (2.3%), calcium carbonate (1.6%), molashine (1.0%), sodium chloride (0.6%), premix (0.3%)), and had access to hay and water *at libitum*. The animals were treated with two injections, with a 24 h interval, into the dorsal neck muscle, containing 200 mg PG in an ethanol based preparation. Urine samples were collected at time intervals of 12 h, starting at the time of the first administration until 24 h after the last. The samples were stored at -85°C in a monitored temperature environment until assay.

Cow C, of the Belgian blue mixed breed, aged six years and weighing 624 kg, was kept on a stable feeding regime, consisting of dried grass and chopped corn, for two weeks before treatment. The animal received three injections into the dorsal neck muscle, with a 24 h interval, containing 1 g PG as an ethanol based preparation.

Urine samples were collected at the time of the treatments and 12 h thereafter. Afterwards, samples were collected daily until 1 week after the first treatment. The samples were stored at -85°C in a monitored temperature environment until assay.

Compliant samples

Eighteen urine samples from non-treated pregnant cows were collected at different farms. Additionally, 14 urine samples from animals, demonstrated as non-treated according to official routine analysis, were included to increase the individual number of the reference population (i.e. compliant). The samples were stored at -85°C in a monitored temperature environment until assay.

Spiked samples

A urine sample containing low concentrations of 5-androstene-3β,17α-diol (AEdiol) (2.7 µg L^{-1}) and exempt from 5β-pregnane-3α,20α-diol (BAA-PD), was spiked with these substances at 100 µg L^{-1}, and divided into 11 subsamples, respectively, to be further used during the method validation.

Samples from cattle treated with testosterone and estradiol

Additionally, urine samples from a bull and heifer treated with a single intramuscular injection of 17β-testosterone propionate and 17β-estradiol benzoate, presented and discussed in a previous publication,[191] were also used for this study.

5.3.3. Instrumentation

HPLC-UV

Two different HPLC setups were used during sample preparation. Reversed phase HPLC was performed on a Waters Alliance 2690 system, coupled to a UV-detector (diode array detector, DAD) measuring from 205 to 235 nm, and subsequently to an automated fraction collector. The system was equipped with a precolumn (Kinetex Security Guard Ultra C18 for 2.1 mm ID) and a C$_{18}$ functionalized analytical column (Kinetex XB-C18; 250 mm x 4.6 mm; 5 µm), held at 50°C. An HPLC method was

developed on the basis of a mobile phase composition (A:B; v/v) of 35:65, with H$_2$O/MeOH (95/5; v/v) as solvent A and MeOH as solvent B. After elution of the analytes, the mobile phase composition (A:B; v/v) was changed to 0:100 as a rinsing phase at the end of the run. A constant flow rate of 0.8 mL min^{-1} and an injection volume of 100 µL were used.

For normal phase HPLC, another Waters Alliance 2690 system, set up with UV detector, fraction collector and two diol functionalized columns (LiChrospher Diol; 250 mm x 4 mm; 5 µm) in series, was used. A constant mobile phase composition of isooctane/isopropanol (85/15; v/v) was preferred for method A and B, but for fraction PDC in method C, as described under 5.3.4, a constant composition of isooctane/isopropanol (95/5; v/v) was used, both with a rinsing phase at the end of the run. A flow rate of 1 mL min^{-1}, a column temperature of 40℃ and an injected volume of 100 µL were selected.

GC-(MS/C-IRMS)

For GC-(MS/C-IRMS) analyses, a Thermo Trace GC Ultra gas chromatograph, equipped with a Thermo Scientific Triplus autosampler, was connected to both a Thermo DSQ II single quadrupole mass spectrometer and a Thermo MAT 253 isotope ratio mass spectrometer, via the Thermo Scientific GC Isolink. To achieve the parallel coupling of the two detectors, the signal was split after GC using two SilFlows (^{TM} SGE Analytical Science). The system was equipped with an Optima 17MS column (Machery Nagel – 30 m; 0.25 mm i.d.; 0.25 µm df) and gas flows were controlled using the Thermo Scientific Conflo IV interface. Helium was used as carrier gas at a flow rate of 1.2 mL min^{-1}. After injection of 8 µL, at 20 µL s^{-1}, the injector temperature was held at 100℃ for 0.10 min, with a vent flow operating at 20 mL min^{-1}. Then, the temperature was increased at 8℃ min^{-1} up to 320℃ and held 2 min. The GC oven was held at its initial temperature of 110℃ for 1.5 min. Next, the temperature was raised to 220℃ at 30℃ min^{-1}, to 270℃ at 6℃ min^{-1}, to 300℃ at 2℃ min^{-1} and finally to 330℃ at 50℃ min^{-1}, which was held for 3 min. The transfer line temperature was set at 300℃. Approximately 5% of the GC eluate was transmitted to a quadrupole mass analyzer for characterization; the steroid signals were acquired in the MS full scan mode (*m/z* 50 to 400) after electron ionization (70

EV). The main split fraction was carried out to a combustion furnace, perfectly conditioned (1 h of oxidation before use) and maintained at 950 °C. Next, by passing through a Nafion membrane, water was removed from the gaseous eluate. Electron ionization was used to convert carbon dioxide (CO_2) into the corresponding ionized species characterized by an odd number of electrons ($CO_2+^\bullet$). Finally, isotopomer's species at *m/z* 44, 45 and 46 were simultaneously measured in separated Faraday cups. From these data, $\delta^{13}C_{VPDB}$ values of the analytes were calculated after calibration of the CO_2 reference gas, which was executed by injection of 17β-testosterone acetate and dehydroepiandrosterone (DHEA) acetate with known and certified $\delta^{13}C_{VPDB}$ values. When steroids were measured after acetylation, the shift of the $\delta^{13}C_{VPDB}$ value, caused by the acetate moieties, was corrected using the formula

$D_{OH} = D_{OAc} + 2m (D_{OAc} - D_{Ac})/n,$

in which D_{OH} is the $\delta^{13}C_{VPDB}$ value of the underivatized steroid, D_{OAc} the $\delta^{13}C_{VPDB}$ value of the acetylated steroids, D_{Ac} the $\delta^{13}C_{VPDB}$ value of the acetic anhydride used for the derivatisation, n the number of carbon atoms in the molecule and m the number of hydroxyl groups which were acetylated. [67,100,101,172,179,191]

5.3.4. Sample preparation

In this study, three different sample preparation procedures were used, method A, method B and method C, of which an overview is given in scheme 5.1. However, the analytical protocol prior to the preparative HPLC steps is identical for all three.

First, the urine samples were centrifuged (15 min, 3113 g) to avoid obstruction of the SPE cartridges later on. Ten milliliters of the sample were hydrolyzed at 37 °C, after addition of 50 µL glucuronidase from *E. coli* and 3 mL phosphate buffer 0.8 M to maintain a pH of 6.8. After 15 h, the sample was loaded onto a C_{18} SPE column, which was previously successively conditioned by 6 mL of MeOH and 6 mL of H_2O. After stationary phase washing with 6 mL of H_2O and 5 mL of H_2O/acetonitrile (ACN) (80/20; v/v), target analytes were eluted with 8 mL of H_2O/ACN (5/95; v/v). Next, the eluted sample was evaporated to approximately 300 µL under a nitrogen stream at 45 °C, before adding 0.5 mL of 1 M sodium hydroxide. Then, a liquid-liquid extraction (LLE) was performed using two times 4 mL of n-pentane/diethyl ether (92.5/7.5; v/v).
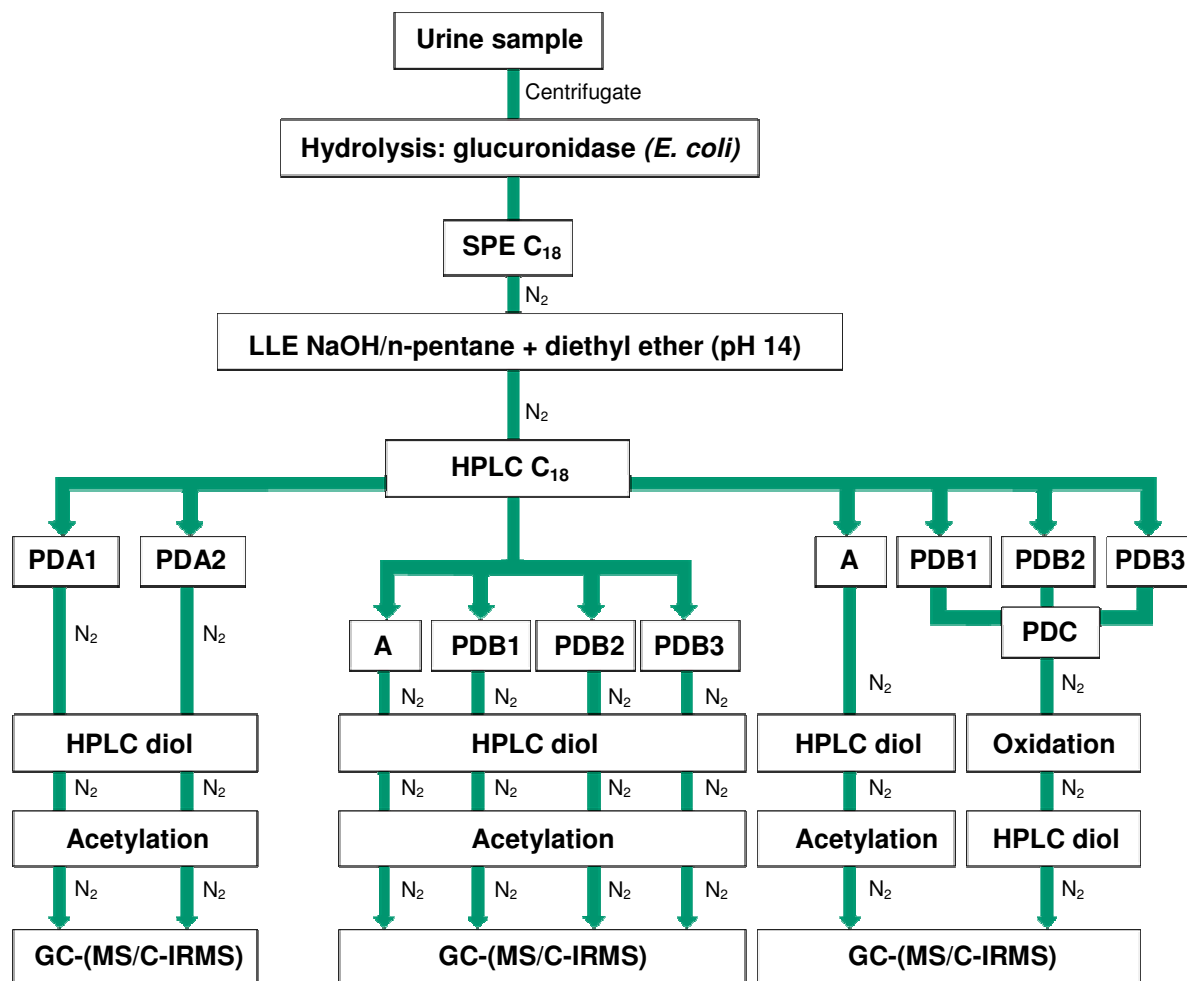
Scheme 5.1. Analytical protocol of the sample preparation for the three used methods, A, B and C.

Content of the fractions (PDA1; PDA2; PDB1; PDB2; PDB3; PDC; A) is defined in table 5.2 and table 5.3. LLE stands for liquid-liquid extraction.

Afterwards, 30 µL of a glycerol solution (10% in methanol) was added to the combined organic layers to serve as a keeper. The sample was evaporated at 37°C under a nitrogen stream, and reconstituted into 80 µL of H_2O and 40 µL of fluoxymesterone in methanol (50 ng/µL), used as internal standard specifically during the reversed phase chromatography step.

The collecting windows for targeted analytes on the reversed phase HPLC system were first determined through three successive injections of a standard solution containing fluoxymesterone, AEdiol, PG and testosterone propionate. The collected fractions for all three methods are defined in table 5.2.

Table 5.2. Description of the collected fractions using reversed phase HPLC.

	Method A	
Fraction	**start time – end time (min)**	**Collected analytes**
PDA1	14.30 – 18.00	ABA-PD; BBA-PD
PDA2	23.00 – 28.30	AAA-PD; AAB-PD; ABB-PD; BAA-PD; BAB-PD; BBB-PD
	Method B and C	
A	9.61 – 11.44	AEdiol
PDB1	15.30 – 18.00	BBA-PD
PDB2	23.30 – 26.30	BAA-PD; BBB-PD
PDB3	26.31 – 28.30	BAB-PD

For method A and B, 20 µL of a PG standard solution in ethanol (10 ng/µL) was added to serve as internal standard during the normal phase HPLC purification. Next, the fractions were evaporated to dryness under a nitrogen stream at 37°C and dissolved in 120 µL of isooctane/isopropanol (90:10; v/v) before injection in the second HPLC system, after calculation of the fractionation windows by a threefold standard injection containing PG and AEdiol. The fractions, collected during the normal phase HPLC purification as described in table 5.3, were evaporated to dryness under a nitrogen stream at 37°C, and 25 µL of both acetic anhydride and pyridine were added. After acetylation overnight at room temperature, the fractions were again evaporated to dryness under a nitrogen stream at 37°C. Finally, the fractions were dissolved in 30 µL of isooctane, containing 19-noretiocholanolone-3-acetate (4 ng/µL) as internal standard, before injection onto the GC-(MS/C-IRMS).

While method A and B only differ in the fractions collected during the HPLC steps, method C employs a different strategy. Fractions PDB1, PDB2 and PDB3, collected during reversed phase chromatography, were recombined and evaporated to dryness at 45°C under a nitrogen stream. The residue was dissolved in 50 µL of acetonitrile, and 50 µL of the oxidizing reagent, $K_2Cr_2O_7$ dissolved at 50 g/L in H_2O/H_2SO_4 (90:10; v/v), was added. After 30 min at 37°C, the oxidation was stopped by adding 900 µL of aqueous Na_2CO_3, at a concentration of 11 g/L. Then, the analytes were extracted using 1.5 mL of isooctane.

The extract was further washed with 250 µL of H_2O and after centrifugation for 3 min at 2300 g, the organic layer was isolated. For the normal phase HPLC purification, 20 µL of a PG standard solution in methanol (10 ng/µL) was added to the sample extract as the internal standard, before evaporation to dryness under a nitrogen stream at 37 °C. After being dissolved in 120 µL of isooctane/isopropanol (95:5; v/v), the extract was injected onto the second HPLC system, loaded with a diol-functionalized column. The fraction labeled "PDC", containing 5β-pregnanedione (B-PDione), was collected as specified in table 5.3. Finally, this oxidized fraction was evaporated to dryness under a nitrogen stream at 40 °C and dissolved in 30 µL of isooctane, containing noretiocholanolone acetate (4 ng/µL) as internal standard, before injection onto GC-(MS/C-IRMS).

Fraction "A", containing AEdiol, was treated as described in method B.

Table 5.3. Description of the collected fractions using normal phase HPLC.

Fraction	start time – end time (min)	Collected analytes
Method A		
PDA1	9.15 – 12.30	ABA-PD; BBA-PD
PDA2	8.65 – 13.30	AAA-PD; AAB-PD; ABB-PD; BAA-PD; BAB-PD; BBB-PD
Method B		
A	11.78 – 13.75	AEdiol
PDB1	9.15 – 11.80	BBA-PD
PDB2	11.15 – 13.30	BAA-PD
PDB3	9.65 – 12.80	BAB-PD
Method C		
A	11.78 – 13.75	AEdiol
PDC	8.15 – 10.80	B-PDione

5.4. Results and discussion

5.4.1. Selection of suitable reference compounds and metabolites

Because the use of IRMS to demonstrate PG treatment in cattle has not yet been described, the first step in the current study was the selection of suitable ERCs and metabolites. For the demonstration of abuse of estrogens and androgens in bovines, both dehydroepiandrosterone (DHEA) and 5-androstene-3β,17α-diol (AEdiol) have been reported as suitable ERCs.[100,101,179,191] Because DHEA is mainly present in urine as a sulpho-conjugate, AEdiol was retained in this study to avoid additional cleanup steps. Unfortunately, very limited information was available regarding the abundance of urinary PG metabolites in bovines, and post-administration data are nonexistent.[32,194] Because pregnanediol glucuronide was reported to be present in bovine urine as an early pregnancy indicator,[195] and since 5β-pregnane-3α,20α-diol (BAA-PD) is known to be a major PG metabolite in human urine, used for doping control purposes since 1999,[118] and currently the mandatory ERC by the World Anti-Doping Agency,[152] pregnanediols (PDs) were selected as candidate target metabolites.

First, endogenous occurrence of the eight PD isomers in urine samples from non-treated animals, 11 pregnant cows, which are expected to have a more active PG metabolism because of its functionality as a pregnancy regulating hormone,[195] and 14 non-pregnant cows, was assessed using method A described under 5.3.4. Although fraction PDA2, of which a chromatogram is shown in figure 5.1, contained too many impurities to determine adequate $\delta^{13}C_{VPDB}$ values, the comparison of the intensities of the different PDs was possible, and the results are visualized in figure 5.2.
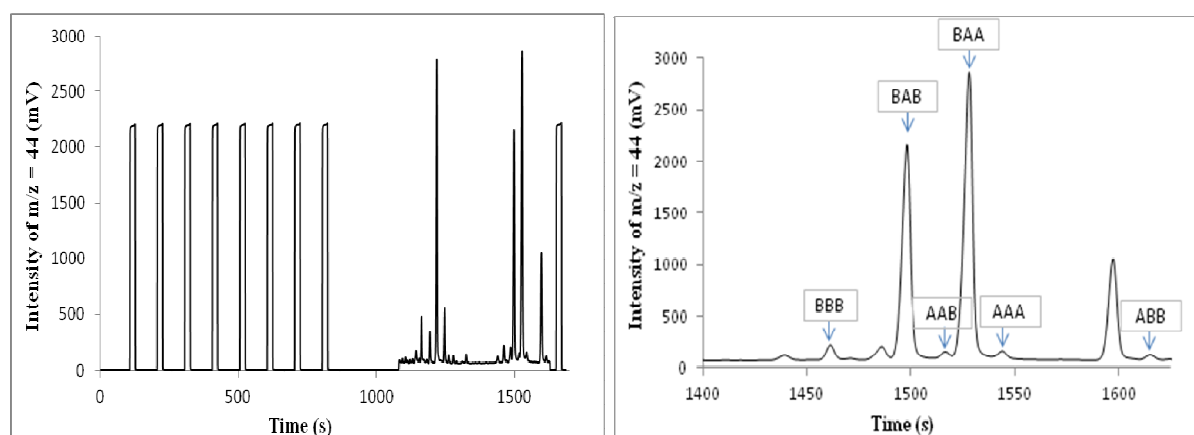
Figure 5.1. IRMS chromatogram (m/z = 44) of fraction PDA2 from a compliant urine sample (left), with a close up of the part containing the six pregnanediols (AAA-, BAA-, AAB-, ABB-, BAB- and BBB-PD) (right).
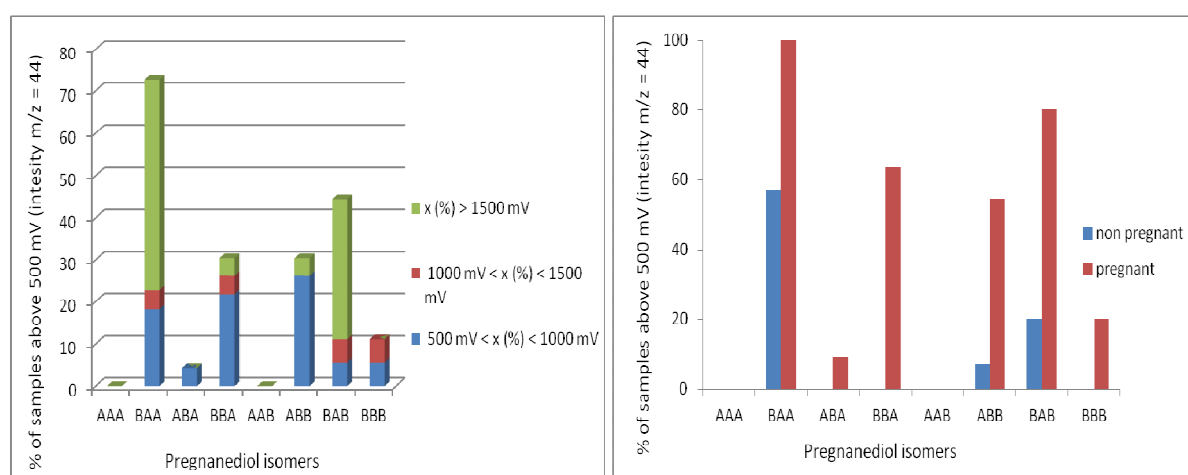
Figure 5.2. Overview of the abundances of the 8 different PD isomers in 25 urine samples from non-treated cows (left), and the difference between samples from pregnant and non-pregnant animals (right).

These indicate that overall, in the endogenous profile, the 5β-PDs are of larger importance than the less abundant 5α-isomers. More specifically, BAA-PD was the most abundant PD-isomer, followed by BAB-, BBA- and ABB-PD, which was later on confirmed by the analysis of the urine samples before and after treatment of cow A, B and C (figure 5.3).
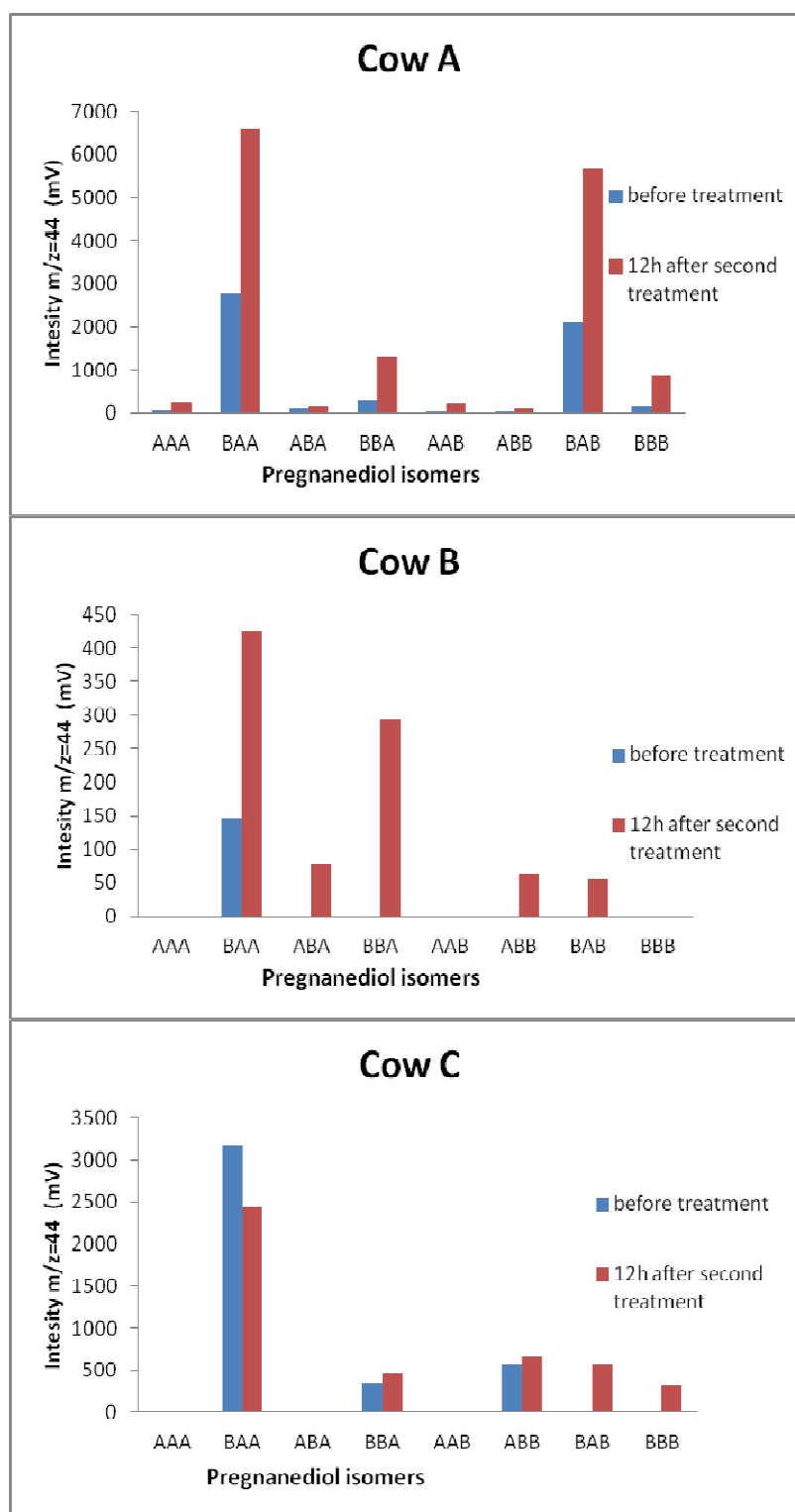
Figure 5.3. Overview of the abundance of the 8 different pregnanediol isomers in urine samples from cow A (upper), B (middle) and C (lower), before and after treatment with progesterone.

However, even though all results point towards the same main metabolites, significant differences between the animals were observed. Although the signal

intensities of the four most abundant PDs clearly increase in samples from cow B after treatment, they are still only present at a low level, most certainly when compared to cow A, in which only ABB-PD remains low in abundance. As for cow C, while being treated with a five times higher dose of PG than the other two animals, the PD-levels remain below those of cow A, with exception of ABB-PD. Finally, it needs to be added that the presence of progesterone in the samples from the treated animals was assessed as well, using the LC-MS/MS method described in a previous publication.[191] The concentrations were comparable with those described for routine samples from bovines, not exceeding 4 µg L^{-1} except for two samples.[77] In urine samples from cow C, elevated progesterone concentrations of 16 and 10.5 µg L^{-1} were measured 12 hours after the first treatment and 72 hours after the third treatment, respectively. Still, this indicated that the progesterone level in urine from treated animals is generally too low to provide accurate IRMS-measurements.

5.4.2. Development and evaluation of two analytical strategies

Method B: measuring 1 ERC and 3 metabolites after acetylation

Most published analytical methods for the detection of steroid abuse with IRMS combine hydrolysis, solid phase extraction (SPE), liquid-liquid extraction (LLE), preparative HPLC and acetylation steps in their sample preparation protocol.[130,172] For the development of method B, as described under 2.4, a similar analytical strategy was adopted for the measurement of AEdiol as ERC, and BAA-, BBA- and BAB-PD as metabolites of PG. A cleanup procedure for AEdiol was already developed,[191] and the described protocol prior to the HPLC steps, as well as the acetylation procedure, proved to be equally suitable for the PDs. To avoid losses during the LLE, the extraction volume of pentane/diethyl ether was raised from one time to two times 4 mL.

For the HPLC purification steps of the PDs, the fractionation windows were established by separate injections of the three PD standards, collecting fractions at regular intervals and evaluating their presence in those fractions using GC-MS. Although all analytes could be collected in separate fractions during reversed phase HPLC, the extracts contained too much co-extracted interferences to be interpretable by GC-(MS/C-IRMS). An additional normal phase HPLC purification of the separate fractions was used to overcome this problem, and allowed the successful

measurement of the $\delta^{13}C_{VPDB}$ values of all four targeted analytes after acetylation, as illustrated in figure 5.4.
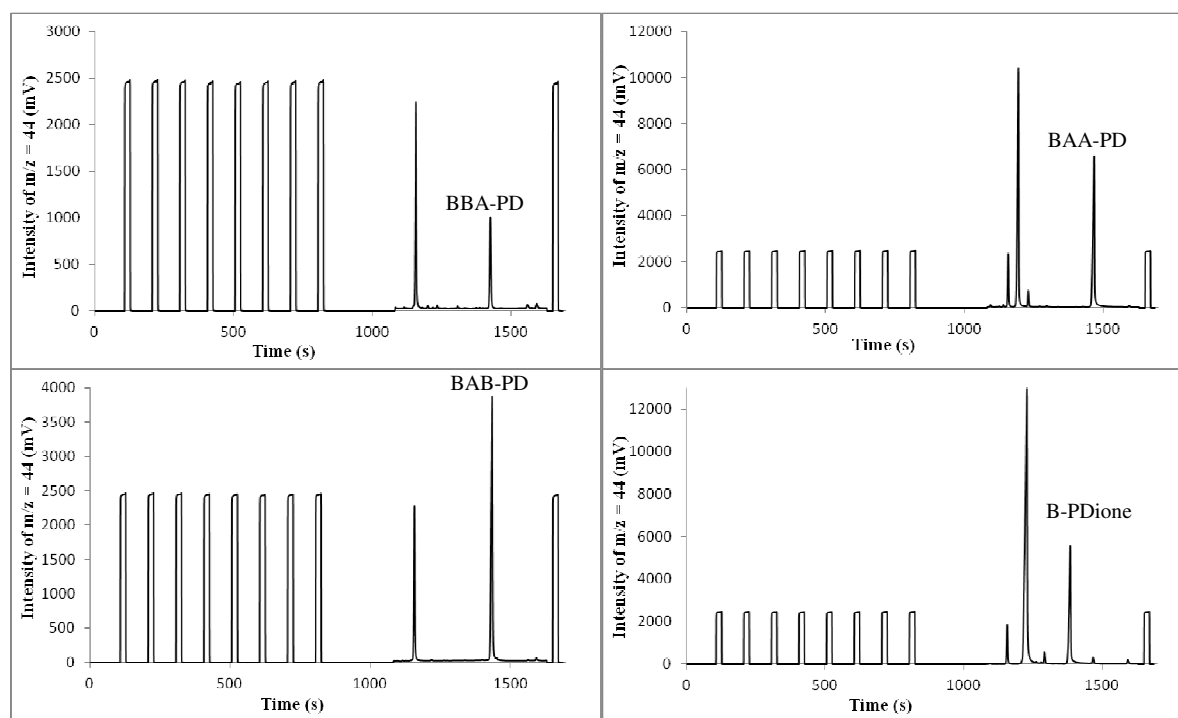
Figure 5.4. IRMS chromatograms (m/z = 44) of a compliant urine sample, showing the fraction containing BBA-PD (upper left), BAA-PD (upper right) and BAB-PD (lower left) obtained using method B, and the fraction containing B-PDione (lower right) obtained after oxidation of the PDs using method C.

Method C: measuring one ERC and the oxidation product of four metabolites

In an attempt to provide a simplified analytical strategy, an oxidation procedure was developed. The oxidation of the targeted analytes holds two important advantages. First, the oxidized steroids do not require a derivatization prior to GC-(MS/C-IRMS) analysis, and therefore eliminate the necessary correction of the $\delta^{13}C_{VPDB}$ values. Second, the eight PD-isomers are converted to two oxidation products, 5α- and 5β-pregnane-3,20-dione (A- and B-PDione, respectively), thus reducing the number of analytes to be investigated.

Since oxidation with $K_2Cr_2O_7$ proved to be effective for the analysis of corticosteroids with GC-C-IRMS,[129,196] the application of a similar procedure was considered relevant. The time optimum, at the given temperature and reagent concentration, was based upon the observations done on AAA-, BAA-, ABA- and BBA-PD. The oxidation was conducted at 2, 5, 10, 15, 20, 40 and 60 min whereas duration of the reaction was assessed from 5 min to 120 min. An optimum was found at 30 min; the absence of underivatized PDs was checked. An additional cleanup step relying on a normal phase HPLC separation was set up to isolate B-PDione. An illustration of an IRMS chromatogram is shown in figure 5.4.

Unexpectedly, the protocol was not suitable for the oxidation of AEdiol. The oxidation of the hydroxyl group at position 17 occurred very rapidly, and was almost complete after two minutes. However, an oxidation of the hydroxyl group at position 3 was not observed. Therefore, a sample preparation procedure for AEdiol identical to method B, using an acetylation, was preferred.

To compare the performance of both methods, six samples, three being collected from untreated bovines and three from a progesterone treated cow, were analyzed using both method B and C. The results, represented in figure 5.5, clearly indicate that both methods are capable of distinguishing between compliant (samples 1-3) and non-compliant samples (samples 4-6): for the non-compliant samples, the difference between the $\delta^{13}C_{VPDB}$ value of AEdiol (ERC) and that of the metabolites clearly exceeds the compliance threshold of 3‰ (described in 3.4.1., and validated for the application of progesterone under 5.4.4.). But more importantly, the $\delta^{13}C_{VPDB}$ values obtained with both methods are highly similar. Remarkably, even though four PDs contribute to the formation of B-PDione, its signal intensity was lower than that of BAA-PD in four of the six samples, and never significantly higher.
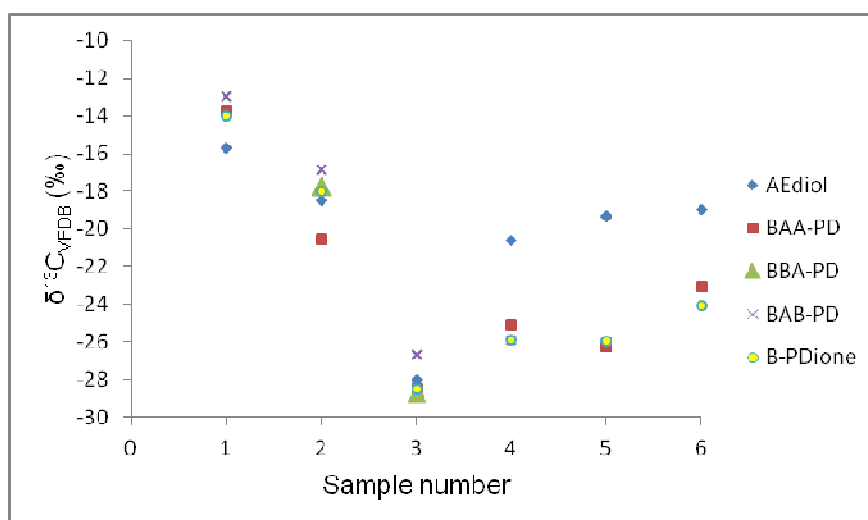
Figure 5.5. $\delta^{13}C_{VPDB}$ values of urine samples from untreated (sample 1-3) and treated (sample 4-6) cows, analyzed with both method B (AEdiol, BAA-, BBA- and BAB-PD) and method C (B-PDione). Measurements below the limit of quantification are not shown.

5.4.3. Method validation

Because BAA-PD was the PD-isomer giving the most intense IRMS signal in all the samples, and because the oxidation procedure eventually provided no added value, it was decided to validate the method based on the measurement of AEdiol as ERC and BAA-PD as metabolite, derived from method B.

The intermediate precision was determined by the measurement of the 11 subsamples of the spiked urine sample, distributed into 7 series of analysis, performed by three different operators over a time span of five months. The sample standard deviation (sd) (n=11) of the $\delta^{13}C_{VPDB}$ values were 0.56‰ and 0.68‰ for AEdiol and BAA-PD, respectively, which is acceptable and similar to published results for other steroids.[117,179,191]

To verify the absence of isotope fractionation during sample preparation, a water sample spiked at 100 µg L^{-1} with both AEdiol and BAA-PD, was included into the 7 series of analysis mentioned above. In each series, the difference between the $\delta^{13}C_{VPDB}$ values of the water sample and those of a standard injection was calculated. The mean of this difference was 0.20‰ and 0.27‰ for AEdiol and BAA-PD, respectively, indicating that the $\delta^{13}C_{VPDB}$ values are not altered in a significant way by the sample preparation.[84,179,191]

For all the spiked samples mentioned above, as well as for all the urine samples described under 5.3.2, the identification of AEdiol and BAA-PD was done according to internationally recognized analytical criteria,[24] by comparison of the abundance of six fragment ion ratios of the analytes with those of a reference standard injected in the same series, identically as discussed in paragraph 3.4.2. Identification of target analytes in every sample was checked, while verification of the absence of coeluting impurities permitted to document the method specificity. This was also verified using AMDIS®, a software application which compares the generated mass spectra from the samples to those of injected standards. It allows the automatic evaluation of the compound identity as well as peak purity. Based on the gathered data, the AMDIS® "Net Match" and "Purity" threshold values were set at 94 and 91, respectively, the lowest obtained values for both analytes, which still corresponded with manually verified correct mass spectra.

To determine the linear range of the IRMS for the analytes, standards were injected in sixfold at different concentrations, from 2.5 up to 80 ng on column. The same criteria as described in chapter III were used to determine the linear range. For AEdiol, the linear range was between 750 and 5500 mV, corresponding with 7 ng and 50 ng on column, respectively.[179,191] For BAA-PD, the measured $\delta^{13}C_{VPDB}$ values were very stable, with a standard deviation of 0.21‰, from approximately 12.5 ng up to 80 ng on column, corresponding to signals from 500 up to 4000 mV (m/z=44), which is similar to previously reported values for androgens and estrogens.[179,191]

Finally, the extraction yield of the method is highly comparable with that of the androgens and estrogens, discussed in paragraph 3.4.1 and 4.4.1, also corresponding with an LOQ of 20 ng mL^{-1}.

5.4.4. Results from treated and untreated cattle

Results from a compliant control population

29 samples from non-treated cows were successfully analyzed using the validated method, of which the results are depicted in figure 5.6. From these results, the threshold $\Delta^{13}C_{VPDB}$ value for compliancy for the ERC - metabolite couple AEdiol - BAA-PD was calculated, as the mean value plus three times the standard deviation.[152] The obtained value was 2.90‰, and therefore, a value of 3‰ could be suggested as a threshold for the presented method. Finally, the low mean $\Delta^{13}C_{VPDB}$

value of 0.24‰ indicates that the additional carbon atoms, at position 20 and 21 of the progestagens, originate from a source with the same $^{13}C/^{12}C$ ratio when compared to androgens or estrogens.
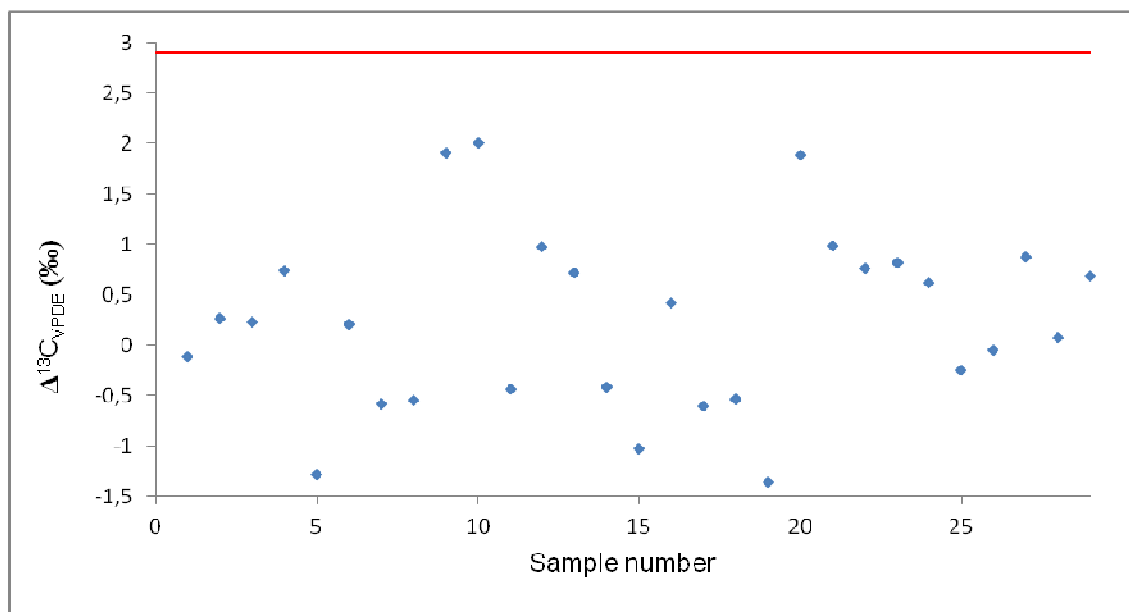
Figure 5.6. $\Delta^{13}C_{VPDB}$ values of urine samples issued from a compliant control population, with AEdiol as ERC and BAA-PD as metabolite of PG. The red line marks the compliance threshold (mean + 3 x SD).

Results from progesterone treated animals

The urine samples from the three cows treated with progesterone were analyzed, and the obtained results are summarized in figure 5.7 and 5.8.
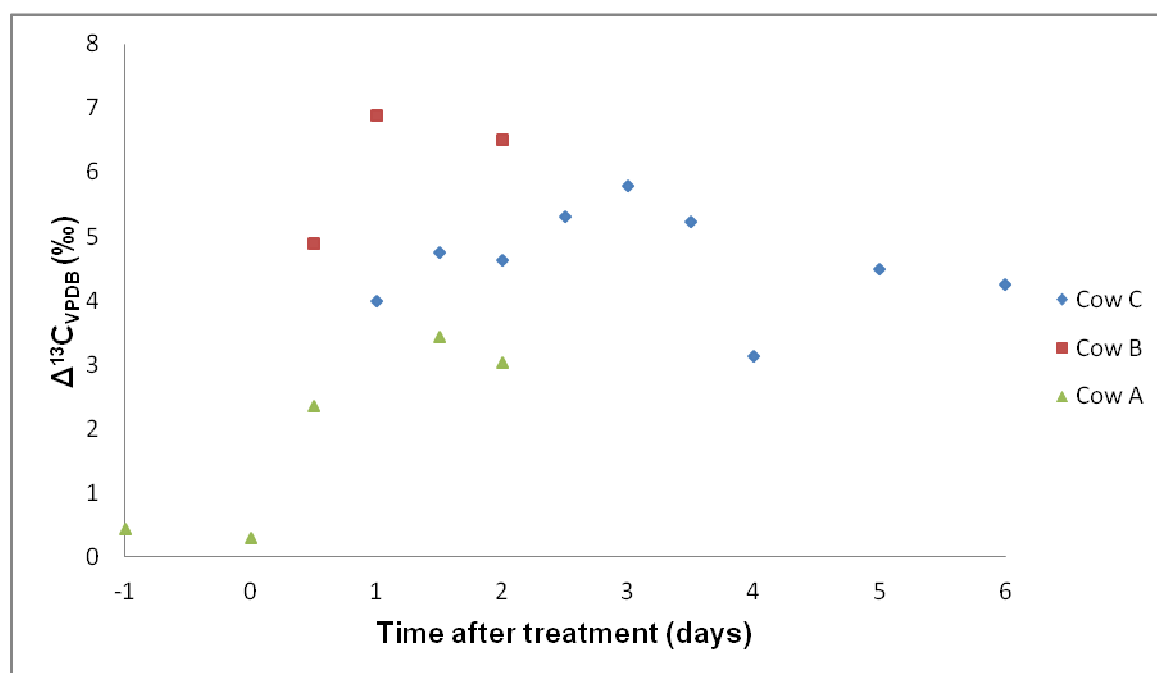
Figure 5.7. $\Delta^{13}C_{VPDB}$ values of urine samples from 3 cows, treated with PG at day 0 and 1 for cow A and B, and at day 0, 1 and 2 for cow C, with AEdiol as ERC and BAA-PD as metabolite. Measurements below the limit of quantification are not shown.

The method developed allowed confirmation of the treatment of all three animals, based on the threshold determined from the compliant control population. However, for cow A, this was only possible after the second intramuscular injection. This can be explained by the higher presence of PDs in this animal before the treatment, as illustrated in figure 5.3, causing a significant endogenous dilution of the measured BAA-PD 12 hours after the first treatment. Remarkably, 24 hours after the first treatment, all pregnanediols were below the limit of quantification, being the lowest concentration still providing a sufficient IRMS signal intensity (20 µg L^{-1}), in the same animal. As for cow C, even though injected with a higher dose of PG, the BAA-PD level only allowed IRMS-measurement and distinction from the control population after 24 h, while for cow B, this was already possible after 12 h. The difference between the treated animals and the control population remained observable until the end of the experiment for the three animals. BAB-PD generally provided fewer measurements within the linear range. However, when it was present in a sufficient amount, the $\Delta^{13}C_{VPDB}$ values were larger than for BAA-PD, except in samples from cow A, in which $\Delta^{13}C_{VPDB}$ (AEdiol - BAB-PD) remained below 3‰.
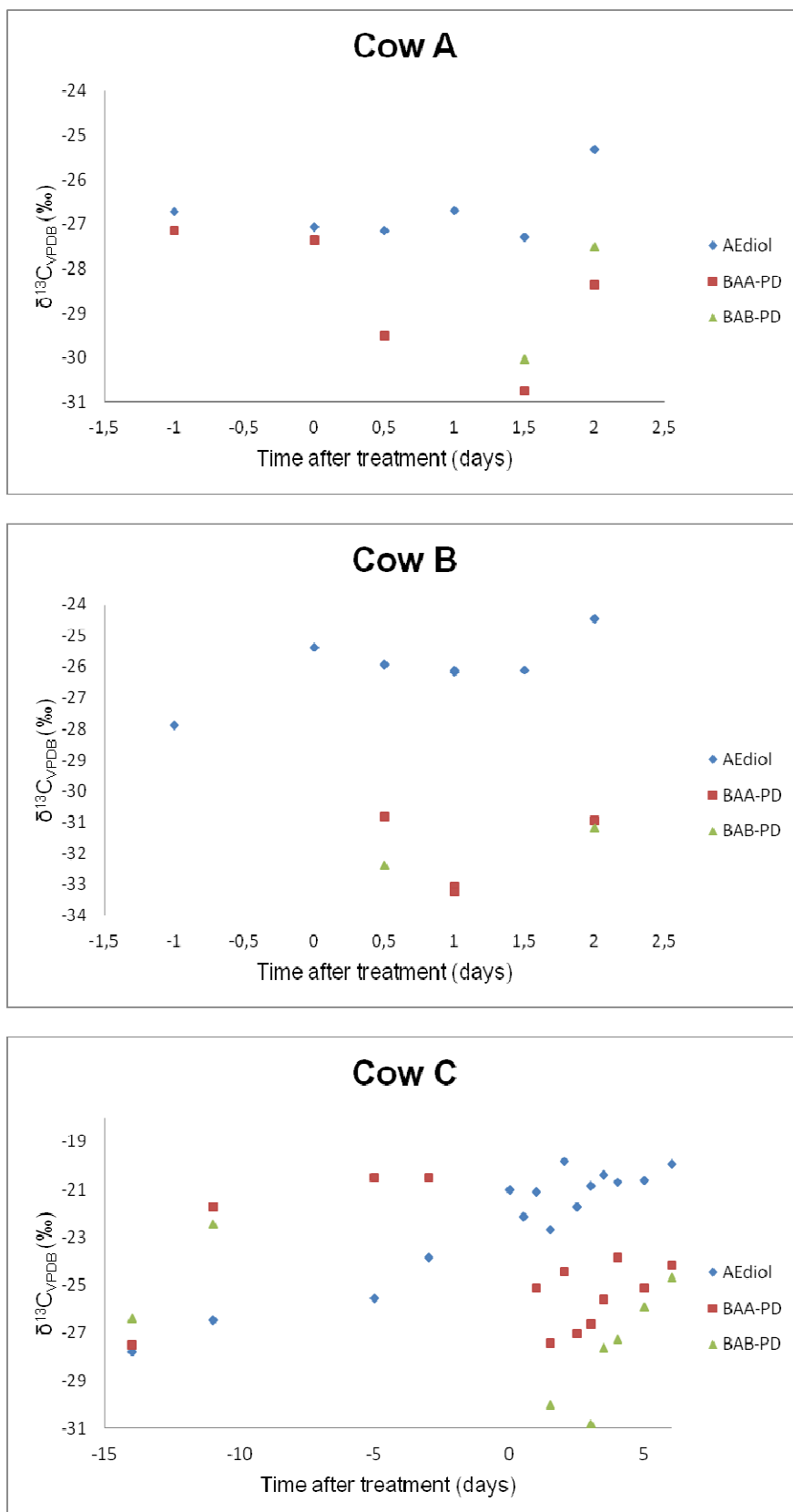
Figure 5.8. $\delta^{13}C_{VPDB}$ values for AEdiol (blue), BAB-PD (green) and BAA-PD (red) of urine samples from cow A (upper), cow B (middle) and cow C (lower), intramuscularly injected with PG. Measurements below the limit of quantification are not shown.

113

It was expected that the elevated dose of PG, in combination with the inclusion of corn into the feeding regime, would result into higher $\Delta^{13}C_{VPDB}$ values for the samples from cow C, when compared to the samples from the other two animals.[101] However, with the highest measurements of 6.89‰ and 5.78‰ for cow B and C, respectively, this was not the case. This observation suggests the presence of a significant endogenous production of PD by cow C, because the diet did have the expected impact on the $\delta^{13}C_{VPDB}$ values of the ERC, which augmented from -27,79‰ up to -19,93‰, as can be seen in figure 5.8. The average $\delta^{13}C_{VPDB}$ values for AEdiol, from T_0 until the end of the experiment, were -26.71‰ and -25.69‰ for cow A and cow B, respectively, representative for a feeding regime very low in C_4 plant material content,[84] while for cow C, this was -20.91‰. Even though the last sample was taken 3 weeks after the feed change, the data suggest that a steady state has not yet been reached at the end of the experiment, which is in line with previous findings, indicating adjustment periods close to 30 days.[84,94] More remarkable is the observed difference between AEdiol and BAA-PD in response to the feeding change. While the values for AEdiol changed gradually over the total time, as previously reported for androgenic compounds,[84,94,191] rising 8.84‰ in total, they already augmented 5.74‰ within the first 3 days for BAA-PD, resulting in a significant offset up to 5.04‰ between AEdiol and BAA-PD before the treatment. This offset could also explain the large impact of the endogenous dilution observed through the relatively small $\Delta^{13}C_{VPDB}$ values obtained after the treatment. A similar impact of a dietary change on BAA-PD in humans has not been reported, although the experimental setup would not have allowed to reveal such an effect since the dietary change was less radical, and time intervals between samples were much larger.[96] It is clear that this observation is a cause for concern if changing from a C_4 to a C_3 plant material based feeding regime would be applied by the farmer. If a sudden feeding regime change of that nature has an impact of the same proportion on the $\delta^{13}C_{VPDB}$ values of the PDs, the calculated threshold $\Delta^{13}C_{VPDB}$ value would be succeeded, and this would result in a false non-compliant result. It is clear that this scenario needs to be further investigated by additional animal experiments.

Eventually, to assess whether the PG administration influences the $\delta^{13}C_{VPDB}$ values of other steroidal compounds, i.e. to assess the specificity of the analytical method, the samples collected after treatment were also analyzed with the previously

published method for the detection of androgen and estrogen abuse.[191] As expected, all the results for the androgens were compliant, with a largest observed $\Delta^{13}C_{VPDB}$ value of 1.38‰ for the ERC – metabolite couple AEdiol – etiocholanolone, which is similar to findings reported for an administration experiment in humans.[197] Unfortunately, the level of 17α-estradiol was too low to provide accurate IRMS-measurements.

Impact of a testosterone/estradiol treatment on BAA-PD

BAA-PD is the ERC of preference for human doping control,[152] and the results from the compliant control population indicate that it might be able to fulfill a similar function in cattle, when no PG was administered. Therefore, 9 urine samples from a bull and 11 from a heifer treated with a single intramuscular injection containing 17β-testosterone propionate and 17β-estradiol benzoate, from the animal experiment which was discussed in detail in chapter IV, were analyzed using method B, consisting of the measurement of AEdiol, BAA-, BAB- and BBA-PD after acetylation, and the data was combined with that obtained during the previous study. Using BAA-PD as ERC, the 17β-estradiol treatment was successfully confirmed in both animals. However, for the 17β-testosterone treatment, this was not the case: the treatment could only be demonstrated in the heifer, and the detection windows were significantly smaller (up to 11 and 19 days using 17α-testosterone and etiocholanolone, respectively). This indicates that BAA-PD would not be a suitable ERC to be used in bovine urine.

When looking at the $\Delta^{13}C_{VPDB}$ values of all the metabolites together, using AEdiol as the ERC, as illustrated in figure 5.9, it becomes clear that the $\delta^{13}C_{VPDB}$ values of BAA-PD are significantly affected by the 17β-testosterone/estradiol treatment, even up to the point where the compliance threshold of 3‰ for PG treatment is crossed, which would indicate a PG-treatment. In the bull, the impact of the treatment on the $\delta^{13}C_{VPDB}$ values is even more significant than for the androgen metabolites. These findings were highly unexpected.
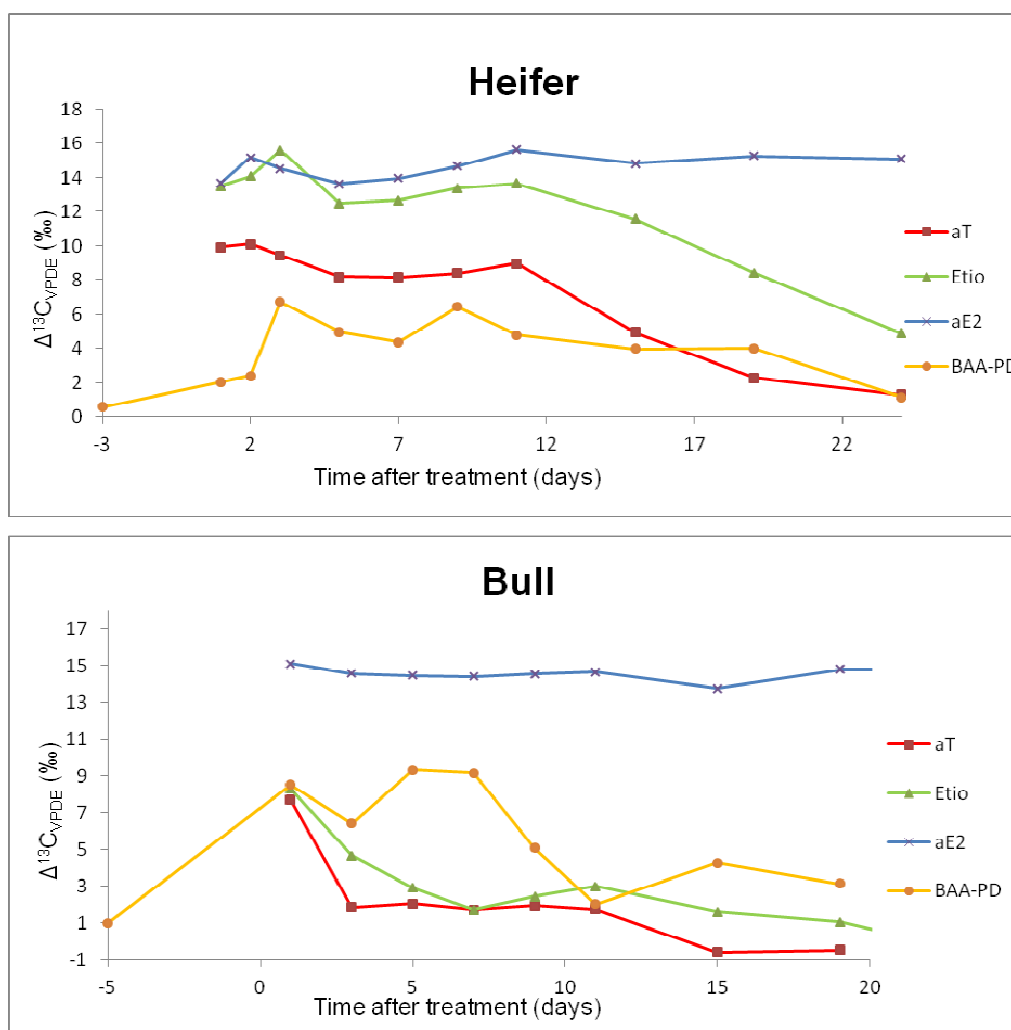
Figure 5.9. $\Delta^{13}C_{VPDB}$ values for 17α-testosterone (aT), 17α-estradiol (aE2) and etiocholanolone (Etio), and BAA-PD, with AEdiol as ERC, in urine samples from a bull (lower) and a heifer (upper), treated with a single intramuscular injection of 17β-testosterone propionate and 17β-estradiol benzoate.

At the moment there is no explanation for this observation, and further study is required to evaluate the specific role of 17β-testosterone and 17β-estradiol in this process, and if these results can be repeated in a new administration study. Therefore, even though the presented method was used to adequately illustrate abuse of PG in cattle, it is advised to include additional analytes into the analytical strategy and create a clearer image regarding possible treatments.

5.5. Conclusions

The ability of GC-(MS/C-IRMS) to differentiate between urine samples from bovines treated intramuscularly with progesterone and a compliant control group has been demonstrated. Through analysis of urine samples from pregnant cows, and verified by results from treated animals, the 5β-pregnane-3,20-diols were selected as promising target metabolites of progesterone. Although 5β-pregnane-3,20-dione, obtained from urine samples as the single oxidation product of the four 5β-pregnane-3,20-diols using $K_2Cr_2O_7$, could be successfully measured using GC-(MS/C-IRMS), it was evaluated that there was not enough gained advantage over a strategy using acetylation. Eventually, a method retaining only the most abundant pregnanediol, 5β-pregnane-3α,20α-diol, as progesterone metabolite, and 5-androstene-3β,17α-diol as endogenous reference compound, was successfully validated. The developed sample preparation was a multistep process, consisting of a hydrolysis, SPE, LLE and two HPLC purification steps before acetylation. Although the treatment could be demonstrated in three progesterone treated cows, with $\Delta^{13}C_{VPDB}$ values up to 6.89‰, the changes in level of the pregnanediols, in response to the treatment, were highly variable between the animals. As a result, the treatment was traceable in a first animal already 12 h after a first administration, whereas in a second this was only after 24 h, and in a third, the treatment was only detected after the second progesterone injection.

However, the impact of a sudden shift in the diet, and of the combined 17β-testosterone/estradiol treatment are a serious cause for concern, and must be studied further through additional animal experiments. It is clear that the presented method, as such, is not adequate to be used for official food safety analyses at the moment, since it would be impossible to conclude if a non-compliant result would be due to a shift in feeding regime or another treatment. However, a deviating result is most certainly atypical, and the integration of BAA-PD into existing methods for the detection of abuse of androgens and estrogens could provide valuable additional information.

6. General discussion and future perspectives

6.1.   Analytical strategy and used sample preparation techniques

As indicated in chapter I, the primary objective of this research was to develop an analytical method for the detection of abuse of synthetic analogues of endogenous sex steroids in cattle using IRMS, starting from knowledge gathered from literature. As thoroughly discussed in chapter II, published methods employ an extensive combination of multiple hydrolysis, SPE and LLE steps, followed by normal phase HPLC fractionation and acetylation. Then, if GC-MS analysis indicates impurities coeluting with the targeted analytes, an additional reversed phase HPLC purification is performed before GC-C-IRMS analysis.[100,101,172] Attempting to provide a method more suitable for routine analyses, a number of adaptations were done to the existing protocol, as discussed in chapter III.[179]

First, the analysis of the sulphate fraction was not included in the current approach. Although at the loss of DHEA as an additional ERC, the gained simplification of the method was significant. Second, through the use of PTV-injection, the sample volume was successfully reduced to half, greatly facilitating the work when dealing with a large number of samples. Third, by coupling a mass spectrometer in parallel to the C-IRMS, separate GC-MS analyses were successfully eliminated from the analytical protocol. However, due to this omission, the HPLC-purification steps needed to be revised. Because of the fact that the identification and purity evaluation are performed simultaneously with the $^{13}C/^{12}C$ measurement, and because all fractions originating from the same sample are preferably measured as subsequent injections to reduce variability on the calculated $\Delta^{13}C_{VPDB}$ values, an additional HPLC-fractionation step after a first GC-(MS/C-IRMS) analysis is not optimal. It was originally hoped that one C_{18} HPLC purification step would be sufficient, as this was described in most analytical methods for routine sports doping control published in the meantime.[130,183,198-200] Unfortunately, the extracts obtained in this way for αT and AEdiol were insufficiently pure, and it was decided to replace one of the SPE steps with an additional HPLC purification using a diol functionalized column for all fractions to assure unhindered IRMS measurements. This strategy proved to be successful, since no coelutions or impurities have been encountered during the analyses until

now. Additionally, the parallel coupled MS proved to be a valuable diagnostic tool upon system failure, allowing to faster pinpoint the exact problem within the setup. Furthermore, the use of an HPLC-based sample preparation strategy has proven its benefits throughout this research. Due to its flexible nature, it provides a relatively open platform, which allows the inclusion of additional steroid analytes through adjustments in mobile phase composition and fractionation windows. This allowed to start off with a method for the detection of estrogen abuse (chapter III), and gradually extent the palette of analytes to include the detection of androgen (chapter IV) and progestagen (chapter V) abuse.

However, even though the presented method provided a number of optimizations compared to previously published ones, the resulting protocol is still extensive and time consuming, resulting in a relatively low sample throughput and a relatively high detection limit (20 ppb). Therefore, a number of suggestions are in order for future ameliorations and explorations in sample preparation, some of which more readily applicable than others.

First of all, analysis of the estrogen fraction of urine samples from the animals treated with βE2, described in chapter IV, with and without the normal phase HPLC purification, provided highly comparable results for αE2, suggesting that the second HPLC preparation step can be omitted from the method for this particular fraction.

Second, the full power of the PTV-injection has not yet been harvested. Due to the long run time of the GC-(MS/C-IRMS) measurement series, usually performed over the weekend, the minimal volume of the final extracts was limited to 30 µL to avoid evaporation of the samples at the end of the run, resulting in failed injections. Using a new type of GC-vials, with a narrow cone shaped interior, the sample volume could be drastically reduced. Of course, amplification of matrix noise is to be expected. However, preliminary results indicated that the extracts are sufficiently pure to allow the sample volume to be reduced to 12 µL without substantial hindrance of background noise, resulting in more than doubled sensitivity.

Finally, the use of selective sorbents could provide the means to shorten the sample preparation strategy. Recently, Doué et al. described that the use of supercritical fluid chromatography (SFC) with a molecularly imprinted polymer (MIP) stationary phase allowed to reduce their sample preparation protocol prior to GC-C-IRMS analysis, for

the detection of abuse of synthetic analogues of endogenous steroids in bovines, with three steps.[201] Although early studies favored the use of preparative HPLC over immunoaffinity chromatography (IAC) because of its flexibility,[113,138,155] this selective antibody-based sorbent could serve a similar purpose, and is already successfully applied as a sample preparation tool for exogenous steroid analyses of urine and fecal samples for almost two decades.[85,202,203]

6.2. A method for official food safety analyses

6.2.1. Method validation

An important objective of this study was to provide an analytical method to be used for official food safety inspections. In Belgium,[204] as in most countries worldwide, this requires the analysis to be performed under ISO 17025:2005 accreditation, the international standard laying down the general requirements for the competence of testing and calibration laboratories.[205] An important requirement is that a thorough and well documented validation study needs to demonstrate the method's potential of providing reliable results.

The performed validation of the developed GC-(MS/C-IRMS) methods was extensive, and consisted of at least ten parts. Criteria for each of the parts were established in advance, based on information found in the literature and specifications provided by the system manufacturer.

The stability and linearity of the ion source of the IRMS apparatus was established using CO_2 pulses giving identical and different peak heights, respectively.

Intermediate precision was assessed through the repeated analysis of a spiked urine sample under reproducibility conditions. The absence of isotope fractionation caused by the sample preparation, or the sample preparation induced bias, was verified by comparing the results of spiked aqueous samples with those of the pure standards used for the spiking. The trueness, or system bias, was evaluated through the measurement of steroid standards with known and certified $\delta^{13}C_{VPDB}$ values. The combination of these three parameters provides the necessary information on the accuracy of the method. The resulting estimated combined measurement uncertainty for AEdiol, αE2, αT and Etio were 0.70‰, 0.99‰, 0.80‰ and 1.24‰, respectively.

The specificity of the method was verified by the evaluation of the mass spectra according to the legal criteria, for every sample analyzed during method validation.[24] Additionally, peak purity was assessed by manual control of the mass spectra throughout the whole of the analyte peak. Additionally, the use of AMDIS, specialized software applying specific algorithms to evaluate analyte purity and identity was validated.

The linear range of the GC-(MS/C-IRMS) system, or the intensity interval in which the instrument provides accurate results, was determined for each analyte specifically through repeated standard injections at different concentrations.

A threshold $\Delta^{13}C_{VPDB}$ value was determined for each ERC-metabolite pair through the analysis of urine samples from a compliant control population. Finally, the aptitude of the method and the adequacy of the determined threshold were verified through the analysis of samples from treated animals.

All the required information could be gathered through four to five analytical series, containing approximately ten samples each. The validation was successfully performed for the estrogen, androgen and progestagen method, and details on the execution and results are unraveled in the previous three chapters.

However, three important shortcomings need to be highlighted. First of all, the level of the spiked urine samples to determine the within lab reproducibility must be revised. Since results are expected to be more variable at the lower end of the linear range, the reproducibility should be determined using urine samples with steroid concentrations at the level of the LOQ.

Second, the procedure to determine the system bias could be seriously improved by using standards of all the analytes targeted in the method, with traceable and certified $\delta^{13}C_{VPDB}$ values obtained through EA-IRMS. This would allow to calculate a measurement uncertainty which is more specific for the individual components, and therefore, together with the above mentioned adaptation to the determination of the within lab reproducibility, would result in a calculated measurement uncertainty closer to reality.

Third, by using a calibration standard mixture with more steroids, of which the $\delta^{13}C_{VPDB}$ values are spread across the entire range of interest, from approximately -14‰ down to -32‰, the calibration can be significantly improved, and the calibration

bias would be more evenly spread across the entire range, whereas now, the higher $\delta^{13}C_{VPDB}$ values are expected to be more biased.

It is clear that the validation procedure is difficult and cumbersome for multiple reasons, which all could be remedied to a certain extent.

The first difficulty lies within the large number of parameters to be validated. However, if adequate reference material were available, under the form of non-compliant urine samples with known $\delta^{13}C_{VPDB}$ values, reproducibility, method bias and system bias could be evaluated together through repeated analysis of this material. This would allow to replace the combined analyses of spiked urine samples, spiked aqueous samples, certified standard injections and non-compliant samples. Together with the data from a compliant control population, and the determination of the linear range, this would provide a complete method validation.

The second difficulty lies within the unclarity regarding the method validation: which parameters should be validated, how should this be done and which criteria need to be applied? Official guidelines should be made available, including these three aspects, to harmonize the validation approach.

For doping control, both aspects are covered by the WADA, which provides reference materials to the official doping laboratories, and which lays down technical requirements for official analyses. With regard to GC-C-IRMS method validation, the currently active WADA technical document indicates the necessity of determining the linear range for each analyte, the analysis of samples from a compliant population to verify the threshold values, and the determination of the combined standard measurement uncertainty, which needs to be below 1‰.[152] The new technical document, which will be in force starting January 2016, will add verification of the linearity of the ion source with CO_2 pulses to the validation requirements, and will provide practical guidelines on how to test and evaluate the linear range per analyte.[153]

In 2014, the EURL published a reflection paper on natural growth promoting substances, encouraging official control laboratories to implement GC-C-IRMS analyses,[29] and is currently working on the development of GC-C-IRMS methods itself. It can be hoped that the EURL can fulfill a similar role for growth promoter

control as the WADA for doping control, providing clear guidance and useful reference materials, which could eventually evolve into inclusion of GC-C-IRMS analysis guidelines in official legislation, the need for which has already been discussed in literature.[30]

6.2.2. Quality control

As a second important requirement to obtain ISO 17025:2005 accreditation, a thorough and well documented quality control system needs to be put in place to allow continuous verification of the reliability of the analytical results in time. The quality control procedure, employed for all analyses until now, is described below.

First of all, a number of samples are added to every analytical series. A urine sample from a treated animal functions as a positive control sample. When no samples from treated animals are available, a urine sample spiked with all the measured metabolites, but not with the ERC, can be used to replace it. Also, a urine sample from the control population is added as a negative control sample. Additionally, two spiked aqueous samples, are included. The first, spiked at 100 µg L^{-1} is used to verify the absence of isotope fractionation during sample preparation. This sample was also spiked with 17β-testosterone glucuronide to evaluate the activity of the used glucuronidase. The second, spiked at the LOQ, 20 µg L^{-1}, is used to verify the efficiency of the sample extraction. Finally, a blank aqueous sample is added to assess possible contamination of the samples during analysis.

Prior to analysis with GC-(MS/C-IRMS), the functionality of the system is evaluated in a three stage process. First, absence of leaks in the system is verified by performing a background scan. Second, linearity and stability of the ion source are evaluated through pulses with the CO_2 reference gas. Finally, a steroid standard is injected to evaluate the functionality of the complete setup.

When all controls are satisfactory, the analytical series can be started. Next to all the separate fractions obtained from the official and control samples at the end of the preparation, a number of additional controls are injected. At the beginning of every series, six standard injections are included to assure stability of the system before measurement of the samples. Next, a solvent blank is injected to evaluate system contamination. Certified steroid standards were included to verify the absence of a system bias. Standards of all measured analytes were included as well for a twofold

purpose: verification of peak identity and purity, and evaluation of sample preparation induced bias by comparison with the spiked aqueous samples.

Processing the GC-(MS/C-IRMS) results from the obtained fractions, after sample purification, comprises four steps. First, the peak purity check and the analyte identification are done through evaluation of the obtained mass spectra. Next, it needs to be verified if the intensity of the obtained IRMS peak is within the linear range. Finally, the measured $\delta^{13}C_{VPDB}$ value needs to be corrected for the acetate moiety, as discussed in the previous chapters.

Besides the controls performed for each analytical series, the calibration of the used CO_2 reference gas using certified steroid standards with known and traceable $\delta^{13}C_{VPDB}$ values, needs to be performed periodically.

The extensive validation and quality control procedures, covering all aspects also included in the WADA doping control prescriptions,[153] resulted in a positive accreditation audit, and the method for the detection of estrogen and androgen abuse have been performed for official control purposes since 2013 and 2014, respectively. However, it became clear that the elaborate quality control resulted in large injection series, and time consuming processing of results. The validation of the AMDIS software to assess peak purity and analyte identification, as discussed in the previous paragraph, proved to be a very helpful automation tool for the processing of large numbers of samples. Still, a number of additional suggestions can be made to streamline the quality control process.

First, the certified steroid standards, included in every analytical series, revealed that the calibration of the CO_2 reference gas remains stable over large periods of time, with the measured $\delta^{13}C_{VPDB}$ values not deviating more than 0.6‰ from the certified value. This corresponds with observations from routine doping analyses,[183] and indicates that calibration of the reference gas only needs to be performed and checked periodically, in accordance with the WADA guidelines. Additionally, the WADA indicates that the linearity check of the ion source is only required periodically as well.[152]

Second, it became clear that, when the same compliant or non-compliant control sample is used over large periods of time, the control charts of these samples were sufficient to indicate isotope fractionation during sample preparation. Hence, the

aqueous sample spiked at 100 µg L^{-1}, included to identify this process specifically, could be omitted from the procedure. Moreover, these control charts proved to be a valuable source of additional information to the method validation. For all measured androgens, AEdiol, αT and Etio, the calculated standard deviations obtained from 14 measurements, of both the compliant, illustrated in figure 6.1, and non-compliant control sample, over a time span of approximately nine months, were close to 0.8‰. This value can be considered a more realistic approximation for the reproducibility of the method than the results obtained during the method validation, which are discussed in chapter III and IV.
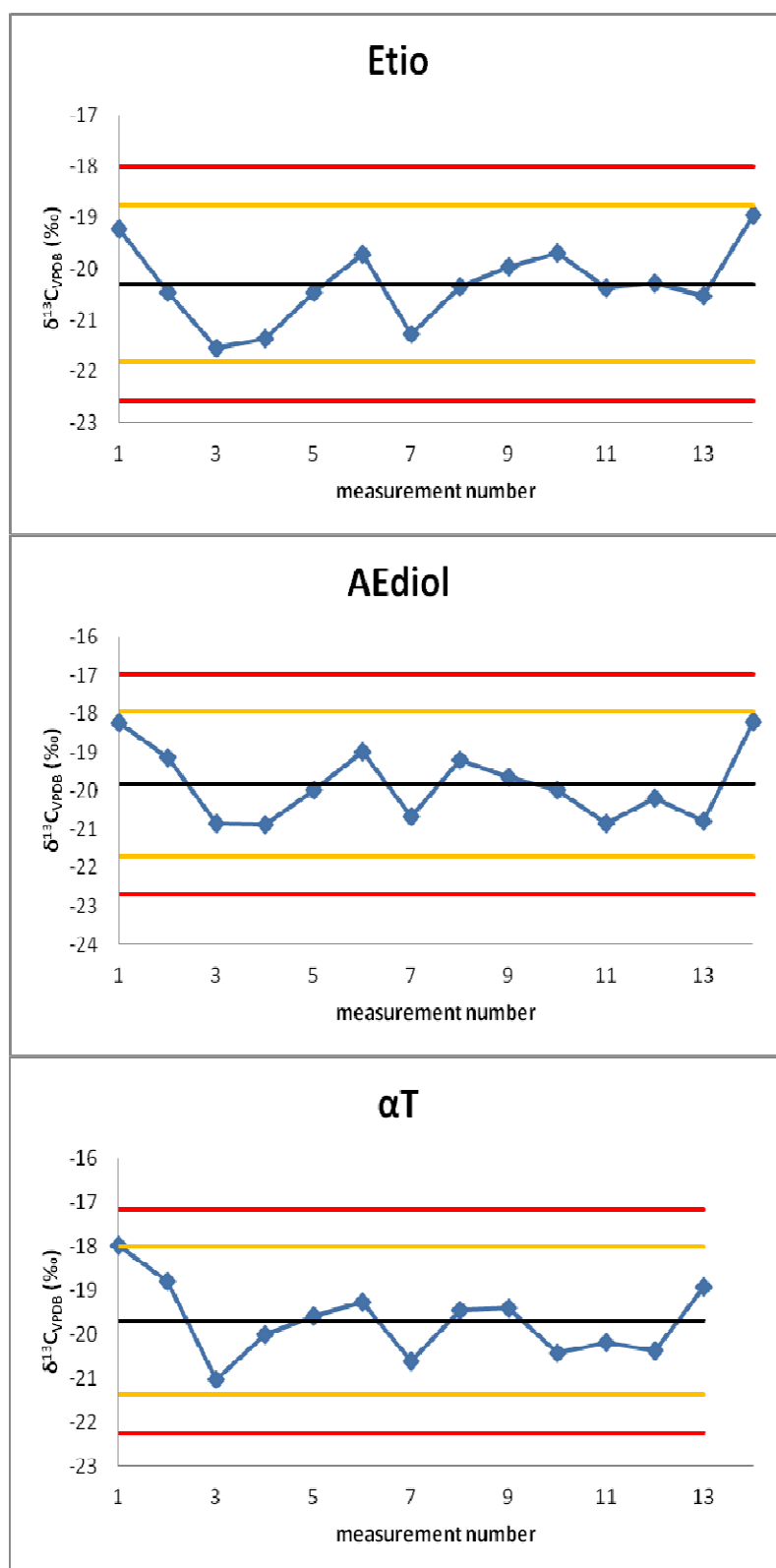
Figure 6.1: Control charts of the compliant control sample, containing the measurements of etiocholanolone (Etio) (upper), 5-androstene-3β,17α-diol (AEdiol) (middle) and 17α-testosterone (αT) (lower). The black line indicates the average, the orange lines indicate the first action level (average ± 2 x standard deviation) and the red lines indicate the second action level (average ± 3 x standard deviation).

Third, through evaluation over time of the six standard injections, performed at the beginning of each injection series to stabilize the system, it is concluded that already after three injections, accurate and stable values were obtained.

Fourth, contamination caused by both the sample preparation and the GC-(MS/C-IRMS) can be simultaneously assessed through one injection by combining all the fractions from the blank water sample, and eliminating the solvent blank. Of course, when a contamination is observed, it will not be possible to locate the source instantaneously, and additional research will be in order. However, through the past three years of official control analyses, contamination has not been observed.

Finally, the analytical procedure prescribes that when a sample is found to be non-compliant, this result needs to be confirmed with a second analysis as a precautionary matter. It can be proposed that, since the first analysis fulfills a screening function, the compliant control sample is only included during the confirmation analysis.

6.2.3. Screening strategies

Since GC-(MS/C-IRMS) analysis remains laborious and time consuming, a screening strategy needs to be in place to select suspicious samples. The original approach was that the samples were selected by the National Investigation Unit (NIU), the inspection unit of the Federal Agency for the Safety of the Food Chain (FASFC) specialized in investigation of hormone abuse in cattle in Belgium, based upon the weight of the animals and other information (prior convictions, police information,…). Additionally, all samples were screened using LC-MS/MS prior to GC-(MS/C-IRMS) analysis, to determine if the targeted analytes were present above 20 µg L^{-1}, the lowest level which still provided a sufficient amount of CO_2, after combustion, to provide an accurate IRMS measurement, or the limit of quantification (LOQ). This screening method, described in chapter IV, was officially included in the analytical strategy and performed under accreditation.

For αE2, this concentration-based screening proved to be sufficient, allowing sifting out 96% of the official samples provided in 2013 and 2014. The applied strategy related closely to the one given by Scarth et al., suggesting GC-C-IRMS analysis after two out of two samples were found above 5 µg L^{-1} and 10 µg L^{-1} for steers and

heifers, respectively.[32] Following the same strategy for the androgens, 65% of the samples was eventually analyzed with GC-(MS/C-IRMS), all giving compliant results.

Regarding IRMS analysis for the detection of abuse of synthetic analogues of endogenous androgens, the above clearly illustrates that research into an adequate and more selective screening technique is of high importance. In chapter I, it was discussed that a lot of screening approaches are under investigation, with the current attention shifting towards multiple indirect biomarker measurement in various types of matrices.[43] However, because of the grave consequences of a non-compliant result for the farmer, a direct confirmatory analysis will remain mandatory.[29] As discussed in chapter IV, alternative confirmation methods are currently limited to analysis of intact steroid esters in hair or plasma, of which the MS detection is a direct proof of administration and therefore require no additional screening approaches. Therefore, a urine-based screening method, including the quantification of the targeted steroids besides an additional, more selective criterion, would be the ideal and directly applicable approach at the moment, worthy of immediate research. Additionally, a powerful screening strategy prior to IRMS analysis significantly reinforces the reliability of a non-compliant result. This is now the fact in anti-doping analyses, where a combined atypical result from the biological passport together with a non-compliant IRMS result provides a very strong statistical result.

6.2.4. Endogenous versus exogenous $\delta^{13}C_{VPDB}$ values

Of the 365 official urine samples, analyzed under accreditation since the beginning of 2013 for the detection of abuse of synthetic analogues of endogenous androgens and/or estrogens, none were found to be non-compliant. The ideal hypothesis to explain this observation would be that none of the sampled animals had been treated with these substances. However, since preparations containing esters of βT and βE2 were still confiscated by the Belgian authorities during the past years, this is not necessarily true. The described developed analytical strategy has a number of critical weaknesses, which could very well be the cause of this.

First of all, the detection window for GC-(MS/C-IRMS), especially with regard to androgen abuse in male animals, is very small, as discussed in chapter IV. Moreover, when an even smaller dosage is used, it is expected that the detection window will be

smaller as well. It could be possible that samples were not taken within the suitable timeframe after treatment. For this reason, the competent authorities have been informed that sampling of live animals at the farm is preferred over sampling at the slaughterhouse for this particular analysis.

Another explanation could be that the diet of the animals influences the outcome of the IRMS analyses. It has been suggested that GC-C-IRMS would be an unsuitable technique to demonstrate illegal treatment in cows from the United Kingdom, because the use of corn in the feeding regime is rather uncommon there. As discussed before, low quantities of C_4 plants in the feeding regime greatly reduce the difference in $\delta^{13}C_{VPDB}$ values between endogenously produced steroids and steroid preparations, which severely compromises a successful detection of abuse.[113] However, when evaluating the obtained $\delta^{13}C_{VPDB}$ measurements for AEdiol from the official control samples, depicted in figure 6.2, ranging from -14.12‰ to -25.97‰ around an average of -19.73‰, this does not seem to be the case.
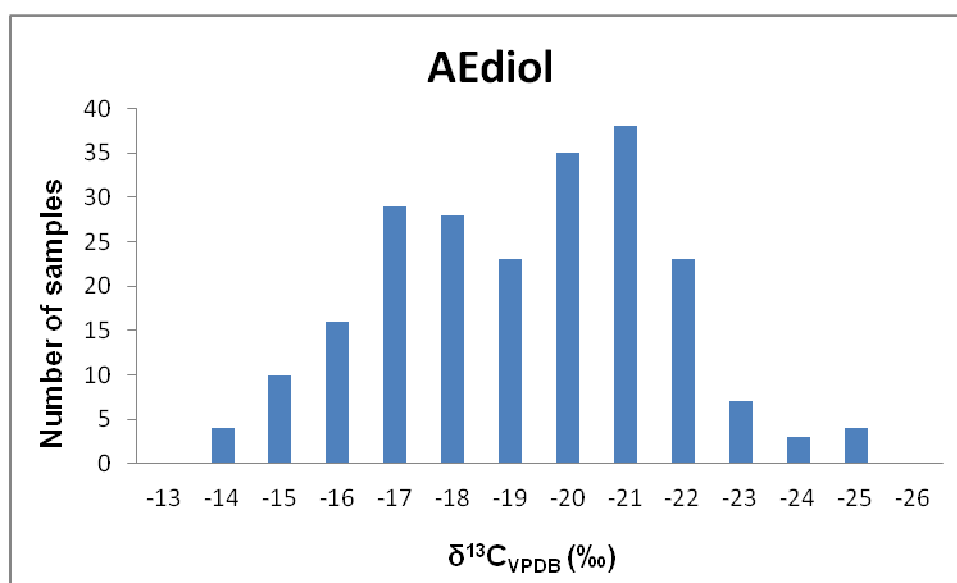
Figure 6.2: Distribution of the $\delta^{13}C_{VPDB}$ values of 5-androstene-3β,17α-diol (AEdiol), measured in official control samples from Belgian cows.

The other way around, the existence has been reported of preparations of synthetic analogues of endogenous anabolic steroids, observed through the analyses of confiscated doping products, having $\delta^{13}C_{VPDB}$ within this range.[88,180-182] Although it was only the case in a minority of the analyzed samples, these unusual $^{13}C/^{12}C$ ratios

are a serious cause for concern, and were also observed in the BAA-PD standard, used at the laboratory.

Unfortunately, the preparations containing synthetic analogues of endogenous sex steroids, confiscated at Belgian farms between 2011 and 2015, were unavailable for analysis due to legal confiscation. Therefore, it is advised that, once they are released from legal custody, analysis of these preparations would be the subject of future research. As for the limited amount of available preparations, confiscated in 2011, all had $\delta^{13}C_{VPDB}$ values below -28‰.

6.3. Results from the animal experiments

When developing an IRMS-based method to demonstrate treatment of bovines with synthetic analogues of endogenous steroids, analyses of real non-compliant samples is a crucial step, since it is the only way to confirm whether the designed strategy is successful. Since non-compliant reference material, being urine samples from treated animals, is not readily available, an administration experiment is indispensable. However, animal experiments are very costly, mainly due to feed consumption, man-hours for collecting samples and destructions of the animals afterwards. This also explains why carbon isotopic data from animal experiments in literature are scarce,[67,84,94,100,101,113] rendering additional administration results into a valuable asset for the community. Even though using young calves could be a way to lower the costs and increase the number of tested animals, older animals were preferred in this research since veal farming is not of equal importance and magnitude in Belgium.
However, it is understandable that it is not possible to obtain statistically significant conclusions from the limited number of treated animals used in the current study. Therefore, conclusions drawn from the animal experiments should be regarded as preliminary, but certainly not invaluable.

The first animal experiment was primarily set up to assess the method's potential to demonstrate treatment of bovines with βT and βE2, injected intramuscularly as a propionate and benzoate ester, respectively. From a zoological point of view, animals

of different sex would receive different concentrations of the steroids, as male animals respond better to female sex steroid treatment and *vice versa*. However, treating a bull and a heifer identically, and varying the amount of maize present in the feed, allowed to take a glance at the influence of gender and diet on the $\delta^{13}C_{VPDB}$ values of targeted ERC and metabolites as well. Finally, the analysis of the large number of urine samples collected up to four weeks after treatment provided more detailed pharmacokinetic data than available from previous studies, and allowed to accurately determine the detection window of the GC-(MS/C-IRMS) method for the treatment.

The results, discussed in detail in chapter IV, confirmed the earlier described impact of a feeding regime, mainly consisting of maize, on the endogenous androgens,[84,94] whose $\delta^{13}C_{VPDB}$ augmented to almost -13‰. The pharmacokinetics revealed a substantially narrower detection window for testosterone treatment in the male animal (three days) compared to the female (24 days), due to a larger endogenous androgen production. The detection window for estradiol treatment (24 days), on the other hand, was not limited by endogenous dilution in both animals, but by the current LOQ of the method.

However, the influence of the nature of the treatment was not included in the described experiment, and provides an interesting topic for future research. By treating animals of the same age and gender with a different dose and/or by varying the type of ester side-chain used for the steroid preparation, interesting insights on the impact on the detection window can be obtained.

The primary objective of the second animal experiment was to evaluate if the combination of AEdiol and BAA-PD would provide a suitable ERC – metabolite pair to confirm PG treatment in bovines. Therefore, three heifers were treated with multiple intramuscular injections of PG, with a 24 h interval, and urine samples were collected before, during and after treatment. Additionally, the third animal was treated with a five times higher dose than the other two, and also the feeding regime of this animal was changed to include more maize, as the influence of these factors on the level and $\delta^{13}C_{VPDB}$ values of PDs in urine had not been studied previously. Unfortunately,

no male animals were available for the experiment, which should be included in future studies.

The results of this experiment, discussed in detail in chapter V, revealed a significantly varying response to the treatment between the animals. In the first animal treated with the lower dose, the $\Delta^{13}C_{VPDB}$ value only became sufficiently high 12 h after the second injection, whereas the treatment could already be elucidated 12 h after the first injection in the second animal. As for the third animal, treated with a higher dose, the treatment could only be detected 24 h after the first injection, since the level of BAA-PD was below the LOQ in the sample taken 12 h after this treatment. Furthermore, the change from a C_3 to a C_4 plant based feeding regime, revealed an abrupt augmentation of $\delta^{13}C_{VPDB}$ values of BAA-PD, whereas this change was more gradual for AEdiol.

This last observation opens important perspectives for future research. First of all, an additional feeding experiment is advised, in which the regime is changed in a different direction, from a C_4 to a C_3 plant based feed. If a comparable but opposite response to this change is observed, the created offset between the $\delta^{13}C_{VPDB}$ values of the ERC and the metabolite could result into a false positive result. It is clear that this study is very necessary, since it is impossible to declare a sample definitively as non-compliant based on the measured $\Delta^{13}C_{VPDB}$ until this issue is clarified.

Circumventing this problem would be to include a reference compound which responds in the same way to the feeding change as BAA-PD. When looking at scheme 2.1, a direct PG precursor such as pregnenolone would provide a likely candidate. However, the limited data available regarding the presence of pregnenolone in bovine urine indicate that its concentration will not be sufficient to make it a suitable ERC.[41] 17α-hydroxypregnenolone, for which no urinary concentration data are currently available, could provide an alternative. Therefore, evaluation of its abundance in routine bovine urine samples would be the first step in evaluating this approach.

Another interesting approach to the detection of progesterone abuse can be suggested. Substantial levels of progesterone have been reported in faecal samples, and are used as a diagnostic marker for pregnancy in cows.[206-208] It is likely that after administration, progesterone would be present in a sufficient amount in faecal

samples, to be used as target metabolite for GC-C-IRMS analysis. In combination, cholesterol could be suggested as an ERC candidate, since it has been reported to be present in bovine faeces in concentrations up to almost 7000 µg g^{-1},[209] and has already successfully been used as an ERC for the detection of testosterone administration in bovines, using bile as matrix.[113]

Finally, measuring the progesterone metabolites in the samples from the first animal experiment, revealed a highly unexpected impact of the combined βE2 and βT treatment on the δ^{13}C$_{VPDB}$ values of BAA-PD. As discussed in chapter V, the difference between the ERC, AEdiol, and BAA-PD exceeded the threshold determined from the analysis of samples from the untreated control population. Therefore, although still a usable metabolite to indicate steroid abuse, BAA-PD does not seem to be specific for the indication of progesterone abuse. To exclude the option that analytical errors are responsible for this observation, some of the samples were reanalyzed using the oxidation protocol, discussed in chapter V, instead of the acetylation, and were send to other laboratories as well, which confirmed the initial findings. This interesting phenomenon raises multiple questions, which can only be answered by additional administration studies.

First of all, it needs to be confirmed that this observation is repeatable.

Second, it should be investigated if either the testosterone, the estradiol, or the combined treatment is responsible. Of course, bovine and human steroid metabolism, although similar, are not identical, but it needs to be noted that a similar observation has never been made in the context of sports doping control, where BAA-PD is a commonly used ERC, but where estradiol is not likely to be used as a doping agent.[130,152]

Third, the mechanism causing this effect needs to be elucidated. Based on the current knowledge of the steroid metabolism, it seems unlikely that testosterone or estradiol themselves would be converted to pregnanediol. Using ^{13}C-labeled preparations in an administration experiment should allow to clarify this.

6.4. Research project AFFIRMS

Over time, evolutions in chromatographic and mass spectrometric techniques revealed the possible endogenous presence of substances which were previously believed to be xenobiotic, such as nortestosterone,[68] and more recently prednisolone in cows and pigs.[45,210] Elucidation of the origin using GC-C-IRMS has been suggested as the confirmatory technique of choice when the presence of these substances is detected in routine samples.[29] However, when these substances are detected in urine samples, their concentrations are generally low, and therefore, IRMS based techniques have not yet been applied for this purpose.

In the context of this problem, a joint research project, for the following four years, was started by the FLSFCG and the CER Groupe under the name AFFIRMS. The objective of the first stage of this project is to investigate the possibility of using IAC as a sample preparation technique prior to GC-(MS/C-IRMS) analysis, with the development of specific antibodies for the ERC and metabolites presented in this work. In the second stage, it will be assessed if IAC provides an extraction which is sufficient to allow GC-(MS/C-IRMS) to be used as a confirmatory analysis when prednisolone is detected above its maximum residue limit (MRL), being 5 µg L^{-1}.[26]

7. References

1. Speight J. Handbook of industrial hydrocarbon processes. *Gulf Professional Publishing, Elsevier (Oxford)* **2010**, p 474.

2. Moss G.M. The nomenclature of steroids (Recommendations 1989). *Eur. J. Biochem.* **1989**, *186*, 429-458.

3. Kime D.E. Steroid nomenclature. *Gen. Comp. Endocrinol.* **1995**, *98*, 119-120.

4. Lange I.G.; Daxenberger A.; Schiffer B.; Witters H.; Ibarreta D.; Meyer H.H.D. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. *Anal. Chim. Acta* **2002**, *473*, 27-37.

5. Reynolds J.E.F. Martindale, the extra pharmacopoeia 29th edition. *The Pharmaceutical Press (London),* **1989**, p 872-902, p 1383-1415.

6. Lone K.P. Natural sex steroids and their xenobiotic analogs in animal production: growth, carcass quality, pharmacokinetics, metabolism, mode of action, residues, methods, and epidemiology. *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 93-209.

7. Dötsch J.; Dörr H.G.; Wildt L. Exposure to endogenous estrogens during lifetime. From the Handbook of Environmental Chemistry Vol. 3, Part L. *Springer-Verlag (Berlin Heidelberg)*, **2001**, p 83-84.

8. Gomes W.R.; Erb R.E. Progesterone in bovine reproduction: a review. *J. Dairy Sci.* **1965**, *48*, 314-330.

9. Stephany R.W. Hormonal growth promoting Agents in Food producing animals. From the Handbook of experimental pharmacology 195**.** Springer-Verlag *(Berlin Heidelberg)* **2010**, p 355-367.

10. Umberger E.J. Products marketed to promote growth in food-producing animals: steroid and hormone products. *Toxicology* **1975**, *3*, 3-21.

11. FDA website: http://www.ecfr.gov/cgi-bin/text-idx?c=ecfr&SID=f44bc2af7b4ac5a3adf170a9e18b9842&rgn=div5&view=text&node=21:6.0.1.1.11&idno=21#21:6.0.1.1.11.0.1.56

12. Song M.K.; Choi S.H. Growth promoters and their effects on beef production – review – *Asian-Aust. J. Anim. Sci.* **2001**, *14*, 123-135.

13. Mader T.L.; Dahlquist J.M.; Sindt M.H.; Stock R.A.; Klopfenstein T.J. Effect of sequential implanting with synovex on steer and heifer performance. *J. Anim. Sci.* **1994**, *72*, 1095-1100.

14. Paisley S.I.; Horn G.W.; Ackerman C.J.; Gardner B.A.; Secrist D.S. Effects of implants on daily gains of steers wintered on dormant native tallgrass prairie, subsequent performance, and carcass characteristics. *J. Anim. Sci.* **1999**, *77*, 291-299.

15. Tretzel L.; Thomas A.; Geyer H.; Gmeiner G.; Forsdahl G.; Pop V.; Schänzer W.; Thevis M. Use of dried blood spots in doping control analysis of anabolic steroid esters. *J. Pharm. Biomed. Anal.* **2014**, *96*, 21-30.

16. Courtheyn D.; Le Bizec B.; Brambilla G.; De Brabander H.F.; Cobbaert E.; Van de Wiele M.; Vercammen J.; De Wasch K. Recent developments in the use and abuse of growth promoters. *Anal. Chim. Acta* **2002**, *473*, 71-82.

17. De Brabander H.F.; Noppe H.; Verheyden K.; Vanden Bussche J.; Wille K.; Okerman L.; Vanhaecke L.; Reybroeck W.; Ooghe S.; Croubels S. Residue analysis: future trends from a historical perspective. *J. Chromatogr. A* **2009**, *1216*, 7964-7976.

18. Imbimbo P.; Castigliego L.; Armani A.; Biolatti B.; Cannizzo F.T.; Gianfaldoni D.; Guidi A. A histologic study on growth promoter target organs of slaughtered beef in Molise region (Italy). *J. Vet. Med. Sci.* **2012**, *74*, 1253-1259.

19. European Council. Council Directive 88/146/EEC of 7 March 1988 prohibiting the use in livestock farming of certain substances having a hormonal action. *Off. J. Eur. Commun.* **1988**, *L70*, 16-18.

20. European Council. Parliament and Council Directive 2003/74/EC of 22 September 2003 amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having hormonal or thyrostatic action and of beta-agonists. *Off. J. Eur. Commun.* **2003,** *L262*, 17-21.

21. European Council. Parliament and Council Directive 2008/97/EC of 19 November 2008 amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having hormonal or thyrostatic action and of beta-agonists. *Off. J. Eur. Commun.* **2008,** *L318*, 9-11.

22. European Council. Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stock farming of certain substances having a hormonal or

thyrostatic action and of β-agonist and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC. *Off. J. Eur. Commun.* **1996,** *L125*, 3-9.

23. European Council. Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. *Off. J. Eur. Commun.* **1996,** *L125*, 10-32.

24. European Commision. Commision Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.* **2002**, *L221*, 8-36.

25. European Commision. Commision Regulation 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off. J. Eur. Commun.* **2010**, *L15*, 1-72.

26. European Commision. Commission Implementing Regulation 406/2013 of 2 May 2013 amending the Annex to Regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, as regards the substance prednisolone. *Off. J. Eur. Commun.* **2013**, *L121*, 42-43.

27. Volksgezondheid en Gezin. Wet van 15 juli 1985 betreffende het gebruik bij dieren van stoffen met hormonale, antihormonale, beta-adrenergische of productie-stimulerende werking. *Belgisch staatsblad* **1985**.
    http://www.ejustice.just.fgov.be/cgi_loi/change_lg.pl?language=nl&la=N&cn=1985071536&table_name=wet accessed on 01/10/2015.

28. http://www.ejustice.just.fgov.be/cgi_loi/loi_l.pl?N=&=&sql=arrexec+contains+%271985071536%27+and+la+=+%27N%27&rech=27&language=nl&tri=dd+AS+RANK&numero=1&table_name=wet&cn=1985071536&caller=arrexec&fromtab=wet&la=N&cn_arrexec=1985071536&dt_arrexec=WET accessed on 01/10/2015.

29. Sterk S.S.; Blokland M.H.; De Rijke E.; Van Ginkel L.A. EURL reflection paper: natural growth promoting substances in biological samples. *Research Report RIKILT*, **2014**, 1-68.

30. Vanhaecke L.; Gowik P.; Le Bizec B.; Van Ginkel L.; Bichon E.; Blokland M.; De Brabander H.F. European analytical criteria: past, present, and future. *J. Ass.Off. Anal. Chem. Int.* **2011**, *94*, 360-372.

31. CRL Guidance Paper of 7th December 2007. CRLs view on state of the art analytical methods for national residuecontrol plans.
http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/EURL_Empfehlungen_Konzentrationsauswahl_Methodenvalierungen_EN.pdf?__blob=publicationFile&v=2 accessed on October 2nd 2015.

32. Scarth J.P.; Clarke A.; Teale P.; Mill A.; Macarthur R.; Kay J. Detection of endogenous steroid abuse in cattle: results from population studies in the UK. *Food Addit. Contam.* **2011**, *28*, 44-61.

33. Nielen M.W.F.; Lasaroms J.J.P.; Essers M.L.; Sanders M.B.; Heskamp H.H.; Bovee T.F.H.; van Rhijn J.H.; Groot M.J. The ultimate veal calf reference experiment: Hormone residue analysis data obtained by gas and liquid chromatography tandem mass spectrometry. *Anal. Chim. Acta* **2007**, *586*, 30-34.

34. Saugy M.; Lundby C.; Robinson N. Monitoring of biological markers indicative of doping: the athlete biological passport. *Br. J. Sports Med.* **2014**, *48*, 827-832.

35. Angeletti R.; Contiero L.; Gallina G.; Montesissa C. The urinary ratio of testosterone to epitestosterone: a good marker of illegal treatment also in cattle? *Vet. Res. Commun.* **2006**, *30*, 127-131.

36. Anizan S.; Di Nardo D.; Bichon E.; Monteau F.; Cesbron N.; Antignac J.-P. ; Le Bizec B. Targeted phase II metabolites profiling as new screening strategy to investigate natural steroid abuse in animal breeding. *Anal. Chim. Acta* **2011**, *700*, 105-113.

37. Blokland M.H.; Van Tricht E.F.; Van Rossum H.J.; Sterk S.S.; Nielen M.W.F. Endogenous steroid profiling by gas chromatography-tandem mass spectrometry and multivariate statistics for the detection of natural hormone abuse in cattle. *Food Addit. Contam.* **2012**, *29*, 1030-1045.

38. Antignac J.; Le Bizec B.; Monteau F.; André F. Study of natural and artificial corticosteroid phase II metabolites in bovine urine using HPLC-MS/MS. *Steroids* **2002**, *67*, 873-882.

39. Maume D.; Le Bizec B.; Pouponneau K.; Deceuninck Y.; Solere V.; Paris A.; Antignac J.; André F. Modification of 17β-estradiol metabolite profile in steer edible tissues after estradiol implant administration. *Anal. Chim. Acta* **2003**, *483*, 289-297.

40. Anizan S.; Bichon E.; Duval T.; Monteau F.; Cesbron N.; Antignac J.-P. ; Le Bizec B. Gas chromatography coupled to mass spectrometry-based metabolomic to screen for anabolic practices in cattle : identification of 5α-androst-2-en-17-one as new biomarker of 4-androstenedione misuse. *J. Mass. Spectrom.* **2012**, *47*, 131-140.

41. Rijk J.C.W.; Lommen A.; Essers M.L.; Groot M.J.; Van Hende J.M.; Doeswijk T.G.; Nielen M.W.F. Metabolomic approach to anabolic steroid urine profiling of bovines treated with prohormones. *Anal. Chem.* **2009**, *81*, 6879-6888.

42. Dervilly-Pinel G.; Weigel S.; Lommen A.; Chereau S.; Rambaud L.; Essers M.; Antignac J.-P.; Nielen M.W.F.; Le Bizec B. Assessment of two complementary liquid chromatography coupled to high resolution mass spectrometry metabolomics strategies for the screening of anabolic steroid treatment in calves. *Anal. Chim. Acta* **2011**, *700*, 144-154.

43. Mooney M.H.; Le Bizec B.; Elliot C.T. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. *Trends Analyt. Chem.* **2009**, *28*, 665-675.

44. Dumas M.-E.; Canlet C.; Vercauteren J.; André F.; Paris A. Homeostatic signature of anabolic steroids in cattle using ^{1}H-^{13}C HMBC NMR metabonomics. *J. Proteome Res.* **2005**, *4*, 1493-1502.

45. De Clercq N.; Van Meulebroek L.; Vanden Bussche J.; Croubels S.; Delahaut P.; Vanhaecke L. The impact of stress on the prevalence of prednisolone in bovine urine: a metabolomic fingerprinting approach. *J. Steroid Biochem. Mol. Biol.* **2015**, *154*, 206-216.

46. Regal P.; Anizan S.; Antignac J.-P.; Le Bizec B.; Cepeda A.; Fente C. Metabolomic approach based on liquid chromatography coupled to high resolution mass spectrometry to screen for the illegal use of estradiol and progesterone in cattle. *Anal. Chim. Acta* **2011,** *700,* 16-25.

47. Gardini G.; Del Boccio P.; Colombatto S. ; Testore G. ; Corpillo D. ; Di Ilio C. ; Urbani A. ; Nebbia C. Proteomic investigation in the detection of the illicit treatment of calves with growth-promoting agents. *Proteomics* **2006**, *6*, 2813-2822.

48. McGrath T.F.; van Meeuwen J.A.; Massart A.-C.; de Pauw E.; Delahaut P.; Buijs J.; Bergwerff A.A.; Elliot C.T.; Mooney M.H. Effect-based proteomic detection of growth promoter abuse. *Anal. Bioanal. Chem.* **2013**, *405*, 1171-1179.

49. Becker C.; Riedmaier I.; Reiter M.; Tichopad A.; Groot M.J.; Stolker A.A.M.; Pfaffl M.W.; Nielen M.F.W.; Meyer H.H.D. Influence of anabolic combinations of an androgen plus an estrogen on biochemical pathways in bovine uterine endometrium and ovary. *J. Steroid Biochem. Mol. Biol.* **2011**, *125*, 192-201.

50. Riedmaier I.; Benes V.; Blake J.; Bretschneider N.; Zinser C.; Becker C.; Meyer H.H.D.; Pfaffl M.W. RNA-sequencing as useful screening tool in the combat against the misuse of anabolic agents. *Anal. Chem.* **2012**, *84*, 6863-6868.

51. Cunningham R.T.; Mooney M.H.; Xia X.-L.; Crooks S.; Matthews D.; O'Keeffe M.; Li K.; Elliot C.T. Feasibility of a clinical chemical analysis approach to predict misuse of growth promoting hormones in cattle. *Anal. Chem.* **2009**, *81*, 977-983.

52. Ludwig S.K.J.; Smits N.G.E.; Cannizzo F.T.; Nielen M.W.F. Potential of treatment-specific protein biomarker profiles for detection of hormone abuse in cattle. *J. Agric. Food Chem.* **2013**, *61*, 4514-4519.

53. Regal P.; Seijas J.A.; Cepeda A.; Fente C. Structure elucidation and HPLC-MS/MS determination of a potential biomarker for estradiol administration in cattle. *Anal. Bioanal. Chem.* **2013**, *405*, 9537-9546.

54. Riedmaier I.; Spornraft M.; Pfaffl M.W. Identification of a potential gene expression biomarker signature in bovine liver to detect the abuse of growth promoters. *Food Addit. Contam.* **2014**, *31*, 641-649.

55. Regal P.; Blokland M.H.; Fente C.A.; Sterk S.S.; Cepeda A.; van Ginkel L.A. Evaluation of the discriminative potential of a novel biomarker for estradiol treatments in bovine animals. *J. Agric. Food Chem.* **2015**, *63*, 370-378.

56. Vascellari M.; Pozza G.; Poppi L.; Capello K.; Angeletti R.; Ravarotto L.; Andrighetto I.; Mutinelli F. Evaluation of indirect biomarkers for detecting corticosteroids used as illegal growth promoters in beef cattle. *Vet. Rec.* **2008**, *163*, 147-152.

57. Groot M.J.; Biolatti B. Histopathological effects of boldenone in cattle. *J. Vet. Med. A* **2004**, *51*, 58-63

58. Shackleton C. Steroid analysis and doping control 1960-1980: Scientific developments and personal anecdotes. *Steroids,* **2009**, *74*, 288-295.

59. Carpenter P.C. Performance-enhancing drugs in sport. *Endocrinol. Metab. Clin. N. Am.* **2007**, *36,* 481-495.

60. De Brabander H.F.; Le Bizec B.; Pinel G.; Antignac J.-P.; Verheyden K.; Mortier V.; Courtheyn D.; Noppe H. Past, present and future of mass spectrometry in the analysis of residues of banned substances in meat-producing animals. *J. Mass Spectrom.* **2007***, 42,* 983-998.

61. Shahidi N.T. A review of the chemistry, biological action, and clinical applications of anabolic-androgenic steroids. *Clin. Ther.* **2001**, *23*, 1355-1390.

62. Hartgens F.; Kuipers H. Effects of androgenic-anabolic steroids in athletes. *Sports Med.* **2004**, *34,* 513-554.

63. European Council. Council Directive 81/602/EEC of 31 July 1981 concerning the prohibition of certain substances having a hormonal action and of any substances having a thyrostatic action, *Off. J. Eur. Commun.* **1981**, *L222*, 32-33.

64. Le Bizec B.; Pinel G.; Antignac J.-P. Options for veterinary drug analysis using mass spectrometry. *J. Chromatogr. A* **2009**, *1216,* 8016-8034.

65. Le Bizec B.; Marchand P.; Maume D.; Monteau F.; André F. Monitoring anabolic steroids in meat-producing animals. Review of current hyphenated mass spectrometric techniques

66. Southan G.; Mallet A.; Jumeau J.; Craig S.; Poojara N.; Mitchell D.; Wheeler M.; Brooks R.V. *Programme and Abstracts of The 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences* **2009**, *306*.

67. Ferchaud V.; Le Bizec B.; Monteau F.; André F. Determination of the exogenous character of testosterone in bovine urine by gas chromatography-combustion-isotope ratio mass spectrometry. *Analyst* **1998**, *123*, 2617-2620.

68. Scarth J.; Akre C.; van Ginkel L.; Le Bizec B.; De Brabander H.; Korth W.; Points J.; Teale P. Presence and metabolism of endogenous androgenic-anabolic steroid hormones in meat-producing animals: a review. *Food Addit. Contam.* **2009**, *26*, 640-671.

69. p. Anizan S.; Bichon E.; Di Nardo D.; Monteau F.; Cesbron N.; Antignac J.-P; Le Bizec B. Screening of 4-androstenedione misuse in cattle by LC-MS/MS profiling of glucuronide and sulfate steroids in urine. *Talanta* **2011**, *86*, 186-194.

70. Buisson C. Developpement d'une stratégie analytique basée sur la spectrométrie de masse de rapport isotopique appliquée au contrôle de l'usage frauduleux

d'hormones steroids en élevage. Thèse de Doctorat, Université de Nantes, Faculté des Sciences et des Techniques, **2005**.

71. Ebeling P.; Koivisto V.A. Physiological importance of dehydroepiandrosterone. *The Lancet* **1994**, *343*, 1479-1481.

72. Scippo M.L.; Gaspar P.; Degand G. ; Brose F. ; Maghuin-Rogister G. ; Delahaut P. ; Willemart J.P. Control of the illegal administration of natural steroid hormones in urine and tissues of veal calves and in plasma of bulls. *Anal. Chim. Acta* **1993**, *275*, 57-74.

73. Biddle S.; Teale P.; Robinson A.; Bowman J.; Houghton E. Gas chromatography-mass spectrometry/mass spectrometry analysis to determine natural and post-administration levels of oestrogens in bovine serum and urine. *Anal. Chim. Acta* **2007**, *586*, 115-121.

74. Smith J.F.; Fairclough R.J.; Payne E.; Peterson A.J. Plasma hormone levels in the cow. *New. Zeal. J. Agr. Res.* **1975**,*18*, 123-129.

75. Aller J.F.; Callejas S.S.; Alberio R.H. Biochemical and steroid concentrations in follicular fluid and blood plasma in different follicular waves of the estrous cycle from normal and superovulated beef cows. *Anim. Reprod. Sci.* **2013**, *142*, 113-120.

76. Vesanen M.; Isomaa V.; Bolton N.J.; Alanko M.; Vihko R. Bovine steroid-hormone and SHBG concentrations postpartum and during the estrous-cycle. *Acta Vet. Scand.* **1990**, *31*, 459-469.

77. Snoj T.; Dolenc J.; Kobal S. Sex steroid levels in urine of cattle of different ages: evaluation of abuse control procedures. *Food Addit. Contam. A* **2014**, *31*, 614-620.

78. Pedro A.; Lehmann F. Early history of steroid chemistry in Mexico. *Steroids* **1992**, *57*, 403-408.

79. Araghiniknam M.; Chung S.; Nelson-White T.; Eskelson C.; Watson R.R. Antioxidant activity of dioscorea and dehydroepiandrosterone (DHEA) in older humans. Life Sciences 1996, 59, PL 147-157.

80. Aguilera R.; Chapman T.; Pereira H.; Oliveira G.; Illanes R.; Fernandes T.; Azevedo D.; Auino Neto F. Drug testing data from the 2007 Pan American Games: $\delta^{13}C$ values of urinary androsterone, etiocholanolone and

androstanediols determined by GC/C/IRMS. *J. Steroid Biochem. Mol. Biol.* **2009**, *115*, 107-114.

81. Lim C.; Evenson G.N.; Perrault W.R.; Pearlman B.A. An environmentally friendly and cost effective synthesis of estradiol featuring two novel reagents: Si(0)/KF and PMHS/hexamethyldisiloxane/pTSA. *Tetrahedron Lett.* **2006**, *47*, 6417-6420.

82. Cawley A.T.; Flenker U. The application of carbon isotope ratio mass spectrometry to doping control. *J. Mass Spectrom.* **2008**, *43*, 854-864.

83. Strahm E.; Emery C.; Saugy M.; Dvorak J.; Saudan C. Detection of testosterone administration based on the carbon isotope ratio profiling of endogenous steroids: international reference populations of professional soccer players. *Br. J. Sports Med.* **2009**, *43*, 1041-1044.

84. Balizs G.; Jainz A.; Horvatovich P. Investigation of the feeding effect on the $^{13}C/^{12}C$ isotope ratio of the hormones in bovine urine using gas chromatography/combustion isotope ratio mass spectrometry. *J. Chromatogr. A* **2005**, *1067*, 323-330.

85. Saugy M.; Cardis C.; Robinson N.; Schweizer C. Test methods: anabolics. *Baillieres Clin. Endocrinol. Metab.* **2000**, *14*, 111-113.

86. De Brabander H.F.; Verheyden K.; Mortier V.; Le Bizec B.; Verbeke W.; Courtheyn D.; Noppe H. Phytosterols and anabolic agents versus designer drugs *Anal. Chim. Acta* **2007**, *586*, 49-56.

87. De la Torre X.; González J.C.; Pichini S.; Pascual J.A.; Segura J. $^{13}C/^{12}C$ isotope ratio MS analysis of testosterone, in chemicals and pharmaceutical preparations. *J. Pharm. Biomed. Anal.* **2001**, *24*, 645-650.

88. Cawley A.; Collins M.; Kazlauskas R.; Handelsman D.J.; Heywood R.; Longworth M.; Arenas-Queralt A. Stable isotope ratio profiling of testosterone preparations. *Drug Test. Analysis* **2010**, *2*, 557-567.

89. Chapelon A.-S.; Moraléda D.; Rodriguez R.; Ollivier C.; Santelli M. Enantioselective synthesis of steroids. *Tetrahedron* **2007**, *63*, 11511-11616.

90. Zhdanov R.I.; Corey E.J. In memorium Igor Torgov: Torgov's way to total steroid synthesis. *Steroids* **2009**, *74*, 723-724.

91. Piper T.; Geyer H.; Gougoulidis V.; Flenker U.; Schänzer W. Determination of $^{13}C/^{12}C$ ratios of urinary excreted boldenone and its main metabolite 5β-androst-1-en-17β-ol-3-one. *Drug Test. Anal.* **2010**, *2*, 217-224.

92. Nes W. Biosynthesis of cholesterol and other sterols. *Chem. Rev.* **2011**, *111*, 6423-6451.

93. Green G.; Aguilera R.; Ahrens B.; Starcevic B.; Kurtzman F.; Su J.; Catlin D. The influence of diet on isotope ratio mass spectrometry values. *Clin. J. Sport. Med.* **2009**, *19*, 287-292.

94. Ferchaud V.; Le Bizec B.; Monteau F.; Andre F. Characterization of exogenous testosterone in livestock by gas chromatography/combustion/isotope ratio mass spectrometry: influence of feeding and age. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 652-656.

95. Flenker U.; Günter U.; Schänzer W. δ^{13}C-Values of endogenous urinary steroids. *Steroids* **2008**, *73*, 408-416.

96. Saudan C.; Kamber M.; Barbati G.; Robinson N.; Desmarchelier A.; Mangin P.; Saugy M. Longitudinal profiling of urinary steroids by gas chromatography/combustion/isotope ratio mass spectrometry: diet change may result in carbon isotopic variations. *J. Chromatogr. B* **2006**, *831*, 324-327.

97. Donova M.V. Transformation of steroids by Actinobacteria: A review. *Appl. Biochem. Microbiol.* **2007**, *43*, 1-14.

98. Aguilera R.; Becchi M.; Casabianca H.; Hatton C.K.; Catlin D.H.; Starcevic B.; Pope Jr. H.G. Improved method of detection of testosterone abuse by gas chromatography combustion isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom.* **1996**, *31*, 169-176.

99. https://wada-main-prod.s3.amazonaws.com/resources/files/WADA-TD2004-EAAS-Reporting-and-Evaluation-Guidance-for-Testosterone%2C-Epitestosterone%2C-T-E-Ratio-and-Other-Endogenous-Steroids.pdf

100. Hebestreit M.; Flenker U.; Buisson C.; André F.; Le Bizec B.; Fry H. ; Lang M. ; Preiss Weigert A. ; Heinrich K.; Hird S.; Schänzer W. Application of stable carbon isotope analysis to the detection of testosterone administration to cattle. *J. Agric. Food Chem.* **2006**, *54*, 2850-2858.

101. Buisson C.; Hebestreit M.; Preiss Weigert A.; Heinrich K.; Fry H.; Flenker U.; Banneke S.; Prevost S.; André F.; Schaenzer W.; Houghton E.; Le Bizec B. Application of stable carbon isotope analysis to the detection of 17β-estradiol administration to cattle. *J. Chromatogr. A* **2005**, *1093*, 69-80.

102. Becue I.; Van Poucke C.; Rijk J.C.W.; Bovee T.F.H.; Nielen M.; Van Peteghem C. Investigation of urinary steroid metabolites in calf urine after oral and intramuscular administration of DHEA. *Anal. Bioanal. Chem.* **2010**, *396*, 799-808.

103. Lieberman S.; Warne P.A. 17-hydroxylase: an evaluation of the present view of its catalytic role in steroidogenesis. *J. Steroid Biochem. Mol. Biol.* **2001**, *78*, 299-312.

104. Aguilera R.; Chapman T.E.; Catlin D.H. A rapid screening assay for measuring urinary androsterone and etiocholanolone $\delta^{13}C$ (‰) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2294-2299.

105. Sano M.; Yotsui Y.; Abe H.; Sasaki S. A new technique for the detection of metabolites labelled by the isotope ^{13}C using mass fragmentography. *Biomed. Mass Spectrom.* **1976**, *3*, 1-3.

106. Schoeller D.A.; Hayes J.M. Computer controlled ion counting isotope ratio mass spectrometer. *Anal. Chem.* **1975**, *47*, 408-415.

107. Barrie A.; Bricout J.; Kozier J. Gas chromatography-stable isotope ratio analysis at natural abundance levels. *Biomed. Mass Spectrom.* **1984**, *11*, 439-447.

108. Craig H. Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. *Geochim. Cosmochim. Acta* **1957**, *12*, 133-149.

109. Zhang Y.; Tobias H.J.; Sacks G.L.; Brenna J.T. Calibration and data processing in gas chromatography combustion isotope ratio mass spectrometry. *Drug Test. Anal.* **2012**, *4*, 912-922.

110. Blackledge R.D. Bad science: the instrumental data in the Floyd Landis case. *Clin. Chim. Acta* **2009**, *406*, 8-13.

111. Meier-Augenstein W.; Brand W.; Hoffmann G.F.; Rating D. Bridging the information gap between isotope ratio mass spectrometry and conventional mass spectrometry. *Biolog. Mass Spectrom.* **1994**, *23*, 376-378.

112. Hall J.A.; Barth J.A.C.; Kalin R.M. Routine analysis by high precision gas chromatography mass selective detector isotope ratio mass spectrometry to 0.1 parts per mil. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1231-1236.

113. Mason P.; Hall S.; Gilmour I.; Houghton E.; Pillinger C.; Seymour M. The use of stable carbon isotope analysis to detect the abuse of testosterone in cattle. *Analyst* **1998**, *123*, 2405–2408.

114. Tsivou M.; Livadara D.; Georgakopoulos D.G.; Koupparis M.A.; Atta-Politou J.; Georgakopoulos C.G. Stabilization of human urine doping control samples: II. Microbial degradation of steroids. *Anal. Biochem.* **2009**, *388*, 146-154.

115. Mazzarino M.; Abate M.G.; Alocci R.; Rossi F.; Stinchelli R.; Molaioni F.; de la Torre X.; Botrè F. Urine stability and steroid profile: Towards a screening index of urine sample degradation for anti-doping purpose. *Anal. Chim. Acta* **2011**, *683*, 221-226.

116. Piper T.; Geyer H.; Schänzer W. Degradation of urine samples and its influence on the $^{13}C/^{12}C$ ratios of excreted steroids. *Drug Test. Anal.* **2010**, *2*, 620-629.

117. Prévost S.; Nicol T.; Monteau F.; André F.; Le Bizec B. GC/C/IRMS to control the misuse of androgens in breeding animals: new derivatization method applied to testosterone metabolites and precursors in urine samples. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2509-2514.

118. Aguilera R.; Catlin D.H.; Becchi M.; Phillips A.; Wang C.; Swerdloff R.S.; Pope H.G.; Hatton C.K. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J. Chromatogr. B* **1999**, *727*, 95-105.

119. Yamada M.; Kinoshita K.; Kurosawa M.; Saito K.; Nakazawa H. Analysis of exogenous nandrolone metabolite in horse urine by gas chromatography/combustion/carbon isotope ratio mass spectrometry. *J. Pharm. Biomed. Anal.* **2007**, *45*, 654-658.

120. Piper T.; Opfermann G.; Thevis M.; Schänzer W. Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids excreted as sulpho conjugates. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3171-3181.

121. Saudan C.; Emery C.; Marclay F.; Strahm E.; Mangin P.; Saugy M. Validation and performance comparison of two carbon isotope ratio methods to control the misuse of androgens in humans. *J. Chromatogr. B* **2009**, *877*, 2321-2329.

122. Sobolevsky T.G.; Prasolov I.G.; Rodchenkov G.M. Carbon isotope mass spectrometry in doping control. *J. Anal. Chem.* **2010**, *65*, 825-832.

123. Piper T.; Mareck U.; Geyer H.; Flenker U.; Thevis M.; Platen P.; Schänzer W. Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2161-2175.

124. Saudan C.; Baume N.; Mangin P.; Saugy M. Urinary analysis of 16(5α)-androsten-3α-ol by gas chromatography/combustion/isotope ratio mass spectrometry: implications in anti-doping analysis. *J. Chromatogr. B* **2004**, *810*, 157-164.

125. Piper T.; Riemann P.; Opfermann G.; Mareck U.; Geyer H.; Vajiala G.; Flenker U.; Schänzer W. Determination of $^{13}C-^{12}C$ ratios of urinary epitestosterone and its main metabolites 5α- and 5β-androstane-3α,17α-diol. *Drug Test. Anal.* **2009**, *1*, 576-586.

126. Shackleton C.; Phillips A.; Chang T.; Li Y. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. *Steroids* **1997**, *62*, 379-387.

127. Brooker L.; Parr M.; Cawley A.; Flenker U.; Howe C.; Kazlauskas R.; Schänzer W.; George A. Development of criteria for the detection of adrenosterone administration by gas chromatography-mass spectrometry and gas chromatography-combustion-isotope ratio mass spectrometry for doping control. *Drug Test. Anal.* **2009**, *1*, 587-595.

128. Gomes R.; Meredith W.; Snape E.; Sephton M. Conjugated steroids: analytical approaches and applications. *Anal. Bioanal. Chem.* **2009**, *393*, 453-458.

129. Bichon E.; Kieken F.; Cesbron N.; Monteau F.; Prévost S.; André F.; Le Bizec B. Development and application of stable carbon isotope analysis to the detection of cortisol administration in cattle. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2613-2620.

130. Piper T.; Emery C.; Saugy M. Recent developments in the use of isotope ratio mass spectrometry in sports drug testing. *Anal. Bioanal. Chem.* **2011**, *401*, 433-447.

131. Benbow T.J.; Frew R.D.; Hayman A.R. Validation of a rapid and simple method for the preparation of aqueous organic compounds prior to compound specific isotope analysis. *Org. Geochem.* **2008**, *39*, 1690-1702.

132. Russell J.M.; Werne J.P. The use of solid phase extraction columns in fatty acid purification. *Org. Geochem.* **2007**, *38*, 48-51.

133. Salvador J.-P.; Sanchez-Baeza F.; Marco M.-P. A high-throughput screening (HTS) immunochemical method for the analysis of stanozolol metabolites in cattle urine samples. *J. Chromatogr. B* **2010**, *878*, 243–252.

134. Caimi R.J.; Brenna J.T. Quantitative evaluation of carbon isotopic fractionation during reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* **1997**, *757*, 307-310.

135. Kenig F.; Popp B.N.; Summons R.E. Preparative HPLC with ultrastable-Y zeolite for compound-specific carbon isotopic analyses. *Org. Geochem.* **2000**, *31*, 1087-1094.

136. Hosogi J.; Tanaka H.; Fujita K.; Kuwabara T.; Ikegawa S. LC-MS/MS coupled with immunoaffinity extraction for determination of estrone, 17β-estradiol and estrone 3-sulfate in human plasma. *J. Chromatogr. B* **2010**, *878*, 222–227.

137. Su P.; Zhang X.; Chang W. Development and application of a multi-target immunoaffinity column for the selective extraction of natural estrogens from pregnant women's urine samples by capillary electrophoresis. *J. Chromatogr. B* **2005**, *816*, 7–14.

138. Aguilera R.; Becchi M.; Grenot C.; Casabianca H.; Hatton C. Detection of testosterone misuse: comparison of two chromatographic sample preparation methods for gas chromatographic/combustion isotope ratio mass spectrometric analysis. *J. Chromatogr. B* **1996**, *687*, 43-53.

139. Gomes R.; Meredith W.; Snape C.; Sephton M. Analysis of conjugated steroid androgens: deconjugation, derivatization and associated issues. *J. Pharm. Biomed. Anal.* **2009**, *49*, 1133–1140.

140. Bowden J.; Colosi D.; Mora-Montero D.; Garrett T.; Yost R. Enhancement of chemical derivatization of steroids by gas chromatography/mass spectrometry (GC/MS). *J. Chromatogr. B* **2009**, *877*, 3237–3242.

141. Parr M.; Schänzer W. Detection of the misuse of steroids in doping control. *J. Steroid Biochem. Mol. Biol.* **2010**, *121*, 528-537.

142. Corr L.; Berstan R.; Evershed R. Optimisation of derivatization procedures for the determination of $\delta^{13}C$-values of amino acids by gas

chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3759-3771.

143. de la Torre X.; Colamonici C.; Curcio D.; Molaioni F.; Pizzardi M.; Botrè F. A simplified procedure for GC/C/IRMS analysis of underivatized 19-norandrosterone in urine following HPLC purification. *Steroids* **2011**, *76*, 471-477.

144. Cawley A.; Trout G.; Kazlauskas R.; George A. The potential of urinary androstdiene markers to identify 4-androstenediol administration in athletes. *Forensic Sci. Int.* **2011**, *208*, 129-138.

145. Sephton M.; Meredith W.; Sun C.; Snape C. Hydropyrolysis of steroids: a preparative step for compound-specific carbon isotope ratio analysis. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 3339-3342.

146. Meredith W.; Gomes R.; Cooper M.; Snape C.; Sephton M. Hydropyrolysis over a platinum catalyst as a preparative technique for the compound-specific carbon isotope ratio measurement of C_{27} steroids. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 501-505.

147. Flenker U.; Hebestreit M.; Piper T.; Hulsemann F.; Schänzer W. Improved performance and maintenance in gas chromatography/isotope ratio mass spectrometry by precolumn solvent removal. *Anal. Chem.* **2007**, *79*, 4162-4168.

148. Meier-Augenstein W.; Watt P.; Langhans C. Influence of gas chromatographic parameters on measurement of $^{13}C/^{12}C$ isotope ratios by gas-liquid chromatography-combustion isotope ratio mass spectrometry. *J. Chromatogr. A* **1996**, *752,* 233-241.

149. Hayes J.M.; Freeman K.H.; Popp B.N.; Hoham C.H. Compound-specific isotopic analyses – a novel tool for reconstruction of ancient biogeochemical processes. *Org. Geochem.* **1990**, *16*, 1115-1128.

150. Meier-Augenstein W. Applied gas chromatography coupled to isotope ratio mass spectrometry. *J. Chromatogr. A* **1999**, *842*, 351-371.

151. Ricci M.P.; Merritt D.A.; Freeman K.H.; Hayes J.M. Acquisition and processing of data for isotope-ratio-monitoring mass spectrometry. *Org. Geochem.* **1994**, *21*, 561-571.

152. https://wada-main-prod.s3.amazonaws.com/resources/files/WADA-TD2014IRMS-v1-Detection-of-Synthetic-Forms-of-Endogenous-Anabolic-Androgenic-Steroids-by-GC-C-IRMS-EN.pdf

153. https://wada-main-prod.s3.amazonaws.com/resources/files/wada-td2016irms-detection_synthetic_forms_eaas_by_irms-en.pdf

154. Mathurin J.-C.; Herrou V.; Bourgogne E.; Pascaud L.; de Ceaurriz J. Gas chromatography-combustion-isotope ratio mass spectrometry analysis of 19-norsteroids: application to the detection of a nandrolone metabolite in urine. *J. Chromatogr. B* **2001**, *759*, 267-275.

155. Desroches M.C.; Mathurin J.C.; Richard Y.; Delahaut P.; De Ceaurriz J. Urinary 19-norandrosterone purification by immunoaffinity chromatography: application to gas chromatography/combustion/isotope ratio mass spectrometry analysis. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 370-374.

156. Aguilera R.; Chapman T.E.; Starcevic B.; Hatton C.K.; Catlin D.H. Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: Controls and athletes with elevated testosterone/epitestosterone ratios. *Clin. Chem.* **2001**, *47*, 292-300.

157. Piper T.; Flenker U.; Mareck U.; Schänzer W. $^{13}C/^{12}C$ Ratios of endogenous urinary steroids investigated for doping control purposes. *Drug Test. Anal.* **2009**, *1*, 65-72.

158. Hilkert A.; Douthitt C.; Schlüter H.; Brand W. Isotope ratio monitoring gas chromatography/mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1226-1230.

159. Piper T.; Thevis M.; Flenker U.; Schänzer W. Determination of the deuterium/hydrogen ratio of endogenous urinary steroids for doping control purposes. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1917-1926.

160. Piper T.; Thomas A.; Thevis M.; Saugy M. Investigations on hydrogen isotope ratios of endogenous urinary steroids: reference-population-based thresholds and proof-of-concept. *Drug Test. Anal.* **2012**, *4*, 717-727.

161. Zhang L.; Kujawinski D.; Jochmann M.; Schmidt T. High-temperature reversed-phase liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 2971-2980.

162. Godin J.; Hopfgartner G.; Fay L. Temperature-programmed high-performance liquid chromatography coupled to isotope ratio mass spectrometry. *Anal. Chem.* **2008**, *80*, 7144-7152.

163. Tobias H.J.; Sacks G.L.; Zhang Y.; Brenna J.T. Comprehensive two-dimensional gas chromatography combustion isotope ratio mass spectrometry. *Anal. Chem.* **2008**, *80*, 8613-8621.

164. Tobias H.J.; Zhang Y.; Auchus R.J.; Brenna J.T. Detection of synthetic testosterone use by novel comprehensive two-dimensional gas chromatography combustion-isotope ratio mass spectrometry. *Anal. Chem.* **2011**, *83*, 7158-7165.

165. Sottas P.-E.; Saudan C.; Schweizer C.; Baume N.; Mangin P.; Saugy M. From population- to subject-based limits of T/E ratio to detect testoterone abuse in elite sports. *Forensic Sci. Int.* **2008**, *174*, 166-172.

166. Sottas P.; Saugy M.; Saudan C. Endogenous steroid profiling in the athlete biological passport. *Endocrinol. Metab. Clin. North Am.* **2010**, *39*, 59-73.

167. Mackay L.; Kazlauskas R. The importance of reference materials in doping-control analysis. *Anal. Bioanal. Chem.* **2011**, *401*, 483-492.

168. Zhang Y.; Tobias H.; Brenna J. Steroid isotopic standards for gas chromatography/combustion isotope ratio mass spectrometry. *Steroids* **2009**, *74*, 369-378.

169. Kioussi M.; Angelis Y.; Cawley A.; Koupparis M.; Kazlauskas R.; Brenna J.; Georgakopoulos C. External calibration in gas chromatography/combustion-isotope ratio mass spectrometry measurements of endogenous androgenic anabolic steroids in sports doping control. *J. Chromatogr. A* **2011**, *1218*, 5675-5682.

170. Munton E.; Murby J.; Hibbert D.; Santamaria-Fernandez R. Systematic comparison of $\delta^{13}C$ measurements of testosterone and derivative steroids in a freeze-dried urine candidate reference material for sports drug testing by gas chromatography/combustion/isotope ratio mass spectrometry and uncertainty evaluation using four different metrological approaches. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1641-1651.

171. http://ec.europa.eu/food/food/chemicalsafety/residues/control_en.print.htm, accessed on 10/09/2013.

172. Janssens G.; Courtheyn D.; Mangelinckx S.; Prévost S.; Bichon E.; Monteau F.; De Poorter G.; De Kimpe N.; Le Bizec B. Use of Isotope Ratio Mass Spectrometry to differentiate between endogenous steroids and synthetic homologues in cattle: a review. *Anal. Chim. Acta* **2013**, *772*, 1-15.

173. Saudan C.; Emery C.; Marclay F.; Strahm E.; Mangin P.; Saugy M. Validation and performance comparison of two carbon isotope ratio methods to control the misuse of androgens in humans. *J. Chromatogr. B* **2009**, *877*, 2321-2329.

174. Pinel G.; Weigel S.; Antignac J.-P.; Mooney M. H.; Elliott C.; Nielen M. W. F.; Le Bizec B. Targeted and untargeted profiling of biological fluids to screen for anabolic practices in cattle. *Trends Anal. Chem.* **2010,** *29*, 1269-1280.

175. Mooney M.H.; Le Bizec B.; Elliott C.T. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. *Trends Anal. Chem.* **2009,** *28*, 665-675.

176. Antignac J.-P.; Courant F.; Pinel G.; Bichon E.; Monteau F.; Elliott C.; Le Bizec B. Mass spectrometry-based metabolomics applied to the chemical safety of food. *Trends Anal. Chem.* **2011,** *30*, 292-301.

177. Dervilly-Pinel G.; Courant F.; Chereau S.; Royer A.-L.; Boyard-Kieken F.; Antignac J.-P.; Monteau F.; Le Bizec B. Metabolomics in food analysis: application to the control of forbidden substances. *Drug Test. Anal.* **2012**, *4*, 59-69.

178. Divari S.; Cannizzo F.T.; Uslenghi F.; Pregel P.; Mulasso C.; Spada F. Corticosteroid hormone receptors and prereceptors as new biomarkers of the illegal use of glucocorticoids in meat production. *J. Agric. Food Chem.* **2011**, *59*, 2120-2125.

179. Janssens G.; Mangelinckx S.; Courtheyn D.; Prévost S.; De Poorter G.; De Kimpe N.; Le Bizec B. Application of gas chromatography-mass spectrometry/combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) to detect the abuse of 17β-estradiol in cattle. *J. Agric. Food Chem.* **2013**, *61*, 7242-7249.

180. Forsdahl G.; Östreicher C.; Koller M.; Gmeiner G. Carbon isotope ratio determination and investigation of seized testosterone preparations. *Drug Test. Analysis* **2011**, *3*, 814-819.

181. Brooker L.; Cawley A.; Drury J.; Edey C.; Hasick N.; Goebel C. Stable carbon isotope ratio profiling of illicit testosterone preparations – domestic and international seizures. *Drug Test. Analysis* **2014**, *6*, 996-1001.

182. Hullstein I.; Sagredo C.; Hemmersbach P. Carbon isotope ratios of nandrolone, boldenone, and testosterone preparations seized in Norway

compared to those of endogenously produced steroids in a Nordic reference population. *Drug Test. Analysis* **2014**, *6*, 1163-1169.

183.   Polet M.; Van Eenoo P. GC-C-IRMS in routine doping controle practice: 3 years of drug testing data, quality control and evolution of the method. *Anal. Bioanal. Chem.* **2015**, *407*, 4397-4409.

184.   Heitzman R.J. The effectiveness of anabolic agents in increasing rate of growth in farm animals; report on experiments in cattle. *Environ. Qual. Saf. Suppl.* **1976**, *5*, 89-98.

185.   Stolker A. A. M.; Groot M.J.; Lasaroms J.J.P.; Nijrolder W.J.M.; Blokland M.H.; Riedmaier I.; Becker C.; Meyer H.H.D.; Nielen M.W.F. Detectability of testosterone esters and estradiol benzoate in bovine hair and plasma following pour-on treatment. *Anal. Bioanal. Chem.* **2009**, *395*,1075–1087.

186.   Kaabia Z.; Dervilly-Pinel G.; Hanganu F.; Cesbrond N.; Bichon E.; Popot M.A. ; Bonnaire Y.; Le Bizec B. Ultra high performance liquid chromatography/tandem mass spectrometry based identification of steroid esters in serum and plasma: An efficient strategy to detect natural steroids abuse in breeding and racing animals. *J. Chromatogr. A* **2013**, *1284*, 126-140.

187.   Rambaud L.; Bichon E.; Cesbron N.; André F.; Le Bizec B. Study of 17 ß-estradiol-3-benzoate, 17α-methyltestosterone and medroxyprogesterone acetate fixation in bovine hair. *Anal. Chim. Acta* **2005**, *532*, 165-176.

188.   Rambaud L.; Monteau F.; Deceuninck Y.; Bichon E.; André F.; Le Bizec B. Development and validation of a multi-residue method for the detection of a wide range of anabolic steroids in hair using gas chromatography-tandem mass spectrometry. *Anal. Chim. Acta* **2007**, *586*, 93-104.

189.   Nielen M.W.F.; Lasaroms J.J.P.; Mulder P.P.J.; Van Hende J.; Van Rhijn J.A.; Groot M.J. Multi residue screening of intact testosterone esters and boldenone undecylenate in bovine hair using liquid chromatography electrospray tandem mass spectrometry. *J. Chromatogr. B* **2006**, *830*, 126-134.

190.   Costain R.M.; Fesser A.C.E.; McKenzie D.; Mizuno M.; MacNeil J.D. Identification of hormone esters in injection site in muscle tissues by LC/MS/MS. *Food Addit. Contam. A* **2008**, *25*, 1520-1529.

191.   Janssens G.; Mangelinckx S.; Courtheyn D.; De Kimpe N.; Matthijs B.; Le Bizec B. Simultaneous Detection of androgen and estrogen abuse in breeding

animals by gas chromatography-mass spectrometry/combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) evaluated against alternative methods. *J. Agric. Food Chem.* **2015**, *63*, 7574-7581.

192. http://ec.europa.eu/food/food/chemicalsafety/residues/monitoring_en.htm (accessed on 2015/09/18)

193. FDA website: http://www.ecfr.gov/cgi-bin/text-idx?c=ecfr&SID=f44bc2af7b4ac5a3 adf170a9e18b9842&rgn=div5&view=text&node=21:6.0.1.1.11&idno=21#21:6.0.1. 1.11.0.1.56 (accessed on 2015/09/18)

194. Scarth J.; Clarke A.; Hands J.; Teale P.; Macarthur R.; Kay J. Validation of a quantitative multi-residue urinary assay for the detection of androgen, oestrogen and progestagen abuse in the bovine. *Chromatographia* **2010**, *71*, 241-252.

195. Yang C.J.; Wu L.S.; Liu S.H.; Lin J.H. Monitoring the reproductive status of dairy cows by urinary pregnanediol glucuronide. *Asian-Aust. J. Anim. Sci.* **2004**, *17*, 460-466.

196. Buisson C.; Mongongu C ; Frelat C. ; Jean-Baptiste M. ; de Ceaurriz J. Isotope ratio mass spectrometry analysis of the oxidation products of the main and minor metabolites of hydrocortisone and cortisone for antidoping controls. *Steroids* **2009**, *74*, 393-397.

197. Wang J.; Yang R.; Yang W.; Liu X.; Xing Y. Impact of progesterone administration on doping test of endogenous steroids. *Anal. Bioanal. Chem.* **2014**, *406*, 6061-6067.

198. Quellet A.; LeBerre N.; Ayotte C. A simplified and accurate method for the analysis of urinary metabolites of testosterone-related steroids using gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 1739-1750.

199. Polet M.; Van Gansbeke W.; Deventer K.; Van Eenoo P. Development of a sensitive GC-C-IRMS method for the analysis of androgens. *Biomed. Chromatogr.* **2012**, *27*, 259-266.

200. De la Torre X.; Colamonici C.; Curcio D.; Molaioni F.; Botrè F. A comprehensive procedure based on gas chromatography-isotope ratio mass spectrometry following high performance liquid chromatography purification for

the analysis of underivatized testosterone and its analogues in human urine. *Anal. Chim. Acta* **2012**, *756*, 23-29.

201.   Doué M.; Bichon E.; Dervilly-Pinel G.; Pichon V.; Chapuis-Hugon F.; Lesellier E.; West C.; Monteau F.; Le Bizec B. Molecularly imprinted polymer applied to the selective isolation of urinary steroid hormones: An efficient tool in the control of natural steroid hormones abuse in cattle. *J. Chromatogr. A* **2012**, *1270*, 51-61.

202.   Dubois M.; Taillieu X.; Colemonts Y.; Lansival B.; De Graeve J.; Delahaut P. GC-MS determination of anabolic steroids after multi-immunoaffinity purification. *Analyst* **1998**, *123*, 2611-2616.

203.   Crooks S.R.H.; Elliot C.T.; Thompson C.S.; McCaughey W.J. Comparison and evaluation of the specificity and binding capacity of commercial and in house affinity columns used in sample preparation for analysis of growth-promoting drugs. *J. Chromatogr. B* **1997**, *690*, 161-172.

204.   http://www.afsca.be/labos/erk-lab/_documents/2012-12-01_LAB-10-P-510-agrement-EXTlabs-without-modifications-v04_EN.pdf accessed on 16/10/2015.

205.   http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=39883 accessed on 16/10/2015.

206.   Isobe N.; Nakao T.; Yamashiro H.; Shimada M. Enzyme immunoassay of progesterone in the feces from beef cattle to monitor the ovarian cycle. *Anim. Reprod. Sci.* **2005**, *87*, 1-10.

207.   Isobe N.; Akita M; Nakao T.; Yamashiro H.; Kubota H. Pregnancy diagnosis based on the fecal progesterone concentration in beef and dairy heifers and beef cows. *Anim. Reprod. Sci.* **2005**, *90*, 211-218.

208.   Kornmatitsuk B.; Thitaram C.; Kornmatitsuk S. Measurement of faecal progesterone metabolites and its application for early screening of open cows post-insemination. *Reprod. Dom. Anim.* **2007**, *42*, 238-242.

209.   Tyagi P.; Edwards D.R.; Coyne M.S. Use of sterol and bile acid biomarkers to identify domesticated animal sources of faecal pollution. *Water Air Soil Pollut.* **2008**, *187*, 263-274.

210.   Delahaut P.; Demoulin L.; Gillard N.; Fichant E.; Courtheyn D. Preliminary study on the presence of prednisolone in porcine urine and liver – how to distinguish endogenous from therapeutically administered prednisolone. *Drug Test. Anal.* **2014**, *6*, 325-335.

8. Summary

The positive effects of steroid hormones on growth and feed conversion are well known since the middle of the past century. However, as a precautionary measure, their use as growth promoting agents in stock farming remains prohibited in the European Union, and is actively monitored. Still, when it comes to synthetic analogues of endogenously produced steroid hormones, detection of abuse remains difficult. Although the capability to elucidate the origin of these compounds in urine samples using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) has been demonstrated since the late 1990s, its application for food safety purposes remained rare. The aim of the current research was to provide fully validated IRMS-based methods for the detection of abuse of synthetic analogues of endogenous sex steroids, which could be implemented as such for official control purposes.

In **chapter I**, an introduction to endogenous steroid hormones, with particular focus on the group of sex steroid hormones, is given. After providing the basic rules of steroid nomenclature, an overview is given of the compounds of interest in the present study. Next, the different types of endogenous steroid hormones are defined, describing their natural functionality in the body, as well as their growth promoting characteristics. Afterwards, information is provided on the currently active legislation, regulating the use of steroid hormones as growth promoters in the European Union, and Belgium specifically. Then, different analytical approaches to screen for abuse of synthetic analogues of endogenous hormones are described, ranging from steroid concentrations and profiling, over holistic fingerprinting techniques, to indirect biomarker screening. Finally, the outline of this doctoral thesis is presented.

In **chapter II**, an extensive literature review is provided, covering all published aspects related to the detection of abuse of synthetic analogues of endogenous steroids in cattle using GC-C-IRMS. First, both the bovine steroid metabolism and the production process of steroid preparations are described, illustrating that the difference in the $^{13}C/^{12}C$ ratio of the respective source materials provides the theoretical basis to distinguish between endogenously and synthetically produced

steroids which are structurally identical. Then, the different factors influencing the steroids' $^{13}C/^{12}C$ ratio, expressed as $δ^{13}C_{VPDB}$ values, are discussed, illustrating the need to measure at least two different steroids in a urine sample: a metabolite of the target compound of which the $δ^{13}C_{VPDB}$ value will be affected by the treatment, and an endogenous reference compound (ERC) of which the $δ^{13}C_{VPDB}$ value will remain stable. The difference between both, the $Δ^{13}C_{VPDB}$ value, will provide the unambiguous indication of the treatment. Next, the analytical application is thoroughly discussed, describing the functionality of the used GC-C-IRMS apparatus, the used sample preparation techniques and the combined analytical strategies. Finally, a link is provided to other IRMS-based detection techniques, and to the field of sports doping control, where GC-C-IRMS is more commonly applied to detect abuse of synthetic analogues of androgenic-anabolic steroids.

In **chapter III**, a confirmation method to detect abuse of synthetic analogues of endogenous estrogens in cattle using gas chromatography coupled to both mass spectrometry and combustion-isotope ratio mass spectrometry in parallel (GC-(MS/C-IRMS)) is presented, which allowed to eliminate the need of additional GC-MS analyses. The sample preparation protocol consisted of a hydrolysis, a solid phase extraction, two liquid-liquid extractions, two HPLC fractionations and an acetylation, after which the $δ^{13}C_{VPDB}$ values of 5-androstene-3β,17α-diol, the ERC, and 17α-estradiol, the metabolite, could be successfully measured. The method was thoroughly validated and allowed to distinguish between samples from treated and untreated animals.

In **chapter IV**, the developed method was fine-tuned and extended to include the detection of abuse of synthetic analogues of androgens, which was possible by additional measurement of 17α-testosterone and etiocholanolone. After thorough validation, the detection method was used to analyze multiple samples from a bull and a heifer, treated intramuscularly with esters of 17β-estradiol and 17β-testosterone. The results from this administration study allowed to adequately determine the detection windows of the method, and provided detailed pharmacokinetic data. Finally, the developed method was directly compared with

alternative confirmatory approaches, being the detection of intact steroid esters in hair and blood samples using other mass spectrometric techniques.

In **chapter V**, the development of a GC-(MS/C-IRMS) method to detect progesterone abuse in bovines is described. First, four pregnanediol isomers were identified as candidate metabolites of progesterone. Next, two analytical methods were developed and evaluated against each other. In the first, three selected 5β-pregnane-3,20-diol isomers were separately measured after acetylation, whereas in the second, the four 5β-pregnane-3,20-diols were converted to one oxidation product, 5β-pregnane-3,20-dione before measurement. Eventually, an approach in which only 5β-pregnane-3α,20α-diol and 5-androstene-3β,17α-diol as metabolite and ERC, respectively, were measured after acetylation was preferred, validated and successfully applied to distinguish between samples from treated and untreated animals. Finally, it was observed that a sudden shift in feeding regime, as well as the treatment with 17β-estradiol and 17β-testosterone, could cause an unexpected and significant offset between the $\delta^{13}C_{VPDB}$ values of the ERC and the metabolite.

In **chapter VI**, the results are discussed in general and a number of suggestions are made for future research. The combination of the developed methods allows to successfully detect abuse of synthetic analogues of endogenous androgen, estrogen and progestagen steroid hormones. However, the sample preparation protocol is still extensive, and perhaps the use of immunoaffinity chromatography could provide a solution. Also, method validation and quality control remain a cumbersome labor. Official guidelines and reference material for GC-C-IRMS analysis of samples from animal origin would provide powerful means for simplification. Finally, additional animal experiments are required to obtain more insight on the unexpected phenomena influencing the $\delta^{13}C_{VPDB}$ values of 5β-pregnane-3α,20α-diol.

9. Samenvatting

De gunstige effecten van steroïdhormonen op groei en voederconversie zijn welbekend sinds het midden van de vorige eeuw. Toch werd uit voorzorg hun gebruik als groeibevorderaars voor voedselproducerende dieren verboden en actief nagegaan en opgevolgd binnen de Europese Unie. De detectie van misbruik van synthetische equivalenten van natuurlijk voorkomende steroïdhormonen blijft echter moeilijk. Hoewel de mogelijkheid om de oorsprong van dergelijke stoffen in urine met behulp van gaschromatografie gekoppeld aan verbrandings-isotoop ratio massaspectrometrie (GC-C-IRMS) aan te tonen reeds gekend is sinds het einde van de negentiger jaren, wordt deze techniek nog steeds slechts zelden gebruikt in het kader van voedselveiligheid. Het doel van het huidige onderzoek was om volledig gevalideerde IRMS-methoden voor de detectie van misbruik van synthetische equivalenten van natuurlijk voorkomende sekssteroïdhormonen te verschaffen, die als dusdanig kunnen geïmplementeerd worden in het kader van officiële controles.

In **hoofdstuk I** wordt een inleiding gegeven rond natuurlijke steroïdhormonen, met verhoogde aandacht voor de groep van sekssteroïdhormonen. Nadat de basisregels rond naamgeving van steroïden worden verschaft, wordt er ook een overzicht gegeven van de verschillende componenten van specifiek belang binnen dit onderzoek. Vervolgens worden de verschillende types van natuurlijke steroïdhormonen besproken, en wordt zowel hun natuurlijke functie in het lichaam evenals hun werking als groeibevorderaar verduidelijkt. Nadien wordt de wetgeving opgesomd die het gebruik van hormonale substanties als groeibevorderaars beperkt, zowel op Europees als specifiek op Belgisch niveau. Daarna worden de verschillende pistes besproken die kunnen worden aangewend voor screeningmethoden, variërend van concentraties en profielen van steroïden, via holistische technieken, tot de screening van indirecte biomarkers. Tenslotte wordt de opbouw van de doctoraatsthesis voorgesteld.

In **hoofdstuk II** wordt een uitgebreid literatuuroverzicht gegeven, dat alle gepubliceerde aspecten rond de detectie van misbruik van synthetische equivalenten van natuurlijk voorkomende steroïdhormonen in vee met behulp van GC-C-IRMS

omvat. Eerst worden zowel het steroïdmetabolisme van runderen als de productieprocessen van steroïdhormoonpreparaten uitgelegd, en wordt er verduidelijkt dat het verschil in het overeenkomstige bronmateriaal, en meer specifiek in de $^{13}C/^{12}C$ verhouding van dat bronmateriaal, de theoretische basis is die toelaat om een verschil waar te nemen tussen natuurlijke en synthetische steroïden die identiek zijn wat betreft hun chemische structuur. Vervolgns worden de verschillende factoren besproken die de $^{13}C/^{12}C$ verhouding van steroïden, die wordt uitgedrukt als $\delta^{13}C_{VPDB}$ waarden, beïnvloeden. Hieruit wordt duidelijk dat op zijn minst twee steroïden moeten gemeten worden in een urinestaal: een metaboliet van de toegediende stof, waarvan de $\delta^{13}C_{VPDB}$ waarde zal worden beïnvloed door de behandeling, en een endogene referentiecomponent (ERC), wiens $\delta^{13}C_{VPDB}$ waarde stabiel blijft. Het verschil tussen beiden, de $\Delta^{13}C_{VPDB}$ waarde, kan de behandeling duidelijk aantonen. Vervolgens wordt de werking van het daartoe gebruikte GC-C-IRMS toestel toegelicht, en worden de individuele staalvoorbereidingstechnieken evenals integrale staalvoorbereidingsprotocols besproken. Tenslotte wordt het verband met andere IRMS-gebaseerde opsporingstechnieken en met het domein van de humane dopingcontrole gelegd, waar GC-C-IRMS een veelgebruikte methode is voor het opsporen van misbruik van synthetische equivalenten van natuurlijk voorkomende anabole steroïden.

In **hoofdstuk III** wordt een bevestigingsmethode voor het opsporen van misbruik van synthetische equivalenten van natuurlijke estrogenen met behulp van gaschromatografie, parallel gekoppeld aan zowel massaspectrometrie als aan verbrandings-isotoop ratio massaspectrometrie (GC-(MS/C-IRMS)) gepresenteerd. De gebruikte opstelling liet toe aanvullende GC-MS analyses te schrappen uit het analyseprotocol. De gebruikte staalvoorbereiding bestond uit een hydrolysestap, gevolgd door een vaste fase extractie, twee vloeistof-vloeistofextracties, twee HPLC opzuiveringen en een acetylering, waarna de $\delta^{13}C_{VPDB}$ waarden van 5-androsteen-3β,17α-diol als ERC, en van 17α-estradiol als metaboliet, succesvol konden worden gemeten. De ontwikkelde methode werd grondig gevalideerd en liet toe om onderscheid te maken tussen stalen van behandelde en onbehandelde dieren.

In **hoofdstuk IV** wordt de ontwikkelde methode verder verfijnd en uitgebreid, zodat eveneens misbruik van synthetische equivalenten van androgenen kan worden opgespoord, wat mogelijk wordt door de toegevoegde meting van 17α-testosteron en etiocholanolon. Na uitgebreide validatie werd de methode aangewend voor de analyse van stalen van een stier en een koe, die intramusculair behandeld werden met 17β-estradiol-3-benozaat en 17β-testosteronpropionaat. De resultaten van deze behandelingsstudie lieten toe om het detectieluik van de methode te bepalen, en verschafte gedetailleerde farmacokinetische data. Tenslotte werd de ontwikkelde methode rechtstreeks vergeleken met alternatieve opsporingsmethoden, zijnde de detectie van intacte steroïdesters in haar- en bloedstalen met behulp van andere massaspectrometrische technieken.

In **hoofdstuk V** wordt de ontwikkeling van een GC-(MS/C-IRMS) methode beschreven die toelaat misbruik van progesteron bij runderen op te sporen. Eerst werden vier pregnaandiol-isomeren geïdentificeerd als kandidaat metabolieten. Vervolgens werden twee analytische methoden ontwikkeld en met elkaar vergeleken. In de eerste methode werden drie geselecteerde isomeren van 5β-pregnaan-3,20-diol apart gemeten na acetylering, terwijl in de tweede methode de vier isomeren van 5β-pregnaan-3,20-diol eerst werden omgezet tot één oxidatieproduct, 5β-pregnaan-3,20-dion, dat vervolgens werd geanalyseerd. Uiteindelijk werd een strategie waarbij enkel 5β-pregnaan-3α,20α-diol, als metaboliet, en 5-androsteen-3β,17α-diol, als ERC, werden gemeten na acetylering. De methode werd gevalideerd en liet toe om onderscheid te maken tussen stalen van behandelde en onbehandelde dieren. Tenslotte werd waargenomen dat een plotse verandering van voederregime, evenals een behandeling met 17β-estradiol en 17β-testosteron, een onverwacht en aanzienlijk verschil kunnen veroorzaken tussen de $\delta^{13}C_{VPDB}$ waarden van de ERC en de metaboliet.

In **hoofdstuk VI** worden de algemene resultaten besproken en worden een aantal suggesties voor verder onderzoek gedaan. De combinatie van de ontwikkelde methoden laat toe om misbruik van synthetische equivalenten van zowel androgene, estrogene als gestagene steroïdhormonen op te sporen. De staalvoorbereiding blijft echter uitgebreid, en mogelijk kan het gebruik van immunoaffiniteitschromatografie

hiervoor een oplossing bieden. Validatie en kwaliteitscontrole blijven eveneens een omvangrijke taak. Officiële richtlijnen en referentiemateriaal voor GC-C-IRMS analysen van stalen van dierlijke oorsprong zouden dit aanzienlijk kunnen vereenvoudigen. Tenslotte wordt duidelijk gemaakt dat aanvullende dierproeven nodig zijn om de onverwachte vaststellingen rond de $\delta^{13}C_{VPDB}$ waarden van 5β-pregnaan-3α,20α-diol te verklaren.

10. Curriculum Vitae

10.1. Curriculum Vitae (Nl)

Geert Janssens werd geboren op 22 oktober 1979 te Leuven. Na het behalen van het diploma hoger secundair onderwijs aan het Don Bosco-Instituut te Haacht (Latijn-Grieks), begon hij in 1997 de studie Bio-ingenieur aan de Katholieke Universiteit Leuven. In 2003 behaalde hij met onderscheiding het diploma Bio-Ingenieur in de Scheikunde (optie voeding).

Vervolgens vatte hij zijn loopbaan aan bij het Sint-Albertuscollege te Haasrode, als leerkracht chemie en fysica in het secundair onderwijs.

In 2004 trad hij in dienst van het Federaal Agentschap voor de Veiligheid van de Voedselketen (FAVV), als inspecteur voor de Nationale Opsporingseenheid (NOE). Binnen deze fraudecel was hij hoofdzakelijk actief in de gerichte bestrijding van hormonenmisbruik in de vetmesting en het opsporen van onregelmatigheden in voedingssupplementen voor humane consumptie.

In 2009 maakte hij de overstap naar het Directoraat-generaal van de Laboratoria van het FAVV, waar hij initieel werd ingezet als kwaliteitsverantwoordelijke voor het Federaal Laboratorium voor de Veiligheid van de Voedselketen te Tervuren (FLVVT). In de daaropvolgende periode was hij als coördinator chemie voornamelijk actief binnen het project ISORA. Dit onderzoeksproject, een actieve samenwerking tussen het FAVV, het 'Laboratoire d'études des résidus et contaminants dans les aliments' (LABERCA) te Nantes en de Vakgroep Duurzame Organische Chemie en Technologie binnen de Faculteit Bio-ingenieurswetenschappen van de Universiteit Gent, had als doelstelling het ontwikkelen van analysemethoden voor het opsporen van misbruik van natuurlijke hormonen in de vetmesting, gebruik makend van gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) binnen het Federaal Laboratorium voor de Veiligheid van de Voedselketen te Gentbrugge (FLVVG).

10.2. Curriculum Vitae (Eng)

Geert Janssens was born on the 22nd of October 1979 in Leuven (B). After graduating from high school at the Don Bosco-institute at Haacht (B) (Latin-Greek), he began the study of Bioscience Engineering at the University of Leuven in 1997. In 2003 he obtained his degree in Bioscience Engineering (option Food chemistry) with honours.

Afterwards, he commenced his career at the Sint-Albertuscollege Haasrode, as a secondary school chemistry and physics teacher.

In 2004, he was recruited by the Belgian Federal Agency for the Safety of the Food Chain (FASFC), as an inspector for the National Investigation Unit (NIU). Within this fraud investigation cell, he was mainly active in the targeted investigation of hormone abuse in stock farming, and the tracing of irregularities regarding food supplements for human consumption.

In 2009, he transferred to the Directorate-general of the Laboratories of the FASFC, were he was initially assigned as the quality manager of the Federal Laboratory for the Safety of the Food Chain at Tervuren (FLSFCT). As chemistry coordinator, he was mainly active within the project ISORA during the following period. This research project, which was a collaboration between the FASFC, the 'Laboratoire d'études des résidues et contaminants dans les aliments' (LABERCA) at Nantes (Fr) and the Department of Sustainable Organic Chemistry and Technology of the Faculty of Bioscience Engineering of Ghent University, was aimed at the development of analytical methods for the detection of abuse of synthetic analogues of endogenous hormones in stock farming, using gas chromatography-combustion-isotope ratio mass spectrometry, and was conducted at the Federal Laboratory for the Safety of the Food Chain at Gentbrugge (FLSFCG)

10.3. Personalia

Name: Geert Janssens

Date of birth: October 22nd, 1979

Place of birth: Leuven, Belgium

10.4. Record of education

2009 – 2016:   PhD researcher in Bioscience Engineering, Department of Sustainable Organic Chemistry and Technology, Ghent University, Ghent, Belgium.

Project: Stable carbon isotope analyses of natural steroid hormones to determine their abuse in cattle.

Promoters: Prof. Dr. ir. Sven Mangelinckx, Prof Dr. ir. Norbert De Kimpe, Prof. Dr. Bruno Le Bizec

1997 – 2003:   Master in Bioscience Engineering, KU Leuven, Belgium.

Option: Food chemistry, honours.

Thesis: effect of the fat content of feed on the intake of polychlorobiphenyls (PCBs) in laying hens.

Promoter: Prof. Dr. ir. Remi De Schrijver.

1991 – 1997:   Secondary School, Don Bosco-Institute, Haacht, Belgium.

10.5. Professional employment

2009 – present: Coordinator chemistry, Directorate-general Laboratories, Federal Agency for the Safety of the Food Chain, Belgium.

2009: Quality manager, Federal Laboratory for the Safety of the Food Chain Tervuren, Belgium.

2004 – 2009: Food safety inspector, National Investigation Unit, Directorate-general Control, Federal Agency for the Safety of the Food Chain, Belgium.

2003 – 2004: Teacher, chemistry and Physics, Sint-Albertus High School, Haasrode, Belgium.

10.6. Peer-reviewed articles

Janssens G.; Mangelinckx S.; Courtheyn D.; De Kimpe N.; Matthijs B.; Le Bizec B. Simultaneous Detection of androgen and estrogen abuse in breeding animals by gas chromatography-mass spectrometry/combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) evaluated against alternative methods. *J. Agric. Food Chem.* **2015**, *63*, 7574-7581.

Janssens G.; Mangelinckx S.; Courtheyn D.; Prévost S.; De Poorter G.; De Kimpe N.; Le Bizec B. Application of gas chromatography-mass spectrometry/combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) to detect the abuse of 17β-estradiol in cattle. *J. Agric. Food Chem.* **2013**, *61*, 7242-7249.

Janssens G.; Courtheyn D.; Mangelinckx S.; Prévost S.; Bichon E.; Monteau F.; De Poorter G.; De Kimpe N.; Le Bizec B. Use of Isotope Ratio Mass Spectrometry to differentiate between endogenous steroids and synthetic homologues in cattle: a review. *Anal. Chim. Acta* **2013**, *772*, 1-15.

10.7. Manuscripts submitted to peer-reviewed journals

Janssens G.; Mangelinckx S.; Courtheyn D.; De Kimpe N.; Matthijs B.; Le Bizec B. The use of gas chromatography-mass spectrometry/combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) to demonstrate progesterone treatment in bovines. Submitted to *J. Chromatogr. A.* **2015**.

10.8. Oral presentations

Janssens G.; Courtheyn D.; De Kimpe N.; Mangelinckx S.; De Poorter G.; Le Bizec B. GC-MS/C/IRMS to prove administration of androgens and estrogens in bovines. Benelux Association of Stable Isotope Scientists Annual Meeting, Nijmegen, The Netherlands, 2014.

10.9. Poster presentations

Janssens G.; Courtheyn D.; De Kimpe N.; Mangelinckx S.; De Poorter G.; Le Bizec B. GC-MS/C/IRMS to prove administration of androgens and estrogens and progestagens in bovines. Seventh International Symposium on Hormone and Veterinary Drug Residue Analysis, Gent, Belgium, 2014.