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ANABOLIC-ANDROGENIC STEROIDS IN HORSES: NATURAL PRESENCE AND UNDERLYING BIOMECHANISMS

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"The universe is full of magic,

Just patiently waiting for our wits to grow sharper"

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NOTATION INDEX

17β-HSD	17β-hydroxysteroid dehydrogenase enzyme
Δ1-SDH	delta-1-steroid dehydrogenase
%RSD	Relative Standard Deviation
αBol	α-Boldenone; 17α-hydroxy-1,4-androstadiene-3-one
AAS	Anabolic-Androgenic Steroids
ABP	Athlete Biological Passport
Ac-CoA	Acetyl-Coenzyme A
ACAT	Acetyl-CoA Acetate Transferase
ACTH	Adrenocorticotropic hormone
ADD	Androstadienedione; boldione; androsta-1,4-diene-3,17-dione
ADF	Acid Detergent Fibre
AED	Androstenedione; androst-4-ene-3,17-dione
AL	Anterior Lobe (of the pituitary gland)
AOAC	Association of Official Analytical Chemists
AORC	Association of Official Racing Chemists
ARB	Australian Racing Board
ASG	Acylated Steryl Glycosides (phytosterol conjugates)
au	Arbitrary Units
BCFIvet	*"Belgisch Centrum voor Farmacotherapeutische Informatie"*
BHI	Brain Heart Infusion
BM1	5β-androst-1-en-17β-ol-3-one
BSE	Bovine Spongiform Encephalopathy
ßBol	ß-boldenone; 17ß-hydroxy-1,4-androstadiene-3-one
ßT	ß-testosterone; 17ß-hydroxy-4-androstene-3-one
CD	Council Decision
CE	Collision Energy
CEC	Capillary Electro Chromatography
CID	Collision Induced Dissociation
CHRB	Canadian Horse Racing Board
CP	Crude Protein
CPB	Cystein Peptone Bouillon
CRH	Corticotrophin-releasing hormone
CRM	Certified Reference Material

DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DM	Dry Matter
DOE	Design of Experiments
d.w.	Dry weight
E_2	Estrogen
EBP	Equine Biological Passport
EC	European Commission
eCG	Equine Chorionic Gonadotrophin
EEC	Expected Endogenous Concentration
EFSA	European Food Safety Authority
EGAs	Estrogens, Gestagens and Androgens
ELISA	Enzyme Linked Immunosorbent Assay
ELSD	Evaporative Light Scattering Detection
EPO	Erythropoietin
ER	Endoplasmic Reticulum
ERC	Endogenous Reference Compound
ESI	Electrospray Ionisation
EU	European Union
FDA	Food and Drugs Agency (US)
FEI	Fédération Equestre Internationale
FFD	Fractional Factorial Design
FID	Flame Ionisation Detection
FS	Free Alcohol phytosterol
FSH	Follicle Stimulating Hormone
FWHM	Full Width at Half Maximum
GC-C-IRMS	Gas Chromatography Combustion Isotope Ratio MS
GC-MS	Gas Chromatography Mass Spectrometry
GC	Glucocorticoids or Gas Chromatography
GCxGC	Comprehensive two dimensional Gas Chromatography
GCT	Granulosa Cell Tumour
GIT	Gastrointestinal tract
GnRH	Gonadotrophin Releasing Hormone
GRAS	Generally Regarded As Safe
hCG	Human Chorionic Gonadotrophin

HDL	High Density Lipoprotein
HMG-CoA	Hydroxy-3-methylglutaryl Coenzyme A
HPA	Hypothalamic-pituitary-adrenal (axis)
HRMS	High-Resolution Mass Spectrometry
IOC	International Olympic Committee
IP	Identification Point
IPP	Isopentenyl pyrophosphate
IR	Infrared (detection)
IS(TD)	Internal Standard
IFHA	International Federation of Horse Racing Authorities
IUPAC	International Union of Pure and Applied Chemistry
LCH	Laboratoire des Courses Hippiques
LCxLC	Comprehensive two dimensional Liquid Chromatography
LDL	Low Density Lipoprotein
LH	Luteinising Hormone
LLE	Liquid-Liquid Extraction
LOD	Limit Of Detection
LOQ	Limit of Quantification
MT/MeT	Methyltestosterone; 17ß-hydroxy-17α-methyl-4-androstene-3-one
MRL	Maximum Residue Limit
MRM	Mutiple Reaction Monitoring
MS/MS	Tandem Mass Spectrometry
NDF	Neutral Detergent Fibre
NMR	Nuclear Magnetic Resonance
NOPS	Naturally Occurring Prohibited Substances
NPC1L1	Niemann-Pick C1-Like 1
NRLs	National Reference Laboratories (EU)
NSAID	Non-Steroidal Anti-Inflammatory Drug
NSW	(Racing) New South Wales
P5	Pregnenolone
PBS	Phosphate Buffered Saline (solution)
R^2	Coefficient of determination
RC	Recommended Concentration
RD	(Belgian) Royal Decree
RMTC	Racing Medication and Testing Consortium, inc (Canada)

RNP	Residual National Program
RSM	Response Surface Modelling
RT	Room Temperature
S/N	Signal to Noise ratio
SCFA	Short Chain Fatty Acids
SE	Steryl Fatty-acid Esters (conjugated phytosterols)
SG	Steryl Glycosides (conjugated phytosterols) or Specific Gravity
SRM	Single Reaction Monitoring
TLC-FL	Thin Layer Chromatography – Fluorescence
TMS	Trimethylsilyl
TU	Thiouracil
U	enzyme unit, converts 1 mole of substrate per second
(U)HPLC	(Ultra) High Performance Liquid Chromatography
VBNC	Viable But Nonculturable
QqQ-MS	Triple Quadrupole Mass Spectrometry
WADA	World Anti-Doping Agency
WAT	White Adipose Tissue

CHAPTER I

General introduction

CHAPTER I

1. STEROIDS

1.1 CHEMICAL STRUCTURE

Steroids comprise a group of **cyclic, organic** compounds whose basis is a characteristic arrangement of seventeen carbon atoms (C_{17}) in a four-ring structure, linked together from three C_6 rings, followed by a C_5 ring. These rings are synthesized by biochemical processes from cyclization of a C_{30} chain, squalene, into lanosterol or cycloartenol (see Chapter I, **1.4 SYNTHESIS AND ABSORPTION OF THE STEROID PRECURSOR CHOLESTEROL**) [1].

The three cyclohexane rings are designated as **rings** A, B and C and the cyclopentane ring as ring D. The three cyclohexane rings (A, B, and C) form the skeleton of a perhydro-derivative of phenanthrene. The D-ring has a cyclopentane structure; hence, though it is uncommon, IUPAC steroids can also be named as various hydro-derivatives of **cyclopentaphenanthrene** (**Figure 1.1.A.**). This 17-carbon compound is also called gonane, the simplest steroid and a substructure present in most steroids.

When the two methyl groups (C-10 and C-13) and 8 C side chain (at C-17) are present, the steroid is said to have a cholestane framework (5α-cholestane, a common steroid core, e.g. **cholesterol, Figure 1.1.B.**). Cholesterol is the precursor of steroids in both humans and animal species.

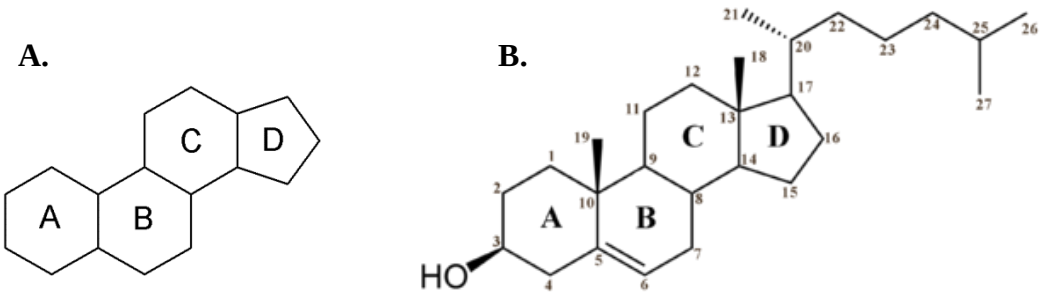

Figure 1.1. (A) Chemical structure of the basic steroid cyclopentaphenanthrene skeleton including IUPAC-approved ring lettering, (B) Chemical structure of cholesterol, including all normally seen branches and atom numbering.

Despite the shared basic steroid skeleton **hundreds of different steroids** can be found in animals, plants and even fungi. They include the sex hormones 17β-estradiol and

testosterone, bile acids, phytosterols, cortisol and drugs such as the anti-inflammatory corticosteroids (e.g. dexamethasone, prednisolone), ergosterols and many more. Individual steroids vary by the oxidation state of the carbon atoms in the rings (single or double bounds) and by the chains and functional groups attached to this four-ring skeleton (**Figure 1.1**., p. 3).

Additionally, steroids can vary more markedly via changes to the ring structure (e.g., via **ring scissions** that produce secosteroids). Secosteroids enhance intestinal absorption of calcium, iron, magnesium, phosphate and zinc. In humans, the most important compounds in this group are **vitamin D3** (also known as cholecalciferol, **Figure 1.2**) and vitamin D2 (ergocalciferol) [2]. Sterols, including cholesterol and phytosterols (see later), are another particularly important form of steroids, having a cholestane-derived framework and a hydroxyl group at the C-3 position (e.g., cholesterol, **Figure 1.1.B**, p. 3) (see also Chapter I, **4. PHYTOSTEROLS**).

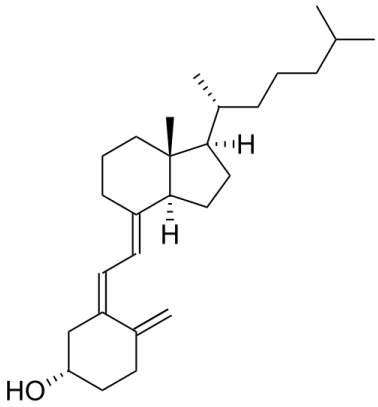

Figure 1.2. Chemical structure of vitamin D3 or cholecalciferol.

1.2 (STEROID) HORMONES AND THEIR ROLE IN THE ENDOCRINE SYSTEM

Hormones are chemical compounds that are naturally produced by animals, human beings and even insects (ecdysteroids) and plants (auxins, gibberellic acid). They have a number of important functions in life, such as reproduction and growth. They act as **signalling** molecules between the different parts of the organism and trigger and modulate key reactions to support and promote life [3]. The most well-known hormones are the **steroid** hormones, e.g. 17β-estradiol, progesterone and testosterone, who are involved in endocrine regulation pathways. Next to influencing **reproduction and growth**, these steroids play other important roles as well. Testosterone regulates protein

synthesis, 17β-estradiol triggers protein disposition and progesterone has an antagonistic role on estrogens.

In mammals, including horses and humans, hormones are secreted primarily by the gonads (testicles of males and the ovaries of females), although small amounts are also secreted by the adrenal glands, the skin and the brain. Other important organs of the **endocrine system** are the pituitary and (para)thyroid gland(s), the pancreas and hypothalamus (**Figure 1.3**) [3].

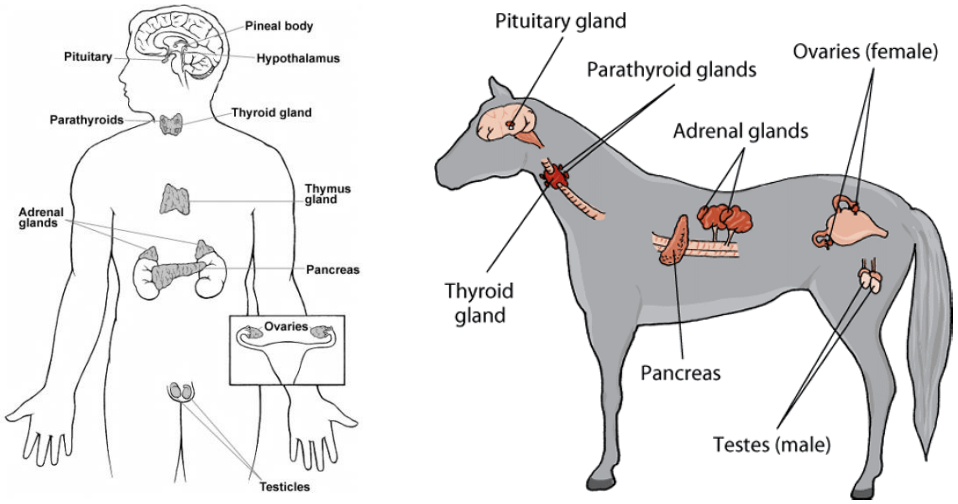

Figure 1.3. Endocrine system of humans and horses. The adrenal glands and gonads (ovaries in females and testicles in males) are involved in steroid biosynthesis**.** Reproduced from DocStock (2014) and the Merck Veterinary Manual (2010).

Within the **testis** the Leydig and Sertoli cells play the most important role. Luteinising hormone (LH) acts on the **Leydig cells** (who are analogous to the cells of the theca internata of antral follicles in the ovary), by binding to the membrane bound receptors for LH. When LH binds to the receptors, Leydig cells produce progesterone, most of which is converted to testosterone (**Figure 1.4,** p. 6). Leydig cells synthesize and **secrete testosterone** less than 30 minutes after the onset of an LH episode. Blood LH is elevated for about 30 to 75 minutes.

The response (testosterone secretion) is short and **pulsatile**, lasting for a period of 20 to 60 minutes. It is believed that the pulsatile discharge of LH is important for two reasons. High concentrations are essential for spermatogenesis and Leydig cells become refractory to sustained high levels of LH. In fact, continual high concentrations of LH result in reduced secretion of testosterone, through the negative feedback loop (**Figure 1.4**, p. 6) [4].

Sertoli cells on the other hand are responsible for spermatogenesis. Spermatogenesis is the process of producing spermatozoa, taking place within the seminiferous tubulus and under the direct control of testosterone. It consists of the sum of all cellular transformation in developing germ cells that occur in the seminiferous epithelium (**Figure 1.4**) [5].

Testosterone in the male is aromatized to estradiol in the brain and estradiol promotes **reproductive behaviour**. Recalling that there is a relatively constant supply of testosterone, and thus estradiol, to the hypothalamus, this allows male to initiate reproductive behaviour any time. In contrast the female experiences high estradiol during the follicular phase only and will display sexual receptivity during estrus only. Under the influence of estrogen, sensory inputs such as olfaction, audition, vision and tactility will lead a fast behavioural response [4].

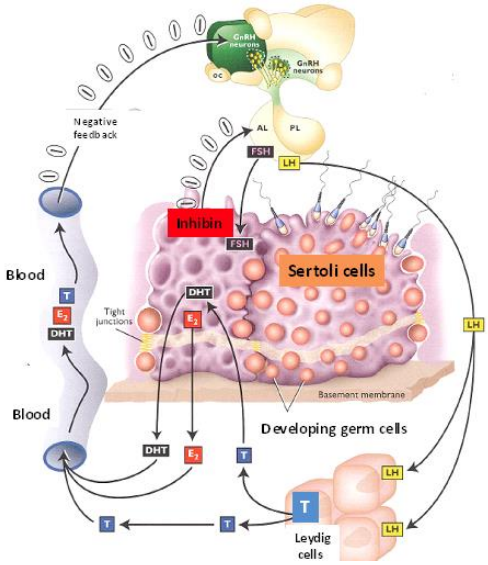

Figure 1.4. Interrelationships among hormones produced by Sertoli cells, Leydig cells, the hypothalamus and the anterior lobe (AL) of the pituitary. T = testosterone, E_2 = estrogen, DHT = dihydrotestosterone, LH = Luteinising Hormone, GnRH = Gonadotrophin Releasing Hormone

Illustration adapted from Senger "Pathways to Pregnancy and Partuition" Second edition, Current Conceptions, Inc. Pullman, Washington (2003) [5]

In addition to the production of testosterone by the Leydig cells, the testes also produce **estradiol (E_2) and other estrogens**. Stallions secrete large amounts of estrogens (both free and conjugated form, see Chapter I, 6.2). In fact, urinary estrogens in the male are significantly higher than urinary estrogens in pregnant mares, but they are of little consequence, as they are secreted as molecules with low physiological activity [4]. In mares the **ovarian** granulosa cells mainly secrete progesterone (P) and estradiol (E_2). Ovarian theca cells predominantly synthesize androgens, including testosterone (T). Ovarian luteal cells secrete progesterone and its metabolite 20α-hydroxyprogesterone [6].

The **adrenal glands** are located just in front of the kidneys and consist of two parts, the cortex and the medulla. The adrenal **cortex** consists of three layers, each of which produces a different set of steroid hormones. The zona glomerulosa (most superficial layer) produces the mineralocorticoids, which help to control the body's balance of sodium and potassium salts (e.g. aldosterone). The zona fasciculata (middle layer) produces glucocorticoids, which are involved in metabolizing nutrients as well as in reducing inflammation (e.g. cortisol). The **zona reticularis** (inner layer) produces sex hormones such as estrogen and progesterone. The adrenal medulla plays an important role in response to stress or low blood sugar (glucose). It releases epinephrine (better known as adrenaline) and norepinephrine, both of which increase heart output, blood pressure, and blood glucose, and slow digestion (Merck Veterinary Manual, 2010).

Next to adrenal and gonadal production of steroids recent papers implied that uterine and **oviductal tissues** can produce steroids as well [7-9]. The equine oviduct is an organ with potential steroidogenic capacities and highly responsive to local changes in progesterone and 17β-estradiol concentrations at the level of morphology, functionality and gene expression of the oviduct. Especially progesterone concentrations were found to be high in oviductal tissue and fluid ipsilateral to the ovulation side during diestrus, whereas other steroid hormone concentrations were not influenced by the side of ovulation [7-9].

These data provide a basis for further studies of the importance of endocrine and paracrine signalling during early embryonic development in the horse. Experiments with porcine uterine slices harvested during both early pregnancy and luteolysis showed that the **uterus** can also produce steroid hormones. The studied cells secreted AED, T, and E_2 *in vitro* and progesterone served as a substrate for steroid synthesis in the uterine cells. Additional research will be needed to confirm these results and to expand them to other species (horses).

Adipose tissue is also no longer considered to be an inert tissue that stores fat. **White adipose tissue (WAT)** is now being recognized as a major endocrine and secretory organ, releasing a wide range of protein factors and signals termed adipokines - in addition to fatty acids and other lipid moieties [10]. These are active in a range of processes, such as control of nutritional intake (leptin, angiotensin), inflammatory process mediators and control of sensitivity to insulin (e.g. “metabolic syndrome”, insulin resistance in horses) [11]. Obesity is a key component in development of the metabolic syndrome and it is

becoming increasingly clear that a central factor in this is the production by adipose cells of bioactive substances that directly influence insulin sensitivity and vascular injury. [10,12].

1.3 BIOSYNTHESIS OF STEROID HORMONES

Biosynthesis of steroid hormones requires a battery of oxidative enzymes located in both mitochondria and **endoplasmic reticulum** (ER). The rate-limiting step in this process is the transport of free cholesterol from the cytoplasm into mitochondria. Within mitochondria, cholesterol is converted to pregnenolone by an enzyme in the inner membrane called CYP11A1.

Pregnenolone (3β-hydroxypregn-5-en-20-one), also known as P5, is the immediate precursor for the synthesis of all of the steroid hormones, including progestogens, mineralocorticoids, glucocorticoids, androgens, and estrogens, as well as the neuroactive steroids (**Figure 1.5**, p. 9). In addition, pregnenolone can be biologically active in its own right, acting as a neurosteroid for treating schizophrenia [13]. Pregnenolone undergoes further steroid metabolism in one of three ways:

1. Pregnenolone can be converted to progesterone (P). The critical enzyme step is two-fold using a 3β-hydroxysteroid dehydrogenase and a Δ4-5 isomerase. The latter transfers the double bond from C5 to C4 on the A ring. Progesterone is the entry into the Δ4-pathway, resulting in production of 17-hydroxy progesterone and androst4-ene-3,17-dione (AED), precursor to testosterone (T) and estrone. Aldosterone (the main mineralocorticoid) and corticosteroids are also derived from progesterone or its derivatives.
2. The enzyme 17α-hydroxylase (CYP17A1) can convert pregnenolone to 17-hydroxy-pregnenolone. Using this pathway, termed Δ5-pathway, the next step is conversion to dehydroepiandrosterone (DHEA) using a desmolase. DHEA is the precursor of AED, and AED in turn is the precursor of testosterone and the estrogens (estradiol, estrone and estriol).
3. Pregnenolone can be converted to androsta-5,16-diene-3β-ol by 16-ene synthetase (not illustrated).

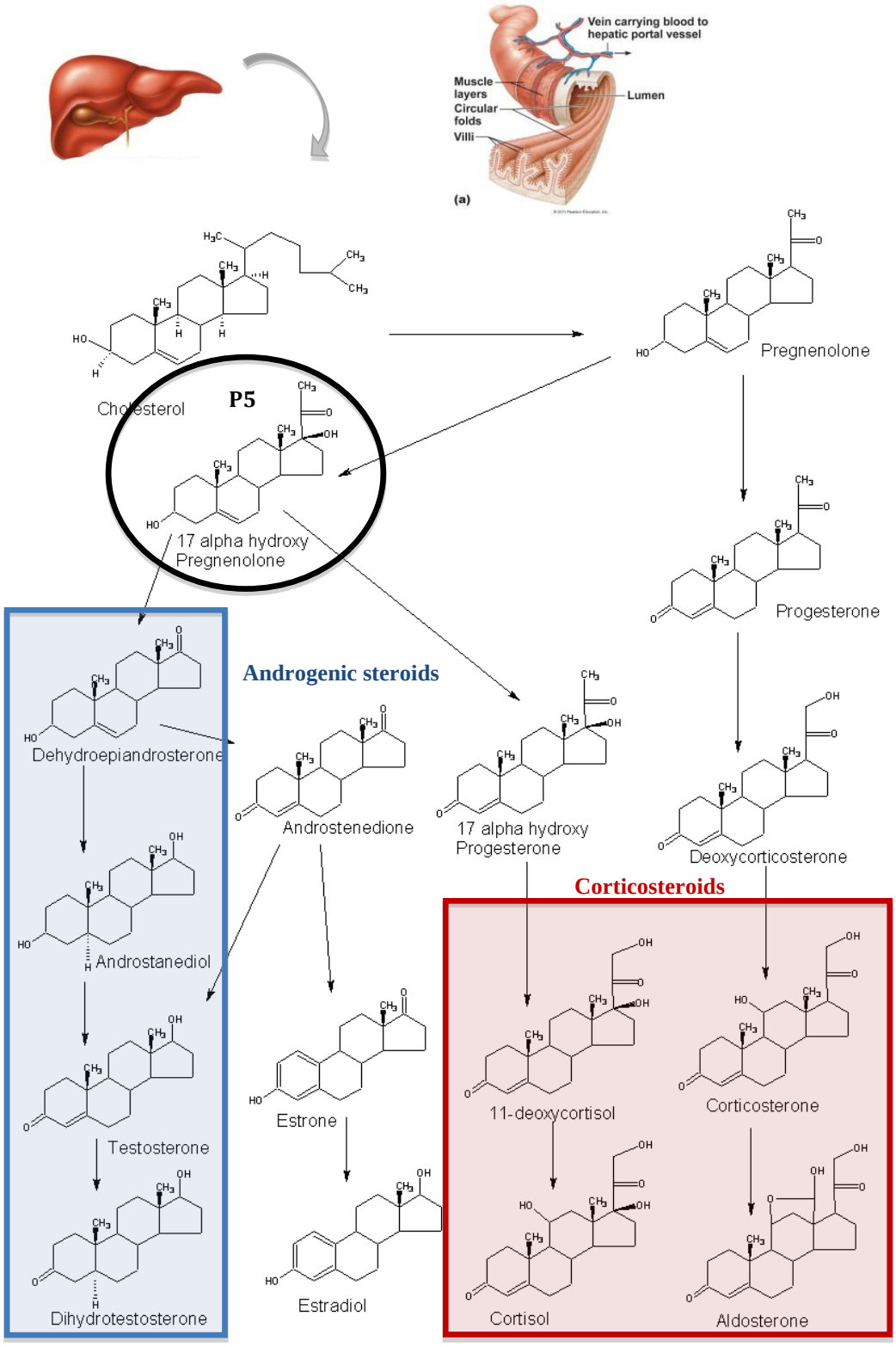

Figure 1.5. The steroid hormone cascade starting from cholesterol and pregnenolone. Cholesterol, the main steroid precursor, is absorbed from the intestines and produced in the liver from acetate (**1.4 SYNTHESIS AND ABSORPTION OF THE STEROID PRECURSOR CHOLESTEROL**). The biosynthesis of steroid hormones takes place in the testicles of males and the ovaries of females, although small amounts are also secreted by the adrenal glands in mammals, including horses and humans (adapted from Pearsson Education Inc. (2011)).

1.4 ANABOLIC-ANDROGENIC STEROIDS (AAS)

The term Anabolic-Androgenic Steroids (AAS) is mostly used to group the naturally occurring male sex hormone testosterone, testosterone precursors and metabolites, and sometimes also (synthetically) produced testosterone variants [14,15]. "Anabolic" refers to the muscle-building capacity, and "androgenic" refers to increased male sexual characteristics while "steroids" refers to the class of compounds (**Figure 1.1.A**., p. 3).

Valid medicinal use of AAS is limited, for example in patients with a negative nitrogen balance, like weakened horses or to accelerate healing after trauma or surgery. Non-therapeutic abuse of AAS however is widespread (Chapter I, 3. and Chapter VII). Anabolic steroids can be given by injection, taken by mouth, or used externally. In humans, AAS are classified as controlled substances, due to the possibility of serious adverse effects and a high potential for abuse as these hormones increase lean muscle mass and can improve athletic performance.

1.5 SYNTHESIS AND ABSORPTION OF THE STEROID PRECURSOR CHOLESTEROL

In humans and animals, cholesterol is the main **precursor** of all steroids, including sex steroids such as testosterone. Cholesterol is **synthesized** in the liver [16,17] and dietary and biliary cholesterol-containing low density lipoproteins can be absorbed from the intestines (**Figure 1.7**, p. 12).

The results of isotope-labelling experiments revealed the source of carbon atoms in cholesterol is **acetate** (acetyl coenzyme A, Ac-CoA [18]. Cholesterol synthesis can be split up into three stages (**Figure 1.6,** p. 11):

1. The synthesis of isopentenyl pyrophosphate **IPP** (**C_5**), an activated isoprene unit that is the key building block of cholesterol, from acetyl CoA. This set of reactions, which takes place in the cytosol, starts with the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetyl CoA and acetoacetyl CoA. This intermediate is reduced to mevalonate and subsequently converted into IPP in three consecutive reactions requiring ATP. The synthesis of **mevalonate** is the committed step in cholesterol formation, making the enzyme catalysing this irreversible step (3-hydroxy-3-methylglutaryl CoA reductase, HMG-CoA reductase) an important control site in cholesterol biosynthesis.

2. The condensation of six molecules of IPP to form **squalene (C_{30})**, by the reaction sequence $C_5 \rightarrow C_{10} \rightarrow C_{15} \rightarrow C_{30}$. Isopentenyl pyrophosphate attacks an allylic carbonium ion formed from dimethylallyl pyrophosphate to yield geranyl pyrophosphate. The resulting C15 compound is called farnesyl pyrophosphate. The same enzyme, geranyl transferase, catalyses each of these condensations. The last step in the synthesis of squalene is a reductive tail-to-tail condensation of two molecules of farnesyl pyrophosphate catalysed by the endoplasmic reticulum (ER) enzyme squalene synthase.

3. Squalene **cyclizes** in an astounding reaction and the tetracyclic product (**lanosterol**) is subsequently converted into cholesterol. Squalene is first activated by conversion into squalene epoxide (2,3-oxidosqualene) in a reaction that uses O_2 and NADPH. Squalene epoxide is then cyclized to lanosterol by oxidosqualene cyclase. Lanosterol in turn is converted into cholesterol in a complex process.

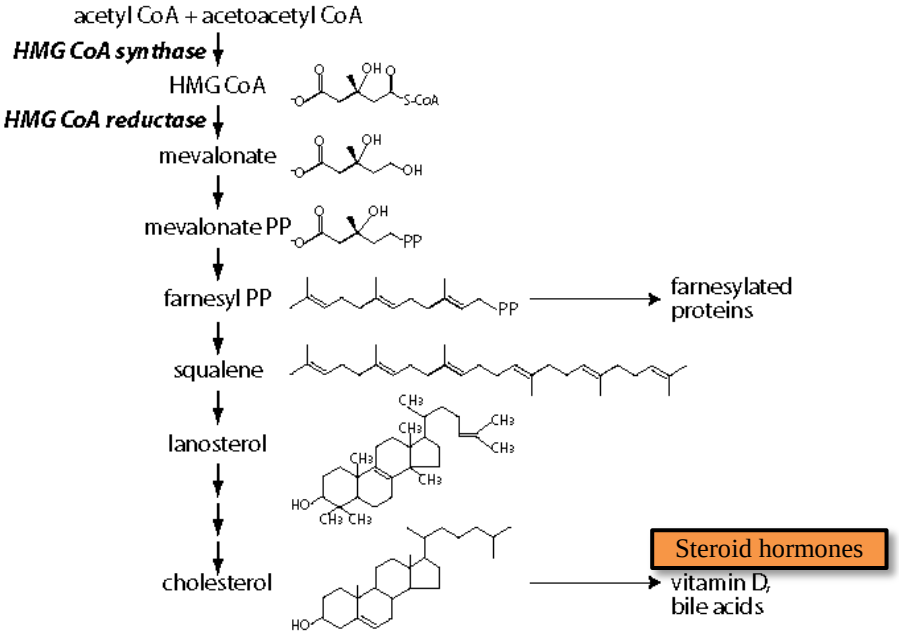

Figure 1.6. Biosynthesis of cholesterol from acetyl CoA.

On the other hand, cholesterol absorption occurs primarily in the duodenum and proximal jejunum at levels of efficiency that vary greatly among individuals [18, 19]. In humans consuming a typical Western diet, about one quarter of the cholesterol entering the lumen of the small bowel is from the diet; the majority of cholesterol in the lumen comes directly from the bile and cells sloughed from the intestinal epithelium [20].

There are two main phases of cholesterol absorption [20]. The first takes place in the lumen and involves digestion and hydrolysis of dietary lipids followed by solubilisation of cholesterol in **mixed micelles** containing bile acid and phospholipids (**Figure 1.7**). This solubilisation facilitates the movement of cholesterol from the bulk phase of the lumen to the surface of the enterocyte. In the second phase, cholesterol crosses the mucosal cell membrane by simple diffusion, and probably by facilitated diffusion as well (NPC1L1, Niemann-Pick C1-Like 1) [21].

Thus far, a specific cholesterol transporter in the microvillus membrane of the enterocyte has not been identified [20, 21]. Within the cell the cholesterol is re-esterified (ACAT, Acetyl-CoA Acetate Transferase) and incorporated into apolipoprotein B-containing nascent lipoproteins (chylomicrons) that are secreted into the lymph. The absorption process is largely specific for cholesterol because plant sterols or phytosterols (see Chapter I, **4. PHYTOSTEROLS**), although structurally similar to cholesterol, are generally absorbed either poorly or not at all [22-25] (**Figure 1.7**).

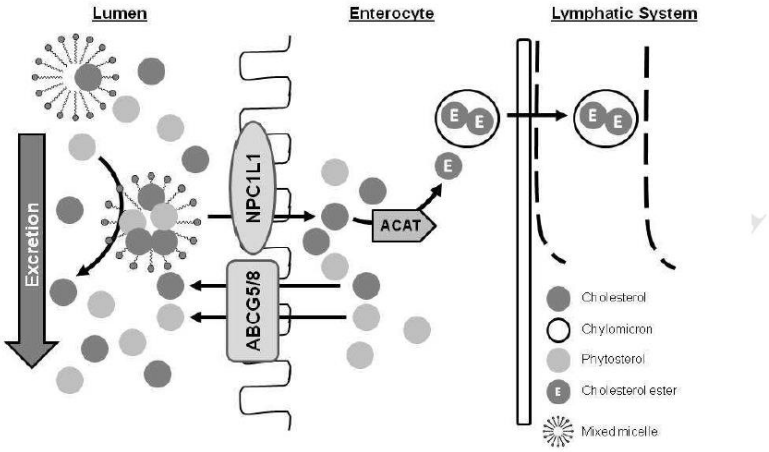

Figure 1.7. Absorption of cholesterol and phytosterols from the intestinal lumen.
Reproduced from Abumweis *et al.* (2014) [22]

2. ANABOLIC STEROID ABUSE

2.1 SCIENTIFIC HISTORY OF ANABOLIC STEROID ABUSE

Back in the middle ages, it was already well-known that after **castration** male animals lose their fertility and most other male characteristics, including behaviour and physical appearance. In 1849 it was known that **hormones** are responsible for these characteristics. Testosterone plays a key role in the development of male reproductive tissues such as the testes and prostate as well as promoting secondary sexual characteristics such as

increased muscle, bone mass, and the growth of body hair [15]. In 1935 a Dutch, pharmaceutical research team was the first to **isolate** and identify the chemical structure of **testosterone** (from bull testicles).

Research teams from Schering and Ciba independently discovered less expensive methods of **synthesizing** testosterone in August 1935. This steroid research was deemed so important that the lead researchers from the Schering and Ciba teams (Butenandt and Ruzicka) ultimately shared the 1939 Nobel Prize in Chemistry for their work on anabolic-androgenic steroid hormones. The discovery of synthetic methods of preparing the anabolic-androgenic steroid known as testosterone was a major breakthrough in the pharmaceutical industry allowing steroid hormone research to flourish.

Charles Kochakian, a synthetic steroid pioneer, made a milestone discovery in the history of steroids, his animal research with testosterone acetate **proved** that testosterone was indeed an **anabolic** hormone in 1936. Kochakian's research group was the first to scientifically document a connection between testosterone and increased muscle mass. In 1938, Allan Kenyon's research group confirmed that the anabolic muscle effects of testosterone propionate occurred in human subjects as well during steroid experiments on eunuchoidal boys, men and women.

In **humans**, the first documented use of testosterone as a performance-enhancing substance in sport was reported in the early **1950s**. Russian weightlifters outcompeted all other athletes and their trainers conceded that they were using testosterone. Once Pandora's Box was open, it was impossible to close. The growth and widespread use of anabolic steroids eventually led the regulatory agents to schedule testosterone and other anabolic agents under control (see Chapter I, **2.3 DOPING REGULATION: HUMANS AND ANIMALS**).

In **animals**, including farm animals (such as cattle, pigs and chicken) and race animals (merely horses, dogs and camels), the range of the issue is comparable. In farm animals, growth promoting agents have been abused on a **regular basis** within the European Union (EU), as it has been reported in a series of European International Symposia and Conferences, such as EuroResidue Conferences on Residues of Veterinary Drugs in Food and the Ghent Symposia on Hormone and Veterinary Drug Residue Analysis, amongst others. The number of active compounds is wide and continuously changing, as observed by the EU National Reference Laboratories (NRLs).

Not only AAS but also estrogenic and gestagenic (EGAs) as well as thyreostatic, corticosteroids and β-agonist compounds, are used alone or in growth promoting **cocktails** with low concentrations of several ones, compromising their detection [3]. As there is no worldwide restriction to the use of AAS as growth promoting and performance enhancing agents AAS are available on the (black) market as food supplements (e.g. DHEA) or even as injectable anabolic preparations (Bol or Bol esters), imported from the US and other regions with less strict regulations regarding AAS [13].

2.2 RESIDUE-ANALYSIS IN FARM ANIMALS: REGULATIONS

The use of AAS leaves trace residues of these hormones in the meat, which can have serious human-health consequences, including amongst others hepatoxicity, infertility and cancer. Because of this potential to threaten public health there have been several European regulations regarding the use of EGAs (including AAS) as animal growth promoters. Initially, the EU issued a ban on the use of estradiol and other natural and synthetic steroid hormones. This ban was already adopted in 1985 but was disputed before the European Court by the United Kingdom. It was annulled because of procedural deficiencies. The ban was finally agreed in 1988 and encompassed a ban on the use of estradiol 17-β, testosterone, progesterone, zeranol, trenbolone acetate and melengestrol acetate within Member States.

Council Directive 96/22/EC (EC, 1996a) elaborated on this by prohibiting the administration of all substances with thyreostatic, estrogenic, androgenic or gestagenic effects and of β-agonists in animal husbandry (while certain therapeutic applications of these drugs were still allowed). Anabolic steroids were included in group A substances according to Annex I of Directive **96/23/EC** (EC, 1996b), which pertains to growth-promoting agents abused in animal fattening and unauthorized substances with no maximum residue limit (MRL).

A **zero-tolerance** policy has been adopted since, and in particular, analytical requirements have been stated in regard to these hormones (EC, 2002; European Commission, Directorate General for Health & Consumers, 2004). However, the possibility of widespread abuse of hormonal substances by unscrupulous farmers and veterinary professionals in some parts of Europe still exists and the use of hormones to promote growth is still a legal practice in some parts of the world, which facilitates the existence of a possible "**black market**" of substances from these areas.

2.3 DOPING REGULATIONS: HUMANS AND HORSES

In humans, anabolic steroids were added to the IOC's (International Olympic Committee) list of **banned** substances in **1975**, as at that time the first test that was considered to be reliable was developed. Consequently, athletes were **first tested** for anabolic steroids during the **1976 Olympic Games** in Montreal. Out of 786 drug tests performed at the 1976 Olympic Games in Montreal, 11 athletes (1.4%) tested positive. Over the following years, thousands of athletes would test positive for the abuse of anabolic steroids in sports, including swimming, baseball, athletics, weight lifting and many other disciplines.

As the abuse continued, additional measurements needed to be taken. In 1990 the American Congress developed the **Anabolic Steroids Control Act**, placing 25 steroids (including boldenone and testosterone) in the same legal class, class III controlled substances, as amphetamines, methamphetamines, opium and morphine. This control act was updated in 2014 ("Designer Anabolic Steroid Control Act of 2014, HR 4771), to include more steroids and designer steroids.

The World Conference on Doping in Sport held in Lausanne in 1999 produced the **Lausanne Declaration** on Doping in Sport. This document provided for the creation of an independent international anti-doping agency to be fully operational for the Olympic Games in Sydney in 2000. Pursuant to the terms of the Lausanne Declaration, the **World Anti-Doping Agency (WADA)** was established on 10 November 1999 in Lausanne to promote and coordinate the fight against doping in sport internationally. WADA was set up as a foundation under the initiative of the IOC with the support and participation of intergovernmental organizations, governments, public authorities, and other public and private bodies fighting against doping in sport. The agency consists of equal representatives from the Olympic Movement and public authorities.

Currently, AAS are still classified as class III controlled substances, they are part of the first section of the **Prohibited List (2015)**, that discusses substances and methods that are prohibited at all times, both in-competition and out-of-competition and any athlete can be tested for these substances at any time. The list of anabolic agents is extensive and even if one is not specifically listed, it is still prohibited if it is a metabolite or has a similar chemical structure or similar biological effect(s) to anabolic agent (WADA 2015 Prohibited Substances List).

Race and sport horses are, just like human athletes, frequently subjected to doping controls to guarantee a safe and fair competition. **FEI** (Fédération Equestre Internationale, responsible for all Olympic disciplines including jumping, dressage and eventing) and **IFHA** (International Federation of Horseracing Authorities) regulations state that "*any use of substances with a potential to affect equine performance, health or welfare and/or with a high potential for misuse is contrary to the integrity of equestrian sport and the welfare of the horses*".

Even as early as the 10th century BC, the Romans used an innocuous mix of water and honey called "hydromel", which was believed to rejuvenate chariot race horses (**Figure 2.1**). The prohibition of hydromel might be the oldest equine anti-doping law. Anti-doping laws in modern equestrian sports date back to the late 19th century.

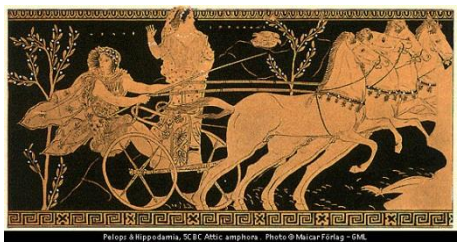

Figure 2.1. Roman chariot racing on a black-figure hydria (510 BC). Chariot racing was the most popular sport in Rome, attracting up to 350.000 spectators for prestigious races.

AAS have been included in the **prohibited substances lists** of equine anti-doping agencies such as FEI and IFHA for several decades, as soon as the influence of AAS on athletic performance had been confirmed. The pharmacological activity of AAS includes increased nitrogen retention, protein synthesis, appetite and the release of erythropoietin in the kidneys [3,16].

In the **United States** it took until 2008 for steroids to be banned from the racing courses. Under the new law, a horse may be given steroids only under certain therapeutic conditions, and a horse may not race for at least 60 days afterwards. Anabolic steroids may never be present in a competing horse (Press release, Kentucky Horse Racing Commission website, Sep. 5, 2008 and RMTC, Racing Medication and Testing Consortium, 2008).

In **Australia** anabolic steroids were only prohibited on race day, but a new total ban (November 1st 2013 and effective from May 1st 2014, Australian Racing Board, ARB) applies to all thoroughbreds from the age of six months, both in and out of competition. This decision has been welcomed by the IFHA and FEI, to further two of their key objectives: to coordinate and harmonize the rules of all member-countries worldwide.

2.4 SEX STEROIDS AS REGISTERED DRUGS

The BCFIvet repertorium (Belgisch Centrum voor Farmacotherapeutische Informatie, "gecommentarieerd geneesmiddelenrepertorium voor diergeneeskundig gebruik") only includes nandrolone as a registered drug. Nandrolone can be used in patients with a negative nitrogen balance, like weakened horses or to accelerate healing after trauma or surgery. Gonadotrophins and drugs that stimulate the secretion of gonadotrophins are registered as well (e.g. eCG, hCG and FSH, to stimulate follicle formation and ripening).

Additionally, EU commission regulation No 37/2010 of 22 December 2009 expands on pharmacologically active substances and their classification regarding maximum residue limits (MRLs) in foodstuffs of animal origin. EU commission regulation 37/2010 lists only a few sex steroids. No anabolic-androgenic steroids (AAS) are included. Progesterone is listed for use in mares, but only for intravaginal therapeutic or zootechnical use and in accordance with the provisions of Directive 96/22/EC. For progesterone no MRL is required. In line with the BCFIvet repertorium gonadotrophins and drugs that stimulate the secretion of gonadotrophins like GnRH, eCG, hCG and FSH (Natural FSH from all species and synthetic analogues) are listed. They can be used in all food producing species and no MRL is required.

Although BCFIvet registration or EU commission regulation No 37/2010 might allow therapeutic use of a few (sex) steroids in production horses, FEI, IFHA or other regulations will imply on these horse as soon as they enter competition. The other way around, sport horses can be treated with other products than the ones listed above, as long as they are non-food producing and in line with FEI/IFHA regulations when entering competition.

3. ENDOGENOUS STEROIDS

3.1 REFERENCE RANGES FOR ENDOGENOUS STEROIDS IN HUMANS

Multiple studies measured the **excreted concentration** of testosterone in **urine** (**Table 3.1**, p. 18). The oldest data date back to the 1960s, when Camacho *et al.* (1963) already stated that the daily excretion of testosterone by normal adult men was many times greater than that of normal adult women [26] (**Table 3.1**, p. 18).

Table 3.1. A) Reference ranges for total testosterone in humans (male).

Reference		Population	Male		
			n	Mean	Outliers
[27]	Futterweit *et al.* (1964)	American	10	171 µg/day	250 µg/day
[28]	Doberne and New (1975)	American	10	84 µg/day	/
[29]	Tresguerres *et al.* (1976)	American	26	150 µg/day	346 µg/day
[30]	Gonzalo-Lumbreras *et al.* (2003)	Spanish	12	125 ng/mL*	191 ng/mL*
[31]	Van Renterghem *et al.* (2010)	Caucasian	2027	37 ng/mL	>100 ng/mL
[32]	Martinez-Brito *et al.* (2013)	Latin-American	2454	60 ng/mL	>200 ng/mL
[33]	Moon *et al.* (2014)	Korean	337	26 ng/mL	>150 ng/mL

*Samples were collected early in the morning, at the maximum of the excretion curves

Table 3.1. B) Reference ranges for total testosterone in humans (female).

Reference		Population	Female		
			n	Mean	Outliers
[27]	Futterweit *et al.* (1964)	American	10	6 µg/day	8 µg/day
[28]	Doberne and New (1975)	American	10	4.2 µg/day	/
[29]	Tresguerres *et al.* (1976)	American	16	24 µg/day	/
[31]	Van Renterghem *et al.* (2010)	Caucasian	1004	12 ng/mL	200 ng/mL
[34]	Pesant *et al.* (2012)	Canadian	155	1.23 nmol/L	1.7 nmol/L
[32]	Martinez-Brito *et al.* (2013)	Latin-American	1181	13 ng/mL	54 ng/mL

On average, in adult males, levels of testosterone are up to ten times as great as in adult females [35]. The reference ranges for blood test of adult males were between 1.8 and 7.5 ng/mL (>50 years old) and 2.90 to 13 ng/mL (<50 years old), while the reference range for adult females was between 0.2 and 0.85 ng/mL (MedlinePlus.gov – National Library of Medicine reference values). As the metabolic consumption of testosterone in males is greater too, the daily production was estimated to be about **20 times greater in men** [36].

3.2 REFERENCE RANGES FOR ENDOGENOUS STEROIDS IN HORSES

Generally, less data are available regarding the normal ranges of excretion of testosterone and its related metabolites in horses, but testosterone and its precursors/metabolites are known to be endogenous in males (stallions and geldings) and females of this species at varying concentrations [98]. In a recent study investigating the effect of γ-oryzanol supplementation on endogenous testosterone levels in horses, the urine **β-testosterone** concentrations were always lower than 1.7 ng/mL for mares and geldings. Popot *et al.* (2008) [37] and Ho *et al.* (2004) [38] measured both ADD and βBol in urine (and faeces) of (male) horses. Testosterone levels measured by Popot *et al.* (2008) were between 71 and 214 ng/mL. If urine samples are being analysed with gas chromatography/mass spectrometry for the identification of cryptorchidism, a cut-off level of 8 ng/mL is held as a marker for cryptorchidism, testosterone levels below 8 ng/mL are regarded normal for

geldings and according to these thresholds, no βBol should be found in geldings [39]. Plasma concentrations for cycling mares vary between 20 and 60 pg/mL and can go up to 245 to 350 pg/mL in bearing mares. Urine concentrations in cycling mares were found to between 1.4 and 20.1 ng/mL [40].

β-boldenone levels measured by Popot *et al.* (2008) in stallions varied between 1.0 and 2.9 ng/mL urine (n = 7) [37]. The range of free and conjugated boldenone determined by Ho *et al.* was between 0.1 and 4.34 ng/mL (n = 63, from 37 male horses), and the mean was 1.27 ± 1.03 ng/mL. Boldenone was not detected in geldings (n = 8), in line with the results of Leung *et al.* (2007) [39]. The mean β-boldenone concentration measured in male horses by Dehennin *et al.* (2003) was 0.34 ng/mL (minimum 0.02, maximum 1.51 ng/mL) (n = 156) [41].

For **progesterone**, a difference needs to be made between mares in anestrus (<2.0 ng/mL) and pregnant mares (60 to 120 pregnant days, 5 to 20 ng/mL) (RIA, college of veterinary medicine, Colorado State University, 2013).

Additionally, as sport horses are frequently subjected to doping analysis, normal levels can be derived from the by anti-doping regulatory organs **accepted levels** (see also **2. ANABOLIC STEROID ABUSE**). Very strict zero-tolerance policies are held for most steroids, but exceptions have been made for the naturally occurring androgenic steroids: boldenone and testosterone (and stanozolol and nandrolone) (**Table 3.2**, p. 20).

As endogenous **β-boldenone** was found in urine and faeces of entire males [37, 38], IFHA (Article 6, 2015) [42], RMTC (Banned Medication List, 2014), and FEI (2015 Equine Prohibited Substances List) [43], abandoned the zero-tolerance for stallions and set a threshold for free and conjugated boldenone of **15 ng/mL**. Despite this threshold for **entire males**, the presence of βBol in urine from mares or geldings is still prohibited.

For **testosterone**, thresholds were set for mares and fillies (unless in foal), up to **55 ng/mL** free and conjugated testosterone in urine and **20 ng/mL** urine for geldings is allowed. For entire male horses "amounts in excess of amounts existing naturally in the untreated horse at normal physiological concentrations" are considered to be non-naturally occurring physiological concentrations. The international threshold for testosterone in **plasma** is 100 pg/mL for geldings. Currently, no threshold for mares is set yet, but it is being suggested to introduce the same threshold of **100 pg/mL** [42,43].

For **nandrolone or nortestosterone** (free and conjugated) the RMTC threshold was set at **1 ng/mL** in urine (geldings, fillies and mares). In male horses other than geldings – **45 ng/mL** of metabolite, 5σ-estrane-3β, 17 σ-diol in urine or a ratio in urine of 5σ-estrane-3β, 17 σ-diol to 5σ-estrene-3β, 17 σ-diol of >1:1 is considered to be indicative for abuse. Only a limited number of regulatory organs (e.g. The Canadian Horse Racing Board, CHRB) set a threshold for **stanozolol**, at **1 ng/mL** urine. For FEI and IFHA for example, stanozolol is listed as a banned substance, and therefore strictly forbidden.

Table 3.2. Anabolic-androgenic steroids (AAS) listed as banned substances according to the FEI 2015 banned substances list. FEI and IFHA set thresholds for two AAS, boldenone and testosterone, accepting that these compounds can be present as endogenous steroids (**black**). Other regulatory organs set thresholds for stanozolol and nandrolone (nortestosterone) as well (**orange**).

17α-hydroxyprogesterone	Dromostanolone	Methandriol	Normethandrolone
Androstenediol	Drostanolone	Methandrostenolone	Oxabolone
Androstenedione (AED)	Epiternbolone	Methenolone	Oxandrolone
Bolandiol	Ethinylestradiol	Methyldienolone	Oxymesterone
Bolasterone	Ethylestrenol	Methylnortestosterone	Oxymetholone
Boldenone*	Fluoxymesterone	Methyltestosterone	Paramethadione
Boldione (ADD)	Formebolone	Methyltrienolone	Prostanozol
Calusterone	Furazabol	Mibolerone	Stanozolol◊
Clostebol	Gestrinone	Nandrolone°	Stenbolone
Danazol	Hydroxytest.	Norandrostenediol	Testosterone∆
Dehydrochloromethyltest.	Mestanolone	Norandrostenedione	Tetrahydrogestrinone
Dehydrochlorotest.	Mesterolone	Norbolethone	Tibolone
Desoxymethyltestosterone	Methandienone	Norclostebol	Trenbolone

* For **boldenone** a threshold has been set at 15 ng free and conjugated boldenone per mL in urine from male horses (other than geldings). Zero-tolerance is held in mares and geldings (FEI, IFHA among others).

∆ For **testosterone** 20 ng free and conjugated testosterone per mL in urine or 100 pg free testosterone per mL in plasma is acceptable for geldings and 55 ng free and conjugated testosterone per mL in urine from fillies and mares (unless in foal) (FEI, IFHA and others).

° For **nandrolone** (nortestosterone) a threshold of 1 ng per mL urine has been set for mares and geldings. For stallions the threshold is significantly higher, at 45 ng per mL urine (RMTC).

◊ Only a limited number of regulatory organs (e.g. The Canadian Horse Racing Board, CHRB) set a threshold for **stanozolol**, at 1 ng per mL urine.

3.3 HORMONAL DISORDERS IN HORSES

A tumour or other abnormal tissue in an endocrine gland (Overview of the endocrine system, **Figure 1.3**, p. 5) often causes it to overproduce hormones, e.g. **granulosa cell tumour (GCT)** of the equine ovary, responsible for the overproduction of **testosterone** (**Figure 3.1**) [44]. If an endocrine gland is destroyed or malfunctioning, underproduction of hormones can be detected, e.g. a malfunctioning thyroid gland is responsible for hypothyroidism in the horse [45].

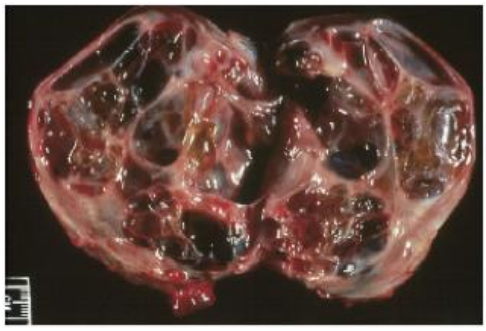

Figure 3.1. **Granulosa Cell Tumour (GCT) with multiple cysts.** GCT can be responsible for the detection of elevated testosterone levels in untreated horses and the with these levels linked male like behavior e.g. flehmen, herding and mounting.

(www.vetsonline.com)

In many cases, the abnormal gland not only overproduces hormones, it also does not respond normally to **feedback signals**. This causes hormones to be released in situations in which the hormone levels would normally be reduced. Sometimes, the overproduction is caused by stimulation from another part of the body. Occasionally, a **tumour outside the endocrine system** can produce a substance similar to a hormone, causing the body to respond as though that hormone were being produced [46].

Figure 3.2. Typical signs of hyperadrenocorticism. Long, wavy coat (hirsutism), muscle wasting along the top line, abnormal fat distribution and increased drinking and urination.

Equine Cushing's disease, also called **hyperadrenocorticism** (PPID or pituitary pars intermedia dysfunction), is the most common endocrine disease in horses. The signs are due primarily to chronic excess of **cortisol** (**Figure 3.2**). Increased cortisol levels may result from several mechanisms, including destruction of a portion of the pituitary gland and overproduction of certain other hormones [47].

4. PHYTOSTEROLS

4.1 BIOLOGICAL ROLE AND CHEMICAL STRUCTURE

Phytosterols are omnipresent in plants, playing a similar role in plants as cholesterol plays in animals: regulating the fluidity of **cell membranes** and featuring in cellular differentiation and proliferation. Over 250 different naturally occurring sterols have been found in higher plants, and as many as 60 different sterols have been described in a single species (e.g. corn). Most phytosterols contain 28 or 29 carbons. Despite this **diversity**, the most frequently occurring phytosterols in nature and in human diets are β-sitosterol (65%), followed by campesterol (30%) and stigmasterol (5%) [48]. They differ in the side chain double bond at C22 and the substituents at C24 (**Figure 4.1**) [49].

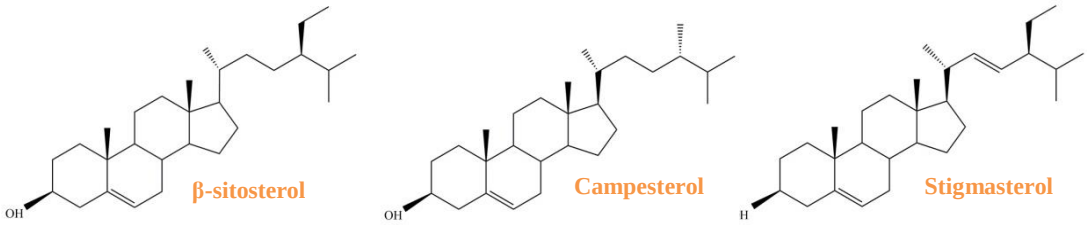

Figure 4.1. Chemical structures of the main phytosterols.

In most plants, other phytosterols and **stanols** such as Δ5/Δ7 avenasterols, cycloartenol and stigmastanol are less abundant. Except for some specific plants such as the Curcurbitaceae, with spinach and cucumbers as the most prominent members, that contain large amounts of Δ7-sterols. Phytosterols can be converted to phytostanols by chemical hydrogenation [50].

Grains are generally regarded as good sources of phytosterols yet different parameters can influence the exact concentration of phytosterols present. **Cultivar and year-to-year variation** of phytosterol content has been shown (e.g. in rye, *Secale cereale* L.). Additionally, the same cultivar can contain different proportions of phytosterols depending on environmental (pH) and climatological factors (temperature, rainfall) affect phytosterol concentration in cereals [49]. Previous research also showed that the different parts of the grain contain different concentrations of phytosterols. The outer layer of corn hulls for example, the pericarp, made up of non-living cell walls, is less ferulate-phytosterol ester rich than the inner layer, the aleurone, that consists of a single layer of living cells, surrounded by thick cell walls [51].

This difference is linked to the anatomical structure of the different seed structures, the proportion of membrane-rich tissues. Fruits and vegetables are generally regarded as low to moderately high in phytosterols [52]. No data are available on the concentrations of phytosterols in feed such as hay, straw and grass (See also Chapter III).

In all plant tissues, phytosterols occur in **five different forms** (**Figure. 4.2**): as free alcohol (FS) (**Figure 4.1**, p. 22), as fatty-acid esters (SE), as steryl glycosides (SG), and as acylated steryl glycosides (ASG). The last three forms (SE, SG, and ASG) are generically called "phytosterol **conjugates**". In free phytosterols (FS), the 3β-OH group on the A-ring of the sterol nucleus is not bound, whereas in the conjugates the OH is covalently bound with other constituents (fatty-acid, sugar or acetyl). The OH group is ester-linked with a fatty acid in SE and linked by a 1-O-β-glycosidic bond with a **hexose**, most commonly glucose, in SG. The third group of phytosterol conjugates (ASG) differ from SG by the addition of a **fatty acid** esterified to the 6-OH of the hexose moiety. Corn, rice and other grains can contain a fourth type of phytosterol conjugate, phytosteryl hydroxycinnamic-acid esters (HSE), in which the sterol 3β-OH group is esterified to **ferulic or p-coumaric acid** (**Figure 4.2**) [50].

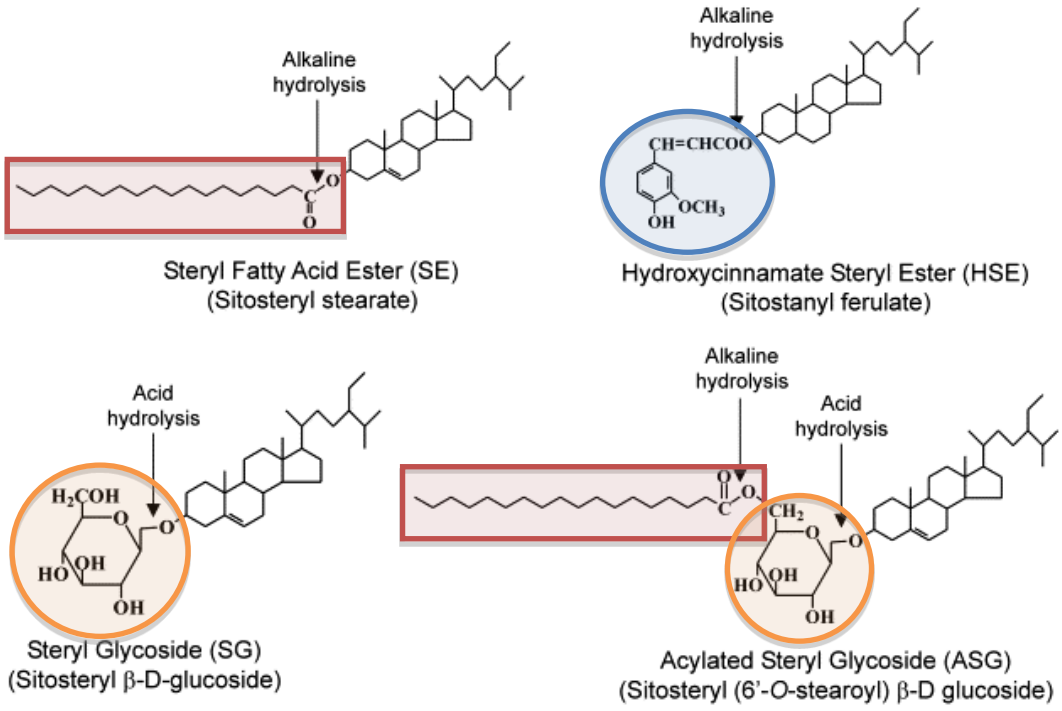

Figure 4.2. Structures of phytosterol conjugates.
The sites of cleavage via alkaline and acid hydrolysis are indicated with arrows (adapted from Moreau *et al.*, 2002) [50].

In humans, phytosterols have been proposed to offer protection against a variety of chronic ailments including cardiovascular diseases, obesity, diabetes, and cancer. As for cancer protection, it has been estimated that diets rich in phytosterols can significantly reduce cancer risk by as much as 20%. They enable more robust antitumour responses, including the boosting of immune recognition of cancer, influencing hormonal dependent growth of endocrine tumours, and altering sterol biosynthesis. In addition, phytosterols have effects that directly inhibit tumour growth, including the slowing of cell cycle progression, the induction of apoptosis, and the inhibition of tumour metastasis [53].

Protection against cardiovascular diseases takes place as consumed phytosterols **compete with cholesterol** for uptake in the mixed micelles (**Figure 1.7**, p. 12), needed for cholesterol absorption by the small intestine. As a result, cholesterol absorption, either from food or from bile salts is lowered by about 50%, leading to a lowering of about 10% of **blood cholesterol level**, despite an increase in hepatic cholesterol synthesis [50]. This reduction is achieved when phytosterols are given either as monotherapy or in addition to statin therapy, a common cholesterol lowering treatment [54]. The average Western diet contains about 400-800 mg of phytosterols per day, while the dose needed for lowering blood cholesterol is about **2 to 3 g per day** [55].

Therefore, many commercial foods, so called functional foods, are supplemented with phytosterols (e.g. margarine, yoghurt drinks). Plant sterols and stanols added to a variety of food products are generally recognized as safe (GRAS) by the FDA (GRAS Notice No. GRN 000112) (2003). Additionally, the Scientific Committee on Foods of the EU (2003) concluded that plant sterols and stanols added to various food products are safe for human use. However, the Committee recommended that intakes of plant sterols and stanols from food products should not exceed 3 g/day because there is no evidence of health benefits at higher intakes and there might be undesirable effects at high intakes.

Phytosterols are also used as **lanolin substitutes** in pharmaceutical and cosmetic formulations, as a less expensive and safe additive. The use of lanolin or wool wax/grease is unfavored because of health damages such as BSE (bovine spongiform encephalopathy) caused by other animal-derived substances in recent years. Plant-derived phytosterols have the same properties as lanolin and form a good substitute.

4.2 LINK TO ANABOLIC STEROIDS

All three main phytosterols (stigmasterol, campesterol and β-sitosterol) make good **raw materials** for the production of steroid hormones because of their typical A-ring molecular structure with a 3β-hydroxyl group and a 5,6-double bond [56]. The C19-steroids, which include AED, ADD and testosterone, are the products of complete (microbial) side chain cleavage of phytosterols (**Figure 4.2**).

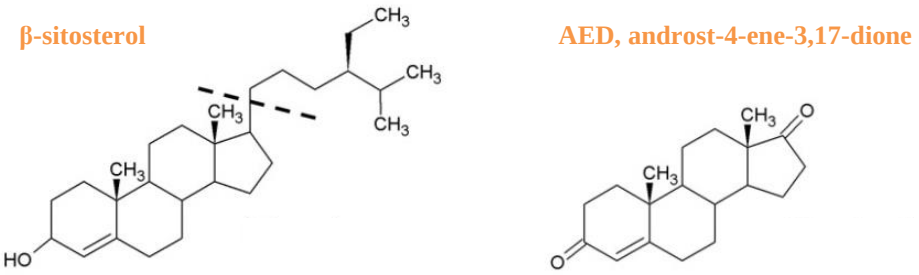

Figure 4.2. Illustration of the closely related chemical structure of phytosterols and anabolic steroids. For the phytosterols, β-sitosterol, the most abundant phytosterol, is shown. The suggested microbial side chain cleavage is indicated with a dashed line (- - -).

Microbial transformation of β-sitosterol into 17-ketosteroids such as androstenones has received much attention, since it allows the use of inexpensive sterols as raw material for the production of steroid intermediates used in the pharmaceutical industry; the microbial side chain cleavage of phytosterols is an alternative to multi-step chemical synthesis. Over 600 relevant patents related to the synthesis and usage of phytosterols and phytostanols have been postulated, highlighting the impact of phytosterols as economically relevant compounds (Espacenet, EU and USPTO, US; 2015).

This microbiological conversion of phytosterols to steroids has been reported for a variety of microorganisms [57, 58] including *Mycobacterium* sp. [59-61], *Arthrobacter* and *Nocardia* sp. [62]. In addition, a number of studies have been devoted to the ability of **invertebrate organisms** to convert phytosterols into anabolic steroids: maggots of *Lucilia Serica* [63], *Crustaceae* [64] and zebra fish [65]. Additionally, Verheyden *et al.* (2010) indicated that feed-related molds might be able to transform phytosterols from feed into androstenones (See also Chapter V).

Other studies indicated that phytosterols might play a role in the detection of steroids in urine and faeces of animals, fed on a phytosterol rich diet. Gallina *et al.* (2007) [66] suggested that stigmasterol might be an α-Boldenone precursor in veal calves and Draisci

et al. (2007) supported this hypothesis by linking the excretion profile of boldenone in urine of veal calves with the consumption of two different milk replacers, with different phytosterol and cholesterol content [67].

Additionally, Song *et al.* (2000) showed that rats, fed a phytosterol rich diet, were able to transform phytosterols, leading to the excretion of ADD, AED and other androstenones in their faeces [68]. Thus far, no studies on the effect of phytosterol consumption on the excretion of steroids in horses have been performed.

5. EQUINE DIGESTIVE TRACT

5.1 ANATOMICAL OVERVIEW

Horses (*Equus Caballus*) are monogastric, hindgut fermenters: the hindgut, consisting of the caecum and colon, comprises roughly two thirds of the volume of the equine digestive tract, making it the most important part of the digestive tract [69] (**Figure 5.1,** p. 27).

Before reaching the hindgut feed passes through different other parts of the intestinal tract [70]:

- Large molar teeth allow the horse to **grind** foodstuffs into small pieces and the act of chewing also stimulates three glands in the mouth to produce liters of saliva. The saliva contains bicarbonate as a buffering agent and amylase, for carbohydrate hydrolysis.
- The **oesophagus**, a one way passage, funnels the feed from the mouth to the stomach. The top of the stomach is known as the squamous, or non-glandular, area whilst the bottom is the gastric, or glandular, region where the digestive secretions, such as HCl, are produced. The small size of the **stomach** is in line with the short retention time. Feed will only spend 30 to 120 min in the stomach before it moves on into the small intestine. Due to the rigid structure of the stomach it cannot accommodate large meals. Instead of stretching it will pass the food through more quickly. As a consequence thereof the digestion process will be less efficient. Roughage will typically spent more time in the stomach, while concentrates are passed through more quickly. Despite this limited capacity, the physiological state of an empty stomach is not advisable, as HCl secretion continues, hence the need for continuous foraging to avoid gastric ulcers [71].

- The **small intestine** consists of three regions: the duodenum, jejunum and ileum and is about 15 to 22 metres long and 7 to 10 cm in diameter in a 500 kg horse. Fat, proteins, most of the vitamins and minerals and about 50 to 70% of soluble carbohydrate is absorbed in the small intestine. Unlike humans, the horse doesn't have a **gall bladder**, bile acids continuously drain from the liver into the small intestine and aid in the breakdown of fats and oil.

On average, feed reaches the large intestine after approximately 3 h and is fermented for 36–48 h [70]. The hindgut itself contains a multitude of different bacterial species but also other **micro-organisms** such as yeasts, anaerobic fungi and protozoa belong to the normal microbiota of the horse. Together they are responsible for breaking down fibres and any soluble carbohydrates that have escaped digestion in the small intestine.

Between 10^3 and 10^5 **protozoa** per mL have been isolated from the caecum and colon of ponies [71]. Approximately 70 species, most of which were ciliates, have been isolated from the large intestine and caecum (including species from the genera *Blepharocorys*, *Buetschlia*, *Cycloposthium* and *Paraisotricha*) [65]. Protozoa assist in the degradation of hemicellulose and pectins and upon removal, dry matter (DM) digestion decreases [71].

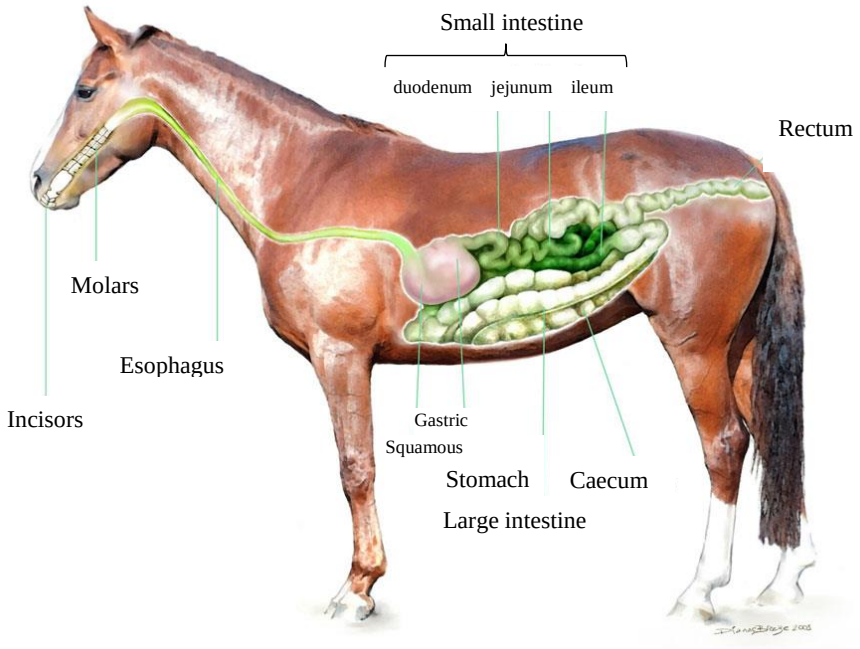

Figure 5.1. Overview of the equine digestive tract.

Anaerobic fungi play an important role in aiding fibre digestion by colonising the fibre particle and using their hyphae to help pull the particle apart, allowing intestinal bacteria

to access and colonise more quickly [74]. The **bacterial flora** exploit both amylolytic, lactate utilising, proteolytic, hemicellulolytic and cellulolytic activity and are responsible for the production of essential amino acids and vitamins B and K, which are essential for metabolism and energy utilisation [72].

The **pH** of the caecum and colon is approximately 6.0 and forms the ideal condition for anaerobic bacteria, fungi and protozoa to degrade hemicelluloses and pectins [75]. Upon **high starch** intake, residual starch may end up in the caecum and colon where it is slowly fermented and may favour the growth of amylolytic bacteria. This results in an increase in SCFAs and **lactic acid** production, leading to a significant decrease in pH [76]. This starch induced hindgut **acidosis** will reduce the growth and activity of the hindgut bacterial and fungal microbiota and consequently impair fibre digestion [71].

Finally, **water** is also absorbed in the colon and the remaining undigested material and bacteria expelled from the colon/caecum are passed into the rectum and excreted as faeces.

5.2 DIET

Horses are anatomically adapted to grazing continually on **marginal forages** [77,78]. In the wild, they travel great distances to obtain food and water. As wild grasses in their natural environment are typically high in fibre but low in energy, the horse grazes for approximately **12 to 16 hours a day** [70]. The relatively small stomach and large hindgut are perfectly suited for this (**5.1 ANATOMICAL OVERVIEW**). The complex plant material is **fermented** by microorganisms in the hindgut to short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which provide 60 to 70% of the daily energy needs of the horse [68]. Ideally, horses should be given free access to hay and/or pasture forages with salt and water ad libitum. A normal size horse (500 kg) will consume 1.5-2.5% of his body weight in **dry matter (DM)** a day [70,79]. If add-libitum foraging of grass is allowed, some horses will even consume up to the absolute max of 3% DM a day [80].

Despite this anatomical predisposition for ad-libitum feeding of low energy forage, today's sport horses are no longer predominantly kept in this most natural state: for practical reasons and to stimulate performance diet has changed significantly. Peak performances (jumping, eventing and racing) or long lasting exercises (like riding school horses, trekking and endurance) might increase the horse's energy requirements to the

point that forage does not suffice. In those cases part of the DM can be supplied through energy rich **grain mixtures**, so-called concentrates, containing oats, barley, corn, wheat and other grains [72]. However, it has been well documented that feeding >50% of the total DM fraction in the form of concentrates increases the risk of colic and laminitis in adult horses. High starch/sugar intake also has been correlated to increased incidence of insulin resistance [71,76]. Linseed, soy or corn oil can be added to the diet to reduce the risk of impaction colic, to improve coat condition or to easily add calories to the diet [81]. The addition of **extra fat** raises the energy density of feeds, which is advantageous for sport horses with high-energy requirements. Ad-libitum foraging in the prairie is rather rare for sport horses. **Fodder** such as hay, silage, straw and pelleted plants are offered to meet up with dry matter and fibre requirements.

Additionally, **feed supplements** are frequently added to the diet of the horse, to optimise his athletic performance. A feed supplement is anything fed to a horse in addition to a natural diet of forage, such as certain **vitamins and minerals**, which might be lacking in the diet. Technically, concentrates can also be considered supplements, but the term evolved to comprise any additional nutrients, besides concentrates. In recent years, a growing number of horse owners have also been feeding **herbal** supplements and various compounds thought to enhance certain aspects of health and performance, such as **hoof, joint or skin problems**. The widespread use of these supplements and the lack of information regarding the composition and purity of these (herbal) supplements can threaten anti-doping policies. Additionally, up until now, only limited research has been performed to study the **influence** of the altered diet of sport horses on the hormonal balance of the horse.

6. ANALYTICAL INSTRUMENTATION

6.1 HISTORY

Both in food residue and sport drug surveillance laboratories **great progress** has been made over the last few decades regarding the detection of residues and forbidden substances (doping) in different matrices. The **analytical methods** specifically used for the detection of steroids have been listed by De Brabander *et al.* (2004) and McKinney *et al.* (2009), but the overall historical evolution can be illustrated as presented in **Figure 6.1.** (p. 30).

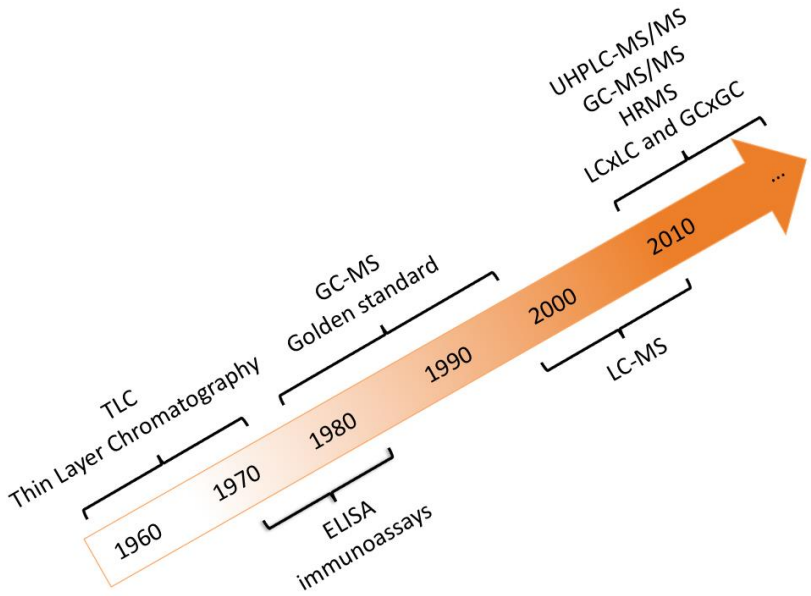

Figure 6.1. Summary of the evolution of analytical techniques used for steroid detection.

In the 60's and early 70's **Thin Layer Chromatography** (TLC) combined with fluorescence detection (TLC-FL) was the most used technique. Later on in the 70's immunoassays such as **ELISA** (Enzyme Linked Immunosorbent Assays) and EIA (Enzyme Immunoassay) were developed and widely used (**Figure 6.1**). Both EIA and ELISA systems are based on the principle of immunoassay linked to an enzyme rather than radioactivity as the reporter label [82].

Mass Spectrometry (MS) was introduced in late 70's, but took until the late 90's to conquer analytical labs worldwide. MS was first coupled to gas and later on to liquid chromatography (GC-MS or LC-MS) [83, 84]. Modern MS instruments are able to perform **MS in series**. The detection is carried out in the same compartment (MS^n) or in different compartments (MS/MS), on both the precursor ion and fragment ions, allowing to reach higher specificity and sensitivity. As of 2010, GC is also gaining back importance, coupled to MS/MS (complimentary to LC-MS/MS). Additionally, comprehensive two dimensional separation techniques, LCxLC or GCxGC are [85,86] (**Figure 6.1**).

High Resolution Mass Spectrometry (HRMS) on the other hand, operating at higher resolutions of 7,500 up to more than 100,000 Full Width at Half Maximum (FWHM), is being optimised now as a screening technique, but also for specific "omics" biomarker approaches such as metabolomics, proteomics and transcriptomics [87-90]. Additionally,

the development of Ultra-High Performance Liquid Chromatography (UHPLC), using **sub-2 µm particles** in the column, allowed higher flow rates and improved separation of compound with similar or identical masses and retention times (e.g. α- and β-isomers of testosterone), while at the same time shortening the run time [89].

The type of **matrix** used for steroid detection varies according to the specific goal of the analysis. Traditionally, **urine and blood** samples are being sampled for the detection of forbidden substances in the light of anti-doping controls but faeces and hair are possible matrices as well [37, 91]. The analysis of equine **mane hair** for the detection of anabolic steroid esters has the potential to greatly extend the time period over which detection of abuse can be monitored, as parent steroids (e.g. testosterone esters) are incorporated into the main hare [91]. In residue analysis meat samples and **skin swabs** are also used [92].

6.2 PHASE I AND II METABOLITES

A problem associated with the detection of boldenone and other related AAS is that they frequently result in little or no excretion of the parent steroid in urine and faeces. Instead, compounds are **metabolized** and excreted as their more hydrophilic phase I and II metabolites [93]. Phase I metabolites are formed through classical **oxidative and reductive** routes. Phase II metabolites, arise from the conjugation of these hydroxyl groups as either **sulphates or ß-glucuronides**, accounting for up to 90% of the excreted metabolites making them an important class for screening [94].

In the case of boldenone metabolism, the 1,4-diene-3-one structure of the A-ring appears to stabilize the steroid for reductive metabolism and boldenone-17ß-sulphate is the major metabolite [95]. Therefore, **extraction** of urine and faeces samples includes **hydrolysis** of both metabolites, releasing the free compounds for detection. Removing conjugates allows to determine the overall concentration (free and conjugated) of the compound, as used to define the thresholds of both the IFHA and FEI (See earlier, **3. ENDOGENOUS STEROIDS**).

6.3 UHPLC-MS/MS

Over the years various methods have been designed as initial screening tools to detect a large number of compounds in different drug classes. GC-MS has been the **gold standard** for the detection of residues and anabolic steroids (in urine) for many years [27,

88-89]. In the past decade, there has been a general shift from **GC-MS** towards LC-MS/MS for drug residue and in doping control testing [96,97]. This is mainly attributed to the **rapid improvement of LC-MS** in recent years, leading to better sensitivity, faster instrument turnaround time, and the ability to handle heat labile and large biomolecules. Recently, this shift has come to a standstill, with even a partial shift back to GC-MS/MS, depending on the type of analysis [98-102]. At the moment, LC and GC techniques can be considered to be **complimentary**, as both techniques have their specific advantages and disadvantages.

Recent work however has proven that UHPLC-MS/MS (Ultra High Performance Liquid Chromatography) instrumentation provides exceptional detection capability of AAS in equine matrices including mane hair [91], plasma [103] and urine [104]. LC-MS/MS is still **widely used** by **anti-doping testing laboratories** for this purpose and several rapid methods have been described to simultaneously detect different classes of compounds [98, 105]. Therefore, throughout this thesis Ultra high performance liquid chromatography (UHPLC) was carried out using an Accela^{TM} autosampler and Accela^{TM} High Speed LC (Thermo Fisher Scientific, San Jose, CA, USA). Detection was carried out on a **TSQ Vantage Triple Stage Quadrupole Mass Spectrometer** (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Heated Electrospray Ionisation probe (HESI-II) or Atmospheric Pressure Chemical Ionisation probe (APCI). **HESI** ionisation has been associated with high sensitivity and background noise upon mass spectrometric analysis. APCI is not as widely used as electrospray ionisation despite the fact that the ionisation of the substrate is very efficient (as it occurs at atmospheric pressure and thus has a high collision frequency). Additionally, by using **APCI** the thermal decomposition of the analyte is significantly reduced because of the rapid desolvation and vaporization of the droplets in the initial stages of the ionisation [106].

Upon ionisation, ions are transferred to the Q0 ion optics, including the Q0 quadrupole and lenses L11 and L12, which transmits the ions from the Q00 ion optics to the mass analyser. The **mass analyser** of the TSQ consists of three **quadrupole rod assemblies** (Q1, Q2, and Q3) and three **lens sets** (L2, L3 and L4) (numbered from the ion source end of the manifold) and separates ions according to their mass-to-charge ratio and then passes them to the ion detection system (**Figure 5.3**, p. 33).

The **rods** of a quadrupole rod assembly opposite to each other are connected electrically (two pairs of two rods each). AC and DC voltages are applied to the rods and these voltages are ramped during scanning. Voltages of the same amplitude and sign are applied to the rods of each pair; the voltages applied to the different rod pairs are equal in amplitude but **opposite in sign** (**Figure 5.2**).

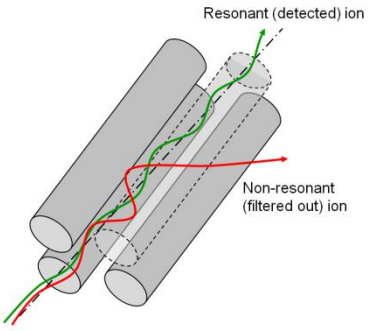

Figure 5.2. Quadrupole rod assembly mass analyzer. (http://chemwiki.ucdavis.edu)

Q2 always acts as an ion transmission device and is also known as the collision cell, as **collision-induced dissociation (CID)** can take place in the chamber that encloses Q2 (if the argon collision gas is present). The Q2 quadrupole rods are bent (90°), preventing the transmission of unwanted neutral species to the detector (**Figure 5.3**). The Q1 and Q3 quadrupoles can act as mass analysers or as ion transmission devices.

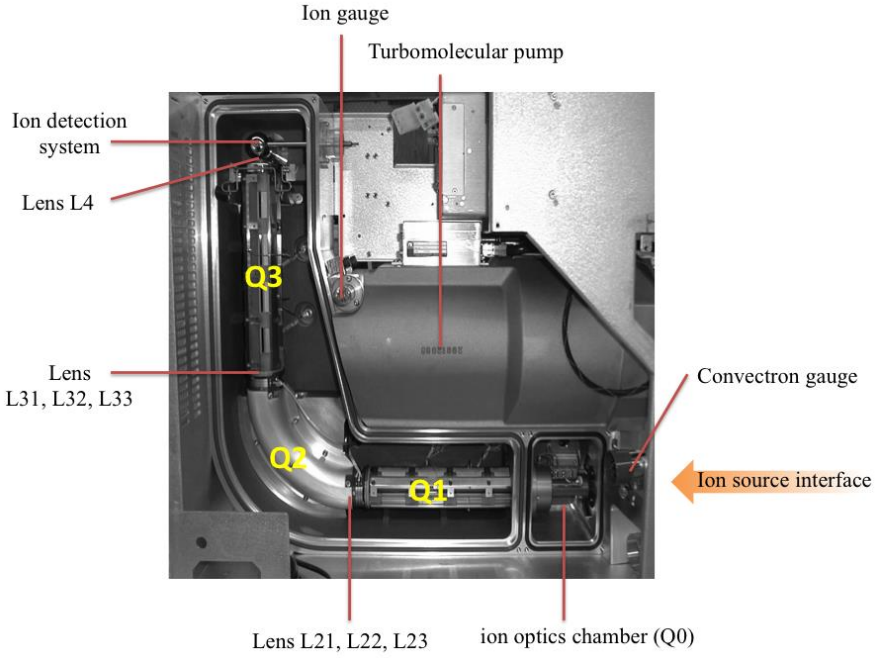

Figure 5.3. Mass analyser, ion detection system and ion optics of the TSQ Vantage Triple Stage Quadrupole Mass Spectrometer. Adapted from the Thermo Fisher Scientific TSQ series Hardware Manual (revised December 2010)

At the rear of the vacuum manifold, behind the mass analyser the TSQ mass spectrometer is equipped with a highly sensitive **ion detection system**. This system produces a high signal-to-noise ratio and allows for voltage polarity switching between positive and negative ion modes of operation and includes a conversion dynode and a channel electron

multiplier. The **electron multiplier** creates a cascade of electrons that finally results in a measurable current at the end of the cathode where the anode collects the electrons. This **current** is proportional to the number of particles striking the cathode, and thus the presence of a certain ion (compound) in the sample of interest [107].

6.4 GC-C-IRMS

Yet still, GC-MS has remained an important tool for analysing saturated steroid metabolites, as they suffer from **poor ionisation**. Alternatively, many urinary screening procedures include hydrolysis of phase II metabolites, releasing the free compounds for detection, allowing to determine the overall concentration of the compound (free and conjugated), as used to define the thresholds of both the IFHA and FEI [42,43] (See also **2. ANABOLIC STEROID ABUSE**).

As already mentioned, the administration of synthetic steroids, especially tackling the **exogenous administration** of steroids of endogenous origin, is an important obstacle for anti-doping regulatory organs. Therefore, doping control laboratories accredited by the WADA require methods of analysis that allow endogenous steroids to be distinguished from their synthetic analogues in urine. To that extend GC is also used in hyphenation with combustion isotope ratio mass spectrometry (**GC-C-IRMS)** a highly specialised instrumental **confirmatory** technique, by measuring the **carbon isotope ratio (Δ^{13}C)** of urinary steroids and confirm their synthetic origin based on the abnormal ^{13}C content [108, 109].

The average isotope ratio of each terrestrial element was fixed around the time of the earth's formation, but localized variations occur based on selective enrichment/depletion of the heavier isotopes (such as ^{13}C) relative to the average values. For example, even though all plants use CO_2 as a carbon source, various factors can influence a plant's ability to enrich or deplete ^{13}C in a process known as fractionation. GC-C-IRMS is capable of measuring these differences in relative ratio of light stable isotopes of carbon (^{13}C/^{12}C), hydrogen (^{2}H/^{1}H), nitrogen (^{15}N/^{14}N) or oxygen (^{18}O/^{16}O) in individual compounds, separated from often complex mixtures of components [110].

Back in 1998 Mason *et al.* already showed that when the isotopic composition of 5β-androstane-3α,17α-diol (the main metabolite of **testosterone** in bile) was normalised with respect to that of an endogenous reference compound (ERC, cholesterol) in the same sample, the metabolite could be used to distinguish between animals treated

intramuscularly with testosterone and untreated animals [111]. Throughout the last decade a variety of different methods has been developed and the number of different steroids under investigation by IRMS has grown considerably. Misuse of **norandrosterone, boldenone, corticosteroids or epitestosterone** can now be detected with the aid of carbon isotope ratios as well [112].

However, in equine anti-doping establishing IRMS as a confirmatory tool is not that straightforward, as one of the factors influencing fractioning is genetic. Monocotyledonous plants (C4 plants), such as corn and desert or marine plants, typically have $\Delta^{13}C$ values varying from -8 to -20‰. Most dicotyledons (C3 plants, including up to 95% of the plants on earth), have $\Delta^{13}C$ values varying from -22 to -35‰. Because animals can only incorporate carbon through the ingestion of plant (or animal matter), the carbon isotope ratios in an animal will reflect the isotope ratios of the food source: **"you are what you eat"** [110]. For horses this implies that $\Delta^{13}C$ values are very close to the ones of exogenous substances, hampering the ability to differentiate between endogenous and exogenously administered compounds (steroids).

Another problem associated with the use of IRMS in horses is the **"third sex": geldings**. If tests are performed to determine if an atypical steroid profile in humans is due to administration of an endogenous steroid androsterone (Andro), etiocholanolone (Etio), and/or the androstanediols (5α- and 5β-androstane-3α,17β-diol) they are typically analysed by IRMS to determine the $\Delta^{13}C$ values. The ratios of these target compounds are compared to the $\Delta^{13}C$ ratio of an endogenous reference compound (ERC) such as 5β-pregnane-3α,20α-diol (Pdiol) [113]. For geldings, it is very difficult to obtain such a, **reliable, endogenous reference compound (ERC).** It is possible to find a reliable ERC for stallions and mares, but this ERC is not consistent with geldings (personal communication, L.C.H.).

In this context, Piper *et al.* (2011) and Cawley and Flenker (2008) also described some of the complexities that can be encountered to obtain valid $\Delta^{13}C$ measurements from GC-C-IRMS and the need for **careful interpretation** of all relevant information concerning an individual's metabolism in order to make an informed decision with respect to a doping violation [112, 114].

7. AIMS

At the start of this project routine horse sport doping control was confronted with the detection of low levels of certain anabolic-androgenic steroids in urine. The question arose if these steroids are of natural origin or residues of exogenously administered drugs? This research project aimed at unravelling the possibility of endogenous prevalence of these steroids in untreated horses and possible explanations for this phenomenon. Based on previous research the digestive biotransformation of phytosterols was suspected to be a possible explanation for the excretion of thus far supposed to be synthetic steroids (e.g. β-boldenone).

The first goal was to get a clear and objective view on the endogenous levels of different AAS and AAS related steroid precursors naturally present in untreated horses. A very sensitive, specific and reliable detection method was needed to this end. To be able to reach this high standard an UHPLC-MS/MS method has been developed and validated according to AORC and EU Council Decision 2002/657. With this method, a population of guaranteed untreated horses (n =105; mares, geldings and stallions) was screened for the excretion of AAS and AAS related steroids (**Chapter II**).

In a second phase of this research, different hypotheses were tested for their possible contribution to the endogenous prevalence of AAS in horses:

1. Which phytosterols are present in feed (forage and concentrates) and are they present at sufficiently high concentration levels? A method was optimised for the extraction and MS/MS detection of phytosterols from feed (**Chapter III**).
2. Can phytosterols from feed be transformed to AAS by the equine microbiota present in the intestinal tract? Are *in vitro* digestion simulations a possible tool to study this biotransformation? Absorption of the transformation products, AAS or precursors, could as such be responsible for the excretion of AAS (**Chapter IV**).
3. Can feed-related molds play a role in the detection of AAS? Can the consumption of molded feed lead to the excretion of AAS (*in vitro*)? (**Chapter V**).
4. Is there a possible correlation between the administration of glucocorticoids, as anti-inflammatory agents, and AAS excretion (both *in vitro* and *in vivo*)? (**Chapter VI**).

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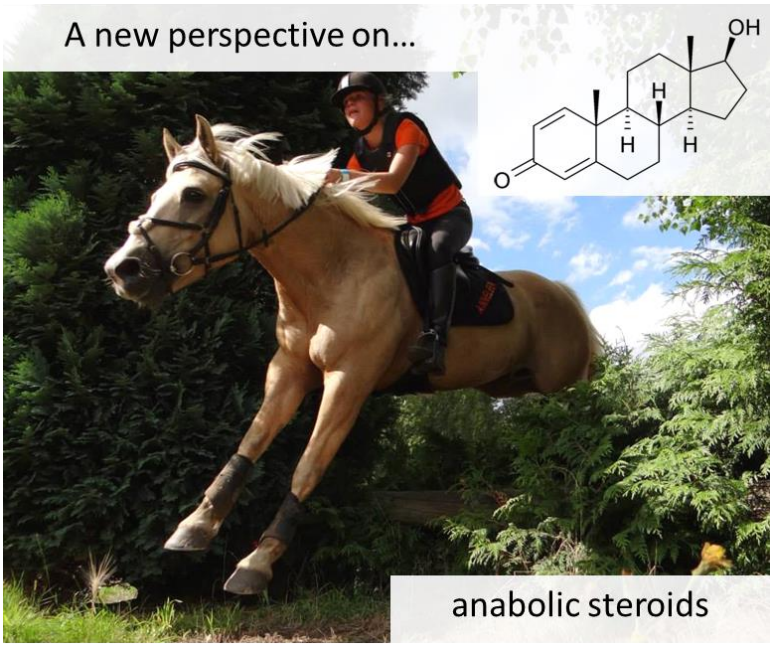
CHAPTER II

Development and validation of a UHPLC-MS/MS method to quantify low levels of anabolic-androgenic steroids naturally present in urine of untreated horses



Adapted from:

"A validated UHPLC-MS/MS method to quantify low levels of anabolic-androgenic steroids naturally present in urine of untreated horses"

By Anneleen Decloedt, Ludovic Bailly-Chouriberry, Julie Vanden Bussche, Patrice Garcia, Marie-Agnes Popot, Yves Bonnaire and Lynn Vanhaecke (2015), *Analytical and Bioanalytical Chemistry*, 407(15):4385-96, DOI 10.1007/s00216-014-8428-x

CHAPTER II

1. ABSTRACT

Doping control is a main priority for regulatory bodies of both the horse racing industry and the equestrian sports. Urine and blood samples are screened for the presence of hundreds of forbidden substances including anabolic-androgenic steroids (AASs). Based on the suspected endogenous origin of some AASs, with β-boldenone as the most illicit candidate, this study aimed to improve the knowledge of the naturally present AAS in horse urine. To this extent, a novel ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was developed and validated according to the Association of Official Racing Chemists (AORC) and European Commission (EC) Council Decision 2002/657, proving the power of this new method. Low limits of detection (0.2 ng/mL), good reproducibility (percentage of standard deviation (%RSD) <10%), high recovery (94.6 to 117.1%), selectivity and specificity, and a linear response (confirmed with R^2 >0.99 and lack-of-fit analysis) were obtained for all included AASs. With this method, urine samples of 105 guaranteed untreated horses (47 geldings, 53 mares, and 5 stallions serving as a control) were screened for β-boldenone and five related natural steroids: androsta-1,4-diene-3,17-dione (ADD), 4-androstene-3,17-dione (AED), α-testosterone (αT), β-testosterone (βT), and progesterone (P). Progesterone, β-testosterone, and α-testosterone were detected in more than half of the horses at low concentrations (<2 ng/mL). Occasionally, not only testosterone and progesterone but also low concentrations of AED, ADD, and boldenone (Bol) were found (0.5–5 ng/mL).

Keywords

AAS - Drug monitoring/screening - Urine

Equine - UHPLC-MS/MS

2. INTRODUCTION

Race and sport horses are, just like human athletes, frequently subjected to **doping controls** to guarantee a safe and fair competition, to protect the welfare of the animal and the integrity of racing, and to allow horses to compete on their inherent merits. Samples (urine, blood, hair, or other matrices) are screened for the presence of hundreds of **forbidden substances** including β2-agonists, stimulants, sedatives/tranquilizers, local anesthetics, anabolic-androgenic steroids (AASs), and others. Because of their popularity as **drugs of abuse** within the horseracing industry and their involvement in equine **reproduction**, research on AAS receives a high focus in the horse [1].

The most **well-known** AAS is **testosterone**, the principal male sex hormone. Closely related to testosterone, both structurally and functionally, are β-boldenone (βBol; androsta- 1,4-diene-3-one-17β-ol or 1,2-dehydrotestosterone), androstadienedione (ADD; androsta-1,4-diene-3,17-dione), and androstenedione (AED; androst-4-ene-3,17-dione) (**Figure 1**, p. 51). AED is a natural intermediate in the synthesis of testosterone [2], and boldenone (Bol), ADD, and Bol esters (e.g. undecylenate ester) are available on the **black market** as anabolic preparations. The pharmacological activity of boldenone is mainly anabolic, with a **low androgenic** potency. The administration of boldenone increases nitrogen retention, protein synthesis, appetite, and the release of erythropoietin in the kidneys [3, 4].

In **humans and livestock**, boldenone was long considered to be a **synthetic** hormone and zero tolerance was maintained [12]. As the occurrence of boldenone in biological samples was rising in different European Union member states, the question arose whether this increased number of boldenone findings was due to illegal treatment of humans and animals or if low concentrations of boldenone could be **endogenously formed** [13, 14]. Indeed, boldenone has been shown to be naturally present in bovine urine and feces [15, 16], whereas Pompa *et al.* [17] described de novo synthesis of boldenone in cattle feces [17] (**Figure 1**, p. 51).

In horses, endogenous boldenone was found in the urine and feces of entire males [18, 19]. As such, today, International Federation of Horseracing Authorities (IFHA) (Article 6, 2015) [20] and Fédération Equestre Internationale (FEI) (2015 Equine Prohibited Substances List) [21] abandoned zero tolerance for **stallions** and set a threshold for free

and conjugated **boldenone** of 15 ng/mL. Despite this threshold for entire males, the presence of boldenone in the urine from mares or geldings is still prohibited. For **testosterone**, thresholds are set as well, up to 55 ng/mL of free and conjugated testosterone in urine for mares and fillies (unless in foal) and 20 ng/mL in urine for geldings.

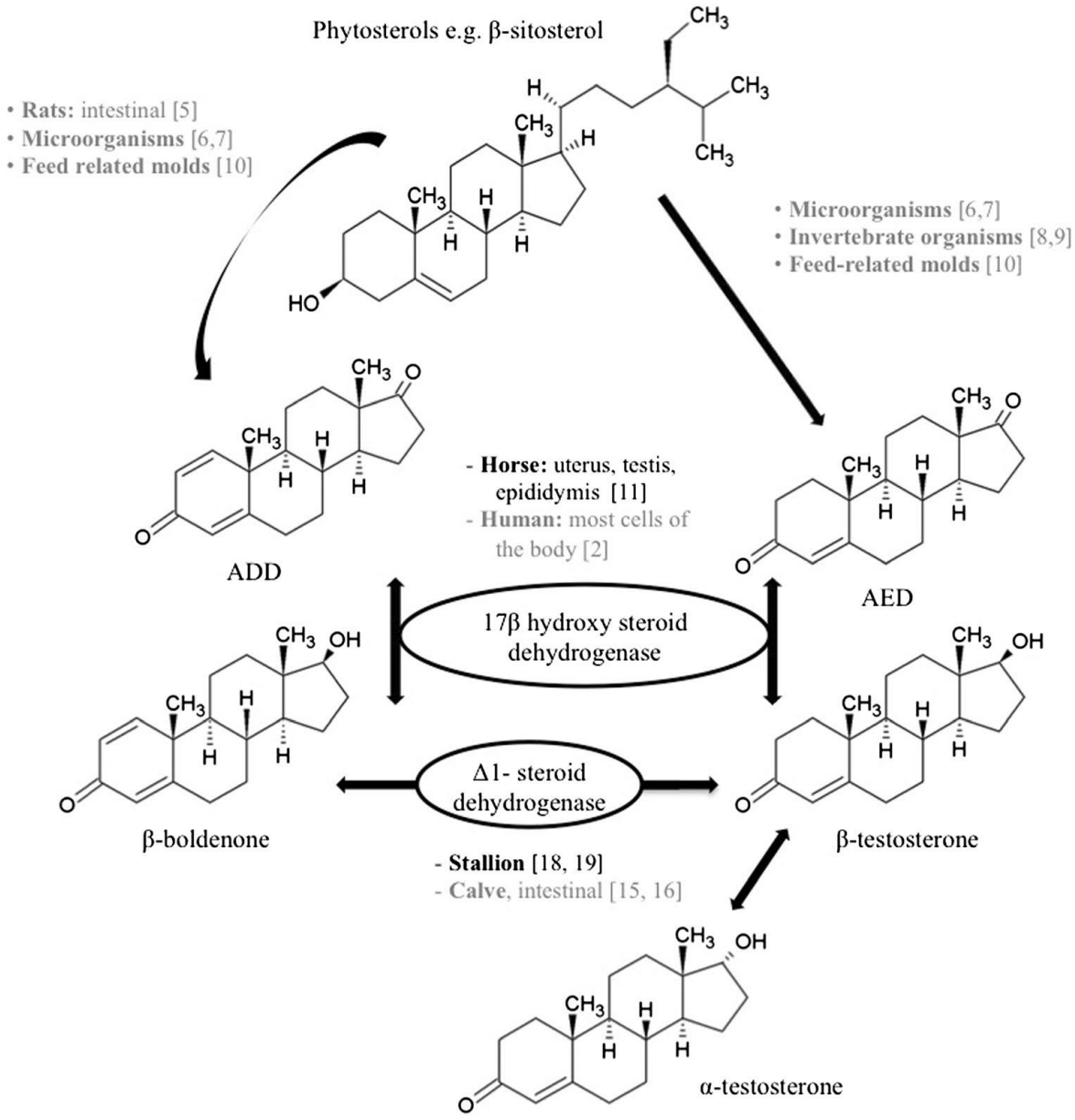


Figure 1. Illustration of the closely related chemical structures of the steroids of interest and summary of proven (in horses) and related pathways of AAS metabolism. Common enzymes are depicted in a **O**. βBol (β-boldenone), ADD (androstadienedione; 1,4-diene-3,17-dione), AED (androstenedione; androst-4-ene-3,17-dione), αT (α-testosterone), βT (β-testosterone) are illustrated.

A problem associated with the detection of boldenone and other related AAS is that they frequently result in little or no excretion of the **parent steroid** in urine. Instead, compounds are **metabolized** and excreted as their more hydrophilic phase I and II metabolites (See also Chapter I, 6.2 Phase I and II metabolites). Phase I metabolites are formed through classical oxidative and reductive routes. The horse tends to secondarily reduce 17 keto groups to form a mixture of 17α- hydroxy and 17β-hydroxy isomers. Phase II metabolites arise from the **conjugation** of these hydroxyl groups as either sulfates or β-glucuronides, accounting for up to 90% of the excreted metabolites [22], making them an important class for screening.

In the horse, there is a trend for **sulphation** to predominate for steroids with a 17β-hydroxyl group, while steroids with a 17α-hydroxyl group tend to form **glucuronide** conjugates [23]. In the case of boldenone metabolism, the 1,4-diene-3-one structure of the A-ring appears to stabilize the steroid for reductive metabolism and boldenone-17β-sulfate is the major metabolite [24]. Therefore, extraction includes **hydrolysis** of both metabolites, releasing the **free compounds** for detection, allowing to determine the overall concentration (free and conjugated) of the compound, as used to define the thresholds of both the IFHA and FEI [20, 21].

Gas chromatography coupled to mass spectrometry (**GC-MS**) has been the gold standard for the detection of anabolic steroids in urine for many years [25–30]. In the past decade however, there has been a shift towards liquid chromatography-tandem mass spectrometry (LC-MS/MS) in doping control testing [31,32]. This is mainly attributed to the rapid improvement of **LC-MS** in recent years, leading to better sensitivity, faster instrument turnaround time, and the ability to handle heat labile and large biomolecules. GC-MS on the other hand offers advantages over LC-MS for the screening of fully saturated steroids (e.g. endogenous steroids such as pregnane-3-α,-20-α-diol, the main metabolite of progesterone in urine). Therefore, LC and GC-MS/MS techniques can be considered to be complimentary, as both techniques have their specific advantages and disadvantages. Recent work however has proven that ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) instrumentation specifically provides exceptional detection capability of AAS in multiple equine matrices including mane hair [33], plasma [34], and urine [35].

However, the studies published so far focus on stallions and/or horses to which steroids have been **administered intramuscularly or orally** [18, 19, 35–39], and not on naturally present endogenous AAS, with β-boldenone as the most illicit candidate for endogenous presence. Therefore, this study focused on healthy, guaranteed untreated, out-of-competition horses, in an attempt to improve the knowledge on the natural **endogenous AAS** in horse urine in general. In total, the urine of 105 guaranteed untreated horses (47 geldings, 53 mares, and 5 stallions) was screened for β-boldenone and five other related steroids: ADD, AED, α-testosterone (αT), β-testosterone (βT), and progesterone (P) via UHPLC-MS/MS. The method was **validated** according to the Association of Official Racing Chemists (AORC) [40] and European Commission (EC) Council Decision 2002/657 [41]. Hypotheses on the **origin and correlations** between occurrences of these AASs in untreated horses are discussed.

3. MATERIAL AND METHODS

3.1 CHEMICALS AND REAGENTS

αT (androst-4-ene-17α-ol-3-one, purity ≥98%), βT (androst-4-ene-17β-ol-3-one, purity ≥98%), methyltestosterone (MT; androst-4-ene-17α-methyl-17β-ol-3-one, purity ≥98%), ADD (androsta-1,4-diene-3,17-dione, purity ≥98%), proteinase type XXIII from *Aspergillus melleus* (3 enzyme units/mg), and β-glucuronidase (*Helix pomatia*, aqueous, >100.000 units/mL) were purchased from Sigma-Aldrich (St. Louis, USA). AED (androst-4-ene-3,17-dione, purity ≥98%) and β-boldenone (androsta-1,4-diene-17β-ol-3-one, β-Bol, purity ≥98%) were obtained from Steraloids (Newport, USA). Progesterone (purity ≥98%) was obtained from Alpha Pharma (Omega Pharma, Zwevegem, Belgium).

Methanol Optima® was bought at Fisher Scientific UK Limited (Leicestershire, UK). Diethyl ether, ethyl acetate, sodium hydroxide, methanol (analytical grade), sulfuric acid (H_2SO_4, 97%, analytical grade), potassium phosphate monobasic (KH_2PO_4), and formic acid (HCOOH, 98–100%, analytic grade) were purchased from VWR (Merck, Darmstadt, Germany). Solvolysis solvent consisted of 900 mL ethyl acetate, 95 mL analytical methanol, and 5 mL H_2SO_4 per liter. The HF Bond Elut-C18 cartridges (6 mL, 500 mg) were obtained from Agilent Technologies (Diegem, Belgium). HPLC grade, ultrapure water was acquired from an in-house water purification system (Arium® 611UV; Sartorius Stedium Biotech, VWR, Haasrode, Belgium).

Stock solutions of each steroid were prepared in methanol Optima® (Fisher Scientific, UK) at 2 µg/mL. Dilutions up to 1 ng/mL were made, and all solutions were kept at 4 °C.

3.2 SAMPLE COLLECTION AND EXTRACTION

As **spontaneously voided samples** were collected and the horses were not given any medication or treatment, according to the latest Belgian and European animal welfare rules (RD 29th of May 2013, published on the 10th of July), they are not considered to be experimental animals. As such, the authors state that they have followed the principles outlined in the **Declaration of Helsinki** for all animal experimental investigations. In addition, informed consent has been obtained from the owners. Horses were owned by the author herself or horses belonging to the faculty (Faculty of Veterinary Medicine, Merelbeke, Belgium); their medical history was known and well documented. Horses were guaranteed to be **untreated with AAS** or other treatments that are known to interfere with the excretion of AAS.

Urine samples were captured in sterile 50-mL tubes, and per horse, two aliquots (A and B) of 3 mL were made and all samples were immediately stored at −20 °C prior to analysis. The **immediate freezing** of collected samples and their instant analysis after thawing is the proposed procedure to prevent all transformations that can occur in stored urine, usually due to microbiological contamination [42].

The sampled population consisted of 47 geldings and 53 mares aged 1 up to 23 years old (average age of **8.9 ± 5.6 years** old), which were all out of competition. No foals (<12 months) were sampled. Five stallions were also sampled for comparison. Horses were on a standard but **non-controlled diet** of concentrate (two meals, 1–3 kg/day), hay, and straw or flax, combined with pasture access for several hours a day. Most of the horses were **Belgian Warmbloods** (B.W.P., 74/105) or **Royal Dutch Sport Horses** (K.W.P.N, 10/105), but some other breeds were included as well: Thoroughbred (seven), Andalusian horse (one), Arabian horse (two), Quarter Horse (two), Gipsy Horse (one), and breedless ponies (seven).

Excessive fluid intake can substantially dilute urinary concentrations and result in false-negative reports. On the other hand, poor drinking can lead to the overestimation of the detected compounds. Different methods for **correction (normalization)** of drug/metabolite concentrations in urine have been put forward [43]. In this study, the

density of the urine samples was measured through the specific gravity (SG) of the urine samples using a **pocket refractometer** (PALUSG(CAT); Atago, Tokyo, Japan). The measured SG was used to correct the detected concentrations using the formula described by Cone *et al.* [44]. All concentrations that are given in the results are SG normalized.

$$Concentration_{SG\ normalized} = Concentration_{specimen} \times (SG_{reference} - 1) / (SG_{specimen} - 1)$$

3.3 HYDROLYSIS OF THE GLUCURO- AND SULFO-CONJUGATED COMPOUNDS

Hydrolysis of the urine samples is preferred over the analysis of unhydrolysed urine to increase the sensitivity of detection. Pooling nonconjugated and released sulfate and glucuronide conjugated fractions allows to increase the detectable concentration of free compounds and, in line with the IFHA and FEI regulations, to evaluate free and conjugated boldenone as a whole.

For the **hydrolysis** of the urine samples, 1 mL of phosphate buffer (1 M KH_2PO_4, pH 6.1) was added to 3 mL of urine. Next, the internal standard methyltestosterone (MT, 5 ng/mL), 50 µL of a protease solution (≥450 units/mL), and 25 µL of **β-glucuronidase** were added. For each sample, the pH was set at 6.1 ± 0.1 (by adding drops of 6, 1 or 0.1 M HCl) and hydrolysis was executed at 55 °C (1 h). After hydrolysis, 3 mL of ultrapure water was added and non-hydrolyzed proteins were removed by agglutinating them at the bottom of the tube by centrifugation (2400 x *g*, 15 min). Finally, the supernatant was filtered over a cotton wool filter before solid phase extraction (SPE).

3.4 SOLID PHASE EXTRACTION, WASHING, AND SOLVOLYSIS

The **cartridges** (6 mL, 500 mg C18, Bond Elut, Isolute) were conditioned with 4 mL methanol and 4 mL ultrapure water. The centrifuged and filtered urinary samples were loaded onto the column and washed consecutively with 7 mL ultrapure water and 7 mL hexane. The cartridges were dried under vacuum (−0.5 bar). Next, the nonconjugated and glucuronide-conjugated fraction was eluted with **diethyl ether** (7 mL) and the sulfate-conjugated fraction with **solvolysis solvent** (7 mL).

The diethyl ether fraction is **washed** instantly. The sulfate-conjugated fraction is first incubated at 55 °C (2 h) to allow solvolysis to take place. Both fractions were washed with 1.5 M sodium hydroxide by turning (8 min, 60 rpm), centrifugation (6 min, 1400×g), and collecting the washed fractions. These fractions were **pooled and dried** under nitrogen (50 °C, 45 min). Each sample was reconstituted in 100 µL of ultrapure methanol

(Optima®), vortexed, and ultrasonicated (3 min). Finally, 100 µL of ultrapure water was added and the sample was vortexed and ultrasonicated (3 min) again. After centrifugation (12,300 × g, 10 min), the sample was transferred to an LC-MS vial with insert for UHPLCMS/ MS analysis

3.5 UHPLC-MS/MS ANALYSIS

UHPLC was carried out using an Accela™ autosampler and a Accela™ High Speed LC (Thermo Fisher Scientific, San Jose, CA, USA) with a Nucleodur™ Sphinx RP column (1.8 µm, 50×2.1 mm, Macherey-Nagel). All analytes could be accurately separated in a total run time of only 9 min (**Figure 2**).

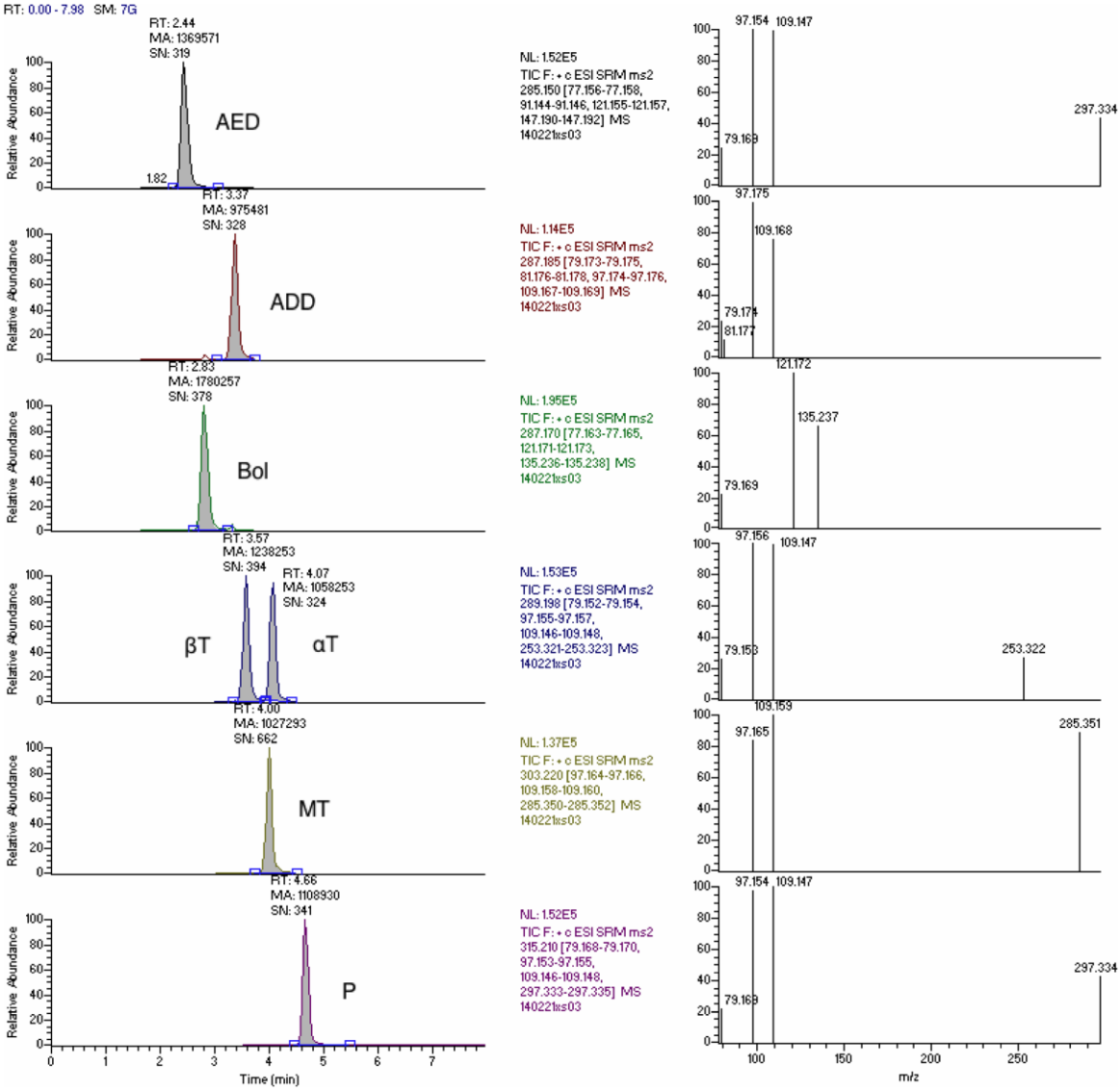


Figure 2. Chromatograms and fragmentation spectra of a standard solution of steroids. (0.1 ng of ADD, AED, Bol, αT, βT, MT, and P was brought on column)

The mobile phase consisted of ultrapure water as solvent A and methanol Optima® as solvent B, both acidified with 0.1% formic acid (26.5 mM). A gradient was run at 300 µL/min, starting with 58% solvent B for the first 2 min, steadily increasing to 100% solvent B at 5.5 min, and then held at 100% solvent B for 1.5 min (up to 7 min). Then, the column was allowed to equilibrate at the initial conditions of 42% solvent A and 58% solvent B for 2 min.

Table 1. SRM specifics for all analytes of interest: precursor ion, product ions, retention time (RT), appropriate S-Lens RF amplitude, and the corresponding collision energy (CE)

Analyte	Precursor Ion (*m/z*)	Product Ions (*m/z*)	(Relative) Retention Time (min)	S-lens (V)	Collision Energy (V)
ADD	285.2	77	2.45	54	51
		91	(0.61)		39
		121			22
		147			15
AED	287.2	79	3.39	70	36
		81	(0.85)		37
		97			21
		109			25
βBol	287.2	77	2.85	56	51
		121	(0.71)		26
		135			17
αT	289.2	79	4.06	70	40
		97	(1.01)		23
		109			27
		253			16
ßT	289.2	79	3.59	70	39
		97	(0.90)		22
		109			27
		253			15
P	315.2	79	4.64	75	38
		97	(1.16)		23
		109			28
		297			13
MT	303.2	97	4.01	73	30
		109	(1.00)		28
		285			15

3.6 STANDARD ADDITION APPROACH AND DATA ANALYSIS

Multiple parameters such as the color, pH, density, and protein content of the urine samples demonstrate the **diverse biochemical composition** of the individual urine samples. Therefore, an individual quantification method was combined with fitting the metabolites' area ratio in a calibration curve. As such, multiple calibration curves were made for the validation of the method (see below), but the standard addition approach

was used as an additional **urine-specific quantification**. The extract of one aliquot of each urine sample (A, blank sample) was reconstituted in methanol/water (50:50). Injection on the UHPLC-MS/MS system resulted in an area under the curve, relative to the present concentration of the analyte in the blank sample. This area was divided by the area under the curve for the internal standard (5 ng/mL MT), gaining the area ratio (Ar_A) of the blank sample for each analyte present. To the other aliquot (B, spiked sample), **5 ng/mL (ρ)** of the six analytes was added before extraction. Reconstitution of this aliquot was also performed in methanol/water (50:50). UHPLC-MS/MS analysis of this aliquot resulted in an area ratio of Ar_B for each analyte. Using the following formula, the unknown concentration of the analyte in the blank sample (C_A) was calculated:

$$\mathbf{C_A = Ar_A / (Ar_B - Ar_A) \times \rho}$$

$\mathbf{C_A}$	Concentration of the analyte in aliquot A (blank) (ng/mL)
$\mathbf{Ar_A}$ **or** $\mathbf{Ar_B}$	Area ratio of aliquot A or B
ρ	Concentration of added analyte (5 ng/mL)

Data were interpreted using Xcalibur 2.1 qualitative and quantitative software (Foundation 1.0.2 Rev. B; Thermo Fisher Scientific, San Jose, USA).

3.7 QUALITY ASSURANCE: VALIDATION OF THE ANALYTICAL METHOD

To confirm the quantitative performance of the used analysis method, the accuracy, precision, linearity, and sensitivity were appraised. Prior to analysis, the individual targeted compounds and standard mixtures were injected to check the selectivity and **operation conditions** of the chromatographic devices and. The different molecules were identified based on their **relative retention time**, relative to the internal standard. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio (S/N) of at least 3, was **5 pg** on the column for all components. All specified product ions (**Table 1**, p. 57) were used for peak integration for quantification purposes. Quantification was performed by standard addition of each individual urine sample, as described above.

The method was validated using EU Council Decision 2002/657 [41] and AORC MS guidelines [40]. Six replicates of each of the three spiked levels (1, 1.5, and 2 ng/mL) were analyzed. Analysis was carried out on **three separate occasions**, using fortified

matrix (pooled urine from three geldings and two mares, 10.0 ± 4.9 years old). This **pool** was a mixture of visually different urine samples from mares and geldings, which are dark and light in color and different in turbidity. As a consequence, the validation allows expecting equal or even better results when using other urine samples.

In this validation process, specificity and selectivity, linearity, limits of detection and quantification, ruggedness, repeatability, within-laboratory reproducibility, and recovery for each compound were determined according to EU Council Decision 2002/657 and AORC guidelines.

4. RESULTS AND DISCUSSION

4.1 Validation study

4.1.1 Specificity

Analysis of 20 non-fortified pooled urine samples (n = 5) was performed to check the specificity of the method and calculate the endogenous concentrations of Bol, ADD, AED, βT, αT, and P. As chromatograms of the non-fortified urine extracts did not contain **interfering matrix peaks** at the respective retention times of the analytes added, good specificity was concluded. Furthermore, the chromatograms of fortified samples displayed a significant **increase in peak area intensity** and showed no other matrix substances interfering at the specific retention time of the compound, when the chromatographic peak of interest had a **S/N of at least 3**. As a result, based on these results and the criteria described in CD 2002/657/EC, the developed method was found to be specific.

In the non-fortified pooled urine used for the validation, only αT was found endogenously at a concentration of 3.5 ± 0.2 ng/mL. Therefore, the endogenous concentration, calculated as the average concentration of 20 non-fortified samples, was subtracted from the calculated total concentrations.

4.1.2 Selectivity

The different compounds were identified on the basis of their specific retention time relative to the retention time of the standard solution. When using high-resolution separation techniques, such as UHPLC, the maximum difference in retention time of the reference standard and the test compound should be within ± **50% of the half-height**

peak width or 3 s, whichever is larger [40]. All compounds were able to meet this **AORC criterion** in every sample analysed.

In accordance to CD 2002/657/EC, a minimum of four **identification points** (IPs) is required for UHPLC-MS/MS of steroids (precursor ion and greater than or equal to two **diagnostic product ions**). AORC regulations demand greater than or equal to three ions. Both criteria were met, as all compounds included in this method were identified through their precursor ion and three or four product ions: two larger, diagnostic, ions (e.g. *m/z* 253 or 285), and one or two smaller ions (e.g. *m/z* 77, 79, 81 or 91) (**Table 1**, p. 57). From a diagnostic point of view these smaller ions are less significant than the larger ions (see also **Figure 2**, p. 56). *m/z* 91 for example is a typical fragment seen after fragmentation of alkyl benzenes. *m/z* 77 is typically corresponding to the phenyl cation. *m/z* 97 (**Figure 3**) on the other hand is considered to be a diagnostic ion for androst-4-en-3-one-based steroids (e.g. testosterone) [45].

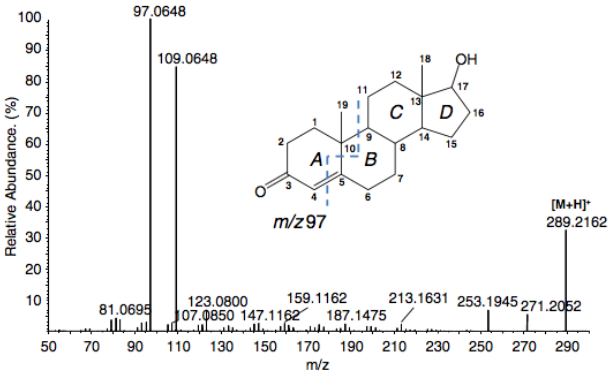


Figure 3. ESI product ion mass spectrum of the protonated molecule $[M + H]^+$ at *m/z* 289 of testosterone. Recorded on an AB SCIEX TripleTOF 5600 with a collision offset voltage of 30 V as part of the structure elucidation of the diagnostic product ion at m/z 97 derived from androst-4-en-3-one-based steroids by ESI-CID and IRMPD spectroscopy [45].

4.1.2 Linearity

For evaluation of linearity, calibration curves were constructed with nine fortification levels (**0.25, 0.5, 1, 2, 4, 6, 8, and 10 times** the recommended concentration (RC) of 1 ng/mL, according to Council Decision 2002/657/) based upon the expected natural presence of the compounds in non-fortified urine samples. Each curve was run twice. The mean slope, R^2, and lack-of-fit linear model were calculated for all calibration curves (n = 8, including the within-laboratory variation).

Slopes for the different compounds ranged between 0.300 and 0.582 with a small variation (0.010–0.020) (**Table 2**, p. 61). The least squares method ($\mathbf{R^2}$) and the lack-of-fit test were used for fitting the calibration curves.

Table 2. Validation parameters of the quantitative performance of the used extraction and detection method for the five compounds (Bol, ADD, AED, αT and ßT).

Analyte	Endogenous concentration* (ng/mL)	Mean slope calibration curve**	Mean R^2**	LOD* (ng/mL)	LOQ* (ng/mL)	Nominal concentration (ng/mL)	Recovery (%)	Precision	
								Repeatability RSD (%)	Intra-lab reproduc. RSD (%)
Bol	NF	0.405 ± 0.011	0.996	0.23	0.76	1.0	102.7	8.0	8.7
						1.5	100.7	6.6	7.0
						2.0	96.3	6.6	6.2
ADD	NF	0.582 ± 0.010	0.997	0.23	0.75	1.0	117.1	5.0	4.9
						1.5	114.9	6.6	7.0
						2.0	106.6	7.4	6.7
AED	NF	0.373 ± 0.010	0.996	0.31	1.02	1.0	100.3	8.5	8.5
						1.5	100.6	7.1	7.0
						2.0	100.2	6.0	5.8
αT	3.5 ± 0.2	0.341 ± 0.013	0.996	0.20	0.68	1.0	106.1	9.3	9.3
						1.5	101.8	8.4	8.9
						2.0	104.5	5.8	6.0
βT	NF	0.301 ± 0.020	0.996	0.16	0.54	1.0	107.0	9.9	9.4
						1.5	105.9	6.2	6.0
						2.0	111.6	7.4	7.7
P	NF	0.243 ± 0.019	0.986	0.26	0.83	1.0	94.6	10.1	10.3
						1.5	97.3	7.9	7.9
						2.0	100.4	7.4	7.2

* Based upon 20 non-fortified samples of pooled urine (individual and intralaboratory replicates)
** Mean slope and mean R^2 for all calibration curves (n = 8, individual and intralaboratory replicates)

The **lack-of-fit test** (F test, α= 0.05) proved linear for all compounds, with F values lower than the F reference value (SPSS 21). The coefficient of determination R^2 was always higher than 0.99, except for progesterone. For progesterone, an average R^2 of 0.986 ± 0.005 was calculated. All other analytical validation parameters were met for progesterone (**Table 2**, p. 61).

4.1.2 Precision

Precision, the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions, is usually expressed in terms of imprecision and computed as a percentage of standard deviation (%RSD) of the test result (definition 1.22 2002/657/EC). Less precision is determined by a larger %RSD.

For one set of samples, 18 aliquots of urine (n = 6×3) were fortified, six samples per level, with 1, 1.5, and 2 times the RC of 1 ng/mL (see "Linearity"). To evaluate the precision of the developed analytical method, repeatability and withinlaboratory reproducibility were determined. Repeatability was confirmed by extracting and analysing the same set of samples (n = 6×3) on three different occasions.

The withinlaboratory reproducibility was confirmed by repeating the above analysis by a different operator on a different occasion under different conditions. Both validation parameters were evaluated by calculating the relative standard deviations (%RSD) (Table 2, p. 61). These were very low (%RSD <10%), confirming the good reproducibility and precision of the developed method.

4.1.3 Trueness

The determination of trueness is assessed using certified reference material (CRM), if available. Alternatively, recovery can be determined during validation, if no certified reference material is available (as described under 4.1.2.1 (2002/657/EC)). Trueness is then expressed as the recovery in spiked samples. **Recovery** is the percentage of the true concentration of a substance recovered during the analytical procedure.

The same set of samples (n = 6×3) was extracted and analysed on four different occasions by two different operators. Recovery was calculated for each analyte in each sample. The mean recovery per level is given in **Table 2 (**p. 61). Recoveries **(94.6–117.1%)** are according to EU Council Decision 2002/657 for substances at concentrations ranging from 10 to 100 ng/mL (20% RSD). For very low concentrations (<10 ng/mL) as used in

this validation, the %RSD is not specified, but extrapolation of the %RSD for higher concentrations would then even allow %RSD up to 25%. The guidelines for performance criteria and validation procedures of analytical methods rely on the level calculated by the Horwitz equation [46]. For 1–10 ng/mL, the Horwitz-based %RSD is even less strict than EU Council Decision 2002/657 (32–45% RSD).

4.1.4 Stability

Urine samples and extracts were immediately **stored at −20 °C**. The immediate freezing of the collected samples and their instant analysis after thawing is the proposed procedure to prevent all transformations that can occur in stored urine, usually due to microbiological contamination [42]. Indeed, if the urine samples were frozen immediately, no differences were seen between the samples extracted at the moment of storage and the samples **extracted 3 weeks later**. The same goes for the **reanalysis of stored extracts** (3 weeks); the measured concentrations were not significantly different from the results obtained after the primary analysis.

4.1.5 Limit of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the mean blank signal as the basis for the calculation of the **LOD and LOQ values**. On each occasion, five non-fortified matrix samples were analysed in parallel to calculate the S/N at the time window in which the analyte is expected. Three times the S/N was used as the LOD and ten times the S/N as the LOQ. For each component, the mean S/N was plotted against the concentration (R^2 >0.99). Based on the fitted curve, the detection limits for the different components were calculated (S/N >3). LODs for the different compounds were between **0.17 and 0.31 ng/mL**. For the LOQ, the same calculation was used, but with the cut-off at S/N >10, and the LOQs amounted between 0.54 and 1.02 ng/mL. All performance characteristics of the validation are presented in **Table 2** (p. 61).

The mathematically determined LODs were confirmed by adding 0.20 ng/mL of each component to a pooled urine sample. The ion chromatograms of a standard injection of boldenone (0.01 ng/µL, **Figure 4.A**, p. 64), a non-fortified urine sample (**Figure 4.B**, p. 64), and the equivalent fortified urine (0.20 ng/mL, **Figure 4.C**, p. 64) are illustrated.

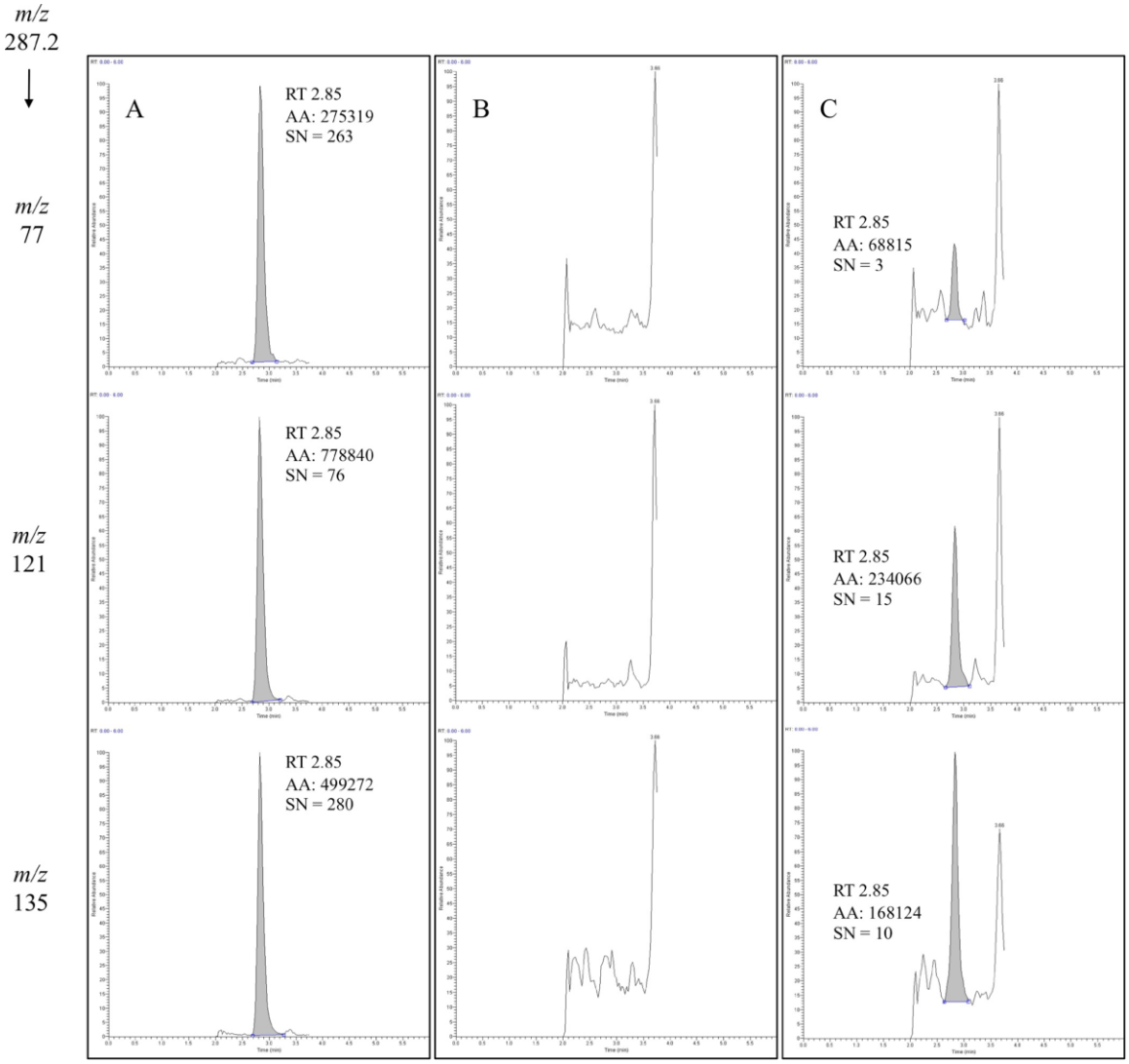


Figure 4. Comparison of the chromatograms obtained using UHPLC-MS/MS on different samples. (A) Standard injection of boldenone (0.01 ng/µL). **(B)** Non-fortified urine. **(C)** Urine fortified with 0.20 ng/mL boldenone (LOD). The three specific transitions of the target compound boldenone (*m/z* 287.2) were monitored as follows: *m/z* 287.2→*m/z* 77, *m/z* 287.2→*m/z* 121, and m/z 287.2→*m/z* 135

In **conclusion**, the full-fledged validation of this method following both CD 2002/657/EC and AORC guidelines exceeds the less extensive quality assurance procedures, such as calibrators and limited calibration curves (three to five points), which are used to confirm comparable methods [18, 47, 48]. The obtained and confirmed LODs are very low compared to other previously published LC-MS/MS methods in urine: the LOD of ADD, for example, (0.2 ng/mL) is very low compared to the described LOD of 10 ng/mL [35]. Good linearity (R^2 >0.99 and lack-of-fit analysis) and low %RSD (<10%) were obtained compared to comparable methods (%RSD >10%) [47]. Additionally, the (relative) retention times of the different compounds are stable over time (<2% variation), whereas

the previously described retention times differ more than 10% [48]. Moreover, the newly developed UHPLC-MS/MS method allows to adequately separate all natural steroids in a 9-min run time, while run times of up to 24 min were used by previous methods, separating even less compounds [15].

4.2 URINE SCREENING OF UNTREATED HORSES

The validated extraction and UHPLC-MS/MS method was put into practice with the urinary screening of 105 guaranteed untreated horses (43 geldings, 57 mares, and 5 stallions). The **density of all the urine samples** was measured as the SG of the urine samples using a pocket refractometer (PAL-USG(CAT); Atago, Tokyo, Japan). The measured SG of each individual urine sample (1.024 ± 0.003) was used to correct the detected concentrations using the formula described by Cone *et al.* [44] (see Chapter II, **3.2 SAMPLE COLLECTION AND EXTRACTION**). All concentrations given here are SG normalized.

Upon analysis of the urine samples from 100 mares and geldings, 81% of them were found to excrete steroids at a certain level. Nevertheless, almost all of them were considered to be negative in the light of horse doping control. However, in one gelding's urine, **β-boldenone** was detected at a concentration of **1.0 ng/mL** (**Table 3**, p. 66). As this horse did not show elevated urinary testosterone levels (0.2 ng/mL) nor any clinical signs or behaviour linked to cryptorchidism [49], the detected β-boldenone residue is suspected to be of **natural origin** (as this horse was not treated in any way).

For c**onfirmation**, three additional aliquots were taken from the same urine sample and analysed. The result of these three replicates (**1.0 ± 0.2 ng/mL**) confirmed the first result. This horse was also resampled 2 months after the first sampling, but no boldenone was found at that moment. These results suggest that the presence of a (low) concentration of boldenone is **variable**. A combination of different factors such as the general condition of the horse, his training status, his gastrointestinal microbial content, the weather, feed and/or seasonal influences might be involved in this transient detection [50, 51]. As direct hydrolysis was applied, i.e. potential 1-dehydrogenation of steroids *ex vivo*, might also be responsible for boldenone detection. Confirmatory analysis with direct detection of the intact boldenone sulphate could be used to support these findings [52].

Table 3. Detection of ADD, AED, Bol, αT and ßT in urine of guaranteed untreated horses (mares, n = 53; geldings, n = 47; and stallions, n = 5). For each compound, the number of horses with the compound detected as well as the average concentration in these horses and the overall average concentration of the group (mares, gelding or stallions) are given. NF = not found (below the respective limit of LOD for this compound, see **Table 2**, p. 61).

Analyte	Gender	Number of Detection	(Average) concentration in detected (ng/mL)	Average concentration group (ng/mL)
ADD	mare	(1/53)	5.5	0.1 ± 0.8
	gelding	(0/47)	NF	NF
	stallion	(2/5)	1.0 ± 0.4	0.4 ± 0.5
AED	mare	(4/53)	1.4 ± 1.3	0.1 ± 0.5
	gelding	(4/47)	0.7 ± 0.6	0.1 ± 0.3
	stallion	(0/5)	NF	NF
Bol	mare	(0/53)	NF	NF
	gelding	(1/47)	1.0	0.0 ± 0.2
	stallion	(2/5)	0.9 ± 0.1	0.4 ± 0.5
αT	mare	(29/52)	2.6 ± 2.7	1.5 ± 2.4
	gelding	(23/47)	4.3 ± 4.2	2.2 ± 3.5
	stallion	(0/5)	NF	NF
ßT	mare	(25/53)	3.2 ± 2.7	1.4 ± 2.4
	gelding	(25/47)	2.5 ± 2.2	1.4 ± 2.0
	stallion	(5/5)	108 ± 14	108 ± 14

ADD and AED, which are chemically closely related to β-boldenone and testosterone (**Figure 1**), were also found in the urine of some horses. Indeed, ADD was found in the urine of a 10-year-old mare horse out of 100 horses at a concentration of **5.2 ng/mL**. Looking at the chromatogram, next to ADD, a large peak of testosterone was present, indicating an elevated concentration of α-testosterone (12 ng/mL), eightfold higher than the average determined for mares in this study (1.5 ± 2.4 ng/mL).

Measuring testosterone levels have been used historically as a diagnostic marker for the presence of ovarian tumours (See Chapter I, **3.3 HORMONAL DISORDERS IN HORSES**). Testosterone concentrations have been reported to be elevated above the normal range of cycling mares in 40 to 50% of mares with **granulosa cell tumours (GCTs)** [53]. Affected mares can show dominant and male-like behaviour (e.g., mounting). No prominent male-like yet some dominant behavior was observed in the mare studied here. Due to the lack of severe symptoms, the ovaries of this mare have, thus far, not been clinically examined to confirm this hypothesis. **Progesterone and β-testosterone** levels for this horse were normal, 2.6 and 2.5 ng/mL, respectively. These levels do not

significantly differ from the average progesterone (1.0 ± 1.6 ng/mL) and β-testosterone concentrations (1.4 ± 2.4 ng/mL) of the mares in this study.

Moreover, all measured testosterone concentrations were **far below** the IFHA international **thresholds** (2015) for geldings (20 ng/mL) and mares (55 ng/mL). In a previous study investigating the effect of γ-oryzanol supplementation on endogenous testosterone levels in horses, the urine β-testosterone concentration was always lower than 1.7 ng/mL. Neither the effect of γ-oryzanol intake nor the difference in gender (mare or gelding) could be proven [54]. The results from the present study confirm this **low concentration** of β-testosterone in both mares (1.4 ± 2.4 ng/mL, n = 53) and geldings (1.4 ± 2.0 ng/mL, n = 47) (**Table 3**, p. 66).

AED was even more present than ADD and was detected in the urine of 8% of the geldings and mares: four geldings (four out of 47) and four mares (four out of 53), and the concentrations were not significantly different according to the gender (1.4 ± 1.3 ng/mL for mares and 0.7 ± 0.6 ng/mL for geldings) (**Table 3**, p. 66). This suggests that the presence of AED is not related to the **gender** of the horse. Interestingly, one of the gelding's urine contained boldenone as well, suggesting a possible correlation between AED and boldenone. This horse has been resampled, and the corresponding sample was negative for both AED and boldenone, sustaining this **potential correlation**. This result supports the hypothesis that external factors may play a role in the anabolic steroid status of the horse, as mentioned earlier.

The urine of all five stallions sampled during this study, contained **large amounts of β-testosterone** (108 ± 14 ng/mL). These concentrations are, as expected, much higher than those for geldings and mares. The average concentration for geldings (1.4 ± 2.0 ng/mL) and mares (1.4 ± 2.4 ng/mL) are 100-fold lower. In two entire males (two out of five) for which concentrations of 120 and 126 ng/mL β-testosterone were found, **boldenone** was found as well at 1.0 and 0.8 ng/mL. This **100-fold difference** between the detected β-testosterone and boldenone level is in accordance with the previous results of Popot *et al*. [19] and Ho *et al.* [18]. The mean boldenone concentration for the five stallions (0.4 ± 0.5 ng/mL) is also in accordance with the mean concentration of boldenone of 0.34 ng/mL (minimum 0.02, maximum 1.51 ng/mL) (n = 156) measured in male horses by Dehennin *et al.* [55]. Interestingly, in two male horses, we also found a low concentration of **ADD (1.0 ± 0.4 ng/mL)**, and these were the two stallions that also showed β-boldenone.

In summary, these results demonstrate that, in contrast to what was expected, all steroids that were included in this method could be shown to be naturally present in urine of at least one horse, and 81% of these horses were found to excrete steroids at a certain level. T and P are found in the urine of most of the horses, both geldings and mares, at low concentrations. AED and ADD, which are closely related to AAS and possible precursors of forbidden substances such as boldenone, can be present in the urine of untreated horses at low concentrations (1 ng/mL).

Reduction of these, or other, precursor steroids might lead to the unexpected detection of **low concentrations of forbidden steroids** such as β-boldenone. The urine of one horse did indeed contain 1 ng/mL β-boldenone. The transformation of ADD to boldenone and AED to testosterone is carried out by the same enzyme, **17β-hydroxysteroid dehydrogenase**, which is found in most cells of the human body [2]. In horses, this enzyme is merely found in the testis and epididymis (stallion) and ovaries and uterus (mare) [11] (**Figure 1**, p. 51). **Further research** is warranted to elucidate the alternative origins of AED and ADD in horses and the possible transformation pathways to forbidden substances such as boldenone.

5. CONCLUSION

Based on the suspected endogenous origin of some AAS, this study aimed to improve the knowledge on the naturally present low levels of AAS in horse urine. A rapid UHPLC-MS/MS method was developed and successfully and thoroughly validated according to AORC guidelines and EU Council Decision 2002/657 and applied on 105 urine samples from guaranteed untreated horses. In contrast to what was expected, all steroids that were included in this method could be shown to be naturally present in urine of at least one horse, and 81% of these horses were found to excrete steroids at a certain level.

5.1 EXECUTIVE SUMMARY

- A selective and precise extraction and UHPLC-MS/MS method was developed and validated according to AORC guidelines and EU Council Decision 2002/657
- The method proved to be very sensitive (LOQ=0.7 ng/mL, 3-mL sample)
- All stallions were shown to produce β-testosterone (108 ± 14 ng/mL), and two of them excreted β-boldenone (0.9 ± 0.1 ng/mL) and ADD (1.0 ± 0.4 ng/mL) as well
- The method was able to detect low concentrations of progesterone, β-testosterone, and α-testosterone (1–5 ng/mL) present in the urine of mares and geldings (41, 52, and 55, respectively, out of 100 horses)
- Occasionally, other steroids (ADD, Bol, and/or AED) were found in the urine of untreated geldings or mares at low concentrations (0.5–5.0 ng/mL).

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CHAPTER III

Optimisation of phytosterol extraction and analysis from feed by D-Optimal design and UHPLC-MS/MS

Adapted from:

"Validated UHPLC-MS/MS method for the extraction and detection of phytosterols in food, feed and beverages"

By Anneleen Decloedt, Anita Van Landschoot and Lynn Vanhaecke, submitted to *Analytical and Bioanalytical Chemistry*

CHAPTER III

1. Abstract

Phytosterols are omnipresent in plants, as they play an important role in cell membrane stability and as signal transducers. Over the last few decades, the scientific interest in phytosterols has significantly increased. Most of the interest has focused on the cholesterol-lowering properties of phytosterols, but they may also interfere with the endogenous steroid hormone synthesis. Despite this dual interest in phytosterols, accurate and fully validated methods for the quantification of phytosterols in food and feed samples are scarce. During this study a new extraction and detection method for the main phytosterols (β-sitosterol, campesterol and stigmasterol) was optimized and fully validated according to EU Council Decision 2002/657 and AOAC guidelines. A fractional factorial design was used to optimize the extraction procedure. The most optimal conditions were perfected for all influential factors. Detection was carried out on a UHPLC-MS/MS apparatus. The newly developed extraction and UHPLC-MS/MS detection method reached all evaluated performance parameters. The individual recoveries ranged between 95 and 102%. Good results for repeatability and intra-laboratory reproducibility (RSD%) were observed (<10%). Excellent linearity was proven based on determination coefficient (R^2 >0.99) and lack-of-fit test (F test, $\alpha = 0.05$). The LODs and LOQs in grain matrices were as low as 0.01-0.03 mg/100 g and 0.02-0.10 mg/100 g for campesterol, stigmasterol and β-sitosterol, respectively. With this method we were able to quantify all main phytosterols in different grains (oats, barley, corn, malt) and it was shown that the method can be used on other solid feed and food samples as well, including new matrices such as straw, hay, grass and yellow peas. Additionally, the method allowed to quantification of campesterol, stigmasterol and β-sitosterol in liquid samples low in phytosterols such as concentrate-based juices, beer and soft drinks.

Keywords

Phytosterols - Ultra-High Performance Liquid Chromatography

Tandem Mass Spectrometry – Validation - Extraction optimisation

2. Introduction

Phytosterols are omnipresent in **plants**, as they play an important role in cell membrane stability and fluidity. They can also participate in the control of membrane-associated metabolic processes, as signal transducers. Over the last few decades, the scientific interest in phytosterols increased significantly, based upon two very distinct properties related to their chemical structure. Most of this interest has focused on the cholesterol-lowering properties of phytosterols and phytostanols [1]. Phytosterols are structurally very **similar to cholesterol** except that they contain a substitution at the C24 position on the sterol side chain (**Figure 1**, p. 79) [2]. A lot of research has been dedicated, in both humans and model animals, to the confirmation and unravelling of the mechanisms responsible for these cholesterol lowering capacities. It is generally assumed that phytosterols reduce cholesterol absorption in the intestinal tract, through the displacement of cholesterol from the micelles [3].

The European Foods Safety Authority (EFSA) and Food and Drugs Agency (FDA) concluded that, relative to a placebo, blood **LDL cholesterol levels** can be significantly reduced by 7 to 10.5% (respectively 5 to 15%) if a person consumes 1.5 to 2.4 grams (1 to 3 grams) of plant sterols and stanols a day [4]. The effect is usually established within the first 2–3 weeks after diet change and could be sustained for more than one year [5]. A daily dietary intake of at least **2 grams a day** of phytosterols (expressed as non-esterified phytosterols) is required to make an authorized health claim relating phytosterol consumption to cholesterol lowering and cardiovascular disease risk [6]. No significant alterations in high-density-lipoprotein (HDL)-cholesterol or triglycerides in general were reported, and the effectiveness of this approach has been positively tested in hypercholesterolaemic patients, as well as in individuals with normal cholesterol levels [7].

On the other hand, phytosterols may interfere with the endogenous steroid **hormone synthesis**. This possible involvement in the synthesis, excretion and detection of steroids in humans and sports or farm animals is currently under discussion. Most anabolic-androgenic steroids (AAS) are strictly forbidden, as they can increase nitrogen retention, protein synthesis, appetite and the release of erythropoietin in the kidneys, leading to unfair competition or illegitimate weight gain. The **conversion** of phytosterols to steroids

has been reported in different biological systems mainly involving a variety of microorganisms such as *Mycobacterium* sp., *Arthrobacter* and *Nocardia* sp. [8-10]. On the other hand, a number of studies have been devoted to the ability of invertebrate organisms to convert phytosterols into anabolic steroids and a study in rats reported the *in vivo* intestinal biotransformation of consumed phytosterols to steroids (ADD, AED and androstanedione) [11]. Additionally, recent work indicated that a novel mechanism of endogenous steroid-synthesis is to be considered: non-toxic **feed-borne fungi**, naturally present on animal feed, were shown capable of converting phytosterols into steroids [12,13]. Complete (microbial) side chain cleavage produces the C19-steroids, which include AED, ADD, boldenone and testosterone (**Figure 1**). Other studies reported similar biotransformation potential in environmental mold species [14,15].

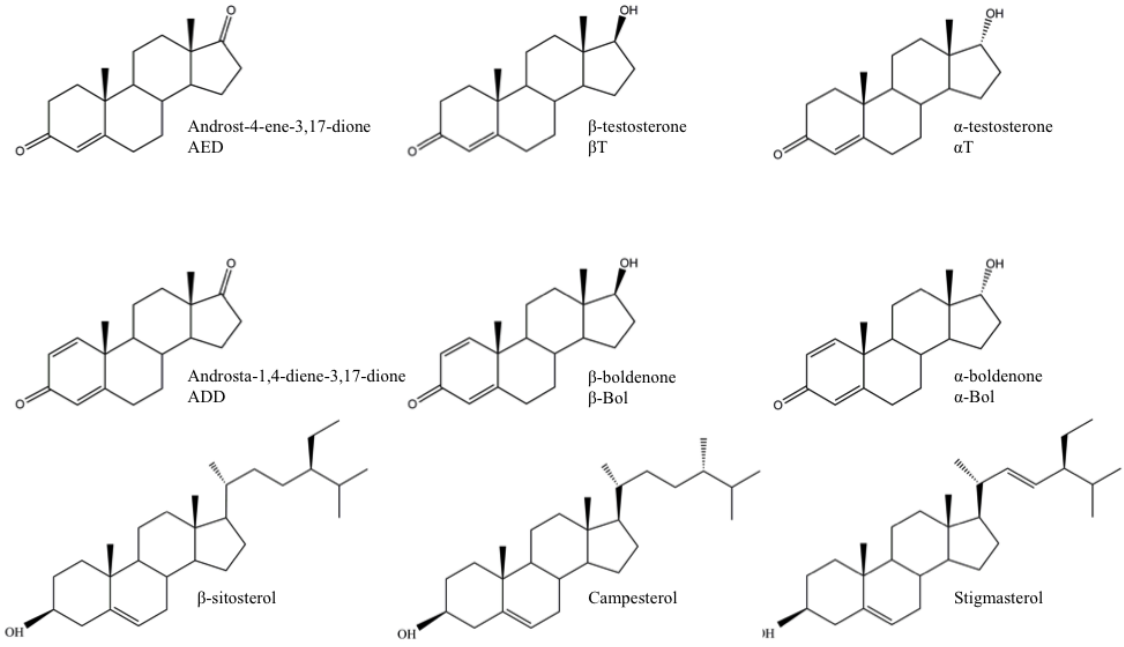

Figure 1. Chemical structure of the three main phytosterols (campesterol, stigmasterol and β-sitosterol) compared to the structure of different C-19 steroids, including β-boldenone.

Despite this dual interest in phytosterols, accurate and fully validated methods for the accurate quantification of phytosterols in food and feed matrices are **scarce** (**Table 1**, p. 80). Different methods can be used to purify, separate and detect phytosterols in food and feed samples. Separation can be achieved with a wide variety of chromatographic techniques including column chromatography (CC), gas chromatography (GC), thin-layer chromatography (TLC), normal-phase high-performance liquid chromatography (HPLC),

reversed-phase HPLC and capillary electro chromatography (CEC). GC can be considered as the most reported separation technique [14]. GC however requires derivatisation of the samples, in most cases silylation of sterols to trimethylsilyl (TMS) ether derivatives is performed prior to analysis. Detection is carried out through flame ionisation detection (FID), UV detection (UV), evaporative light scattering detection (ELSD), infrared detection (IR), nuclear magnetic resonance detection (NMR) or mass spectrometry (MS) [16]. The use of **advanced MS techniques** such as High Resolution MS or MS/MS for the quantification of phytosterols in feed and food has thus far not been explored. Additionally, most methods published so far only focus on food samples, including fruits, vegetables, cereals, nuts and berries, and not on feed (**Table 1**).

It should be noted that **no official reference method** has been developed for the analysis of phytosterols or phytostanols in food and feed. Some international reference methods exist for the analysis of sterol fractions of fats and oils, such as the ISO 6799, IUPAC methods 2.401 and 2.403, ISO 12228 and AOCS Ch 6-91. Also, Codex Stan 210 refers to ISO 6799 and IUPAC 2.403 methods. All these methods have been developed for the analysis of sterols as natural minor food components, implying that the total sterol contents are 1% or lower [28].

Table 1. Different methods for the quantification of phytosterols in food (1999-2014).

	Authors	Year	Matrix	Method	Quality control
[24] [26]	Normén *et al.*	1999 2002	Cereal foods	GC-FID/GLC	recovery ISTD Theoretical LOD
[31]	Toivo *et al.*	2001	Sunflower, corn, onion, oil	GC-MS	Calibration curves, LOD ISTD dicholesterol Recovery, %RSD
[21]	Piironen *et al.*	2002	Cereals and cereal products	GC-MS	
[22]	Piironen *et al.*	2003	Vegetables, fruit and berries	GC-MS	
[32]	Phillips *et al.*	2004 2005	Cereals, nuts, fruits Nuts and seeds	GC-FID	Recovery Commercial reference samples
[33]	Sorenson *et al.*	2006	Saw palmetto raw materials Dietary supplements	GC-MS	Single-laboratory validation
[34]	Nair *et al.*	2006	Supplements, health care	HPLC-ELSD	Full validation, incl. LOD/LOQ in solvent
[35]	Winkler *et al.*	2007	Corn distiller's dried grain	GC-MS	Unknown
[18]	Ryan *et al.*	2007	Grains	HPLC-PAD (UV) GC-FID	Unknown
[19]	Mo *et al.*	2013	Edible oils	APCI LC-MS/MS	Calibration curves, recovery, LOD/LOQ

Within this study a **new extraction and detection method** was optimised and fully validated according to EU Council Decision 2002/657 and AOAC (Association of Analytical Chemists) MS guidelines. The method allowed quantifying campesterol, stigmasterol and β-sitosterol in different grains (e.g. corn, oats, barley and malt) and it was shown that the method can be used on other solid feed and food samples as well including grass, hay, straw, yellow peas, apple, hop and tomato. Additionally, the method allows quantifying campesterol, stigmasterol and β-sitosterol in liquid samples low in phytosterols such as concentrate-based juices, beer and soft drinks as well.

3. MATERIAL AND METHODS

3.1 LC-MS^2 REAGENTS AND CHEMICALS

Methanol Optima® (HPLC grade) and chloroform (analytical grade) were purchased from Fisher Scientific (Leicestershire, UK). Sodium hydroxide and methanol (analytical grade) were purchased from VWR (Merck, Darmstadt, Germany). HPLC grade, ultrapure (UP) water was acquired from an in-house water purification system (Arium® 611UV, Sartorius Stedium Biotech, VWR, Haasrode, Belgium).

Cholesterol (≥99%, from lanolin), β-sitosterol (≥97%, from soy beans) and stigmasterol (≥97%) were purchased from Fluka (Sigma Aldrich, St-Louis, USA). Campesterol (≥99%) was obtained from Applied Science Laboratoria (Bedford, USA). Stock solutions of each component were made in methanol Optima® at 200 ng/µL. Dilutions up to 0.1 ng/µL were made in methanol Optima®. All solutions were kept at 4 °C.

3.2 FEED AND FOOD SAMPLES

Oats (*Avena sativa*) were kindly gifted by Canadian Oats Milling (Alberta, Canada). Yellow peas (*Lathyrus aphaca*) were obtained from JM Grain Inc (North Dakota, USA). Apples (*Malus domestica* 'Jonagold') were bought at a nearby grocery shop. Corn (*Zea mays*) crops were harvested straight from the field (East-Flanders, Belgium). Hop (*Humulus lupulus*) was a Hallertau magnum variety, dried, grinded and distributed by Simon H. Steinerhop (Mainburg, Duitsland). Barley (*Horleum vulgare*) and pale malt (*Horleum vulgare*) were obtained through mouterij Dingemans NV (Stabroek, Belgium). Straw and hay samples were obtained from DB fourage (Knesselare, Belgium). Gras samples were collected straight from the field (*Lolium Perenne*, Ghent, Belgium).

100-g homogenous subsamples were **freeze-dried** (-0,020 mBar at -50 °C, 24 h) in a Christ Alpha 1-4 LSC freeze dryer (SciQuip, Shropshire, UK) and samples were thoroughly mixed (Moulinette, Moulinex, Belgium) and grinded with a mortar and pestle and/or grinded in a rotating laboratory disc mill DLFU certified by EBC, MEBAK and IOB (0.1-0.2 mm) (Buhler Miag, Benelux).

All phytosterol concentrations listed are expressed per **dry weight**, unless indicated differently. Samples were subsequently stored under dry and dark conditions at -18 °C. Liquid samples were obtained from different producers including the "Brouwersverzet" (Old Brown beer, Anzegem, Belgium), "Brouwerij Timmermans" (Old Kriek lambic beer, Itterbeek, Belgium), AB Inbev (Jupiler, Leuven, Belgium) and Ghent University College (Bijloke, Ghent, Belgium). Orange juice, apple juice and an orange based soft drink were purchased from The Coca-Cola Company (Ghent, Belgium).

3.3 EXTRACTION PROTOCOL OPTIMISATION

Previous research has shown that is possible to extract phytosterols from different food matrices (**Table 1**, p. 80). Most extraction protocols described use a liquid-solid extraction from grinded and freeze-dried material with an apolar solvent (hexane) or chloroform-methanol, (2:1 v/v). Based on these existing protocols an initial protocol was formulated: 200 mg subsamples were weighted and spiked with 5 ng/mg cholesterol (100 µL of a 10 ng/µL standard solution). Six ml **chloroform:methanol** (2:1 v/v) was added to the sample and thoroughly vortexed (30 sec), ultrasonicated (10 min) and centrifuged (4,400 x *g*, 10 min). With a glass pipette the organic extract was separated from the powder and transferred to a new recipient (50 mL sterile, plastic tube). The powder was re-extracted with 6 ml chloroform:methanol (2:1 v/v) and again thoroughly vortexed (30 sec), ultrasonicated (10 min) and centrifuged (4,400 x *g*, 10 min).

Subsequently, the organic solvent was **washed** with 5.5 ml 0.1 M NaCl, shaken and left to separate into two distinct phases (30 min). The upper, watery phase was removed and the chloroform fraction dried with 1 g Na_2SO_4, shaken and incubated overnight (16h). The following day, the extract was filtered over a SPE filter cartridge (Isolute MSPD reservoir) under vacuum and 1 ml chloroform was added to the filter cartridge, to elute the remaining sample. The entire filtrate was evaporated to dryness under a gentle nitrogen stream (2 bar, 50 °C for 45 min). Upon analysis, the extract was reconstituted in 180 µl methanol Optima®; vortexed (30 sec) and ultrasonicated (10 min). Afterwards, 20

µl ultra-pure water was added and the sample was vortexed (30 sec), ultrasonicated (3 min) and vortexed (30 sec). After centrifugation (12,300 x *g*, 10 min) the sample was transferred to an LC-MS vial with insert for UHPLC-MS/MS analysis.

This initial, literature based protocol was further optimized as a feed (grain) extraction procedure for phytosterols using a **fractional factorial design** (FFD). All dependent variables that might significantly affect the extraction efficiency were screened to elucidate the significant factors (D-Optimal Design), which subsequently could be optimized in a surface response modeling approach (SRM), designed with Modde 5.0 Pro software, a tool for design of experiments (DOE) and subsequent data analysis (Unimetrics, MKS Company, Sweden).

The FFD and SRM approach allow a quicker and more cost-effective optimisation, compared to one-at-the-time experiments. In general, a FFD is a good **screening approach** as the amount of possible scenarios (trials) produced by the model is significantly lower than that for full factorial designs. Unlike full n factorial designs, which require all combinations of n versions of each of k variables, a FFD only needs $n^{(k-1)}$ [17].

3.4 UHPLC–TRIPLE QUADRUPOLE MS/MS ANALYSIS

Ultra-high performance liquid chromatography (U-HPLC)-MS/MS detection was used to quantify phytosterols. **Separation** was carried out using an Accela™ High Speed LC (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Thermo Scientific™ Hypersil GOLD™ Column (particle size: 1.9 µm, 50 x 2.1 mm I.D.). The mobile phase consisted of ultra-pure water as solvent A and methanol Optima® as solvent B.

The gradient was run at 300 µl/min, starting with a linear gradient of 90% solvent B for the first 2 min, increasing to 100% solvent B at 5.5 min, and then held at 100% solvent B for 1.5 min (up to 7 min). Afterwards, the column was allowed to equilibrate at the initial conditions of 10% A and 90% B for 2 min. The divert valve was used to load the detector from 1.5 to 4.5 min. All analytes could be accurately separated in a total run time of only 10 min (**Table 2**, p. 84).

Table 2. SRM specifics for all analytes of interest: precursor ions, product ions, (relative) retention time (RT), appropriate S-Lens RF-amplitude and the corresponding collision energy (CE).

Analyte	Precursor Ion (*m/z*)	Product ions (*m/z*)	(Relative) Retention Time (min)	S-lens (V)	Collision Energy (eV)
Campesterol	383.3	81	2.38	86	35
		91	(1.11)		49
		95			34
		105			43
Stigmasterol	395.3	81	2.40	59	37
		91	(1.12)		52
		105			44
		297			18
β-sitosterol	397.3	91	2.61	88	47
		95	(1.22)		35
		105			40
		147			28
Cholesterol* *internal standard	369.3	91	2.14	84	52
		95	(1.00)		34
		105			40

Detection was carried out on a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an Atmospheric Pressure Chemical Ionisation probe (APCI). Injection volumes were 10 µL each and the **APCI** source was operated in the positive ion mode. The discharge current was set at ± 4 µA. The sheath, sweep and auxiliary gas pressure were set at 20, 2 and 10 arbitrary units respectively, the capillary temperature at 300 °C and the vaporizer temperature at 320 °C. The collision gas pressure was kept at 1.5 mTorr and the cycle time was 0.8 s. Data were acquired in the **multiple reaction monitoring** (MRM) mode. The resolution of the quadrupole mass filter (Q1) was set with a peak width of 0.2 Da at half height, the Q3 filter at 0.7 Da at half height. All specified product ions (**Table 2**) were used for peak integration and quantification purposes.

Data were interpreted using Xcalibur 2.1. w/Foundation 1.0.2 Rev. B qualitative and quantitative software (Thermo Electron, San Jose, USA). Area ratios were calculated relative to the **internal standard cholesterol**, that was added to both calibration and unknown samples, to compensate for losses during sample preparation or variability during the analytical determination. Cholesterol can be considered as a good internal standard as it is very similar to phytosterols (calibrated analytes), chemically and in

retention time, but chromatographically distinguishable, cheap and not endogenously present in plant-derived samples.

3.5 DATA ANALYSIS AND QUALITY ASSURANCE OF THE ANALYTICAL METHOD: VALIDATION PROCEDURE ACCORDING TO EU COUNCIL DECISION 2002/657 AND AOAC MS GUIDELINES

To confirm quantitative performance of the newly developed extraction and detection method a validation process was performed. Specificity and selectivity, linearity, limits of detection and quantification (LOD and LOQ), ruggedness, repeatability, within-laboratory reproducibility and recovery (accuracy) for each compound were determined according to **EU Council Decision (2002/657/EC)**. Prior to analysis, the individual targeted compounds and standard mixtures were injected to check the selectivity and **operation conditions** of the chromatographic devices. The different molecules were identified based on their relative retention time, relative to the internal standard (ISTD) cholesterol. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio (S/N) of at least 3 was <0.1 ng on column for all components.

The method was **validated** according to EU Council Decision 2002/657/EC and AOAC MS guidelines. Six replicates of each of the three spiked levels were analyzed. These spiked levels were set as the **expected endogenous concentration (EEC)** and 1.5 and 2 times this EEC. Analysis was carried out on three separate occasions, using fortified barley matrix. Previous research states that barley is moderately high in phytosterols (50 ± 2 mg/100 g) compared to other grains [18]. As a consequence, the validation allows expecting equal or even better results when using other feed (or food) samples.

3.6 QUANTITATIVE ANALYSIS OF DIFFERENT FEED AND FOOD SAMPLES

Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard. In each matrix, **two calibration curves** were constructed based upon nine fortification levels (0, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 times the EEC). The EEC was preliminary determined by standard addition and/or based upon available, reference values in literature. At least three non-fortified samples were extracted together with a nine-point calibration curve, at two different occasions ($n \geq 24$ samples per matrix). The calibration was run twice, before and after the experimental, non-fortified samples. Unknown samples were quantified by fitting the metabolites' area ratio in the corresponding calibration curve.

4. RESULTS AND DISCUSSION

4.1 EXTRACTION PROTOCOL OPTIMISATION

Different parameters of the extraction protocol were evaluated during the extraction optimisation: the solvent used the volume of the extraction solvent, temperature for drying under nitrogen, single or double liquid-liquid extraction, drying with Na_2SO_4 and the inclusion of an additional hydrolysis step. A fractional factorial D-optimal design was chosen, optimizing the seven variables through 16 trial and 3 central point samples. A significantly higher response was seen after extraction with **methanol:chloroform**, compared to the extraction with the strictly apolar solvent hexane, which was used by Mo *et al.* (2013) among others [19]. This can be explained by the highly effective solvolysis conducted by methanol and chloroform, compared to the less invasive hexane treatment.

Additionally, a larger extraction volume (**15-20 mL**) was found to be more successful at extracting the phytosterols from the sample than the lower volumes initially used (based upon previously described methods). On the other hand, the added acidic hydrolysis step before extraction did not lead to a significant increase in response, the solvolysis effect of methanol:chloroform hydrolysis makes acidic hydrolysis redundant (**Figure 2**). Drying with **Na_2SO_4** and **washing** were not found to be of significant positive influence on the detected response, for any of the phytosterols.

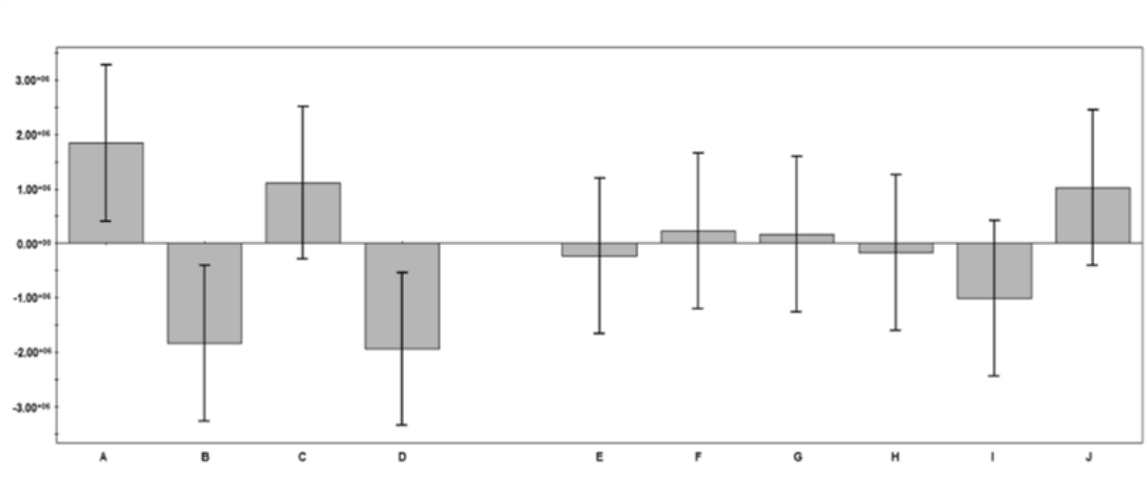

Figure 2. Coefficient plot obtained for stigmasterol after FFD D-Optimal Design (Modde 5.0). A = solvent (chloroform:methanol); B = solvent (hexane); C = extraction volume; D = drying temperature under nitrogen; E = single LLE; F = double LLE; G = drying with Na_2SO_4; H = no drying with Na_2SO_4; I = hydrolysis before extraction; J = no hydrolysis before extraction.

A **Response Surface Modeling** (RSM) approach following a Central Composite Face (CCF) design was used to confirm the influence of Na_2SO_4 for drying and 0.1 M NaCl as a washing solution. Both parameters were found to have a negative influence on the phytosterol response detected. Therefore, these time-consuming steps were not included in the optimized protocol.

An additional RSM approach was used to optimize the two significant quantitative parameters: **drying temperature** and **extraction volume** (illustrated for campesterol, **Figure 3A**). The optimal drying temperature was found to be at **46 °C**, instead of 50°C, higher or lower temperatures led to a decrease in response, for stigmasterol, β-sitosterol, campesterol and cholesterol (**Figure 3B**). Based upon the surface modeling plot, all extraction volumes of more than 12.5 mL seemed to be equally efficient. However, when looking into detail at the volume – response correlation, **16 mL** was found to be the ideal volume (50 mg d.w. sample) (**Figure 3C**).

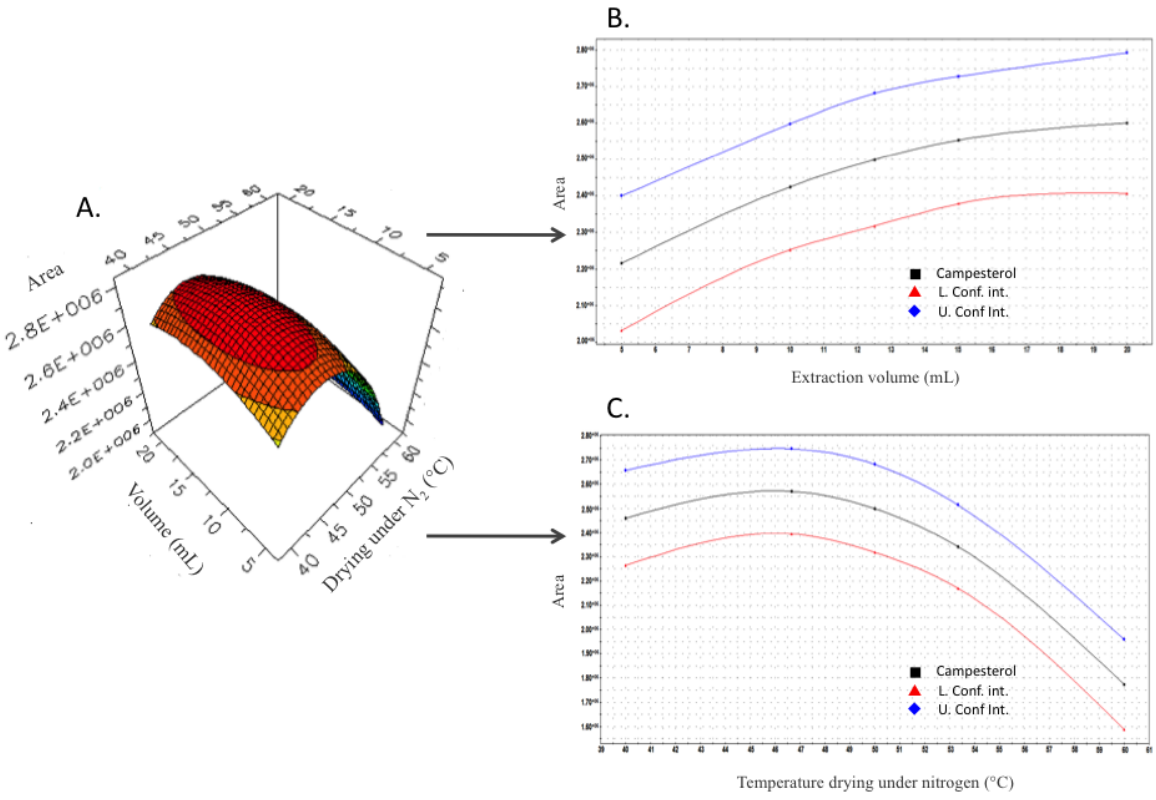

Figure 3. (A) Response surface modelling plot for campesterol. **(B)** Response prediction plot for campesterol, in relation to the applied drying temperature under nitrogen (°C). **(C)** Response prediction plot for campesterol in relation to the extraction volume used (mL) (Modde 5.0, Conf. level = 95%).

Volumes above 16 mL did show slightly higher responses (**Figure 3. B**, p. 87), but the extraction volume was limited to 16 mL (2:1 v/v chloroform:methanol) to limit the use of chloroform and thus the release of chloroform into the environment, as biodegradation in (surface) water and soil is slow. WHO assessed the potential effects of indirect exposure in the general environment on human health as well as environmental effects (International Chemical Assessment Document 58 on chloroform, 2014) and found that chloroform is metabolized rapidly in the liver by the cytochrome P-450 enzymes, but with the formation of phosgene (by oxidation) and by reduction to the dichloromethyl free radical. Both chloroform and/or these metabolites can cause depression of the central nervous system (CNS), hepatotoxicity, nephrotoxicity and cancer.

For **liquid samples**, the protocol was left unchanged to allow the parallel extraction of liquid and solid samples. The optimal sample volume for liquids was determined using a small full factorial design. Full factorial design was preferred over FFD as optimizing just one parameter already significantly limits the number of trials needed. Different sample volumes (2.5-25 mL) were tested (in triplicate) and 5 mL was found to be the optimal sample volume, both in relative response per mL and signal-to-noise.

4.2 DATA ANALYSIS AND QUALITY ASSURANCE OF THE ANALYTICAL METHOD: VALIDATION PROCEDURE ACCORDING TO EU COUNCIL DECISION 2002/657 AND AOAC GUIDELINES

The analytical method was **validated** according to the criteria specified in CD 2002/657/EC for quantitative confirmation, including quantitative performance of the used analysis method, accuracy, specificity, precision, linearity, LOD and LOQ and the AOAC MS criteria.

4.2.1. Specificity

Twenty non-fortified corn and barley samples (n = 20) were analyzed as well as 20 samples of each fortified with the three most relevant phytosterols (β-sitosterol, campesterol and stigmasterol) and the ISTD cholesterol. Standard injections did not show any **interference** between the different components. In all grain samples (corn, oats and barley), a peak was found preceding the stigmasterol peak. Optimisation of UHPLC separation allowed to accurately distinguish this peak from the true stigmasterol peak based on the difference in retention time (relative retention time difference of minimally

10%). The newly developed UHPLC-MS/MS method was found specific for all three phytosterols and the ISTD in the presence of matrix components.

4.2.2. Selectivity

Analytes were identified based upon their relative retention time and a system of **identification points (IPs)**. The most stringent EU Council Decision 2002/657 guidelines were used as the golden standard: they require a minimum of four IPs for confirmatory analysis. Both the precursor ion and product ions can be used as IPs. A LR-MS precursor ion can earn 1 IP and each transition product or product ion can earn 1.5 IP.

For each component included in this UHPLC-MS/MS method, the precursor ion and at least three product ions were evaluated, amply meeting the identification point criterion. Additionally, the method yielded individual relative retention times with coefficients of variation smaller than 1%, well within the stated tolerance level of 2.5% for LC according to the 2002/657/EC and AOAC guidance documents.

4.2.3. Calibration curves

The **linearity** of the chromatographic response was assessed with calibration curves using barley samples fortified to produce nine calibration points. Based upon the minimal EEC in grains, as determined with preliminary standard addition experiments and previously published data, samples were spiked with 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 times the minimal EEC in barley (1 mg for stigmasterol, 2.5 mg for campesterol and 10 mg/100 g for β-sitosterol). Linearity was evaluated through the coefficient of determination (R^2) and lack-of-fit (F-test, SPSS 21, IBM, USA). A linear correlation (R^2 >0.99) was seen between the added concentration of phytosterols and the detected chromatographic response for all compounds, for each nine-point calibration curve (n = 8). Additionally, the regression model equations, resulting from the lack-of-fit test, indicated linearity for all compounds, with F values lower than the F reference value (F-test, $\alpha = 0.05$).

4.2.4. Trueness: recovery

The determination of trueness is assessed using **certified reference material** (CRM), if available. Alternatively, recovery can be determined during validation, if no certified reference material is available, as described under 4.1.2.1 (2002/657/EC). Trueness is then expressed as the recovery in spiked samples. **Recovery** is the percentage of the true concentration of a substance recovered during the analytical procedure.

Table 3. Validation parameters of the quantitative performance of the used extraction and detection method for the three major phytosterols (stigmasterol, campesterol and β-sitosterol).

Analyte	Endogenous concentration (mg/100 g)*	Mean slope calibration curve*	Mean R^{2*}	LOD (mg/100 g)	LOQ* (mg/100 g)	Nominal concentration (mg/100 g)	Recovery (%)	Precision	
								Repeatability RSD (%)	Intra-lab reproduc. RSD (%)
Stigmasterol	2.8 ± 0.4	5.20 ± 0.50	0.995	0.02° 0.17◊	0.07 0.27	1	100 ± 6	5	8
						1.5	102 ± 5	4	6
						2	102 ± 4	5	7
Campesterol	8.1 ± 0.8	26.9 ± 2.7	0.998	0.01° 0.02◊	0.02 0.05	2.5	95 ± 6	8	8
						3.75	99 ± 3	3	2
						5.0	98 ± 2	3	1
β-sitosterol	31 ± 3	26.0 ± 2.3	0.998	0.03° 0.02◊	0.10 0.05	10	101 ± 7	9	9
						15	99 ± 8	9	5
						20	101 ± 4	4	3

* Mean slope and mean R^2 for all calibration curves (n = 8, individual and intralaboratory replicates)

° Based on the S/N ratio, a cut-off of S/N>3 was used to determine the LOD, S/N>10 as the cut-off for the LOQ

◊ Based on S.D. of response and slope: LOD respectively LOQ is then 3.3 or 10*σ/*s* with *s* the slope of the calibration curve of the respective compound and σ the S.D. of response

The same set of samples (n = 6x3) was extracted and analyzed on four different occasions by two different operators. Recovery was calculated for each analyte in each sample. The mean recovery per level is given in **Table 3** (p. 90). Recoveries (**95-102%**) are according to the strictest EU Council Decision 2002/657 guidelines for substances at concentrations ≥10 μg/kg (such as phytosterols), a mean recovery of 80 to 110% should be reached (interval -20 to +10%).

4.2.5. Precision

Precision, the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions and usually expressed in terms of imprecision and computed as standard deviation (%RSD) of the test result (definition 1.22 2002/657/EC). Less precision is determined by a larger **%RSD** or coefficient of variance (CV). One set of samples consisted of 18 aliquots of (n = 6x3) fortified with 1, 1.5 and 2 times the component specific EEC (6 samples per level, *see linearity*).

To evaluate the precision of the developed analytical method, **repeatability and within-laboratory reproducibility** were determined. Repeatability was confirmed by extracting and analyzing the same set of samples (n = 6x3) on three different occasions, by the same operator. The intralaboratory reproducibility was confirmed by repeating the above analysis (n = 6x3) by a different operator on a different occasion under different conditions in the same lab.

Both validation characteristics were evaluated by calculating the **relative standard deviations** (%RSD) (**Table 3**, p. 90). These were within the ranges set by the CD 2002/657/EC based upon the Horwitz equation, one of the acceptability criteria for many of the recently adopted chemical methods of analysis of AOAC International, the European Union, and other European organizations dealing with food analysis [20]. The Horwitz equation states that $\%PRSD = 2^{(1-0.5\log C)}$ where C is the concentration found or added, expressed as a mass fraction. For concentrations exceeding 1 mg/kg ($C = 10^{-6}$), maximal %RSD allowed is 16%. All %RSD calculated were below the postulated value, confirming good reproducibility and precision of the developed method. The low %RSD obtained (<10%) were significantly better compared to comparable methods, where %RSD easily exceeded 10% or they were not determined (**Table 1,** p. 80).

4.2.6. Limit of detection and quantification

The **limit of detection** (LOD) and limit of quantification (LOQ) were determined according to ICH (International Conference on Harmonisation) 1) visual observation, 2) S/N ratio and, 3) based on the S.D. (σ) of response and slope. LOD respectively LOQ is then 3.3 or 10*σ/s with s the slope of the calibration curve of the respective compound and σ the S.D. of response, which can be obtained by standard deviation of "blank response", residual standard deviation of the regression line, standard deviation of the y-intercept of the regression line or Sy/x i.e. standard error of estimate.

Based upon the S/N ratio LODs for the different compounds were between **0.01 and 0.03 mg/100 g d.w.**. For the LOQ, the same calculation was used, but with the cutoff at S/N >10, and the LOQs amounted between **0.02 and 0.10 mg/100 g d.w.**. LODs and LOQs calculated based on the S.D. (σ) of response and slope are presented in **Table 3** (p. 90), together with all other performance characteristics of the validation procedure performed.

As no phytosterol-free, vegetable matrix was available, the mathematically determined LODs could not be confirmed by adding the corresponding concentration of each component to a pooled phytosterol-free sample. The obtained and confirmed LODs were very low compared to other previously published GC-MS/GC-FID or PAD methods. The LOD of 0.01- 0.03 mg/100 g is significantly better than the LOD of 0.5 mg/100 g and 0.16 mg/100 g described by other authors [20,22]. Other authors only calculated the limits of detection (LOD) and quantification (LOQ) in solvent. Nair *et al.* (2006) determined that the LOD and LOQ were 2 and 5 μg/ml, respectively, which is more sensitive than obtained by photo diode array detection (5 and 7 μg/ml), however far less sensitive than the LODs and LOQs in solvent, as determined for our **UHPLC-MS/MS method (0.01 μg/mL)**. Additionally, the LOQs in liquid (beer) samples in our study were lower than 0.05 μg/mL.

In conclusion, the validation of this method following both Council Decision 2002/657/EC and AOAC guidelines exceeds the less extensive quality assurance procedures used by other authors (**Table 1**, p. 80). Additionally, the (relative) retention times of the different compounds are very stable over time (<2% variation). Due to the structural similarity of phytosterols, long HPLC separations (up to 80 min) are often required to separate these compounds [23]. However, the UHPLC and selectivity of

tandem mass spectrometric (MS-MS) detection can facilitate quantitative analysis of overlapping peaks and thereby allow **shorter chromatographic runs** (10 min) [24].

4.3 QUANTITATIVE ANALYSIS OF DIFFERENT FEED AND FOOD SAMPLES

The applicability of this newly optimised method was confirmed by analysing various grains and other solid **feed and food samples** (**Table 4**, p. 94) and **liquid samples**, such as fruit concentrate-based juices, a soft drink and different beer samples (**Table 5**, p. 95). To ensure exact and correct quantification all feed samples were rigorously **homogenized** and identical subsamples were taken. If possible, obtained phytosterol concentrations were compared to available GC-MS and GC-FID results. Samples were extracted on two different occasions and on each occasion a nine-point calibration curve with three additional blank samples was extracted (n =2x12 samples) for quantification purposes. The newly developed extraction and detection method was found to be **applicable** on different complex feed and food matrices, allowing to quantify phytosterols present in different matrices, including matrices that have been evaluated in previous studies, but also not yet investigated matrices [25].

Grains have been a matter of study for the past decade, as they are generally regarded as good sources of phytosterols. Normen *et al.* (2002) reported 28.1, 37.0, 22.5, and 68.3 mg of total phytosterols per 100 g of wheat flour, corn flour, rice flour, and rye flour [26]. Ryan *et al.* (2007) extracted finely ground grains with hexane/isopropanol (3:2, v/v) and found total phytosterols in corn, barley, millet, rye, and buckwheat to be 43.6, 50.4, 57.8, 75.9, and 106.5 mg per 100 g of the grains, respectively [18].

Importantly, when different **cultivars and harvest years** were being compared total phytosterols (in rye) were in the range of 76.1–100.7 mg per 100 g flour [27], compared to 75.9 mg (Ryan *et al.*, 2007) and 68.3 mg per 100 g flour (Normén *et al.*, 2002), highlighting the influence of the harvest year, cultivar and analytical method used on the detected concentrations of phytosterols in grains. These differences were confirmed by Piironen *et al.* (2002), who used GC with a mass spectrometer (MS) for identification, when two cultivars of rye, wheat, barley, and oats grown in the same year were compared. The **highest** plant sterol content was observed in **rye** (mean content 95.5 mg/100 g), whereas the total sterol contents (mg/100 g) of wheat, barley and oats were 69.0, 76.1, and 44.7, respectively. In addition, the comparison of 10 rye cultivars and breeding lines had total sterol contents of 70.7–85.6 mg/100 g [21]. These results are **in**

line with the results obtained in the current study, using the newly developed and validated extraction and UHPLC-MS/MS detection method for barley as well as for oats and corn (**Table 4**).

Table 4. Concentrations of β-sitosterol, stigmasterol and campesterol detected in different grains (oats, barley, corn and malt), feed (grass, hay, straw and yellow peas) and food samples (apple, tomato and hop), determined using the newly optimised and validated extraction and UHPLC-MS/MS method.

Matrix	Compound	Endogenous concentration (mg/100 g d.w.)	Endogenous concentration (mg/100 g f.w.)	R^{2*}
Barley 96% d.w. (*Horleum vulgare*)	Stigmasterol	2.8 ± 0.4	2.7 ± 0.4	0.995
	Campesterol	8.1 ± 0.8	7.8 ± 0.8	0.998
	β-sitosterol	31 ± 3	30 ± 3	0.998
Barley malt 96% d.w. (*Horleum vulgare*)	Stigmasterol	2.2 ± 0.2	2.1 ± 0.2	0.990
	Campesterol	8.9 ± 0.4	8.5 ± 0.4	0.993
	β-sitosterol	39 ± 2	37 ± 2	0.997
Corn 94% d.w. (*Zea mays*)	Stigmasterol	4.0 ± 0.5	3.8 ± 0.4	0.993
	Campesterol	18 ± 1	17 ± 1	0.995
	β-sitosterol	60 ± 3	56 ± 3	0.991
Oats 96% d.w. (*Avena sativa*)	Stigmasterol	0.6 ± 0.1	0.6 ± 0.1	0.999
	Campesterol	3.0 ± 0.1	2.9 ± 0.1	0.999
	B-sitosterol	20 ± 2	19 ± 2	0.999
Grass 26% d.w. (*Lolium perenne*)	Stigmasterol	1.6 ± 0.1	0.4 ± 0.0	0.993
	Campesterol	16 ± 1	4.2 ± 0.3	0.991
	β-sitosterol	113 ± 8	29 ± 2	0.967
Straw 90% d.w. *(Triticum)*	Stigmasterol	17 ± 1	15 ± 1	0.990
	Campesterol	14 ± 1	13 ± 1	0.992
	β-sitosterol	48 ± 4	44 ± 4	0.994
Yellow peas 87% d.w. (*pisum sativum L.*)	Stigmasterol	4.7 ± 0.3	4.1 ± 0.3	0.994
	Campesterol	10 ± 0	8.7 ± 0.3	0.996
	β-sitosterol	67 ± 3	58 ± 3	0.992
Hay 92% d.w. (*mixed*)	Stigmasterol	17 ± 1	16 ± 1	0.987
	Campesterol	33 ± 2	31 ± 2	0.983
	β-sitosterol	141 ± 9	130 ± 8	0.990
Hop 93% d.w. (*Humulus lupulus*)	Stigmasterol	3.2 ± 0.2	3.0 ± 0.2	0.996
	Campesterol	9.8 ± 0.3	9.1 ± 0.3	0.997
	β-sitosterol	127 ± 8	118 ± 7	0.999
Red tomato 6% d.w. (flesh) *(Solanum lycopersicum)*	Stigmasterol	5.4 ± 0.4	0.3 ± 0.0	0.997
	Campesterol	1.5 ± 0.1	0.1 ± 0.0	0.993
	β-sitosterol	4.3 ± 0.5	0.3 ± 0.0	0.990
Apple 17% d.w. (flesh) *(Malus domestica 'Jonagold')*	Stigmasterol	0.3 ± 0.0	0.05 ± 0.01	0.999
	Campesterol	1.5 ± 0.2	0.26 ± 0.04	0.999
	β-sitosterol	65 ± 5	11 ± 1	0.999

* Mean R^2 of two calibration curves extracted at two different occasions

Table 5. Concentration of β-sitosterol, stigmasterol and campesterol detected in concentrate-based drinks and beers, using the newly optimised and validated extraction and UHPLC-MS/MS method.

Matrix	Compound	Endogenous concentration (µg/100 mL)	R^{2*}
Orange juice from concentrate	Stigmasterol	20 ± 2	0.995
	Campesterol	71 ± 5	0.994
	β-sitosterol	421 ± 38	0.990
Apple juice from concentrate	Stigmasterol	3 ± 0	0.998
	Campesterol	27 ± 3	0.994
	β-sitosterol	207 ± 10	0.994
Soft drink 6% orange concentrate	Stigmasterol	19 ± 1	0.992
	Campesterol	73 ± 3	0.990
	β-sitosterol	479 ± 34	0.997
Lager beer 5.2% vol	Stigmasterol	3 ± 1	0.998
	Campesterol	24 ± 5	0.993
	β-sitosterol	200 ± 38	0.992
Old brown beer 6% vol	Stigmasterol	3 ± 0	0.991
	Campesterol	26 ± 3	0.996
	β-sitosterol	182 ± 39	0.992
Kriek lambic beer 6% vol 400 g/L wild cherries	Stigmasterol	3 ± 0	0.990
	Campesterol	25 ± 2	0.991
	β-sitosterol	179 ± 26	0.993
Ale 8% vol Double hop	Stigmasterol	4 ± 0	0.996
	Campesterol	30 ± 3	0.998
	β-sitosterol	255 ± 20	0.999

* Mean R^2 of two calibration curves extracted at two different occasions

Dry peas (*Pisum sativum L.*) (**Table 4**, p. 94) have the largest production volume of all special crops in Canada and the US. Thus far, claims have been made regarding the cholesterol lowering properties of these dry peas, yet limited data was available regarding the exact phytosterol content. According to EC guidelines seedlings of peas contain 0.1 – 0.2% phytosterols [28]. The yellow peas analysed in this study showed to contain respectively 4.7 ± 0.3 mg stigmasterol, 10 ± 0 mg campesterol and 67 ± 3 mg β-sitosterol per 100 g f.w., proving that yellow peas are indeed a good source of phytosterols, containing comparable levels of phytosterols as grains.

Apples (*Malus domestica* "Jonagold") were found to contain stigmasterol (0.05 ± 0.01 mg/100 g f.w.), campesterol (0.3 ± 0.0 mg/100 g f.w.) and β-sitosterol (11.0 ± 0.8 mg/100 g f.w.). These values are slightly lower than the concentrations measured by Piironen *et al.* (2002). They detected but were not able to quantify stigmasterol and found 0.9 ± 0.0

mg campesterol and 15.7 ± 0.4 mg β-sitosterol /100 g f.w. This can be due to the difference between "Jonagold" and "BORKH" apple species and the fact that the values obtained for *Malus domestica* "Jonagold" only included the eatable flesh of the fruit and not the peel and seeds. Apple seeds and apple seed oil are frequently sold as phytosterol rich supplements. Other sources refer to **12 mg total phytosterols/100 g f.w.** as the reference value for apples [16], and the latter is very close to the sum of the concentrations found for apples in this study, again confirming the applicability of the newly developed extraction and detection method.

Previous research by Muller *et al.* (2007) and Rapota and Tyrsin (2015) indicated that sitosterol from **beer** (**Table 5**, p. 95) and **hop** (**Table 4**, p. 94) could also potentially compete with cholesterol for protein binding and uptake. However, Rapota and Tyrsin were only able to qualitatively determine the presence of phytosterols in malt and hop, no quantitative data were reported, yet included in the future perspectives [29]. Muller *et al.* (2007) on the other hand was able to proof the presence of β-sitosterol in four beers, but the concentrations found were below or very close to the LOD of the in this study applied extraction and detection method (5 ng/g freeze-dried beer). In Weiss beer they found 7 ng β-sitosterol/g freeze dried beer [30].

With the new UHPLC-MS/MS method we were able to quantify β-sitosterol, campesterol and even stigmasterol in all beers tested so far (**Figure 4**, p. 97). Moreover, we were also able to accurately measure phytosterols in hop, barley and barley malt.

Other beverages such as **juices** (orange and apple juice) and an orange concentrate-based soft drink were also put to the test. Orange juice and an orange based soft drink were found to contain the highest amounts of phytosterols, compared to the apple juice and beers, which contained similar concentrations of phytosterols (**Figure 5**, p. 98). The highest concentration of phytosterols in beers was measured in the artisanal Ale (Bijloke, Ghent, Belgium), probably due to the fact that the original extract of this beer (17.3 °P), in most cases a good indicator of the **grain bill**, is significantly higher than the original extract of the other beers (13.4 ± 1.6 °P).

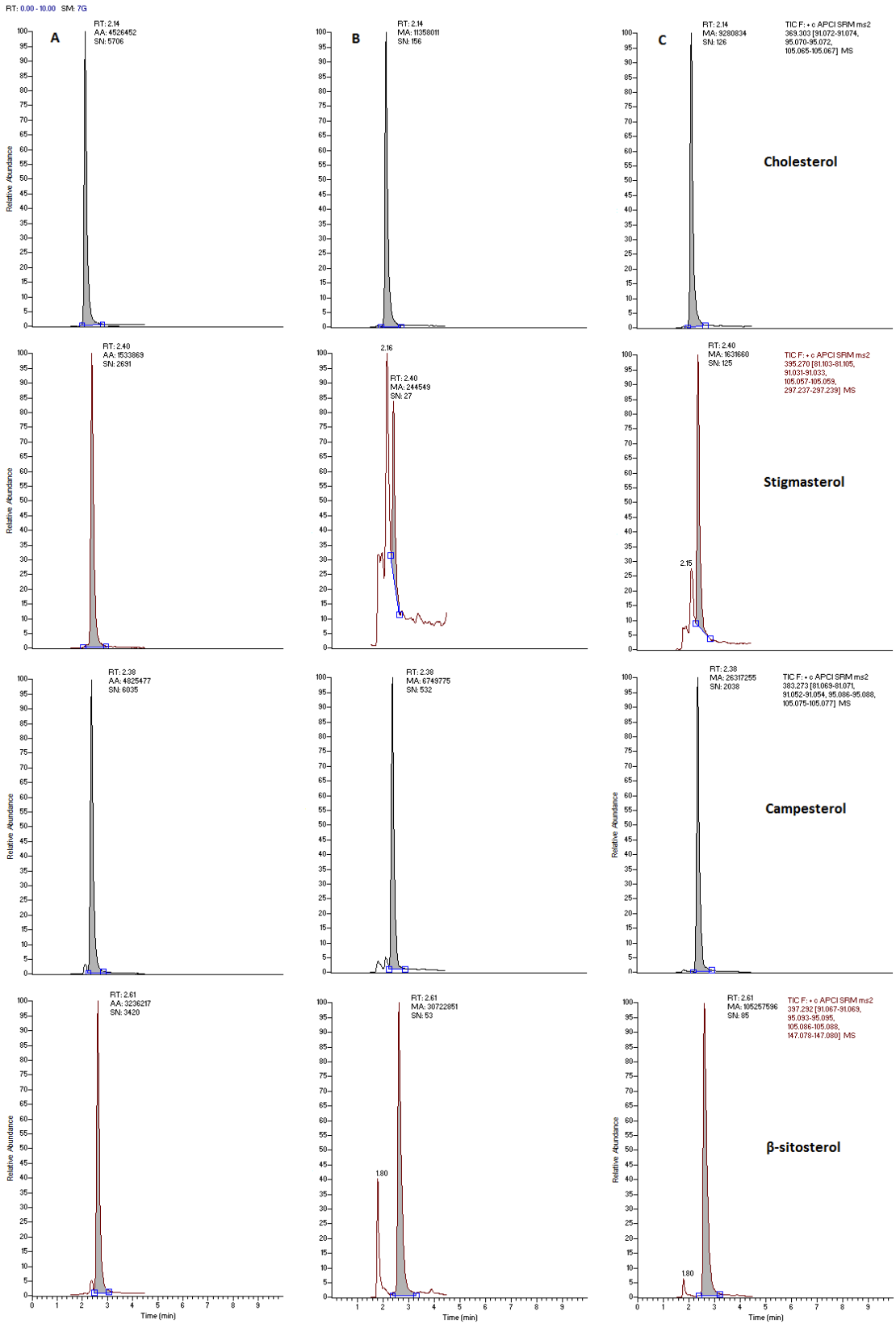

Figure 4. Comparison of the chromatograms obtained using UHPLC–MS/MS. (A) Standard injection of cholesterol, stigmasterol, campesterol and β-sitosterol, **(B)** Blank lager beer sample with added cholesterol (ISTD, 0.2 µg/mL) **(C)** Lager beer sample fortified with stigmasterol (0.18 µg/mL), campesterol (0.9 µg/mL) and β-sitosterol (6 µg/mL).

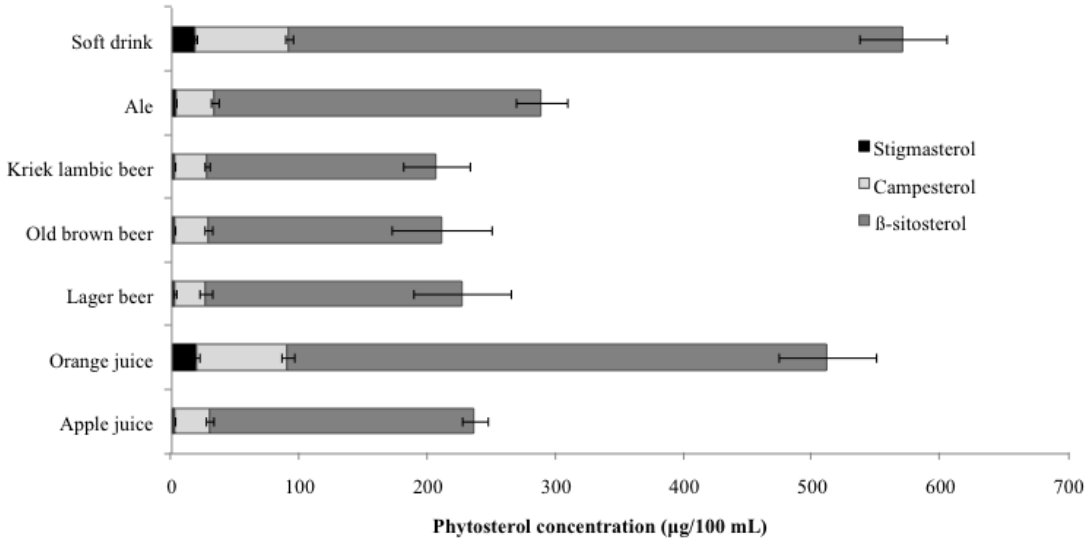

Figure 5. Concentrations of β-sitosterol, stigmasterol and campesterol detected in fruit concentrate-based juices and beers, determined using the new detection method.

5. CONCLUSION

In this study, A FFD statistical design was successfully applied to optimize phytosterol extraction from food and feed samples. This resulted in the discrimination of seven influential factors for which the most optimal conditions were perfected. Compared to previous methods, the optimized extraction protocol is generic, less labor intensive and no derivatisation with e.g. tri-methylsilylether is needed. Moreover, a new UHPLC-MS/MS method, allowing to adequately separate the main phytosterols in a 10-min run time, was developed. The combined extraction and UHPLC-MS/MS detection method was fully validated according to EC/2002/657 and AOAC MS guidelines and complied to all evaluated performance characteristics. The individual recoveries in fortified samples ranged between 95 and 102%. Good results for repeatability and intra-laboratory reproducibility (RSD%) were observed (<10%). Excellent linearity was proven based on determination coefficient (R^2 >0.99) and lack-of-fit test (F test, $\alpha = 0.05$). The calculated LOD and LOQ were very low, 0.01 to 0.03 and 0.02 to 0.10 mg/100g, respectively, compared to previously published methods. Additionally, it was shown our new method allows to quantify the main phytosterols in different grains and in other, both liquid and solid, feed and food samples. Also in some novel matrices, which had not been analysed for phytosterols before, such as hop, yellow peas, grass, hay, straw and different beers and concentrate-based fruit juices, all three phytosterols could be quantified propitiously, even in the latter low-in-phytosterol matrices.

5.1 HIGHLIGHTS

- Optimisation of phytosterol extraction using a Fractional Factorial Design
- Full-fledged validation of phytosterol extraction and detection
- UHPLC-MS/MS as a valid alternative for less sensitive GC methods
- Confirmation of previously determined phytosterol levels in grains
- Phytosterol quantification in yet unexplored matrices such as roughage

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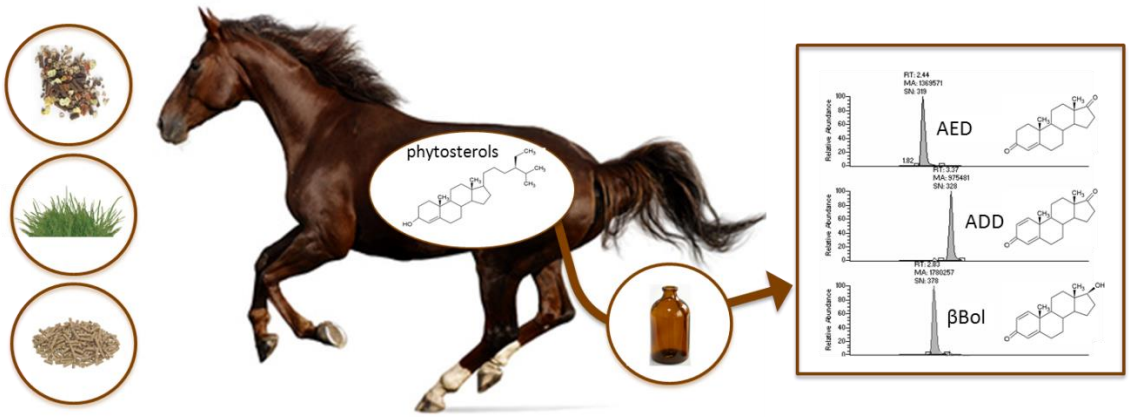
CHAPTER IV

In vitro simulation of the equine hindgut as a tool to study the influence of phytosterol consumption on the excretion of anabolic-androgenic steroids



Adapted from:

"*In vitro* digestion simulation of the equine hindgut as a tool to study the influence of phytosterol consumption on the excretion of anabolic-androgenic steroids in horses"

By Anneleen Decloedt, Ludovic Bailly-Chouriberry, Julie Vanden Bussche, Patrice Garcia, Marie-Agnes Popot, Yves Bonnaire and Lynn Vanhaecke (2015), *Journal of Steroid Biochemistry and Molecular Biology*, 151 p. 180-192 http://dx.doi.org/10.1016/j.jsbmb.2015.06.001

CHAPTER IV

1. ABSTRACT

Traditionally, most androgenic steroids other than testosterone, and testosterone metabolites and precursors, are considered to be synthetic, anabolic steroids. Nevertheless, in stallions, it has been shown that βBol can originate from naturally present testosterone. Other precursors, including phytosterols from feed, have been put forward to explain the prevalence of low levels of steroids (including βBol and ADD) in urine of mares and geldings. However, the possible biotransformation and identification of the precursors has thus far not been investigated in horses. To study the possible endogenous digestive transformation, *in vitro* simulations of the horse hindgut were set up, using fecal inocula obtained from eight different horses. The functionality of the *in vitro* model was confirmed by monitoring the formation of short-chain fatty acids and the consumption of amino acids and carbohydrates throughout the digestion process. *In vitro* digestion samples were analyzed with a validated UHPLC–MS/MS method. The addition of βBol gave rise to the formation of ADD (androsta-1,4-diene-3,17-dione) or αT. Upon addition of ADD to the *in vitro* digestions, the transformation of ADD to βBol was observed and this for all eight horses' inocula, in line with previously obtained *in vivo* results, again confirming the functionality of the *in vitro* model. The transformation ratio proved to be inoculum and thus horse dependent. The addition of pure phytosterols (>50% β-sitosterol) or phytosterol-rich herbal supplements on the other hand, did not induce the detection of βBol, only low concentrations of AED, a testosterone precursor, could be found (0.1 ng/mL). As such, the digestive transformation of ADD could be linked to the detection of βBol, and the consumption of phytosterols to low concentrations of AED, but there is no direct link between phytosterols and βBol.

Keywords

In vitro digestion - Hindgut - Equine

Anabolic steroids - Endogenous – Phytosterols

2. INTRODUCTION

Uncovering doping abuse is a hot topic in today's high-level horse sport industry, including Olympic disciplines and racing, to prevent unfair advantages and to prevent horses from further damaging themselves by masking pain, or competing above their personal limits. Therefore, the presence of hundreds of **forbidden substances** such as narcotics, psychoactive drugs, corticosteroids and anabolic steroids is being controlled. Of the steroids the natural androgenic steroid testosterone is best known to the public. Closely related to testosterone (βT) in terms of chemical structure are, epitestosterone (αT), AED (androst-4-ene-3,17-dione), ADD (androsta-1,4-diene-3,17-dione) and βBol (androsta-1,4-diene-3-one-17β-ol or 1,2-dehydrotestosterone) (**Figure 1**).

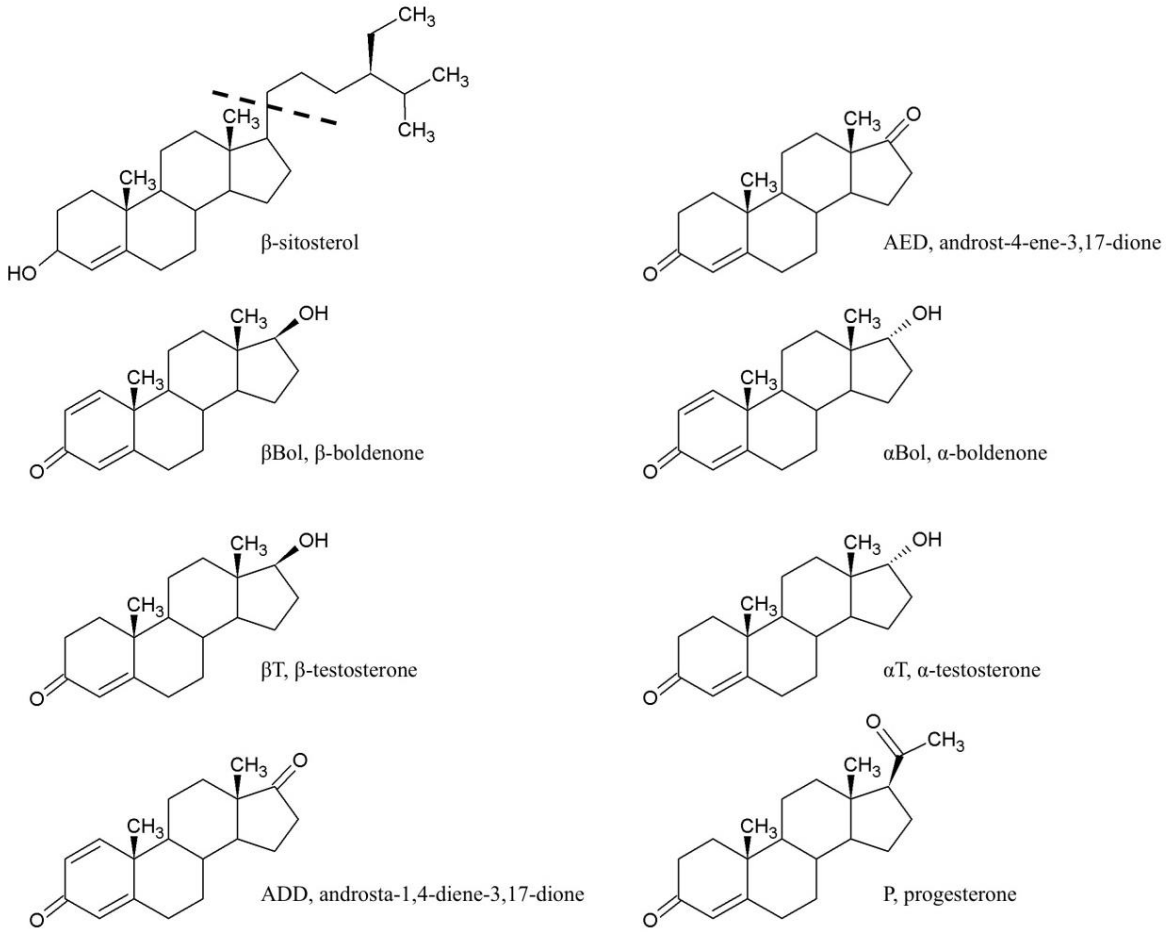


Figure 1. Illustration of the closely related chemical structure of phytosterols and anabolic steroids. For the phytosterols, β-sitosterol, the most abundant phytosterol, is shown. The suggested microbial side chain cleavage is indicated with a dashed line (- - -). Campesterol and stigmasterol differ from β-sitosterol in the side chain double bond at C22 and the substituents at C24.

For a very long time **boldenone** was considered to be a synthetic hormone and zero-tolerance was maintained. As the number of boldenone-positive urine samples was increasing, partly thanks to the constantly improving analytical capabilities (limit of detection) of the European laboratories as a function of time. The question arose whether this was also due to illegal treatment of animals or if boldenone could be of natural origin [1]. As mentioned in chapter III, boldenone could be formed from, the chemically closely related, phytosterols naturally present in plant material (feed). In this respect, the substitution in animal feed of animal fat by fat from vegetable origin, due to the crises from bovine spongiform encephalopathy and polychlorobiphenyl, might be related.

Indeed, boldenone has been shown to be naturally present in bovine urine and feces [1–3] whereas Pompa *et al.* (2006) described *de novo* synthesis of boldenone in cattle feces [4]. Moreover, βBol has been detected in urine from entire male horses [5,6]. According to these findings the IFHA (International Federation of Horseracing Authorities) and FEI (Fédération Equestre Internationale) set a **threshold** for boldenone at **15 ng** free and conjugated boldenone per milliliter in urine from male horses (other than geldings) [7,8]. The presence of boldenone in urine from **mares or geldings** is however still illegal.

Nevertheless, our previous research has demonstrated that AED and ADD, which are next to testosterone the main suspected precursors of boldenone [9], can be present in urine of both untreated geldings and mares at low parts-per-billion levels [10]. In the urine of one AED producing gelding even a low concentration (1.0 ng/mL) of boldenone was found (See also chapter II). AED and ADD may originate from the microbial side chain cleavage of phytosterols (e.g. β-sitosterol) (**Figure 1**, p. 106).

Phytosterols are omnipresent in plants, regulating the fluidity of cell membranes and featuring in cellular differentiation and proliferation, just like cholesterol in animals [11,12]. The three major phytosterols are β-sitosterol, stigmasterol and campesterol (**Figure 1**, p. 106); they differ in the side chain double bond at C22 and the substituents at C24. All three types make **good raw materials** for the production of steroid hormones because of their typical A-ring molecular structure with a 3β-hydroxyl group and a 5,6-double bond. The C19-steroids, which include AED, ADD and testosterone, are the products of complete (microbial) side chain cleavage of phytosterols.

This **conversion** of phytosterols to steroids has been frequently reported in different biological systems, mainly involving a variety of microorganisms [13] such as *Mycobacterium* sp. [14–16], *Arthrobacter* and *Nocardia* sp. [17]. In addition, a number of studies have been devoted to the ability of invertebrate organisms to convert phytosterols into anabolic steroids: maggots of *Lucilia Sericata* [9], *Crustaceae* [18] and zebra fish [19]. However, this biotransformation of phytosterols to steroid hormones has thus far not been demonstrated in horses. For other compounds, it has been proven that the oral uptake of **feed contaminants** can lead to the detection of these contaminants in the horse's urine. Selection of feed materials appears to be of great importance to prevent involuntary positive result to anti-doping tests [20].

To elucidate the endogenous origin of AED or ADD in horses and the possible transformation pathways to forbidden substances such as boldenone, a good experimental set-up was needed to study the horse's digestive metabolism. Horses are hindgut fermenters: the **hindgut**, caecum and colon, comprises roughly two thirds of the volume of the equine digestive tract [21]. As such, horses are especially adapted to grazing continually on marginal forages [22]. Complex plant material is fermented by microbes in the hindgut to short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which provide 60–70% of the daily energy needs of the horse [23].

***In vitro* incubation systems** have been developed to simulate the gastrointestinal tract (GIT) of humans [24] and many different animals [25]. Such *in vitro* digestions were used to monitor digestion and passage rates [26] and transformations of different compounds e.g. phenolic compounds [27]. Generally, using an *in vitro* batch system enhances **reproducibility**; unlike when using an *in vivo* set-up, reaction parameters can be standardized. Though the use of these types of batch cultures has its **limitations**, i.e. absence of gastrointestinal absorption and lack of interaction with the host colonic mucosa, *in vivo* studies were not considered here due to lack of versatility in terms of mechanistic explorative potential as well as time consuming and costly nature [28]. Using *in vitro* digestion simulations allows to avoid the use of living animals, limiting experimental costs and ethical constraints.

In this case, an *in vitro* incubation system allows studying transformation reactions taking place in the hindgut. As **no standardized horse gut simulation protocol** was available [29], the *in vitro* digestive simulation protocol applied during this study was adapted from

previously reported *in vitro* models, simulating the upper and lower gastrointestinal tract of bovines, porcines and humans [30–34]. A recent study however confirmed the valuable use of *in vitro* assays to study the horse hindgut, using an *in vitro* digestion assay to identify lactate-utilizing bacteria that differentially respond to starch induction, in the light of laminitis in horses [23]. The by Biddle *et al.* (2013) *in vitro* obtained results were in line with previous *in vivo* results described by other authors [35] and the by Biddle *et al.* (2013) applied *in vitro* incubation protocol [36] was very similar to the protocol used in the current study.

The specific **advantages** of batch cultures for this study lie with the fact that they allow to focus on the fermentation reactions taking place in the hindgut, and they enable the use of different fecal inocula (horses) in a short time frame, without ethical constraints. The *in vitro* batch incubations performed only simulate the hindgut fermentation processes, as they are thought to be responsible for the endogenous origin of AED and ADD: complex fermentation reactions only take place in the caecum and colon, the enzymatic activity measured in the foregut of horses [37] is fairly low: the α-amylase activity was estimated between 10 and 50 U/g [38] compared to 3500 U/g for other species such as humans and pigs [39].

As such, this study aimed to evaluate the use of *in vitro* digestions as a tool to imitate *in vivo* hindgut fermentation and, by varying the **supplied precursors** (steroids, phytosterols or entire feed) and using inocula from eight different horses, to elucidate the possible endogenous digestion transformation pathways taking place in the horse's hindgut. Samples were analyzed with a new, sensitive UHPLC–MS/MS method, validated according to EC (EU Council Decision 2002/657) and AORC (Association of Official Racing Chemists) guidelines [10].

3. MATERIAL AND METHODS

3.1. LC–MS^2 REAGENTS AND CHEMICALS

α-testosterone (androst-4-ene-17α-ol-3-one, αT, purity ≥99%), β-testosterone (androst-4-ene-17β-ol-3-one, βT, purity ≥99%), methyltestosterone (androst-4-ene-17α-methyl-17β-ol-3-one, MT, purity ≥99%), androstadienedione (androsta-1,4-diene-3,17-dione, ADD, purity ≥99%), proteinase type XXIII from *Aspergillus melleus* (3 enzyme units per

milligram) and β-glucuronidase (*Helix pomatia*, aqueous, >100.000 units per milliliter) were purchased from Sigma–Aldrich (St-Louis, US). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥99%), α-boldenone (androsta-1,4-diene-17α-ol-3-one, αBol, purity ≥99%) and β-boldenone (androsta-1,4-diene-17β-ol-3-one, βBol, purity ≥99%) were obtained from Steraloids (Newport, US). Progesterone (purity ≥99%) was obtained from Alpha Pharma (Omega Pharma, Zwevegem, Belgium). Methanol Optima, was bought at Fisher Scientific, UK Limited (Leicestershire, UK). Diethyl ether, ethyl acetate, sodium hydroxide, methanol (analytical grade), H_2SO_4, KH_2PO_4 and formic acid (98–100%, analytic grade) were purchased from VWR (Merck, Darmstadt, Germany). Solvolysis solvent consisted of 900 mL ethyl acetate, 95 mL methanol and 5 mL H_2SO_4 per liter. The HF Bond Elut-C18 cartridges (6 mL, 500 mg) were obtained from Agilent Technologies (Diegem, Belgium). HPLC grade, ultrapure water was acquired from an in-house water purification system (Arium 611UV, Sartorius Stedium Biotech, VWR, Haasrode, Belgium).

Stock solutions of each steroid were made in methanol Optima at 1000 and 200 ng/mL. Dilutions up to 1 pg/mL were made in methanol Optima. All solutions were kept at 4 °C.

3.2 *IN VITRO* DIGESTION: BATCH INCUBATIONS

3.2.1. Buffers and broths

Digestion and fecal inoculum buffers were prepared in ultrapure water and **autoclaved** (121 °C, 15 min, 1 atm). All further handlings of the *in vitro* digestion were done in a laminar flow cabinet. Fecal inoculum buffer contained K_2HPO_4 (8.8 g/L), KH_2PO_4 (6.8 g/L) (Merck, Darmstadt, Germany), and sodium thioglycolate (1.0 g/L) (Sigma–Aldrich, Steinheim, Germany). BHI (Brain Heart Infusion) broth (Oxoid, Hampshire, England) ready to use powder was dissolved in ultrapure water (37 g/L). L-cysteine (0.5 g/L) (SAFCSupply Solutions, St. Louis, MO) was added to improve anaerobicity [40]. CPB (Cysteine Peptone Bouillon) consists of a mixture of 5 g yeast extract (AppliChem, Darmstadt, Germany), 1 g peptone (Oxoid, Hampshire, England), 8.5 g NaCl (Merck, Darm-stadt, Germany), 0.5 g L-cysteine and 10 mL haemine solution (Sigma–Aldrich, Steinheim, Germany) in 1 L ultrapure water (**Table 1**, p. 111) [41].

Table 1. Formula of CPB compared to BHI medium.

Formula	CPB	BHI
Yeast extract	5.0 g	/
Beef Heart Infusion	/	5.0 g
Brain infusion solids	/	12.5 g
Peptone	1.0 g	10.0 g
NaCl	8.5 g	5.0 g
Na_2HPO_4		2.5 g
L-cysteine	0.5 g	0.5 g
haemine	2 mg	/
NAD	4 mg	/
UP H_2O	1 L	1 L

Haemine solution was prepared by adding 2 mL concentrated NH_4OH solution (25%, Merck, Darmstadt, Germany) and 0.1 g haemine to 500 mL sterile ultrapure water and sterilized afterwards (121 °C, 15 min, 1 atm). After filtration over a 0.45 mm filter (Merck, Darmstadt, Germany), pH was adjusted to 7.0 and sterilized again. Under sterile conditions 0.25 mL of nicotinamide adenine dinucleotide (Sigma–Aldrich, Steinheim, Germany) solu-tion in water (2 mg/mL) was added. The total solution was mixed and stored at 4 °C until use. BHI agar plates were prepared with ultra-pure water and 37 g/L BHI, 12 g/L agar and 0.5 g L cysteine (Oxoid, Hampshire, England).

3.2.2. Fecal inoculum

To simulate the hindgut fermentation reactions in the *in vitro* batch incubation, a fecal inoculum is required. **Fresh fecal matter** was collected from adult mares and geldings (8–18 years old) during spontaneous discharge. As spontaneously voided samples were collected, and the horses were not given any medication or treatment, according to the latest Belgian and European animal welfare rules (RD 29th May 2013, published on the 10th of July), they are not considered to be experimental animals. As such, the authors state to have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations. In addition, informed consent has been obtained from the owners, horses were owned by the author herself or horses belonging to the faculty (Faculty of Veterinary Medicine, Merelbeke, Belgium); their medical history was known and well documented. Horses were guaranteed to be **untreated with AAS** or other treatments that are known to interfere with the excretion of AAS.

All **horses** were Belgian Warmblood Horses (B.W.P.) or Royal Dutch Sport Horses (K.W.P.N) and on a standard but non-controlled diet of concentrate (2–4 kg/day), hay (8–

10 kg/day) and straw as their bedding material, combined with pasture access for several hours a day. The fresh fecal matter was kept anaerobic (in a firmly closed bag) to protect it from oxygen exposure. Samples were at 37 °C during transport, before further processing in the lab. **Fecal slurry** was made by adding 1/5 fecal phosphate (v/v) buffered saline and homogenization in a stomacher for 10 min. The suspension was transferred into 50 mL falcon tubes and centrifuged at 500 x *g* for 5 min, removing large fibers. To the supernatant glycerol (99.5%) (Analar Normapur, Fontenay-sous-Bois, France) was added at a 20% (v/v) ratio, which was gently mixed under atmospheric conditions, before storage at -80 °C [42].

3.2.3. Digestion protocol and sampling

Dark, autoclaved 125 mL **penicillin flasks** were used to avoid light and UV influences. To 45 mL medium (CPB or BHI broth), 5 mL of the fecal inoculum was added. The following supplements (see below for complete list) were added depending on the experimental set-up: steroids, phytosterols or phytosterol-rich herbal supplements. When adding phytosterols (50% β-sitosterol), 0.2% (v/v) Tween 80 was added to the *in vitro* digestion to increase solubility of the hydrophobic phytosterols [43]. The flasks were capped and **anaerobic conditions** were established using a flush system for 1 h, alternating every 2 min between N_2 (1 bar) and vacuum suction. The anaerobic conditions in flasks were confirmed using resazurin-saturated test strips. The flasks were then incubated for 72 h (37 ± 0.5 °C, 150 rpm) (Innova 42 series, incubator shaker series, New Brunswick Scientific), conform the maximal expected *in vivo* retention time in the hindgut (48 to 72 h) [44].

Samples (3 mL) were taken after 0, (12), 24, 48 and 72 h of incubation. Sampling was done as secure and standardized as possible using syringes, causing as little disturbance as possible to the bacterial environment. Incubations were gently stirred to homogenize before sampling. Samples were stored at -20 °C prior to extraction.

3.2.4. Validation of the in vitro *digestion protocol*

To confirm the **functionality of the *in vitro* model** confirmatory experiments have been performed assessing the microbiological growth and bioactivity of the hindgut flora in the *in vitro* digestion simulation, validating the use of *in vitro* digestion batch incubations as a tool to study equine hindgut fermentations. Microorganism-associated activities were monitored as described by Kiebooms *et al.* (2012), Biddle *et al.* (2013) and Molly *et al.*

(1994). Molly *et al.* (1994) used the same confirmatory methods to validate the Simulator of the Human Intestinal Microbial Ecosystems (SHIME) *in vitro* reactor [23,30,45].

Short-chain fatty acids (SCFA) (acetic acid, phenylacetic acid, propionic acid, 3-phenylpropionic acid, 3,4-dihydrophenylpropionic acid, butyric acid, isobutyric acid, 3-hydroxybutryric acid, valeric acid, isovaleric acid and 4-methylvaleric acid) were monitored in samples taken during the *in vitro* batch incubations (0 and 72 h) and analysed using an ultra-high performance liquid chromatograph (UHPLC, 2.1 x 150 mm Acquity UPLC HSS T3 1.8 µm) coupled to an **Orbitrap high-resolution mass spectrometer** (Thermo Fisher Scientific, Exactive^{TM}), according to Vanden Bussche *et al.*, 2014 [46]. The production of SCFAs throughout the *in vitro* digestion simulation was used as a quality control parameter by Biddle *et al.*, (2013), for an equine *in vitro* digestion simulation, and Molly *et al.* (1994), to validate the SHIME reactor system [23,45].

Amino acids (L-lysine, L-threonine, L-alanine, L-serine and L-phenylalanine) were monitored in samples during the *in vitro* batch incubations (0 and 72 h) and analyzed using an ultra-high performance liquid chromatography (UHPLC, 2.1 x150 mm Acquity UPLC HSS T3 1.8 µm) coupled to an Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Exactive^{TM}). Cadaverine, the decarboxylation product of the amino acid lysine, was monitored as well (Vanden Bussche *et al.*, 2014) [46].

Carbohydrate metabolism was also monitored during the *in vitro* batch incubations (0 and 72 h) using an ultra-high performance liquid chromatograph (UHPLC, 2.1 x 150 mm Acquity UPLC HSS T3 1.8 µm) coupled to an Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Exactive^{TM}). Glucose levels were confirmed quantitatively by analyzing the *in vitro* digestion samples with a Dionex Ion Chromatograph (ICS-3000, Thermo Scientific^{TM} Dionex^{TM}) method developed by Vanbeneden *et al.* (2006) [47].

To evaluate the **rate of bacterial fermentation**, **pH** of the digestive samples was monitored over time (Seven Easy pH, Mettler Toledo, Schwerzenbach, Switzerland). Absorbance or optical density measured at a wavelength of 600 nm (**OD600**) was used as a measurement of biomass over time, an indication of cell density (Eppendorf BioPhotometer, Hamburg, Germany). All samples were diluted (2, 4, 8–16 times) as the

relationship between OD and biomass concentration only approximates to linearity at low OD values (<0.5). Recalculated OD values were then plotted against incubation time.

Additionally, **total anaerobic bacteria** were counted after 48 and 72 h of fermentation. One milliliter of *in vitro* digestion sample was serially diluted (10-fold) using a sterile peptone solution (1 g/L peptone, 0.4 g/L agar, 8.5 g/L NaCl, and 0.5 g/L cysteine), and spirally plated (EddyJet, IUL instruments, Barcelona, Spain) onto BHI agar plates (37 g/L BHI, 12 g/L agar and 0.5 g L cysteine) (Oxoid, Hampshire, England). After incubating the plates for 48 h at 37 °C (Led Techno lab equipment, Termaks incubator, Eksel, Belgium), colony-forming units (CFU) were counted and expressed as **log10 CFU/mL** *in vitro* digestion and compared to *in vivo* reference values. BHI agar is an enriched non-selective medium for the isolation and cultivation of most anaerobic bacteria and other fastidious microorganisms. The basic nutritive properties are brain heart infusion from solids as well as meat peptones, with the addition of yeast extract. This medium was supplemented with haemin and vitamin K1 as growth factors for most anaerobic bacteria.

3.2.5. In vitro *digestion supplements*

Steroids that were used as **supplements** to the *in vitro* digestion were of analytical quality (see **3.1**, purity ≥99%), including α-testosterone (αT), β-testosterone (βT), androsta-1,4-diene-3,17-dione (ADD), androst-4-ene-3,17-dione (AED), β-boldenone (βBol) and Progesterone (P). Phytosterols (50% β-sitosterol) were purchased from Sigma–Aldrich (St-Louis, US). Polysorbate 80 (Tween 80) is a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid. By adding 0.2% Tween 80 (v/v) to the medium up to 2 g/L of the hydrophobic substrate β-sitosterol can be solubilized [49].

For horses, the consumed concentration of phytosterols can be calculated based on the **average diet** of a normal size horse (500 kg), consuming 3% of his body weight in dry matter a day (15 kg) [50]. For sport horses this dry matter is mostly (up to 60%) supplied through energy rich grain mixtures, the so-called concentrates (9 kg/day) [51]. Those grains can be very rich in phytosterols. Corn, which is often used in horse feed, contains up to 1.3 g of phytosterols per kg dry weight (d.w.) [52]. Even a simple apple or carrot contains respectively 1.5 and 1.4 g/kg d.w.[53]. Additionally, linseed-, soy- or corn oil (5–10 g phytosterols per kilogram) is frequently added to the diet to prevent impaction colic, to improve the coat condition or to easily add calories to the diet [54]. The addition of extra fat raises the energy density of feeds, which is advantageous for sport horses with

high-energy requirements. As such, a sport horse will easily take in more than 10 g of **phytosterols a day**. As the average hindgut of a horse has 125 L content and the feed stays there for 24–72 h, adding **10 mg** of phytosterols to 50 mL of *in vitro* digestion was used to simulate the intake of a normal sport diet, **100 mg** for the simulation of a phytosterol-enriched diet. Two commercial phytosterol rich supplements were tested as well. Both supplements claim to support digestion, activate the saliva production, improve intestinal motility, and therefore improve the availability of nutrients, and anti-inflammatory effects. The producer advises to dose 1 g/day per horse (500 kg body weight).

3.2.6. Bacterial strains

Mycobacterium sp. DSM 2966 (NRRL B-3683) and ***Mycobacterium*** sp. DSM 2967 (NRRL B-3805) were vacuum dried cultures delivered by the Leibniz Institute DSMZ (German Collection of Microorganism and Cell Cultures). Reactivation was executed in nutrient broth (Oxoid) at 30 °C (7 days), according to DSMZ guidelines for these two strains. This process was repeated three times to remove any stabilizing agents. In a glass vial, 1 mL of the final bacterial suspension was added to 3 mL glycerol, vortexed and stored at -80 °C. Hereof, 100 ml was added per 10 mL broth to inoculate the incubations with *Mycobacterium* sp. DSM 2966 or 2967.

3.3. EXTRACTION OF *IN VITRO* DIGESTION AND URINE SAMPLES

3.3.1. Hydrolysis

For the hydrolysis of *in vitro* digestion samples, 1 mL of phosphate buffer (1 M KH_2PO_4, pH 6.1 ± 0.1) was added to 3 mL of sample. Next, the internal standard methyltestosterone (MT, 5.0 ng/ mL), 50 ml of a ≥450 units/mL **protease solution** and 25 ml **β-glucuronidase** were added. Of each sample the pH was set at 6.1 ± 0.1 (by adding 1 M HCl) and the hydrolysis was executed at 55 °C (1 h). After hydrolysis, 3 mL of ultrapure water was added and large, non-hydrolyzed proteins were removed by agglutinating them at the bottom of the tube through centrifugation (2400 x *g*, 15 min). Finally, the supernatant was filtered over a cotton wool filter before solid phase extraction (SPE).

3.3.2. Solid phase extraction (SPE), liquid–liquid extraction and solvolysis

The SPE cartridges (6 mL, 500 mg C18, Bond Elut, Isolute) were conditioned with 4 mL methanol and 4 mL ultrapure water. The centrifuged and filtered samples were loaded

onto the column and washed with consecutively 7 mL ultrapure water and 7 mL hexane. The cartridges were dried under vacuum (-0.5 bar). Next, the non-conjugated and glucuronide-conjugated fractions were eluted with **diethyl ether** (7 mL) and the sulfate-conjugated fraction with **solvolysis solvent** (7 mL). Solvolysis of the sulfate conjugated fraction is executed at 55 °C (2 h). Both fractions were washed with 1.5 M sodium hydroxide (5 mL) by turning (8 min, 60 rpm) and centrifugation (6 min, 1400 x *g*).

3.3.3. Pooling and reconstitution

The washed fractions were pooled and dried under nitrogen (50 °C, 30 min). Each sample was reconstituted in 100 ml of ultrapure methanol, vortexed and ultrasonicated (3 min). Finally, 100 ml of ultrapure water was added, vortexed and ultrasonicated as well. After centrifugation (12300 x *g*, 10 min) the sample was transferred to an LC–MS vial with insert for UHPLC–MS/MS analysis.

3.4. UHPLC–TRIPLE QUADRUPOLE MS/MS ANALYSIS

The ultra-high performance liquid chromatography (U-HPLC) MS/MS detection was performed according to Decloedt *et al.* [10]. Separation was carried out using an Accela™ High Speed LC (Thermo Fisher Scientific, San Jose, CA, USA) with a Nucleodur™ Sphinx RP column (1.8 µm, 50 x 2.1 mm, Macherey-Nagel). The mobile phase consisted of ultra-pure water containing 0.1% formic acid (26.5 mM) as solvent A and methanol Optima1 with 0.1% formic acid (26.5 mM) as solvent B. All analytes could be accurately separated in a total run time of only 9 min.

Detection was carried out on a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Heated Electro Spray Ionization probe (HESI-II). Injection volumes were 10 mL each and the HESI source was operated in the positive ion mode. It was found that positive ion mode afforded better sensitivity [55]. Data were acquired in the multiple reaction monitoring (MRM) mode. The precursor ions selected and their collision energies are listed in **Table 2** (p. 117). All specified product ions were used for peak integration for quantification purposes. Data were interpreted using Xcalibur 2.1. w/Foundation 1.0.2 Rev. B qualitative and quantitative software (Thermo Electron, San Jose, USA).

The internal standard methyltestosterone (MT, 5 ng/mL) was added to both calibration and unknown *in vitro* digestion samples, to compensate for losses during sample

preparation or variability during the analytical determination. Methyltestosterone is a good internal standard as it is very similar to the calibrated analytes, chemically and in retention time, but chromatographically distinguishable, cheap and not endogenously present. Unknown *in vitro* digestion samples were quantified by fitting the metabolites' area ratio in a calibration curve.

Table 2. SRM specifics for all analytes of interest: precursor ions, product ions, retention time (RT), appropriate S-Lens RF-amplitude, and the corresponding collision energy (CE).

Analyte	Precursor Ion (*m/z*)	Product Ions (*m/z*)	(Relative) Retention Time (min)	S-lens (V)	Collision Energy (eV)
ADD	285.2	77	2.93	54	51
		91	(0.68)		39
		121			22
		147			15
AED	287.2	79	3.77	70	36
		81	(0.87)		37
		97			21
		109			25
αBol	287.2	77	3.90	56	51
		121	(0.90)		26
		135			17
ßBol	287.2	77	3.33	56	51
		121	(0.77)		26
		135			17
αT	289.2	79	4.38	70	40
		97	(1.01)		23
		109			27
		253			16
ßT	289.2	79	3.93	70	39
		97	(0.91)		22
		109			27
		253			15
P	315.2	79	4.93	75	38
		97	(1.14)		23
		109			28
		297			13
MT* *Internal standard	303.2	97	4.33	73	30
		109	(1.00)		28
		285			15

3.5. QUANTIFICATION: DATA ANALYSIS AND QUALITY ASSURANCE OF THE ANALYTICAL METHOD

Quantitative performance of the used analytical method, accuracy, precision, linearity and sensitivity were previously validated in urine for αT, βT, AED, ADD, P and βBol [10] (See also Chapter II). Prior to each analysis, the individual targeted compounds and standard mixtures were injected to check the **selectivity and operational conditions** of the chromatographic devices. The different metabolites were identified based on their

relative retention time, relative to the internal standard. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio of at least 3 was 5 pg on column for all analytes of interest. All specified product ions were used for peak integration for quantification purposes.

Extracts of a blank *in vitro* digestion (45 mL CPB + 5 mL fecal inoculum) fortified with different levels of a standard solution were injected to validate the method for *in vitro* digestion samples. Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard. The validation was performed with 18 aliquots of blank ***in vitro*** **digestion suspension fortified** (6 samples per level) with 1, 2 and 4 ng/mL of each analyte. In parallel, a calibration curve was constructed based upon nine fortification levels (0, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 ng/mL) and this curve was run twice, before and after validation and experimental samples. Two additional calibration curves were extracted on two other occasions, by two other operators, to check **intralaboratory reproducibility**.

The R^2, mean slope and lack of fit to a linear model were calculated (SPSS 21) for all **calibration curves**. Fitting the area ratios of the samples in the nine-point calibration curve allowed quantification of the samples. Three additional blank samples were analyzed as well, to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise ratio was used as the detection limit (LOD) ten times the signal to noise ratio as the quantification limit (LOQ). In line with Decloedt *et al.* (2015), for the quantification of the urine samples an individual quantification method was combined with fitting the metabolites' area ratio in a calibration curve, to avoid the urine composition from biasing the quantification. As such, the standard addition approach was used for urine specific quantification. One aliquot was left blank, while the other was fortified with 5.0 ng/mL of each analyte [10].

4. RESULTS

4.1. QUANTIFICATION: DATA ANALYSIS AND QUALITY ASSURANCE OF THE ANALYTICAL METHOD

The **mean slope** for each component was calculated based upon all calibration curves. Slopes for the different compounds ranged between 0.134 and 0.307 with a small

variation (0.004–0.020). The least squares method was used for fitting the calibration curves and evaluating linearity of the method. For each individual analyte, the obtained correlation coefficients (R^2) of the calibration curves were always higher than 0.99, showing good linearity in the range of 0.25–10 ng/mL. The **lack of fit** (F-test, $\alpha = 0.05$) proved linear for all compounds, with F values lower than the F reference value. Recoveries for all compounds were checked based upon the 18 aliquots of blank *in vitro* digestion that were fortified (6 samples per level) with 1, 2 and 4 ng/mL of each compound (ADD, AED, βBol, αBol, αT, βT and P) (**Table 3**).

Table 3. Validation parameters of the quantitative performance of the used extraction and detection method for the seven steroids of interest, in *in vitro* digestion samples.

Analyte	R^2	LOD (ng/mL)	LOQ (ng/mL)	Nominal level (ng/mL)	Recovery (%)	Repeatability RSD%
αBol	0.992	0.33	1.07	1.0	81.7	13.6
				2.0	95.4	5.8
				4.0	119.3	8.3
ßBol	0.999	0.23	0.76	1.0	95.4	5.5
				2.0	100.5	4.2
				4.0	109.7	12.1
ADD	0.998	0.10	0.33	1.0	96.2	6.3
				2.0	98.8	5.3
				4.0	107.0	11.6
AED	0.992	0.11	0.38	1.0	87.4	5.4
				2.0	94.8	10.9
				4.0	100.7	8.8
αT	0.998	0.22	0.72	1.0	104.9	6.0
				2.0	101.6	2.3
				4.0	107.5	11.7
ßT	0.993	0.14	0.46	1.0	91.1	6.9
				2.0	101.3	3.9
				4.0	114.9	12.2
P	0.991	0.23	0.76	1.0	123.3	8.0
				2.0	106.7	17.7
				4.0	102.0	16.2

As the method was already fully validated in urine, matrix effects can be compared. On average, the UHPLC–MS/MS is **two times more sensitive** in *in vitro* digestion samples than in urine. Other validation parameters (recovery, precision, repeatability) were consistent between urine and *in vitro* digestion matrix (**Table 3** and **table 2**, p. 61).

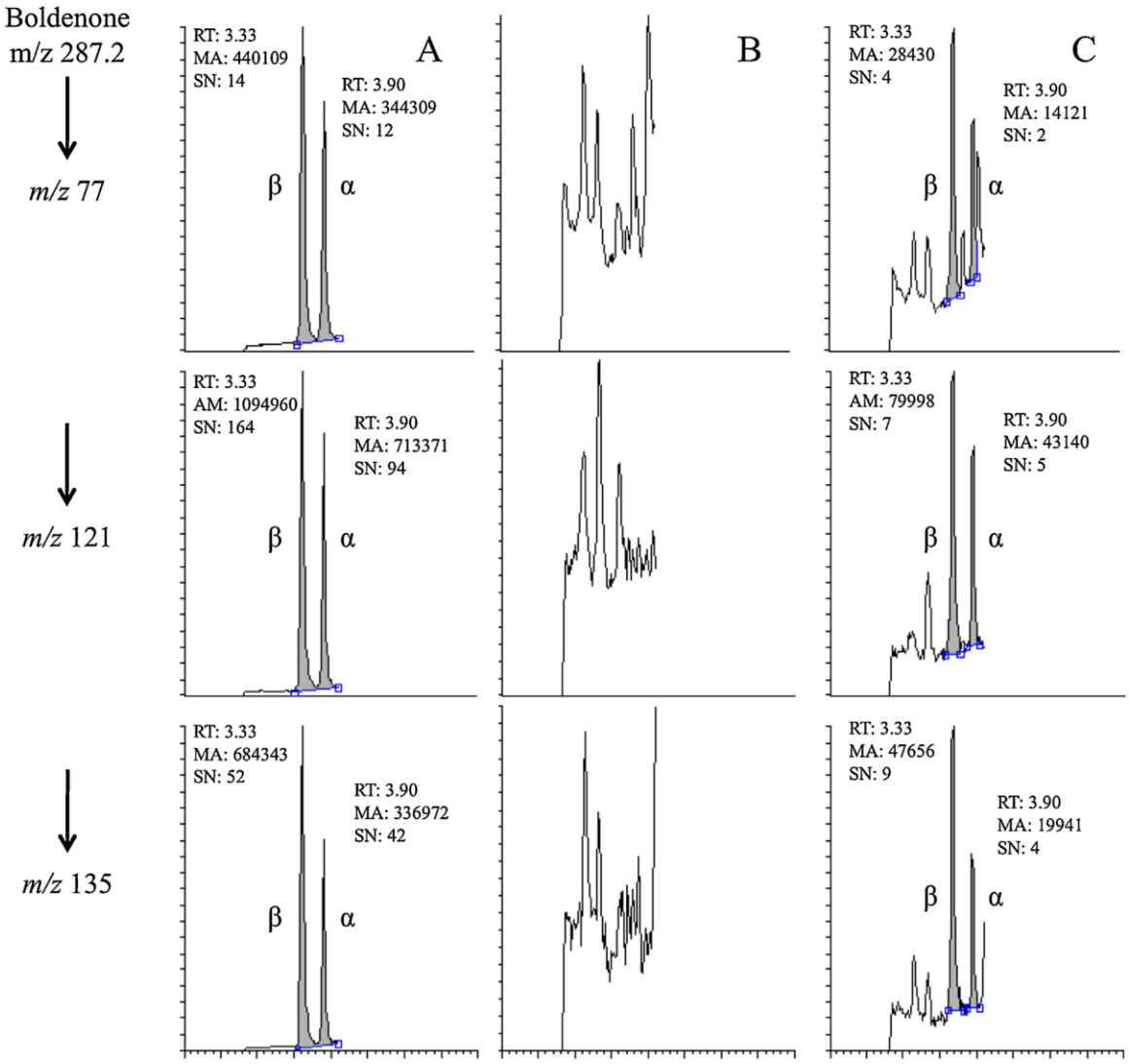


Figure 2. Comparison of the chromatograms obtained using UHPLC–MS/MS. (A) Standard injection of αBol and βBol (0.001 ng/mL), **(B)** blank *in vitro* digestion sample and **(C)** blank *in vitro* digestion sample fortified with 0.10 ng/mL αBol and βBol (LOD for βBol). The LOD for αBol is at 0.3 ng/mL. Three specific transitions of the target compound boldenone (*m/z* 287.2) were monitored: *m/z* 287.2 → *m/z* 77, *m/z* 287.2 → *m/z* 121, and *m/z* 287.2 → *m/z* 135.

The sensitivity of the method was confirmed by adding **0.10 ng/mL** of each component to a blank *in vitro* digestion sample (Illustrated for α and βBol, **Figure 2**). For the other compounds (AED, ADD, αT, βT, P, αBol) comparable results were obtained (not illustrated). We can conclude that the method is sensitive (up to 0.10 ng/mL), specific, precise and a linear quantification is possible. Therefore, this validated method was found adequate to analyze *in vitro* digestion samples of the following experiments.

4.2. *IN VITRO* DIGESTION: PROOF OF PRINCIPLE

Visually, the change in turbidity and the production of volatile fatty acids was the first sign of bacterial growth and fermentation taking place. The pH of the *in vitro* digestion amounted 7.0 ± 0.0 at the start, dropping to 5.5 ± 0.1 and slightly increasing back to 6.0 ± 0.5 by the end of the incubation (72 h) (full lines, **Figure 3**). The **pH drop** was in line with the exponential increase in biomass (cell density) in the first 24 h, measured as the absorbance at 600 nm (OD600) (dashed lines, **Figure 3**). OD600 and pH changes over time were similar for all three inocula in both CPB and BHI medium, but the absolute values differed according to the medium used. The pH drop and biomass accumulation were less pronounced in CPB than in the energy-rich medium (BHI) (**Table 1**, p. 111). The content of CPB was adapted from van de Kerkhof (2002) who was able to confirm that boldenone can be naturally occurring in humans, with an *in vitro* digestion experiment [41]. Therefore, CPB was tested as an alternative for the general-purpose BHI broth [33].

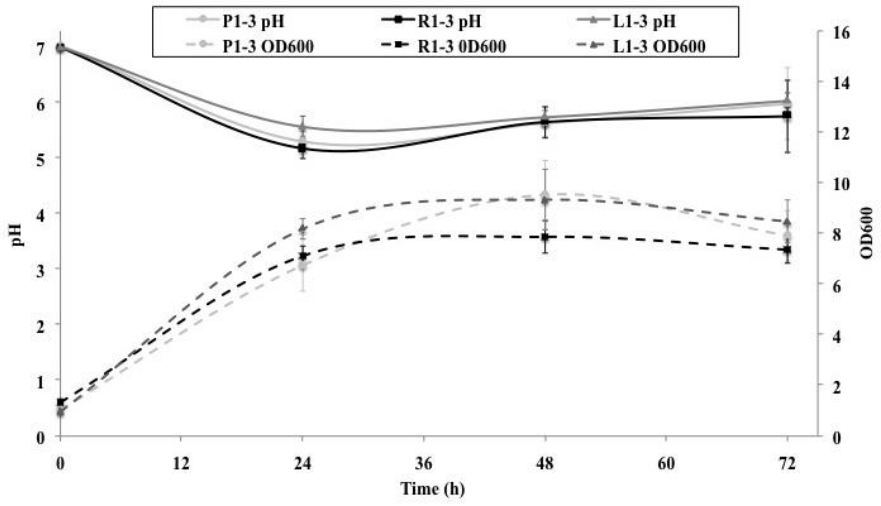


Figure 3. OD600 and pH of the *in vitro* digestions over time (0 to 72 h).
Three different inocula (P,R and L) were tested in triplicate.

Total anaerobic bacteria counts (CFU/mL *in vitro* digestion plated onto BHI agar) confirmed these results. After 48 h, 8.2–8.4 log10 CFU/mL were found in the BHI medium based *in vitro* digestions, while 7.0–7.2 log10 CFU/mL were present in the CPB based *in vitro* digestions. In line with the slight pH increase and biomass decrease after 36–48 h, the CFU/mL counts after 72 h were slightly lower, at respectively **7.6–8.2 log10** and **6.3–6.7 log10 CFU/mL**, for BHI and CPB medium, respectively (three different

inocula, in triplicate), in line with reference values for feces and colon, 7.6– 8.0 and 6.3–8.0 log10 CFU/mL respectively (diet dependent) [48].

Relative quantitative data, T0 compared to T72 and to non-inoculated media (respectively CPB and BHI), were obtained for carbohydrates, amino acids and SCFA. **GENE-E software**, a matrix visualization and analysis platform designed to support visual data exploration, was used to visualize the obtained date (**Figure 4**).

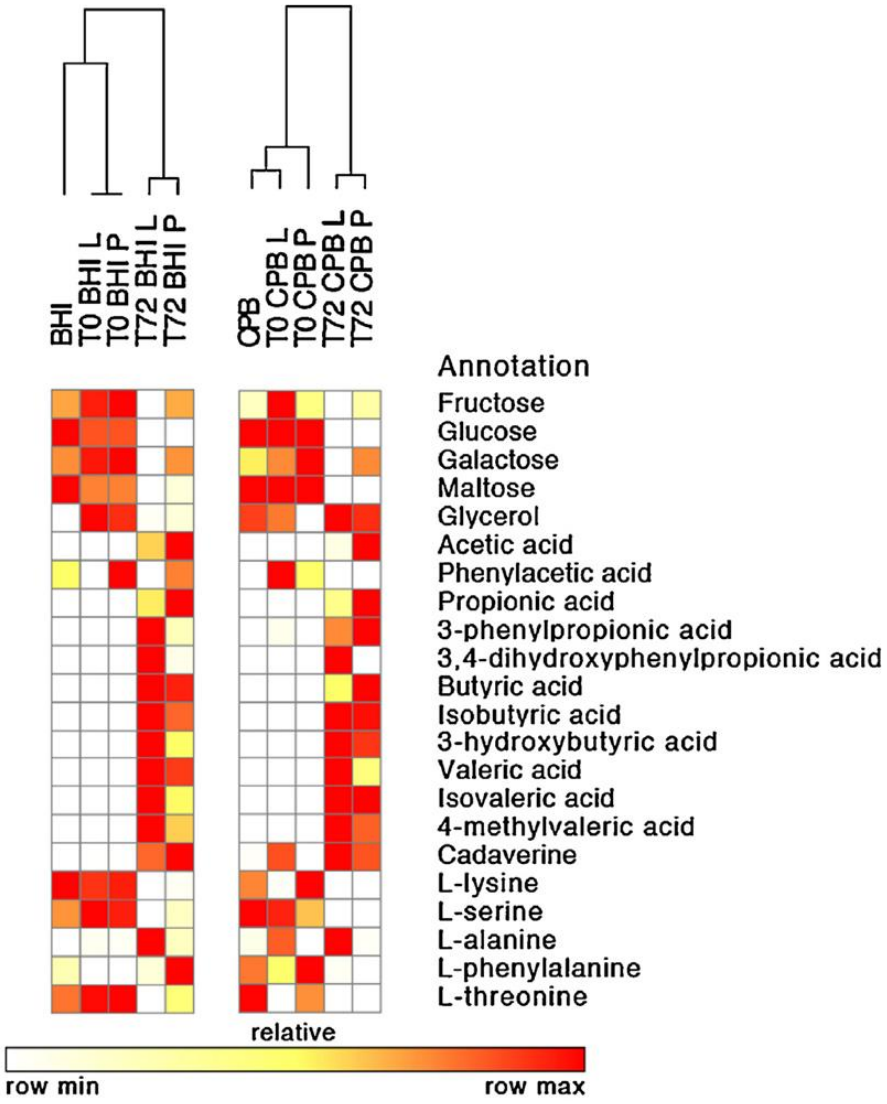


Figure 4. Relative quantification of short-chain fatty acids (SCFA), carbon sources and amino acids at the start and at the end of the *in vitro* digestion. Samples after 0 or 72 h of incubation, with two different media (BHI and CPB) and using different equine inocula (P and L) are illustrated (GENE-E generated heatmap, One Minus Pearson Clustering).

GENE-E software includes heat mapping, filtering and clustering algorithms. The in vitro digestion data were clustered according to a Minus One Pearson Correlation clustering algorithm (GENE-E software, Broads Institute). The T72 samples clustered together

while the non-inoculated BHI medium was clearly clustered with the non-incubated (T0) samples from both horses (L and P), as these samples were high in carbon sources, high in amino acids and no SCFAs could be detected. The T72 samples on the other hand were typically very high in SCFAs, as can be expected from *in vivo* results, and low in amino acids and carbon sources. A similar trend could be observed for the CPB based *in vitro* digestions.

For **glucose**, the relative quantitative data (**Figure 4**) were confirmed quantitatively by analyzing the *in vitro* digestion samples with a **Dionex Ion Chromatography** (ICS-3000, Thermo Scientific™ Dionex™) method described by Vanbeneden *et al.* (2006) [47]. The glucose concentration initially present in the *in vitro* digestions with BHI medium was 1.67 ± 0.01 g/L and 0.13 ± 0.01 g/L with CPB medium (compared to an eight-point calibration curve, 0.25 to 10 g/L). At the end of the *in vitro* digestions (T72), glucose was no longer detectable in the incubations with CPB medium while 0.13 ± 0.00 g/L was left in the *in vitro* digestions with BHI medium (in triplicate, for three different inocula). These results were in line with the results obtained through UHPLC–Orbitrap–MS analysis (**Figure 4**, p. 122; glucose).

On the other hand, ***Mycobacterium* sp.** DSM 2966 (NRRL B-3683) and *Mycobacterium* sp. DSM 2967 (NRRL B-3805) are known to be able to catalyze the biotransformation of phytosterols to AED and ADD [15–17,43,49,56]. This allows using them as a **positive control** to test the biotransformation promoting operational conditions of the *in vitro* digestion. In each medium (20 mL, CPB or BHI) three replicate cultures of strain 2966 and 2967 were made with added pure phytosterols (>50% β-sitosterol, 10 mg). As such we could check which of the strains is the most interesting in the light of the transformation to AAS, and which medium is preferred.

Interestingly, both strains were able to **produce βBol**, even within 24 h, and both strains tended to transform β-sitosterol into AED, αT and βT and low concentrations of P as well, but no α-Bol was detected. The transformation efficiency of both strains was however very different. Within 72 h (**Figure 5**, p. 124) strain 2967 produced more than 1049 ± 155 ng/mL AED and 380 ± 15 ng/mL ADD, while strain 2966 only produced respectively 10 ± 5 and 30 ± 5 ng/mL AED and ADD.

Therefore, **strain 2967** was preferred as the positive control for further biotransformation experiments. Negative controls without *Mycobacterium* sp. 2966 or 2967 did not lead to the detection of any AAS. *Mycobacterium* sp. 2966 and 2967 did produce some AAS without the addition of phytosterols, yet more than 100 times less efficient than the respectively replicates with added phytosterols (not illustrated). In this case, it is most likely that cholesterol from the medium was used as precursor.

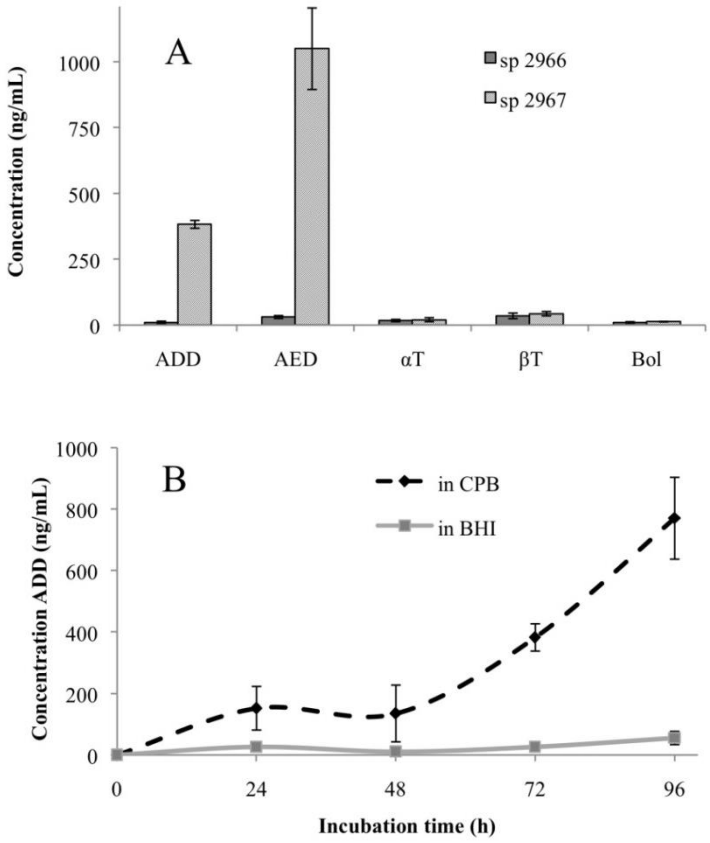


Figure 5. Biotransformation of phytosterols (>50% β-sitosterol) (A) By both *Mycobacterium* strains (DSM sp. 2966 and 2967) in CPB medium (72 h incubation). **(B)** by *Mycobacterium* sp. 2967 to ADD in CPB and BHI medium, over time (96 h).

Mycobacterium sp. 2967 can transform phytosterols into steroids in both CPB and BHI, but up to nine times more ADD is found when using **CPB medium**, compared to BHI (**Figure 5B**). This can be explained by the fact that CPB is a less rich medium compared to BHI, stimulating bacteria to metabolize the more complex, thus less energy efficient phytosterols. CPB medium has a lower, yet sufficient energy content than BHI per milliliter, CPB does not contain added glucose and less amino acids and fats from peptone and organic derived solids than BHI (**Table 1**, p. 111). This might trigger the hindgut fermenters to exploit their enzymatic capacity to the maximum, metabolizing less

favorable compounds as well (e.g. the precursors added, phytosterols), without limiting their basic survival. Together with the positive results obtained by Van de Kerkhof *et al.* [41] with CPB medium for the *in vitro* endogenous boldenone detection in humans, CPB was the medium used in the hindgut simulating *in vitro* digestions.

4.3. BLANK *IN VITRO* DIGESTION

Fecal inocula from two untreated, healthy horses (horse n° 1, a 9 years old gelding and horse n° 2, an 8 years old mare) were used. Urine of the gelding contained βT (4.8 ng/mL), αT (1.5 ng/mL) and P (6.1 ng/mL). The mare was excreting βT (6.0 ng/mL), αT (2.5 ng/mL) and P (2.6 ng/mL) (**Table 4**, p. 128). Samples from the *in vitro* digestive simulations (with CPB medium) were taken after 0, 24, 48 and 72 h. No ADD, AED, αT, βBol or αBol were found during this experiment. At the start of the incubation both digestions showed **traces of P** (1.1 ± 0.1 and 0.9 ± 0.1 ng/mL for the gelding and mare, respectively). This concentration was maintained throughout the experiment. The digestive fluids contained low concentrations of βT as well, amounting respectively 0.6 ± 0.1 ng/mL for the gelding and 0.3 ± 0.2 ng/mL for the mare. **βT disappeared within 24 h** (**Figure 6A**, gelding).

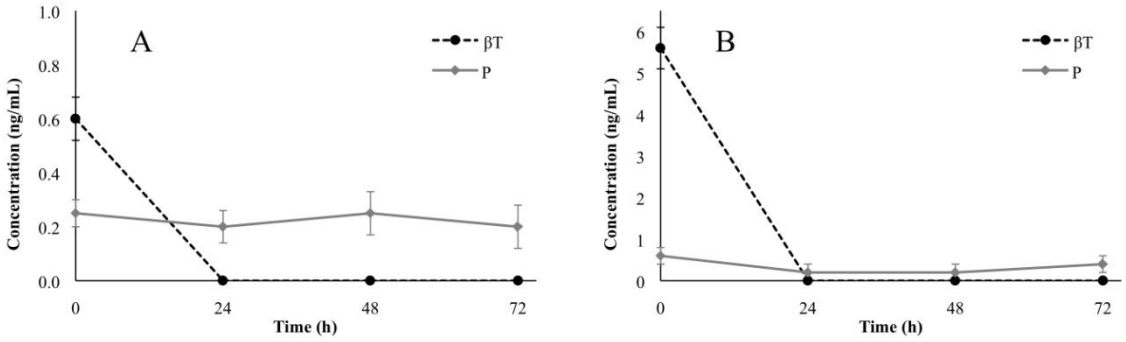


Figure 6. Detected AAS-related steroids during *in vitro* digestion in CPB medium using a gelding's fecal inoculum. (A) Blank *in vitro* digestion, **(B)** fortified with 5 ng βT per mL at the start of the incubation (time = 0 h).

4.4. *IN VITRO* DIGESTIONS WITH ADDED STEROID PRECURSORS

To improve the knowledge on the steroid transformation possibilities in the equine hindgut, the *in vitro* digestions were fortified with low concentrations of different steroids (βT, αT, P, βBol, AED or ADD at 5.0 ng/mL). The reaction products were monitored. The same inocula (horse n° 1, a 9 years old gelding and horse n° 2, an 8 years old mare, **Table 4**, p. 128) as in the previous experiment were used. In both the mare and gelding

added **αT and βT** (5.0 ng/mL) were no longer detectable shortly after the start of the digestion. **Within 24 h** the concentrations dropped below the limit of detection (<0.1 ng/mL) (**Figure 6B**, gelding, p. 125) and no other AAS were detected throughout the experiment. When adding βBol (5.0 ng/mL) to the digestion, a different reaction pathway was initiated. In the mare's digestive fluids (**Figure 7A**), βBol concentrations declined rather slowly: detection of βBol was possible until 48 h (1.4 ± 0.3 ng/mL). After 24 h, the conversion of βBol to ADD was detectable (1.4 ± 0.2 ng/mL), lasting until the next sampling after 48 h (1.5 ± 0.2 ng/mL), with a subsequent decline below the LOD after 72 h of incubation. Very low concentrations of progesterone and βT were found, similar to the results for blank and βT spiked incubations. In the gelding (**Figure 7B**), **βBol** concentrations declined faster (below LOD after 24 h), and a different transformation product (αT) was found after 24 h, lasting up until 48 h (0.3 ± 0.2 ng/mL), with a subsequent decline below the LOD after 72 h of incubation.

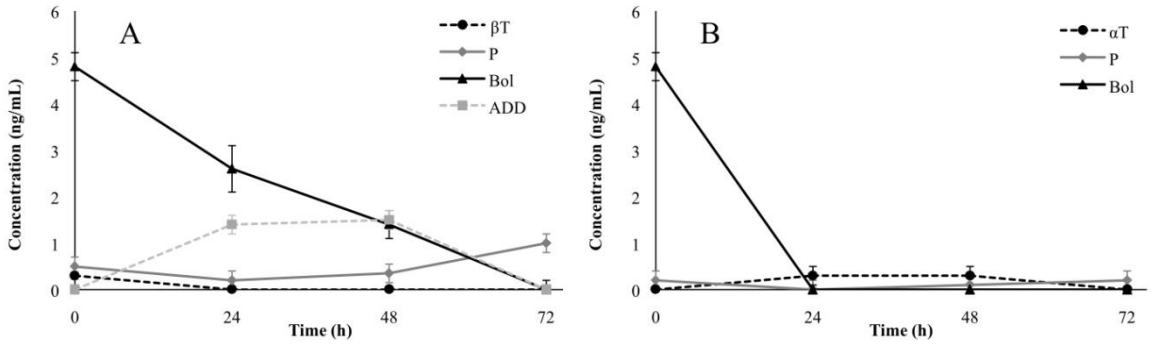


Figure 7. Detected anabolic steroids during the β-boldenone fortified in vitro digestion in CPB medium. (A) Using a mare's inoculum, **(B)** using a gelding's inoculum. Both digestions were fortified with 5.0 ng β-boldenone per milliliter at the start of the incubation (time = 0 h).

ADD and AED are chemically closely related to βBol and our previous research has shown that the urine of some geldings and mares contains low concentrations of endogenous AED (4 geldings 1.4 ± 1.3 ng/mL and 4 mares 0.7 ± 0.6 ng/mL) or ADD (one mare, 5.5 ng/mL) [10] (See also Chapter II). Inocula from the same mare and gelding as the previous experiment were used and fortified with AED (5.0 ng/mL). **AED** concentrations declined rapidly and no transformation to one of the other AAS was detected.

When adding **ADD** (5.0 ng/mL) to the *in vitro* digestion, the ADD concentration diminished throughout the digestion, resulting in the formation of βBol (**Figure 8,** p.

127). As βBol is the forbidden steroid with the most elicit suspected endogenous origin [1,57], this **transformation** capacity was studied thoroughly. Feces samples from six additional horses were used and the *in vitro* digestions were done in triplicate with the addition of ADD at three different levels (0 ng/mL = blank, 5.0 ng/mL = low level and 50 ng/mL = high level). An additional sample was taken **after 12 h**. βBol concentrations in all digestive simulations (eight horses) were highest after 12–24 h (**Figure 8**) and for most of the incubations detectable up until 72 h. No αBol was detected in any of the *in vitro* digestions. Urine samples from the eight horses used in this experiment were analyzed in parallel (**Table 4**, p. 128). Horse n° 5 (mare) produced ADD (5.5 ng/mL) and demonstrated an elevated αT concentration (13 ng/mL). Horse n° 6 (gelding) produced βBol (1.0 ng/mL) and AED (1.5 ng/mL).

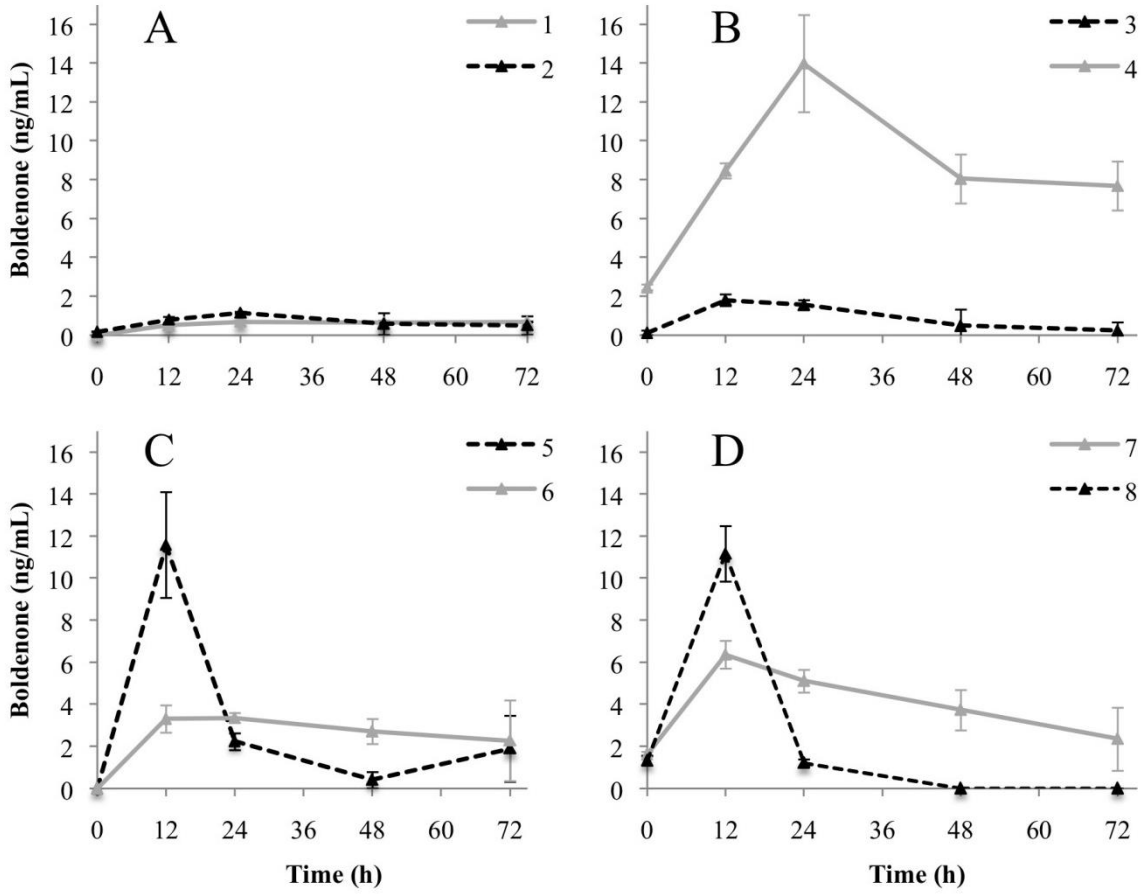


Fig. 8. Detection of β-boldenone during *in vitro* digestion of ADD in CPB medium. (A) Using inocula from the previously used horses: a mare's (horse n° 2) and a gelding's inoculum (horse n°1). **(B)**, **(C)**, and **(D)** using six other inocula from three mares (horse n°3, 5 and 8) and three geldings (horse n° 4, 6 and 7). All digestions illustrated were fortified with 50 ng ADD per milliliter digestion at the start of the incubation (time = 0 h). Results from digestions with the mares' inocula are indicated with a dashed line, geldings with a connected line. The *in vitro* digestions for these 8 horses were done in triplicate.

With two inocula (in triplicate, horse n° 1 and n° 2; **Table 4**), the limiting effect of BHI medium on the formation of βBol from ADD was confirmed, as previously deduced from the *Mycobacterium* DSM 2966 and 2967 experiments (**Figure 5B**, p. 124). ADD concentrations were declining more slowly and the formation of βBol was significantly lower.

Table 4. Detected AAS and related steroids in urine of the horses used for *in vitro* digestion. NF = not found, concentration below the limit of detection in urine (<0.2 ng/mL) [10].

Horse n°	Sex	Age (years)	Urinary concentration (ng/mL)						
			ADD	AED	αBol	βBol	αT	ßT	P
1	gelding	9	NF	NF	NF	NF	1.5	4.8	6.1
2	mare	8	NF	NF	NF	NF	2.5	6.0	2.6
3	mare	12	NF	NF	NF	NF	2.2	2.8	6.0
4	gelding	18	NF	NF	NF	NF	8.2	0.6	3.5
5	mare	8	5.5	NF	NF	NF	13.0	2.6	2.7
6	gelding	8	NF	1.5	NF	1.0	NF	0.3	NF
7	gelding	11	NF	NF	NF	NF	4.0	8.4	4.7
8	mare	18	NF	NF	NF	NF	14.7	1.7	4.2

3.5. *IN VITRO* DIGESTIONS WITH ADDED PHYTOSTEROLS (>50% B-SITOSTEROL) OR PHYTOSTEROL-RICH HERBAL SUPPLEMENTS

Both AED and ADD can originate from the **microbial side chain cleavage** of phytosterols (e.g. β-sitosterol). However, this biotransformation of phytosterols to steroid hormones has thus far not been demonstrated in horses while the microbial conversion of phytosterols to steroids has been frequently reported in other biological systems, mainly involving a variety of micro-organisms [15,17,58]. The addition of phytosterols (10 or 100 mg, >50% β-sitosterol) to the *in vitro* digestion (horse n° 1, 2, 4 and 6; **Table 4**) did not lead to the detection of boldenone (in triplicate) or any of the other AAS. For one horse (horse n°4) we did detect a low concentration of AED (0.10 ± 0.02 ng/mL or 0.5 ± 0.1 mg/g β-sitosterol).

Interestingly, a trace of **AED** was already present at the start of the incubation, and the concentration augments with the incubation time, confirming the biotransformation of the added phytosterols by the fecal micro-organisms. Two different phytosterol-rich herbal supplements that claim to support digestion and metabolism were tested as well. They were individually added to the *in vitro* digestion system (in triplicate). During these

digestions no transformation to AAS was detected. One of the supplements was however found to contain traces of **αT (37 ± 3 ng/g**, the advised daily dosage for a 500 kg body weight horse).

5. Discussion

In some cases, low concentrations of **βBol, ADD and AED** (<5 ng/mL) have been detected in **urine** of horses that were never in contact with synthetic AAS (See also Chapter II) [10]. As a possible explanation for this, phytosterols and phytosterol transformation products have been put forward, since it has been proven that steroid hormone intermediates could be produced from phytosterols through microbial transformation [13,16,17], and/or feed-related molds [59].

In this study, the addition of pure phytosterols (>50% β-sitosterol) to *in vitro* simulations of the equine hindgut indeed led to the detection of a **low concentration of AED** (0.10 ± 0.02 ng/ mL *in vitro* digestion or 0.5 ± 0.1 mg/g β-sitosterol) for one horse (1/4). This transformation has been previously confirmed in rats fed with phytosterols, excreting steroids (AED, ADD and androstanedione) in their feces [60]. However, the *in vitro* digestions with added phytosterols did not lead to the detection of α or βBol, ADD, or α or βT. Based on these results, we cannot directly link the digestive transformation of consumed phytosterols to the detection of βBol. These results are in line with the data obtained in humans by Verheyden *et al.* (2009) [61]. In this report, male and female volunteers were asked to consume a phytosterol rich yogurt drink during the first three weeks of the study. βBol was not detected in any of the urine samples. Still, ADD and AED were detected in the urine of some of the volunteers.

The presence of **ADD** is interesting as additional *in vitro* digestions allowed us to identify ADD as a precursor of βBol when present in the hindgut. When adding ADD to the *in vitro* digestion, all of the performed digestions (eight different fecal inocula) resulted in the **formation of βBol**. This is in line with *in vivo* experiments which demonstrated that when ADD is orally administered to a horse, βBol is found in the feces, within 24 h, and demonstrates the ability to simulate the *in vivo* hindgut fermentation reactions with in vitro incubations, making *in vitro* digestions a useful tool to study the fermentation reactions that take place in the horse's hindgut [6]. Multiple hypotheses and different inocula can be tested, with reproducible results.

Interestingly, the horse that was able to transform phytosterols into low concentrations of AED (0.10 ± 0.02 ng/mL) was also the one with the highest capacity to transform ADD to βBol (14 ± 2 ng/mL). As AED is also closely related to Bol, and both AED and ADD are possible microbial transformation products of phytosterols, AED was added to the **different *in vitro* digestions** as well. Upon addition of AED to the digestion it was degraded rapidly but no other steroids of interest were detected. When other steroids (αT, βT or P) were added to the *in vitro* digestions, a similar fast microbial degradation could be observed.

There was no correlation between the gender of the horses and the detected concentrations of βBol after the addition of ADD. The **interindividual variation** between the different horses outweighed the gender effect between mares and geldings, supporting the crucial impact of the individual hindgut flora on the enzymatic transformation capacity. Therefore, it is interesting to keep in mind that it has been reported that diet differences significantly influence the individual microbial composition of the horse's hindgut, leading to an adapted, horse-specific hindgut flora [23,50]. Traditionally, the equine digestive tract is anatomically and physiologically adapted to a relatively **nomadic lifestyle** of continually browsing for low-starch and fiber-rich feed [51]. In the healthy horse, anaerobes within the caecum ferment this fiber-rich feed to volatile fatty acids and lactate [35].

The dietary energy obtained from naturally available nutrient sources (grasses, rushes, sedges and perhaps occasional cereals) is however inadequate for daily, high intensity- and prolonged exercise. Therefore, the modern **domesticated sport horse** requires an increased quality and quantity of **feed intake**. This can be supplied by feeding energy rich grain mixtures, full of rapidly fermentable carbohydrates in the form of starch or sugars. Additionally, vegetable oils are frequently added to the diet [54]. The addition of extra fat easily raises the energy density of feeds, which is advantageous for sport horses with high-energy requirements. Thus far, this dietary influence on the individual hindgut flora has only been studied in the light of fermentative laminitis, acidosis, colic, and stomach ulcers [62], but not regarding the enzymatic transformation capacity of the hindgut.

When **βBol** was added to the digestion, different reaction pathways were initiated depending on the inoculum. The reaction products were either ADD or αT. Moreover, when βBol is slowly degraded by the mare's inoculum, more time seems to be at hand for

transformation, leading to a higher concentration of ADD. The maximal transformation ratio βBol:ADD amounted 29%, while for the gelding's inoculum the ratio βBol:aT was less than 7%. Fecal inocula from two untreated, healthy horses (horse n° 1, a 9 years old gelding and horse n° 2, an 8 years old mare) on a moderate grain-rich diet (4 kg a day) were used as the negative control experiments. The digestive fluids of these **blank *in vitro* digestion** incubations contained low concentrations of βT and P. βT disappeared within 24 h, due to microbial breakdown by the hindgut bacteria (gelding). The presence of P and βT in the *in vitro* digestion simulation is in line with the presence of P and βT in the urine of the horses. αT was also present in urine but was not detected in the digestive fluids. These results demonstrate that, without specific additives, the hindgut of these healthy, untreated horses does not exert metabolic activity that directly leads to the formation of AAS or AAS-related precursors.

Interestingly, when using this gelding's inoculum, the detected concentration of βT at the start exactly summed the added concentration of **βT** and the concentration detected at the start of the blank incubation. This low concentration of βT is not detected when using the mare's inoculum, indicating that it must originate from the **gelding's inoculum**. Steroid hormones circulating in the bloodstream are metabolized in the liver and passed with bile into fecal matter [63], resulting in their detection in feces [6]. The passage of steroids from plasma to feces takes about 24 h [64]. Fecal androgen concentrations have been merely assessed in stallions [6]. Fecal estrogen concentrations on the other hand, have been used to monitor pregnancy [65,66] and diagnose cryptorchidism [67].

The other way around, it has been proven that, together with nutritional compounds, **contaminants**, possibly including steroids or steroid precursors, can be absorbed from the intestinal tract [20]. Passage by the liver leads to the formation of type II metabolites, steroid glucuronide- and sulphate conjugates, which are excreted and detected in urine [68]. Other transformation reactions, the so called **type I transformations**, can also take place in the liver. To study these type I transformation reactions, *in vitro* set-ups have been developed as well. Scarth *et al.* (2010) for example, used equine liver microsomes and S9 tissue fractions to study the metabolism of the androgenic/anabolic steroid stanozolol [69]. Using high-resolution accurate mass full scan analysis on the Orbitrap, equine liver microsome and S9 *in vitro* fractions were found to generate all the major type I metabolites observed following *in vivo* administrations.

Wong *et al.* (2011) on the other hand, confirmed the use of **homogenized liver** and, in addition to the previously reported *in vitro* metabolites, some additional known *in vivo* metabolites in the equine could also be detected for testosterone (βT) and epitestosterone (αT) [70]. Unfortunately, to our current knowledge, the **equine liver metabolism of AED** has not yet been tested. Labrie *et al.* (1997) did confirm that one of the 17β-hydroxysteroid dehydrogenase isoenzymes (17β-HSD) is able to control the last step in the formation of testosterone from AED in all rhesus monkeys and human peripheral intracrine tissues examined. Types 3 and 5 17β-HSD, respectively catalyze the formation of testosterone from AED in the testis and peripheral tissues [71].

Additionally, **predigestive effects** (e.g. UV radiation, oxidation, heat, moist and feed-related molds) might affect the phytosterol side chain stability in feed [59]. These parameters could trigger local hotspots for transformation in a feed batch, especially when self-heating is involved [72]. When a horse consumes such hotspots, this could lead to the transient and temporary detection of low concentrations of AAS in the horse's urine. Further research must be conducted to confirm or to disprove these hypotheses.

6. CONCLUSION

The use of *in vitro* batch incubations to simulate the equine hindgut was validated by performing a series of quality controls, including monitoring the formation of short chain fatty acids, the depletion of amino acids and carbon sources and the transformation of ADD, the suspected βBol precursor. Upon addition of ADD the *in vitro* transformation of ADD to βBol could be detected for eight different horses. The transformation capacity was fecal inoculum- and thus horse dependent, but no αBol was found in any of the *in vitro* digestion samples. These results are in line with *in vivo* results, proving that *in vitro* digestions are a useful tool to study the fermentation reactions that take place in the equine hindgut.

The addition of phytosterols to the *in vitro* digestion simulations led to the detection of AED, an important steroid precursor. As such, the digestive transformation of consumed phytosterols cannot be directly linked to the detection of βBol, but, by providing the necessary precursors, they might form an intermediate step in the systemic steroid biotransformation pathway. Predigestive influences such as UV radiation, oxidation, heat and feed-borne molds might contribute to the predigestive degradation of phytosterols, increasing the release of Bol, ADD, T or other AAS related precursors, including AED, in the hindgut. This new research question will be the upcoming challenge to unravel the detection of low concentrations of forbidden, and considered to be synthetic, steroids such as the mere suspect βBol, in untreated horses.

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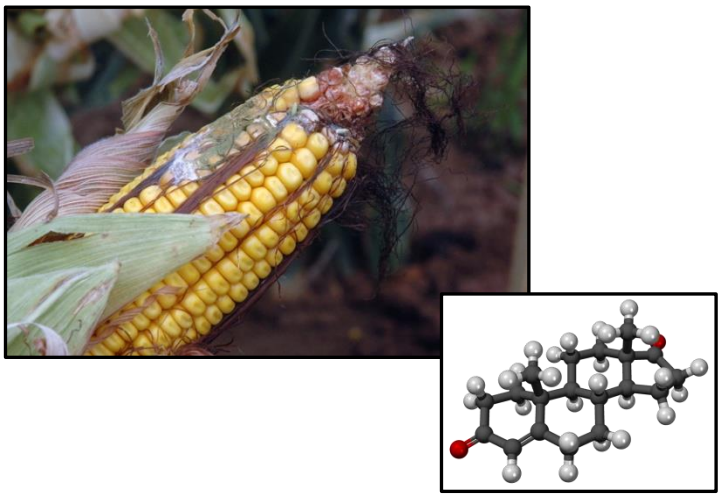
CHAPTER V

Moldy feed, a possible explanation for the excretion of anabolic-androgenic steroids in horses?



Adapted from:

"Moldy feed, a possible explanation for the excretion of anabolic-androgenic steroids in horses?"

By Anneleen Decloedt, Ludovic Bailly-Chouriberry, Julie Vanden Bussche, Patrice Garcia, Marie-Agnes Popot, Yves Bonnaire and Lynn Vanhaecke (2015), *Drug Testing and Analysis* (submitted)

CHAPTER V

1. ABSTRACT

To ensure fair competition and to protect the horse's welfare, horses have to compete on their own merits, without any unfair advantage that might follow the use of drugs. Therefore, regulatory authorities list all substances that are not allowed in competition, including most anabolic-androgenic steroids. As zero-tolerance is retained, the question arose if the consumption of moldy feed could lead to the excretion of steroids, due to the biotransformation of plant phytosterols to steroids. A rapid UHPLC-MS/MS analytical method, previously validated according to AORC (Association of Official Racing Chemists) and EU Council Decision 2002/657 guidelines, was used to measure steroids in different sample types. Multiple moldy feed samples were tested for the presence of steroids. The effect of digestion was tested by *in vitro* simulation of the horse's hindgut in batch incubations. In most feed samples no steroids were detected, even when the products were molded. Moldy corn however showed to contain up to 3.0 ± 0.4 µg/kg AED (androst-4-ene-3,17-dione), the main testosterone precursor. This concentration increased when moldy corn with added phytosterols was digested *in vitro*. One herbal phytosupplement also showed to contain α-testosterone (up to 37 ± 3 ng/g). These results demonstrate that it is important to caution against the consumption of any feed or (herbal) supplement of which the detailed ingredients and quantitative analysis are unknown. Especially the consumption of moldy corn should be avoided, not only from a horse health and welfare point of view, but also to avoid possible inadvertent positive doping results.

Keywords

UHPLC-MS/MS – Feed – Horse

Anabolic-androgenic steroids - Molds

2. INTRODUCTION

To ensure fair competition and to protect the horse's welfare, horses have to compete on their own merits, without any unfair advantage that might follow the use of performance enhancers. Therefore, regulatory authorities such as IFHA (International Federation of Horseracing Authorities) and FEI (Fédération Equestre Internationale) list and control all substances that are not allowed in and out of competition. Anabolic-androgenic steroids (AAS) are part of this list as they can increase nitrogen retention, protein synthesis, appetite and the release of erythropoietin in the kidneys, making them very popular as drugs of abuse. To the public, the **natural, androgenic steroid testosterone** is the best known AAS. Closely related to testosterone (βT) in terms of chemical structure are, epitestosterone (αT), AED (androst-4-ene-3,17-dione), ADD (androsta-1,4-diene-3,17-dione), β-boldenone (β-Bol, androsta-1,4-diene-3-one-17β-ol or 1,2-dehydrotestosterone) and α-boldenone (α-Bol, androsta-1,4-diene-one-17α-ol) (**Figure 1**).

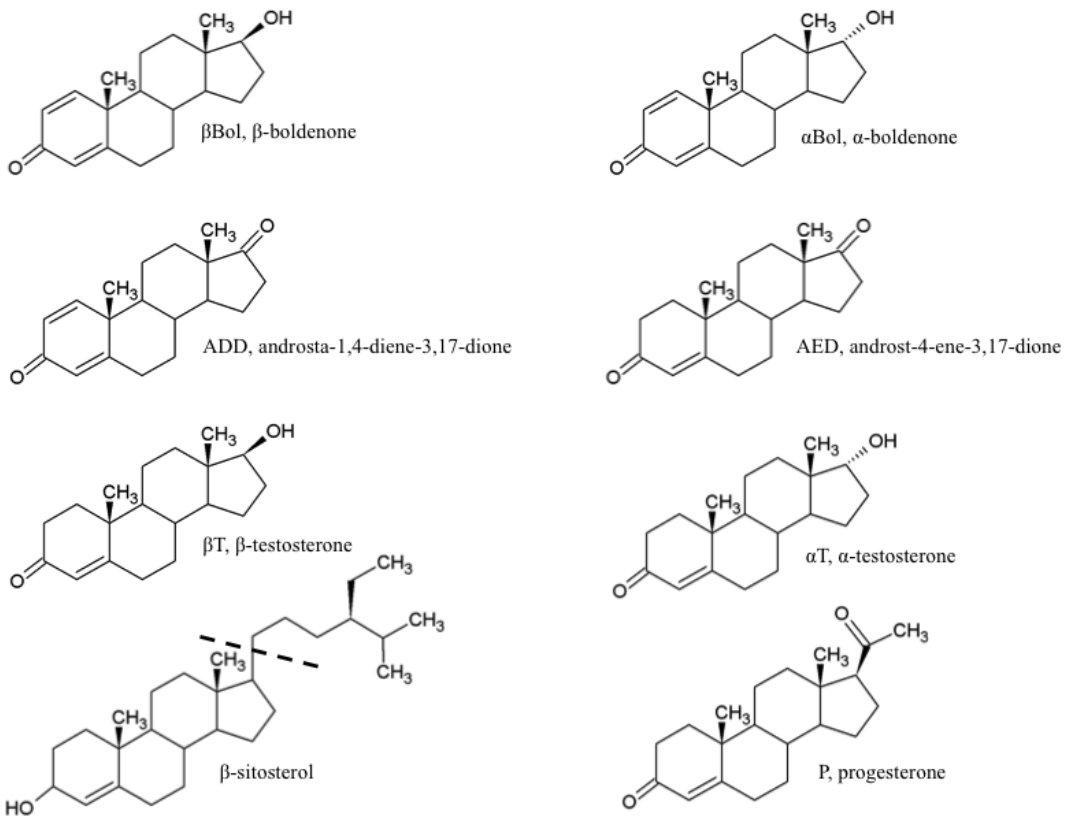


Figure 1. Illustration of the closely related chemical structures of phytosterols and anabolic steroids. For the phytosterols, β-sitosterol, the most abundant phytosterol, is shown. The suggested microbial side chain cleavage is indicated with a dashed line (- - -). Campesterol and stigmasterol differ from β-sitosterol in the side chain double bond at C22 and the substituents at C24.

For a very long time **boldenone** was considered to be a **synthetic hormone** and zero-tolerance was maintained. As the number of boldenone-positive urine samples was increasing, the question arose whether this was due to illegal treatment of animals or if boldenone is an endogenous steroid [1]. By using new and more sensitive analytical methods, boldenone has been shown to be naturally present in bovine urine and feces [2-4] and low concentrations of β-boldenone have been detected in the urine from untreated intact male horses [5-7]. According to the latter findings the IFHA (International Federation of Horseracing Authorities) and FEI (Fédération Equestre Internationale) set a threshold for boldenone at 15 ng free and conjugated boldenone per mL in urine from male horses (other than geldings) [8,9]. The presence of boldenone in mares or geldings is however still prohibited. This zero-tolerance is very strict as, with the current extensive extraction protocols and high-end mass spectrometry detection methods, traces of up to **0.1 ng/mL** could be detected [10].

Previous research showed that the **consumption of NOPS** (Naturally Occurring Prohibited Substances, e.g. xanthine or morphine), even at levels much below the effective dosage, may be responsible for an inadvertent positive anti-doping urine analysis [11]. Additionally, recent work indicated that a novel mechanism of endogenous steroid-synthesis is to be considered: **non-toxic feed-borne fungi**, naturally present on animal feed, might be capable of converting phytosterols into steroids [12]. Complete (microbial) side chain cleavage produces C19-steroids. Other studies reported similar biotransformation potential in environmental mold species, isolated from soil [13]. In some cases, this has been held as an argument to defend urinary detection of anabolic steroids (e.g. “Moldy oats blamed for positive swabs”, The Southland Times, March 2012, New-Zealand). Unfortunately, the study mentioned earlier only focused on cattle feed and the effect of mold enzymes on the feed was not measured over time nor compared to a relevant negative control (e.g. mold-free feed). Moreover, they did not study the actual influence of digestion of contaminated feed on cattle or, in this case, horses [12].

The **conversion of phytosterols** to steroids has been reported in other biological systems [14], mainly involving a variety of microorganisms [15] such as *Mycobacterium* sp. [16-18]), *Arthrobacter* and *Nocardia* sp. [19]. In addition, a number of studies have been devoted to the ability of invertebrate organisms to convert phytosterols into anabolic steroids: maggots of *Lucilia Serica* [20], *Crustaceae* [21] and zebra fish [22]. One study

reported the intestinal *in vivo* biotransformation of consumed phytosterols to steroids (androsta-1,4-diene-3,17-dione, androst-4-ene-3,17-dione and androstane-3,17-dione) by rats [23]. However, the biotransformation of phytosterols to steroid hormones has thus far not been studied in relation to the equine consumption of moldy phytosterol-rich feed.

The **term "moldy"** is used to describe the diseased appearance resulting from infection by one or more, parasitic, fungal species [24]. It is a major biotic constraint to grain production worldwide, especially present when grain development coincides with wet and warm weather conditions [25]. Due to their parasitic nature, molds are very interesting **biotransformation candidates** as they are capable of adapting to different substrates. Generally, microorganisms capable of degrading hydrophobic hydrocarbons, such as phytosterols, possess different physiological properties, including active transporters, cell wall adaptations and the excretion of biosurfactants [26-28]. Molds developed their own efficient strategy to cope with these difficult substrates, by producing a wide variety of extracellular enzymes and biosurfactants [24], to promote the extracellular transformation of these difficult substrates.

As a result, it may be **hypothesized** that aerobic storage of moldy feed can lead to the formation of steroids or steroid precursors. Alternatively, the contamination of feed with known phytosterol converting bacteria could possibly lead to the transformation of phytosterols from feed. On the level of the horse, the consumption of moldy or bacterially contaminated feed could as such lead to the detection of endogenous steroid levels. As zero-tolerance is retained for most AAS, this could have serious consequences.

To tackle these different research questions, a **full-fledged *in vitro* approach** was set-up. Due to ethical constraints, it is not appropriate to test the consumption of moldy feed *in vivo*. *In vitro* batch incubations were performed following a previously validated *in vitro* digestion model [29] (See also chapter IV) to simulate the hindgut fermentation of moldy feed, as they are thought to be responsible for the biotransformation reactions from phytosterol metabolites to AAS or related precursors. All samples were analyzed with a recently developed and validated, sensitive and robust UHPLC-MS/MS method [10].

3. MATERIALS AND METHODS

3.1 LC-MS/MS REAGENTS AND CHEMICALS

α-testosterone (androst-4-ene-17α-ol-3-one, αT, purity ≥99%), ß-testosterone (androst-4-ene-17ß-ol-3-one, ßT, purity ≥99%), methyltestosterone (androst-4-ene-17α-methyl-17ß-ol-3-one, MT, purity ≥99%), androstadienedione (androsta-1,4-diene-3,17-dione, ADD, purity ≥99%), proteinase from *Aspergillus melleus* (3 enzyme units per mg) and β-glucuronidase (*Helix pomatia*, aqueous, >100.000 units per mL) were purchased from Sigma-Aldrich (St-Louis, US). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥99%), α-boldenone (androsta-1,4-diene-17α-ol-3-one, α-Bol, purity ≥99%) and ß-boldenone (androsta-1,4-diene-17ß-ol-3-one, ß-Bol, purity ≥99%) were obtained from Steraloids (Newport, US). Progesterone (P, purity ≥98%) was obtained from Alpha Pharma (Omega Pharma, Zwevegem, Belgium).

Methanol Optima®, was bought at Fisher Scientific, UK Limited (Leicestershire, UK). Diethyl ether, ethyl acetate, sodium hydroxide, methanol (analytical grade), H_2SO_4, KH_2PO_4 and formic acid (98-100%, analytic grade) were purchased from VWR (Merck, Darmstadt, Germany). Solvolysis solvent consisted of 900 ml ethyl acetate, 95 ml methanol and 5 ml H_2SO_4 per liter. Phytosterols (>50% β-sitosterol) were purchased from Sigma-Aldrich (St-Louis, US). Polysorbate 80 (Tween 80) is a nonionic surfactant and emulsifier derived from polyethoxylatedsorbitan and oleic acid. By adding 0.2% Tween 80 (v/v) to the medium up to 2 g/L of the hydrophobic substrate ß-sitosterol can be solubilized [30]. The HF Bond Elut-C18 cartridges (6 ml, 500 mg) were obtained from Agilent Technologies (Diegem, Belgium). HPLC grade, ultrapure (UP) water was acquired from an in-house water purification system (Arium® 611UV, Sartorius Stedium Biotech, VWR, Haasrode, Belgium).

Stock solutions of each steroid were made in methanol Optima® at 1000 and 200 ng/µL. Dilutions up to 1 pg/µL were made in methanol Optima®. All solutions were kept at 4 °C.

3.2 BUFFERS, BROTHS AND FEED SAMPLES

All buffers and broths were prepared in ultrapure water, autoclaved (121 °C, 15 min, 1 atm) and further handlings were done in a **laminar flow cabinet**. Phosphate buffered saline (PBS, pH 7) contained NaCl (8 g/L), KH_2PO_4 (0.134 g/L) and K_2HPO_4 (1.12 g/L). Fecal inoculum buffer contained K_2HPO_4 (8.8 g/L), KH_2PO_4 (6.8g/L) (Merck, Darmstadt, Germany), and sodium thioglycolate (1.0 g/L) (Sigma-Aldrich, Steinheim, Germany), as

a reducing agent. CPB (Cysteine Peptone Bouillon) consists of a mixture of 5 g yeast extract (AppliChem, Darmstadt, Germany), 1 g peptone (Oxoid, Hampshire, England), 8.5 g NaCl (Merck, Darmstadt, Germany), 0.5 g L-cysteine and 10 mL haemine solution (Sigma-Aldrich, Steinheim, Germany) in 1 L ultrapure water [31]. **L-cysteine** (0.5 g/L) (SAFCSupply Solutions, St. Louis, MO) was added to improve **anaerobicity** [32]. Haemine solution was prepared by adding 2 mL concentrated NH_4OH solution (25%, Merck, Darmstadt, Germany) and 0.1 g haemine to 500 mL sterile ultrapure water and sterilized afterwards (121 °C, 15 min, 1 atm). After filtration over a 0.45 µm filter (Merck, Darmstadt, Germany), pH was adjusted to 7.0 and the medium was sterilized again. Under sterile conditions 0.25 mL of nicotinamide adenine dinucleotide (Sigma-Aldrich, Steinheim, Germany) solution in water (2 mg/mL) was added. The total solution was mixed and stored at 4 °C until use.

Sabouraud Dextrose Agar was obtained from Oxoid LTD (Basingstoke, Hampshire, England) and prepared according to the manufacturer's guidelines with 30 g/L Sabouraud Dextrose Liquid Medium and 1.5% Agar (15 g/L). Moldy and mold-free feed were collected from the field (corn) or obtained from a nearby horse shop (carrots and commercial grain mix). Two commercial phytosupplements were bought in a specialized horse shop.

***Mycobacterium* sp.** DSM 2966 (NRRL B-3683) and *Mycobacterium* sp. DSM 2967 (NRRL B-3805) were vacuum dried cultures delivered by the Leibniz Institute DSMZ (German Collection of Microorganism and Cell Cultures). Reactivation was executed in nutrient broth (Oxoid) at 30 °C (7 days), according to DSMZ guidelines for these two strains. This process was repeated three times to remove any stabilizing storage agents. In a glass vial, 1 mL of the final bacterial suspension was added to 3 mL glycerol, vortexed and stored at -80 °C until use.

3.3 AEROBIC INCUBATION OF MOLDY FEED

Moldy feed (carrots, field corn and a commercial grain mix) were mixed (10 min, Moulinette, Moulinex, Berkshire, UK) until a **homogenous mash** or powder was obtained. Dark, autoclaved 125 mL penicillin flasks were used to exclude light and UV influences. To 45 mL medium (CPB broth), 5 g of the moldy feed was added. The feed was incubated at two different temperatures (37 °C and 21 °C: room temperature, RT) to simulate the normal storage temperature (RT) and a higher temperature (37 °C), to

simulate the influence of **self-heating** in moldy hot spots [33]. Samples (3 mL) were taken after 0, 24, 48, 72 and 96 h.

1 mL of phosphate buffer (1M KH_2PO_4, pH 6.1 ± 0.1) was added to 3 mL of sample. Next, the internal standard MeT (5 ng/mL), 50 µl of a ≥450 units/mL protease solution and 25 µl ß-glucuronidase were added. Of each sample the pH was set at 6.1 ± 0.1 (by adding 1M HCl) and **hydrolysis** was executed at 55 °C (1 hour). After hydrolysis, 3 mL of ultrapure water was added and large, non-hydrolyzed proteins were removed by agglutinating them at the bottom of the tube through centrifugation (2400 x *g*, 15 min). Finally, the supernatant was filtered over a cotton wool filter before solid phase extraction (SPE).

The **SPE cartridges** (6mL, 500 mg C18, Bond Elut, Agilent) were conditioned with 4 mL methanol and 4 mL ultrapure water. The centrifuged and filtered samples were loaded onto the column and washed with consecutively 7 mL ultrapure water and 7 mL hexane. The cartridges were dried under vacuum (-0.5 bar). Next, the non-conjugated and glucuronide-conjugated fractions were eluted with diethyl ether (7 mL) and the sulfate-conjugated fraction with solvolysis solvent (7 mL). **Solvolysis** of the sulfate conjugated fraction was executed at 55 °C (2 hours). Both fractions were washed with 1.5 M sodium hydroxide (5 mL) by turning (8 min, 60 rpm) and centrifugation (6 min, 1400 x *g*).

The **washed fractions** were pooled and dried under nitrogen (50 °C, 30 min). Each sample was reconstituted in 100 µl of ultrapure methanol, vortexed and ultrasonicated (3 min). Finally, 100 µl of ultrapure water was added and the sample vortexed and ultrasonicated again (3 min). After centrifugation (12300 x *g*, 10 min) the sample was transferred to an LC-MS vial with insert for UHPLC-MS/MS analysis.

3.4 AEROBIC INCUBATION OF A COMMERCIAL GRAIN MIX WITH *MYCOBACTERIUM* SP.

100 µL *Mycobacterium* stock sp. DSM 2966 and 2967 (glycerol, stored at -80 °C) was added per 10 mL broth (CPB). After a **preculturing step** of three days (150 rpm, 37 °C) the *Mycobacteria* culture (5 mL) was used to inoculate the commercial grain mix powder (5 g) in 40 mL CPB medium. The incubation was kept aerobically and monitored for 72 h; samples were taken after 0, 12, 24, 48 and 72 hours. Samples were extracted according to the extraction protocol described above.

Additionally, to fully study the possibilities of *Mycobacterium* sp (DSM 2967) as a

phytosterol transforming agent, the **oxygen dependence** for growth and enzymatic activity was tested. To create anaerobic conditions, the flasks were capped and anaerobic conditions were established using a flush system for 1 h, alternating every 2 min between N_2 (1 bar) and vacuum suction.

3.5 ACTIVITY OF EXTRACELLULAR ENZYMES EXCRETED BY CORN-RELATED MOLDS

Moldy corn crops were collected from the fields (East-Flanders, autumn). The moldy corn kernels were pooled and mixed thoroughly (10 min, Moulinette, Moulinex, Berkshire, UK) until a homogenous, yellowish corn powder was obtained. This powder was diluted in sterile UP water (1:3) and plated onto Sabouraud Dextrose Agar plates under a laminar flow cabinet. These agar plates were incubated for 4 days (28 °C). All **morphologically different mold colonies** were isolated on new Sabouraud Dextrose Agar plates until pure isolates were obtained (**Figure 2**). Subsequently, through this procedure obtained mold isolates were classified based upon their morphology.

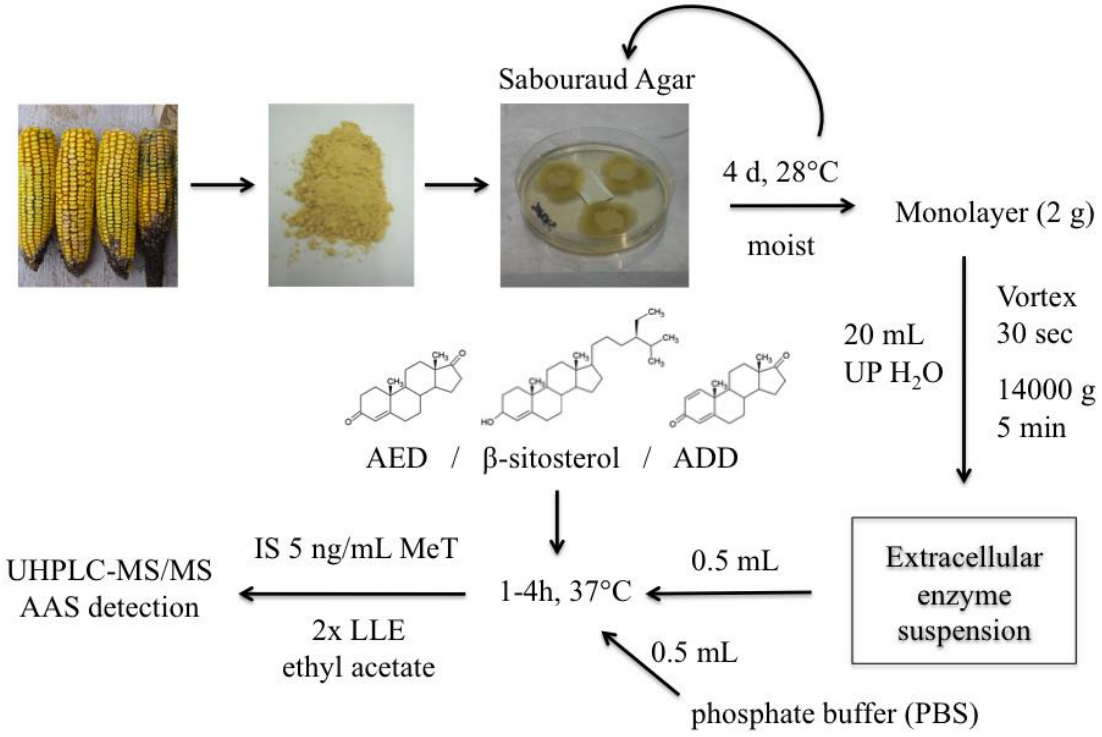


Figure 2. Isolation procedure and experimental set-up to test the biotransformation potential of extracellular enzymes excreted by mold species isolated from moldy corn.

The different isolated mold strains were individually grown under moist conditions at 28 °C until a **monolayer** was obtained (4-7 days). The mycelium (2 g) was transferred

into a 50 mL tube; vortexed in sterile, UP water (20 mL) and the mycelium was separated from the extracellular enzyme suspension by centrifugation (14000 x *g*, 5 min, 4 °C). In a 15 mL tube 0.5 mL of the supernatant was added to 0.5 mL PBS buffer and incubated with or without an excess of the suggested precursors (1 µM AED, ADD, phytosterols) at 37 °C for 1 to 4 hours. Samples were extracted immediately after the incubation period was finished (**Figure 2**, p. 150).

Extraction of the extracellular-enzyme suspension samples was based on the method described by De wasch *et al.* (2002) for *Neomysis integer* biotransformation experiments [34] (**Figure 2**, p. 150). The internal standard (methyltestosterone, MT) was added at 5 ng/mL prior to extraction. The metabolites were extracted from the medium by liquid-liquid extraction (LLE) using 2 x 4 mL **ethyl acetate**. After centrifugation (5 min, 4 °C, 14000 x *g*) the organic phase was withdrawn and the ethyl acetate fractions were combined and vacuum evaporated to dryness under nitrogen (50 °C, 15 min).

Each sample was reconstituted in 100 µl of ultrapure methanol, vortexed and ultrasonicated (3 min). Finally, 100 µl of ultrapure water was added and the sample was vortexed and ultrasonicated (3 min). After centrifugation (12300 x *g*, 10 min) the sample was transferred to an LC-MS vial with insert for UHPLC-MS/MS analysis. The obtained results were compared per treatment (different steroid precursor added) and per mold species as compared to the control samples, with **independent samples t-tests** (SPSS Statistics 21.0.0., SPSS Inc, Chicago, IL) (P=0.05).

3.6 IN VITRO DIGESTION OF MOLDY FEED

To simulate the hindgut fermentation reactions that take place in the equine hindgut, ***in vitro* batch incubations** were set up. Previous research (See also Chapter IV) showed that *in vitro* digestion simulations are a good tool to study *in vivo* metabolic transformations in the equine hindgut [29,35]. For these incubations a fecal inoculum was required. Fresh fecal matter was collected from an 18-year old, well-trained **gelding during spontaneous discharge**. This horse was selected based upon his high natural anabolic-androgenic steroid profile in urine (8.2 ng/mL α-testosterone, 0.6 ng/mL β-testosterone and 3.5 ng/mL progesterone).

Additionally, previous research proved that this horse's microbiome has **high biotransformation capacity**, especially at transforming added phytosterols to AED (0.10 ± 0.02 ng/mL) and added ADD (50 ng/mL) to β-Bol (up to 14 ± 2 ng/mL) [29]. The horse

was fed on a **standard but non-controlled diet** of concentrate (4 kg/day), hay (10 kg/day) and straw. The horse was a Royal Dutch Sport Horse (K.W.P.N) and guaranteed to be untreated with AAS or other treatments that are known to interfere with the excretion of AAS, his medical history was well known and documented. As spontaneously voided urine and faecal samples were collected, and the horse was not given any medication or treatment, according to the latest Belgian and European animal welfare rules (RD 29th May 2013, published on the 10th of July), he was not considered to be an experimental animal. As such, the authors state that have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations. In addition, informed consent from the owner was obtained.

The fresh fecal matter was kept **anaerobic** (in a firmly closed bag) during transport to protect it from oxygen exposure and at 37 °C, until further processing in the lab. Fecal slurry was made by adding 1:5 (20% w/w) phosphate buffered saline followed by a homogenization step in a stomacher (Stomacher 400 Classic Laboratory Blender, Seward, West Sussex, UK) for 10 min. The **faecal slurry** was transferred into 50 mL falcon tubes and centrifuged at 500 x *g* for 5 min, removing large fibers. To the supernatant, glycerol (99.5%) (Analar Normapur, Fontenay-sous-Bois, France) was added at a 20% (v/v) ratio, which was gently mixed under atmospheric conditions, before storage at −80 °C.

Dark, autoclaved 125 mL penicillin flasks were used to avoid light and UV influences. To 45 mL medium (CPB broth), 5 mL of the fecal inoculum was added. For each condition, **three *in vitro* digestions** were done in parallel (replicates). As such the effect of moldy and mold-free corn on the detection of steroids could be tested. For both moldy and mold-free corn, the effect of adding additional phytosterols (corn + phytosterols and moldy corn + phytosterols) was tested as well. One negative control (blank *in vitro* digestion) was done in parallel. To complete the set-up, a control with only pure phytosterols as the phytosterol source was included as well (>50% β-sitosterol) (**Table 1**, p. 153). The medium volume was adjusted to obtain a constant total *in vitro* digestion volume in the flask of 50 mL for each condition. When adding pure phytosterols, 0.2% (v/v) Tween 80 was added to the *in vitro* digestion to solubilize the hydrophobic phytosterols [30].

The flasks were capped and anaerobic conditions were established using a flush system for 1 h, alternating every 2 min between N_2 (1 bar) and vacuum suction. The flasks were then **incubated for 72 h** (37 ± 0.5 °C, 150 rpm), conform the expected *in vivo* retention

time in the hindgut of 48 to 72 h [36]. Samples (3 mL) were taken after 0, (12), 24, 48 and 72 h of incubation. Sampling was done as secure and standardized as possible using syringes, causing as little disturbance as possible to the bacterial environment. Incubations were gently stirred to homogenize before sampling. Samples were stored at -20 °C prior to extraction. Extraction was performed according to the previously described extraction procedure (See **3.3 AEROBIC INCUBATION OF MOLDY FEED**).

Table 1. Experimental set-up of the *in vitro* digestion of moldy corn (n=3)

Name *in vitro* digestion	Faecal inoculum (mL)	CPB medium (mL)*	Corn (g)	Moldy corn (g)	Phytosterols (g)
Blank *in vitro* digestion	5	45	/	/	/
Phytosterols	5	45	/	/	0.1
Corn + phytosterols	5	40	5	/	0.1
Moldy corn + phytosterols	5	40	/	5	0.1
Corn	5	40	5	/	/
Moldy corn	5	40	/	5	/

*The total *in vitro* digestion volume was kept constant at 50 mL

3.7 UHPLC–TRIPLE QUADRUPOLE MS/MS ANALYSIS

Ultra-high performance liquid chromatography (U-HPLC) MS/MS detection was performed according to Decloedt *et al.* (2015) [10,29]. **Separation** was carried out using an Accela™ High Speed LC (Thermo Fisher Scientific, San Jose, CA, USA) with a Nucleodur™ Sphinx RP column (1.8 µm, 50 x 2.1 mm, Macherey-Nagel). The mobile phase consisted of ultra-pure water containing 0.1% formic acid (26.5 mM) as solvent A and methanol Optima® with 0.1% formic acid (26.5 mM) as solvent B. A gradient was run at 300 µl/min, starting with a linear gradient of 58% solvent B for the first 2 min, increasing to 100% solvent B at 5.5 min, and then kept at 100% solvent B for 1.5 min (up to 7 min). Afterwards, the column was allowed to equilibrate at the initial conditions of 42% A and 58% B for 2 min. All analytes could be accurately separated in a total run time of only 9 min [10].

Detection was carried out on a **TSQ Vantage Triple Stage Quadrupole Mass Spectrometer** (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Heated Electrospray Ionization probe (HESI-II). Injection volumes were 10 µL each and the HESI source was operated in the positive ion mode. It was found that positive ion mode afforded better sensitivity [37]. An ESI Ion Spray Voltage of 3 kV was applied. The sheath and auxiliary gas pressure were set at 45 and 15 arbitrary units respectively, the

capillary temperature at 310 °C and the heater temperature at 370 °C. Data were acquired in the multiple reaction monitoring (MRM) mode. The resolution of the quadrupole mass filter (Q1) was set with the peak width of 0.2 Da at half height, the Q3 filter at 0.7 Da at half height.

All specified product ions were used for peak integration for quantification purposes [10,29]. Data were interpreted using Xcalibur 2.1. w/Foundation 1.0.2 Rev. B qualitative and quantitative software (Thermo Electron, San Jose, USA). Area ratios were calculated relative to the **internal standard methyltestosterone** (MT, 5 ng/mL), that was added to both calibration and unknown samples, to compensate for losses during sample preparation or variability during the analytical determination. Methyltestosterone can be considered as a good internal standard as it is very similar to the calibrated analytes, chemically and in retention time, but chromatographically distinguishable, affordable and not endogenously present.

3.8 DATA ANALYSIS, QUANTIFICATION AND QUALITY ASSURANCE OF THE ANALYTICAL METHOD

Quantitative performance of the used analysis method, accuracy, precision, linearity and sensitivity was previously validated in different matrices (urine and *in vitro* digestion samples for αT, βT, AED, ADD, α-Bol and β-Bol) [10,29] (See also chapter II and IV).

Prior to each new analysis, the individual targeted compounds and standard mixtures were injected to check the **selectivity and operational conditions** of the chromatographic devices. The different metabolites were identified based on their relative retention time, relative to the internal standard. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio of at least 3 was 5 pg on column for all analytes of interest.

Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard. A **calibration curve** was constructed based upon ten fortification levels (0, 0.125, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 ng/mL) and this curve was run twice, before and after the experimental samples. Unknown samples were quantified by fitting the metabolites' area ratio in the calibration curve.

4. RESULTS

4.1 AEROBIC INCUBATION OF MOLDY FEED

Different moldy and mold-free feed samples were tested for the presence of AAS and related steroid precursors. Most of them did not contain steroids, yet one of the herbal phytosupplements did contain α-testosterone (up to 37 ± 3 ng/g). Although three different moldy feeds were monitored for 96 h (5 sampling points), at two different temperatures (RT and 37 °C) and in two different media (CPB and PBS), **mostly AAS free samples** were obtained. Upon incubation of moldy carrots and a spoiled commercial grain mix no AAS were detected. Although the aerobic incubation of **moldy corn** did not lead to the transformation of phytosterols to ADD, boldenone or βT, traces of αT and P (<0.5 µg/kg) were formed at one time point (24 h) and **AED** was formed throughout the entire incubation, in both CPB and PBS, at a comparable concentration (3.0 ± 0.4 µg/kg). At the higher temperature (37 °C, in PBS) the detected concentration was stable after 48 h, while the concentration tended to decline at RT (**Figure 3**).

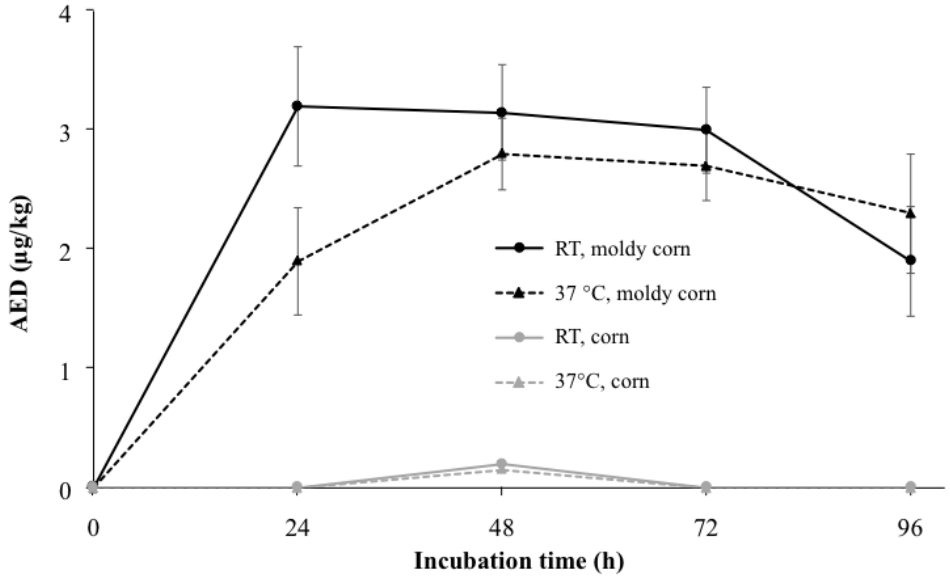


Figure 3. Aerobic transformation of phytosterols from moldy and mold-free corn to AED in CPB medium, at two different temperatures (RT and 37 °C).

4.2 AEROBIC INCUBATION OF A COMMERCIAL GRAIN MIX WITH *MYCOBACTERIUM* SP

In feed deliberately contaminated with *Mycobacterium* sp. 2967, a known phytosterol transforming agent (instead of spontaneously present molds), **βT was detected**, with both media (CPB and PBS) and at both temperatures (RT and 37 °C). ADD, AED, α-Bol, β-Bol, αT and P were not found. At the start of the incubation traces of βT were already

present in the samples and **no significant increase** was observed throughout the experiment. Therefore, we concluded that the feed itself was contaminated with βT, and the detection was not related to the incubation with *Mycobacterium* sp. 2967. As a result, the deliberate inoculation of feed with a known phytosterol transforming bacteria, *Mycobacterium* sp. 2967, did not lead to the production of AAS.

To ensure that the ingestion of *Mycobacterium* sp. and the resulting possible presence of these microorganisms in the equine hindgut could not influence the *in vivo* excretion of AAS, the **oxygen dependence** of mycobacterial cell growth and enzymatic activity was tested. Based upon the previous results (See also chapter IV), *Mycobacterium* sp. 2967 was chosen as the test strain, and only CPB medium was used [29]. The biotransformation capacity was evaluated under aerobic versus anaerobic conditions for a prolonged period of time (1.5 to 6 days).

Under anaerobic conditions, the bacteria did not grow and no transformation of phytosterols was observed either. Enzymatic activity and thus metabolism ceased immediately upon **oxygen restriction**. Only the positive control, with pure phytosterols added as the substrate and under aerobic conditions, kept its transformation potential. This implies that the potent phytosterol transforming *Mycobacteria* sp 2967 is strictly aerobic and as such can't be held responsible for the *in vivo* transformation of phytosterols to detectable AAS in the digestive system of the horse.

4.3 ACTIVITY OF EXTRACELLULAR ENZYMES EXCRETED BY CORN-RELATED MOLDS

Three morphologically different mold strains could be isolated from the moldy corn powder: a prominent ***Mucor* species** and two other less dense types that were present after prolonged incubation, a yellow and a white species (**secondary contributors** to the molding process). The extracellular enzymes from these three mold isolates and an additional negative control (UP H_2O) were tested for their biotransformation capacity of AAS precursors (AED/ADD/β-sitosterol) in PBS buffer (phosphate Buffered Saline). The endogenous mold levels were also measured, by incubating the extracellular enzymes without any added precursors.

To include the **effect of incubation time** on the biotransformation capacity, the biotransformation reaction was monitored at two different time points, after an incubation period of **1 and 4 h**. A negative control (PBS + UP H_2O), without any extracellular enzymes, was tested as well. Within 1 hour, the exposure of ADD (1 µM) to the

extracellular enzymes of the *Mucor* sp led to the **detection of β-Bol** (3.6 ± 0.8 ng/mL), while no β-Bol was detected when AED or pure phytosterols (>50% β-sitosterol) were added, nor in the negative control, without added precursors. No α-Bol was detected either. This concentration increased when exposure time was prolonged (up to 5.8 ± 0.1 ng/mL after 4 h). A low, yet significant, concentration of βT (0.15 ± 0.05 to 0.23 ± 0.01 ng/mL) was detected as well, while βT was not found when ADD was exposed to the extracellular enzymes excreted by the other isolates (**Table 2**).

Table 2. Detected steroids after exposure of ADD to the extracellular enzymes of three isolated corn-related mold species. No α-Bol, P or αT were found (n=4).

Species	βBol (ng/mL)		AED (ng/mL)		βT (ng/mL)	
	1h	4h	1h	4h	1h	4h
Blank	0.09 ± 0.01^{a}	0.08 ± 0.00^{a}	1.5 ± 0.1^{a}	1.4 ± 0.1^{a}	NF^{a}	NF^{a}
***Mucor* sp**	3.6 ± 0.8^{c}	5.8 ± 0.1^{d}	1.7 ± 0.5^{a}	1.2 ± 0.1^{a}	0.15 ± 0.05^{b}	0.23 ± 0.01^{b}
Yellow mold	0.2 ± 0.1^{b}	0.23 ± 0.02^{c}	2.2 ± 0.6^{b}	1.7 ± 0.1^{b}	NF^{a}	NF^{a}
White mold	0.2 ± 0.0^{b}	0.15 ± 0.00^{b}	2.6 ± 0.2^{b}	2.1 ± 0.1^{b}	NF^{a}	NF^{a}

NF = not found (<0.01 ng/mL)
^{abcd} = significant difference between mold strains and blank control samples (t-test, p = 0.05)

When **AED** was added as precursor the **detection of βT** (14 ± 3 ng/mL after 1 h and 20 ± 3 ng/mL after 4h) was the most prominent, and traces of ADD and αT could be detected as well (**Table 3**). When AED was added to the negative control (PBS + H_2O) a low concentration of βT could be detected within one hour (1.5 ± 0.1 ng/mL) and this concentration was maintained when prolonging the incubation time (1.4 ± 0.0 ng/mL). A low concentration of αT (<0.2 ng/mL) was also detected.

Table 3. Detected steroids after exposure of AED to the extracellular enzymes of three isolated corn-related mold species. No P, β- or α-Bol were found (n=4).

species	ADD (ng/mL)		βT (ng/mL)		αT (ng/mL)	
	1h	4h	1h	4h	1h	4h
blank	NF	0.05 ± 0.01^{a}	1.5 ± 0.1^{a}	1.4 ± 0.0^{a}	0.16 ± 0.01^{a}	0.15 ± 0.00^{a}
***Mucor* sp**	NF	0.04 ± 0.01^{a}	14 ± 3^{c}	20 ± 3^{c}	0.18 ± 0.01^{a}	0.18 ± 0.01^{b}
Yellow mold	NF	0.05 ± 0.01^{a}	2.0 ± 0.3^{b}	1.7 ± 0.1^{b}	0.17 ± 0.01^{a}	0.14 ± 0.02^{a}
White mold	NF	0.04 ± 0.01^{a}	2.4 ± 0.1^{b}	1.9 ± 0.2^{b}	0.18 ± 0.01^{a}	0.15 ± 0.02^{a}

NF = not found (<0.01 ng/mL)
^{abc} = significant difference between mold strains and blank control samples (t-test, p = 0.05)

The **addition of pure phytosterols** (>50% β-sitosterol) to the extracellular enzymes led to the detection of ADD and AED at comparable concentrations (<0.1 ng/mL). These small alterations in AED concentration were not significantly different from the negative control (**Table 4**). The addition of phytosterols to the *Mucor* sp. extracellular enzymes did lead to a low yet significant detection of ADD after 4h (0.02 ± 0.00 ng/mL) (in triplicate), which was not observed for any of the other strains nor in the negative control.

Table 4. Detected steroids after exposure of pure phytosterols (>50% β-sitosterol) to the extracellular enzymes of three isolated corn-related mold species.
No α-Bol, β-Bol, αT, βT or P were found (n=4).

Species	ADD (ng/mL)		AED (ng/mL)	
	1h	4h	1h	4h
blank	NF[a]	NF[a]	0.05 ± 0.03[a]	0.05 ± 0.02[a]
***Mucor* sp**	NF[a]	0.02 ± 0.00[b]	0.03 ± 0.02[a]	0.04 ± 0.02[a]
Yellow mold	NF[a]	NF[a]	0.04 ± 0.02[a]	0.04 ± 0.02[a]
White mold	NF[a]	NF[a]	0.07 ± 0.05[a]	0.06 ± 0.02[a]

NF = not found (<0.01 ng/mL)
[ab] = significant difference between mold strains and blank control samples (t-test, p = 0.05)

4.4 *IN VITRO* DIGESTION OF MOLDY FEED

Predigestive biotransformation of phytosterols in moldy feed (carrots, corn and commercial grain mix) showed that aerobically incubated moldy corn can contain up to 3.0 ± 0.4 µg/kg AED, an important anabolic-steroid precursor (**Figure 3**, p. 155). Therefore, this feed was subjected to the *in vitro* digestive simulation, a tool to study *in vivo* digestion, of which the functionality has been proven in previous studies [29,35]. No AAS were detected in the blank *in vitro* digestion without added precursors. When adding (moldy) corn and/or phytosterols (>50% β-sitosterol) to the *in vitro* digestion, AED was detected throughout the entire digestion period (0 to 72 h). The highest concentrations of AED were obtained for the combination of moldy feed with added phytosterols (0.28 ± 0.02 ng/mL) (**Figure 4**, p. 159).

The detected concentration was higher than the concentration detected when no additional phytosterols were added to the digestion (0.20 ± 0.05 ng/mL). The addition of mold-free corn and/or pure phytosterols (>50% β-sitosterol) led to low concentrations of AED in the digestion (0.05-0.12 ng/mL).

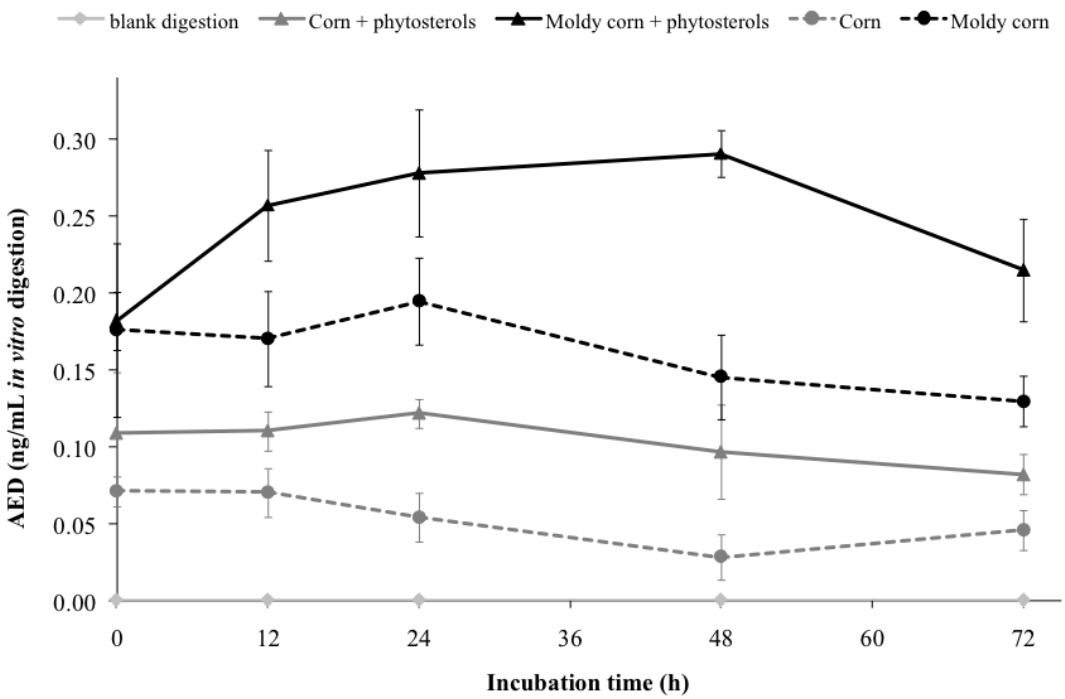


Figure 4. Detection of AED during the *in vitro* digestion of (moldy) corn.

5. DISCUSSION

Different moldy and mold-free feed samples were tested for the presence of AAS. Most of them did not contain AAS, yet one of the herbal phytosupplements showed to contain α-testosterone. In addition, the aerobic incubation of moldy corn showed that AED is formed and this in both PBS buffer and CPB medium, at a comparable concentration. Traces of αT and P were also detected. The detected concentration of **AED** was slightly higher at room temperature. As the majority of fungal species are **mesophiles**, growing at temperatures within the range 0–35 °C with the optimum growth temperature being 25–30 °C, this can be linked to the difference in biotransformation. The optimum growth temperature is closer to RT than to 37 °C [29]. On the other hand, neither moldy carrots nor the moldy commercial grain mix led to the direct production of AAS.

Two complementary explanations can be formulated to explain the difference between the different feed types. At the one hand, the **phytosterol content** differs between feedstuffs. Grains, and especially corn, are known to contain high levels of phytosterols (>1200mg per kg fresh weight) [38], while carrots only contain 100-200 mg of phytosterols per kg fresh weight [39]. The phytosterol concentration present in commercial grain mix was unknown (no analytical data available). Based on the

composition of the product it can be estimated as in between both values (500-1000 mg per kg fresh weight). On the other hand, corn was harvested directly from the field, while carrots and the commercial grain mix were bought in a shop. A different mold **contamination process** might as such be responsible for the moldy appearance, and as different mold species can vary in their (extra)cellular enzymes, this can differentially affect the phytosterol biotransformation potential [40]. The latter was confirmed by the extracellular enzyme experiments executed in this study.

The ***Mucor* species**, naturally present on corn, was up to ten times more efficient at transforming phytosterols to AAS precursors (AED and ADD), and AED and ADD to respectively βT and β-Bol, than the other two isolates. Studying more different isolates would allow monitoring the variation between different mold species and will be important to take into account the range of enzymes excreted by different mold species. Different species differ in their ability to degrade different substrates and few organisms have the potential to degrade all available plant cell wall components [41,42].

Generally, microorganisms adapted to use hydrophobic hydrocarbons, such as phytosterols, need to possess the following **physiological properties**: **(1) lipophilic cell walls** and adaptive changes in surface properties allowing direct adhesion to hydrophobic substrates, **(2) high affinity uptake systems** such as active transporters and membrane-associated enzymes for initial degradation, **(3)** the ability to excrete **biosurfactants** or bio-emulsifiers to increase the bioavailability of phytosterols.

Based on these properties, molds are interesting biotransformation candidates as they are capable of adapting to different substrates, due to their parasitic nature and capability to produce a wide variety of extracellular enzymes. The enzymatic potential of **fungi** has already been a matter of study for industrial applications of fungal extracellular enzymes e.g. microbial biodegradation of pollutants, including hydrocarbons (e.g. oil) [27,43,26]. Extracellular enzyme production supplements the direct uptake of nutrients and is linked to nutrient availability and environmental conditions.

Interestingly, for some mold species, including *Mucor* sp., it has been described that they can produce **biosurfactants**, compounds of microbial origin that exhibit surfactant properties [26,44]. These biosurfactants are specifically interesting in the light of phytosterol conversion, as phytosterols are difficult to solubilize, highly hydrophobic substrates that are as such difficult to access enzymatically. The industrial large-scale,

biological production of steroids from phytosterols therefore uses chemical surfactants such as ethoxylated sorbitan esters (Tween 60 or 80®) to create micro-emulsions, enhancing phytosterol solubilization and availability. Therefore, the excretion of biosurfactants is a crucial parameter in the identification of molds as possible phytosterols transforming agents.

Based on the results obtained with moldy feed samples, moldy and mold-free corn were used to study the effect of digestion of moldy feed on the horse's steroid profile. Due to ethical constraints, it was not appropriate to test the consumption of moldy feed *in vivo*. Alternatively, ***in vitro*** **incubation** systems have been developed to simulate the gastrointestinal digestion of horses [29,35], humans [45,46] and many other animals [47-49]. Though these types of *in vitro* batch cultures do have their limitations, i.e. absence of gastrointestinal absorption and lack of interaction with the host colonic mucosa, using an *in vitro* batch system enhances reproducibility; unlike when using an *in vivo* set-up, reaction parameters can be standardized.

For **horses**, results obtained *in vitro* showed to be in line with previously described *in vivo* results [50], proving that *in vitro* digestions are a useful tool to study the fermentation reactions that take place in the equine hindgut [29]. The focus is on the hindgut as complex fermentation reactions only take place in the caecum and colon, the enzymatic activity and digestion measured in the foregut of horses is fairly low [51], the α-amylase activity was estimated between 10 and 50 U/g [52] compared to 3500 U/g for other species such as humans and pigs [53].

When adding (moldy) corn and/or phytosterols (>50% β-sitosterol) to the *in vitro* digestion, AED was detected throughout the entire digestion period. The highest concentrations of AED were obtained for the combination of moldy feed with added phytosterols. In the latter case, the phytosterols were transformed by the combination of mold and hindgut microbial activity. **The combination of mold enzymatic activity and the *in vitro* digestion fermentation** showed to be crucial to power the transformation of phytosterols into AED, the main testosterone precursor.

Digestion of **mold-free corn and/or added pure phytosterols** (>50% β-sitosterol) gave rise to AED in the digestion as well, but at very low concentrations (≤0.1 ng/mL). As these concentrations were very close to or below the detection limit [29], they were not considered significant. The digestion of pure phytosterols, mold-free corn, and the blank

in vitro digestion without any added precursors did not lead to the significant detection of AED or other steroids, while the concentration of AED produced through the digestion of moldy corn (with and without added phytosterols) was significant.

The difference between moldy corn **with and without added phytosterols** indicates that the conversion with added phytosterols is higher, probably due to the fact that the bioavailability of these phytosterols exceeds the bioavailability of the phytosterols embedded in corn. *In vivo,* the stomach and small intestinal digestion will contribute to this bioavailability. When adding phytosterols to the extracellular enzymes of all three corn-related mold isolates, AED peaks were detected as well, but not to a significant level. As a result, it may be deduced that the extracellular enzymes were not able to directly transform β-sitosterol into AED, but that the combination of mold enzymes and fermentation was crucial to detect low, yet significant, levels of AED.

The **extracellular enzyme experiments** with the *Mucor* corn isolate revealed the formation of ADD, the main boldenone precursor, from pure phytosterols (>50% β-sitosterol), but at a very low rate. ADD was not detected after 1 hour of incubation but 4 hours of incubation with phytosterols did lead to the detection of ADD. Studying the effect of a longer **incubation time** could be useful to reveal the full biotransformation potential of the *Mucor* species. Additionally, the isolated *Mucor* species showed to be very efficient at transforming ADD to boldenone, supporting the possibility of a two-step reaction from pure phytosterols to ADD to β-Bol. When supplied with AED, the *Mucor* sp isolate could form up to 20 ng/mL βT (after 4 hours).

Both other secondary, mold isolates were also able to transform **ADD to boldenone**, but less efficiently. Their biotransformation capacity was barely higher than that of the negative control. In line with the *Mucor* sp isolate, the other two isolates were able to form βT and traces of αT out of AED. However, the detected concentrations for these two isolates were 3 to 10 times lower than those obtained with the *Mucor* species. Still, qualitatively, the results were quite comparable.

Additionally, the formation of certain steroids in the blank enzyme suspension (PBS buffer + H_2O, without any enzymes) was low, yet present. Minor impurity of the added supplements (≥99% purity) or spontaneous oxidation or reduction in the presence of oxygen and water could be responsible for this **baseline detection**. This confirms the

importance of comparing the extracellular mold enzyme results to the results of control samples, as marked in the introduction.

The suit of processes taking place between consumption of feed to urinary excretion and detection of steroids is very **complex**, involving many different contributors and side-reactions. In this study we focused on biotransformations taking place in the feed itself, catalyzed by mold enzymes, and the effect of the equine hindgut digestive processes (including microbial fermentation) on the digestion of (moldy) feed. Other metabolic pathways can additionally contribute to the formation of intermediate precursors (e.g. AED to testosterone). Recent work by Fabregat *et al.* (2015) confirmed the formation of 1,4-androstadien-3,17-dione (ADD), 4,6-androstadien-3,17-dione, 17β-hydroxy-4,6-androstadiene-3-one and 17β-hydroxy-1,4-androstadiene-3-one (boldenone) upon **hepatic phase I metabolism** of T. Analogously to T, this pathway leading to the formation of Δ1 and Δ6 metabolites, might also prevail for other steroids, opening the possibility of targeting additional biomarkers [54].

Moreover, compounds and phase I metabolites may be further reduced and/or modified during **phase II catabolism** in the liver. The set of phase I and II products could in turn be transformed in the hindgut by the wide range of microorganisms, under aerobic or mainly anaerobic conditions. This enterohepatic circulation might additionally increase the yield of biochemical modifications by reprocessing the "flow-through" [55]. Yet unexplored and thus unchartered reactions and reaction products of the enterohepatic circulation should be taken into account, when considering the connection to the urinary excretion of steroids.

This biological complexity is extremely difficult to reproduce *in vitro*. Based on the results obtained in this study, additional ***in vivo* research** is warranted to further unravel the correlation between the controlled consumption of phytosterol rich or enriched feed, albeit exposed to mold enzymes, and the urinary and faecal excretion of steroids in horses.

6. CONCLUSION

Different moldy and mold-free feed samples were tested for the presence of anabolic-androgenic steroids. Moldy corn showed to contain low levels of AED, the main testosterone precursor. The effect of digestion of moldy corn was tested by *in vitro* simulation of the horse's hindgut. The *in vitro* digestion of moldy corn with phytosterols showed the highest AED concentrations, suggesting that AED was produced as a result of the phytosterols being subjected to the combined effect of fermentation and mold-related extracellular enzyme activity.

However, in most other feed samples no AAS were detected, even when the products were molded. These results prove that plant phytosterols cannot be held directly responsible for the excretion of AAS in horses, but it is important to caution against the consumption of any feed or herbal supplement of which the detailed ingredients and quantitative analysis are unknown. Especially the consumption of moldy corn should be avoided, not only from a horse health and welfare point of view, but also to avoid a possible inadvertent positive doping result, as these crops can contain low levels of steroids.

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CHAPTER VI

Influence of glucocorticoid treatment on the excretion of anabolic-androgenic steroids in equine urine: *in vivo* case study and *in vitro* simulations

Adapted from:

"Influence of glucocorticoid treatment on the excretion of anabolic-androgenic steroids in equine urine: *in vivo* case study and *in vitro* simulations"

By Anneleen Decloedt, Sander Damen and Lynn Vanhaecke, *Equine Veterinary Journal*, submitted

CHAPTER VI

1. ABSTRACT

Reasons for performing study. Anabolic-androgenic steroids (AAS) are strictly forbidden in equine sports because of their stimulating effect on muscle growth and performance. Nevertheless, low levels of AAS have been found in some untreated horses. Glucocorticoids (GC), used as an anti-inflammatory therapy and structurally related to AAS, might play a role in this phenomenon.

Objectives. In this study the influence of glucocorticoid treatment on the excretion of low levels of AAS in horse urine was studied both *in vivo* and *in vitro*, in order to unravel the possible correlation between glucocorticoid treatment and the detection of AAS.

Study Design. The *in vivo* effects of glucocorticoid treatment on the AAS excretion profile were investigated by analysing urine samples collected from a gelding treated intra-articular with betamethasone (11.4 mg). Additionally, multiple *in vitro* digestion simulations were set up to study the possibility of a direct biotransformation of natural and synthetic glucocorticoid to AAS, by the microbiota of the equine hindgut.

Methods. Urine and *in vitro* digestion samples were extracted and analysed with fully validated UHPLC-MS/MS and UHPLC-Orbitrap-HRMS analytical methods. *In vitro* digestion simulations were performed according to a previously validated protocol.

Results. A significant influence of betamethasone treatment on the urinary excretion of α-testosterone (αT), β-testosterone (βT) and androsta-1,4-diene-3,17-dione (ADD) was seen. αT-concentrations up to 20 ng/mL were detected. ADD was not found before treatment but could be detected up to 93 days post-treatment. Cortisone and cortisol also peaked (>30 ng/mL) between day 37 and 48 post-treatment. The *in vitro* digestion results revealed no direct biotransformation of glucocorticoids to AAS by the microbiota of the equine hindgut.

Conclusions. This study shows that an intra-articular glucocorticoid treatment can trigger the excretion of AAS in urine, not by direct biotransformation upon gastrointestinal digestion, but more likely by influencing the hypothalamic-pituitary-adrenal axis.

2. INTRODUCTION

Given their potential to increase physical performances, **anabolic-androgenic steroids** (AAS) are frequently being abused in equine sports [1-3]. The abuse of these steroids has pushed regulatory institutes, like FEI (Fédération Equestre International) and IFHA (International Federation of Horseracing Authorities), to develop appropriate and reliable analytical methods to confirm abuse of these prohibited substances. For most compounds, zero-tolerance policy is held. In other cases, international thresholds can be adopted for substances endogenous to the horse [4, 5].

17β-Testosterone (βT) (**Figure 1**) for example is generally accepted as an endogenous androgenic-anabolic steroid [2, 6]. Other AAS, with 17β-boldenone (ß-Bol or 1-dehydrotestosterone, **Figure 1**) as a mere suspect, might be of endogenous origin as well and are under further investigation. ß-Bol has been demonstrated to be naturally present in entire male horses [7, 8]. Other steroids, such as androsta-1,4-diene-3,17-dione (ADD or boldione) and androst-4-ene-3,17-dione (AED), can function as a precursor for 17β-boldenone and 17β-testosterone, respectively, in various animal species [7], including horses [8-10].

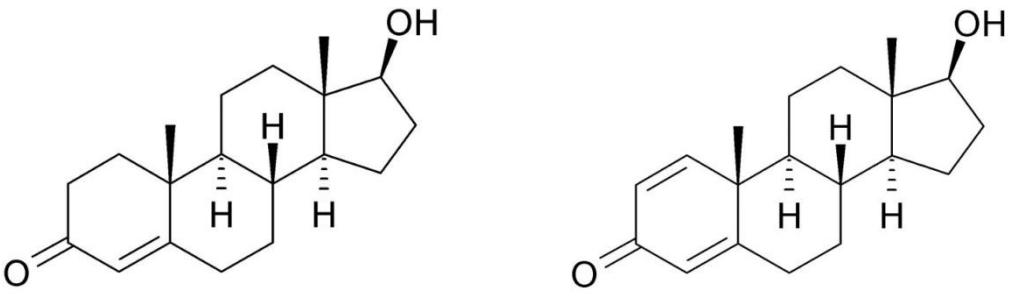

Figure 1. Chemical structures of 17β-testosterone (ßT) and 17β-boldenone (ß-Bol). ß-Bol differs from βT only by one double bond at the 1-position.

The current **FEI prohibited substances** list (2015) includes an international threshold of 15 ng ß-Bol (free and conjugated) per mL urine of entire male horses, whereas the presence of ß-Bol in urine from mares and geldings is still prohibited [4, 5]. Nevertheless, low concentrations (<5ng/ml) of AED, ADD and ß-Bol have been reported in routine analysis (personal communication) and after urinary screening of untreated horses [9].

The potentially **endogenous presence** of 17β-boldenone and related compounds might be a complicating factor in doping control [11]. Proper investigation on the origin of these AAS is required to guarantee adequate doping policies in the future.

Earlier studies have shown that dietary contaminants in feed can cause horses to get a positive doping result in urine analysis [12], pointing at the **equine enteric tract** as a potential source of ß-Bol or related steroids. Hence, the hypothesis that glucocorticosteroids (GC), being structurally related to the group of AAS, can be transformed to AAS by the microbiota of the equine colon was formulated.

Glucocorticoids are endogenously secreted by the adrenal cortex, under control of the hypothalamic-pituitary-adrenal axis (HPA axis). Hypothalamic corticotrophin-releasing hormone (CRH) initiates the secretion of adrenocorticotropic hormone (ACTH) in the pituitary gland. Glucocorticoid receptors in the hypothalamus inhibit CRH-secretion, thereby establishing a classical endocrine regulatory negative feedback loop [13].

Endogenous glucocorticoids are classically known to be secreted as a reaction to **stress** [14]. At rest (n = 50) cortisol levels are at 24 ng/mL. Reference values for cortisol levels after race (n = 100) are 70 ng/mL, and after endurance (n = 50) cortisol levels can go up to 65 ng/mL (personal communication L.C.H.). Other biological functions include the control of energy homeostasis and the suppression of inflammation [15]. Because of these **anti-inflammatory** effects, glucocorticoid treatments are frequently used in equine medication [16].

The **structural resemblances** between glucocorticoids and AAS increase the probability of a possible transformation, as compounds belonging to both groups contain the same steroid (sterane) skeleton and have common precursors [17]. Glucocorticoids though possess 21 C-atoms in total (**Figure 2**, p. 170), whereas the androgenic steroids only have 19 C-atoms (androstanes) (**Figure 1**) [18]. This structural difference is in line with the glucocorticoid character, requiring a C11-hydroxyl group, and respectively androgenic character of the components [19].

Supporting this hypothesis is the fact that the detection of anabolic-androgenic steroids (ß-Bol, AED and/or ADD) in urine often coincided with a glucorticoid administration in the weeks or months before sampling [9] (See also chapter II).

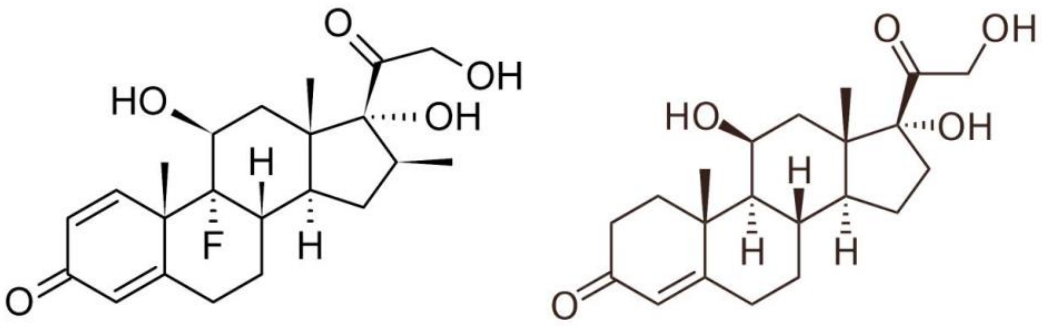

Figure 2. Chemical structures of hydrocortisone (cortisol, endogenous GC) and betamethasone (synthetic GC). The anti-inflammatory activity of the synthetic corticoid betamethasone has proven 25 times higher than the relative activity of the endogenous corticoid cortisol (hydrocortisone).

3. MATERIAL AND METHODS

3.1 IN VIVO EXPERIMENTAL SET-UP

To elucidate the *in vivo* effects of a glucocorticoid treatment on a horse's urinary AAS excretion profile urine samples were collected from a gelding with mild bone spavin treated with 1 mL Chronodose (Celestone®) in each hock. The term '**bone spavin**' describes degenerative osteoarthritis of the hock [20]. As in most cases, the two most distal joints were affected (*art. centrodistalis* and art. *tarsometatarsea*). Intra-articular injection of betamethasone is valuable in this regard for its anti-inflammatory and analgesic properties.

Chronodose is a frequently used formulation in equine medicine that contains two forms of betamethasone: **betamethasone-acetate and bethamethasone-disodiumphosphate**. Betamethasone-acetate (3 mg/mL = 2.7 mg/mL betamethasone) is responsible for the medicament's long term effects. By esterification, a depot effect is created, out of which betamethasone is slowly released over time, whereas betamethasone-disodiumphosphate (4 mg/mL = 3 mg/mL betamethasone) renders the glucocorticoid activity on the short term [21].

Urinary samples were collected and analysed on a regular basis to monitor the excretion of AAS, such as ß-Bol or its precursors, and (endogenous) glucocorticoid, over time. Three samples were collected before treatment (day -10, -2 and 0) and 36 samples over the following four months post-treatment (up to day 122). All samples were midstream

urine samples captured in sterile 50 mL-tubes and stored at -20 °C until extraction and further analysis. All samples were taken in the evening (**between 7 and 9 p.m**), limiting the influence of diurnal rhythm related variation of cortisol and testosterone concentrations (See also chapter I).

As **spontaneously voided samples** were collected and the horse was treated for medical reasons, and not specifically in the light of this study, according to the latest Belgian and European animal welfare rules (RD 29th of May 2013, published on the 10th of July), this horse was not considered to be an experimental animal. As such, the authors state that they have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations. In addition, informed consent had been obtained from the owner of the horse. External influences on the ACTH-axis were kept to a minimum as both diet and environmental conditions were controlled. Throughout the entire follow-up period (pre- and post-treatment) the horse was kept on a normal, non-controlled **diet** of grass and hay (24 h/day), in a **stable herd**. Throughout the entire period of monitoring, the horse did not suffer from any other condition other than the mild bone spavin the horse was initially treated for.

3.2 IN VITRO SIMULATION OF THE EQUINE HINDGUT: BIOTRANSFORMATION OF GLUCOCORTICOIDS

In order to investigate the **possible biotransformation** of glucocorticoids into AAS by microbial fermentation, *in vitro* simulations of the equine hindgut were set up. Faecal inocula from four different horses were incubated *in vitro* to simulate equine hindgut digestion, following a previously described protocol, of which the functionality had been shown by full-fledged validation: all results obtained following this protocol were in line with *in vivo* results, proving that these *in vitro* digestions are a useful tool to study the fermentation reactions that take place in the equine hindgut [22, 23].

In this set-up a variety of glucocorticoids was tested. **Cortisol** or hydrocortisone (**Figure 2**, p. 170) was chosen based on its natural presence under stress. Hydrocortisone is the main endogenous glucocorticoid in large mammals, hydrocortisone blood levels rise when the horse is exposed to an acute or chronic stressor [24, 25]. **Cortisone, prednisolone and betamethasone** were also tested as these are frequently used by veterinarians as anti-inflammatory treatment. Cortisone is specifically interesting as it is the inactive form of cortisol/hydrocortisone and therefore suitable as a cortisol-prodrug

[19, 26]. Betamethasone was also included as it is a highly potent anti-inflammatory drug, often used in joint disease therapy (e.g. the horse studied in the *in vivo* section of this study) [20, 27].

Spontaneously discharged, **fresh faeces** was collected from four healthy, untreated horses, fed on controlled diet of concentrate (4 kg/day), hay (10 kg/day) and straw bedding. Faecal slurry was made by adding 1/5 (w/w) phosphate buffered saline and homogenization in a stomacher. Glycerol (99.5%, Analar Normapur, Fontenay-sous-Bois, France) was added at 20% (v/v) ratio, after which the inoculum was stored at -80 °C.

CPB (**Cysteine Peptone Bouillon**) consists of a mixture of 5 g yeast extract (AppliChem, Darmstadt, Germany), 1 g peptone (Oxoid, Hampshire, England), 8.5 g NaCl (Merck, Darmstadt, Germany), 0.5 g L-cysteine (SAFC Supply Solutions, St. Louis, Missouri, added to improve anaerobicity), and 10 mL haemin solution (Sigma-Aldrich, Steinheim, Germany) in 1L ultrapure water [28, 29]. The CPB broth was autoclaved at 121 °C for 15 min (1 atm), and stored at 4 °C until usage. All handlings of the *in vitro* digestion were done in a laminar flow cabinet.

Dark, autoclaved 125 mL **penicillin flasks** were used to avoid light and UV influences. 45 mL CPB medium and 5 mL faecal inoculum (horse n° 1-4) was added. Cortisone, prednisolone, cortisol or betamethasone were separately added to 3 flasks each (same digestion in triplicate). An additional negative control was included for each horse, without adding a precursor. The flasks were capped and anaerobic conditions were established using a flush system for 1 h, alternating every 2 min between N_2 (1 bar) and vacuum suction. The flasks were then incubated for 72 h (37 ± 0.5 °C, 150 rpm). Samples (3 mL) were taken after 0, 12, 24, 48 and 72 h of incubation. Incubations were gently stirred to homogenize before sampling. Samples were stored at -20 °C prior to extraction.

3.3 LC-MS REAGENTS AND CHEMICALS

α-testosterone (androst-4-ene-17α-ol-3-one, αT, purity ≥99%), ß-testosterone (androst-4-ene-17ß-ol-3-one, ßT, purity ≥99%), methyltestosterone (androst-4-ene-17α-methyl-17ß-ol-3-one, MeT, purity ≥99%), androstadienedione (androsta-1,4-diene-3,17-dione, ADD, purity ≥99%), cortisol (hydrocortisone or 17-hydroxycorticosterone, purity ≥98%), cortisone (17-hydroxy-11-dehydrocorticosterone, purity ≥ 98%), dihydrocortisone, prednisone, methylprednisolone, prednisolone (1-dehydrohydrocortisone, purity ≥99%), betamethasone (9α-Fluoro-16β-methylprednisolone, purity ≥98%), cortisol-d4 (purity

≥99%) proteinase type XXIII from *Aspergillus melleus* (3 enzyme units per mg) and β-glucuronidase (*Helix Pomatia*, aqueous, >100.000 units per mL) were purchased from Sigma-Aldrich (St-Louis, US) and prednisolone-d8 from TRC (Canada). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥99%), α-boldenone (androsta-1,4-diene-17α-ol-3-one, αBol, purity ≥99%) and ß-boldenone (androsta-1,4-diene-17ß-ol-3-one, ßBol, purity ≥99%) were obtained from Steraloids (Newport, US). Progesterone (purity ≥99%) was obtained from Alpha Pharma (Omega Pharma, Zwevegem, Belgium). Methanol Optima® was bought at Fisher Scientific, UK Limited (Leicestershire, UK). Diethyl ether, ethyl acetate, sodium hydroxide, methanol (analytical grade), H_2SO_4, KH_2PO_4 and formic acid (98-100%, analytic grade) were purchased from VWR (Merck, Darmstadt, Germany).

Solvolysis solvent consisted of 900 mL ethyl acetate, 95 mL methanol and 5 mL H_2SO_4 per liter. The HF Bond Elut-C18 cartridges (6 mL, 500 mg) were obtained from Agilent Technologies (Diegem, Belgium). HPLC grade, ultrapure water was acquired from an in-house water purification system (Arium® 611UV, Sartorius Stedium Biotech, VWR, Haasrode, Belgium). (Glucocortico)steroid stock solutions were made in methanol Optima® at 25 ng/µL and kept at 4 °C.

3.4 EXTRACTION OF URINE AND *IN VITRO* DIGESTION SAMPLES

3.4.1 Hydrolysis

Hydrolysis **increases the sensitivity** of detection, as pooling non-conjugated and released sulphate and glucuronide-conjugated fractions allows to increase the detectable concentration of free compounds. 1 mL of phosphate buffer (1M KH_2PO4, pH 6.1 ± 0.1) was added to 3 mL of sample. Next, the internal standard (methyltestosterone, 5 ng/mL), 50 µl of a ≥450 units/mL protease solution and 25 µl ß-glucuronidase were added. Of each sample the pH was set at 6.1 ± 0.1 (by adding 1M HCl) and hydrolysis of the **glucuronide-conjugated** steroids was executed at 55 °C (1 hour). Afterwards, 3 mL of ultrapure water was added and large, non-hydrolysed proteins were removed by agglutinating them at the bottom of the tube through centrifugation (4000 *x* g, 15 min). Finally, the supernatant was filtered over a cotton wool filter before solid phase extraction (SPE).

3.4.2. Solid phase extraction (SPE)

SPE cartridges (6 mL, 500 mg C18, Bond Elut, Isolute) were conditioned with 4 mL methanol and 4 mL ultrapure water. The centrifuged and filtered samples were loaded onto the column and washed with consecutively 6.5 mL ultrapure water and 6.5 mL hexane. The cartridges were dried under vacuum (-0.5 bar). Non-conjugated and glucuronide-conjugated fractions were eluted with diethyl ether (7 mL) and the sulphate-conjugated fraction with solvolysis solvent (6.5 mL). Solvolysis of the **sulphate-conjugated steroids** was executed at 55 °C (2 hours). Both fractions were washed with 1.5 M sodium hydroxide (5 mL) by turning (8 min, 60 rpm) and centrifugation (6 min, 3000 *x* g).

3.4.3. Pooling and reconstitution

Washed fractions were pooled and dried under nitrogen (50 °C, 45 min). Each sample was reconstituted in 100 µl of ultrapure methanol, vortexed and ultrasonicated (3 min). Finally, 100 µl of ultrapure water was added, vortexed and ultrasonicated as well. After centrifugation (9000 *x* g, 10 min) the sample was transferred to a LC-MS vial with insert for analysis.

3.5 ANALYTICAL METHODS (UHPLC-MS/MS AND UHPLC-ORBITRAP-HRMS)

Of each sampling point (39 in total) three 3 mL aliquots were extracted and analysed with two different analytical methods: a UHPLC-MS/MS and an Orbitrap-MS method. Mean concentrations and the standard deviation on the mean concentrations were evaluated.

The **UHPLC-MS/MS method** used an Accela™ autosampler and Accela™ High Speed LC (Thermo Fisher Scientific, San Jose, USA) coupled to a MS/MS TSQ Vantage Triple Stage Quadrupole™ benchtop mass spectrometer (UHPLC-MS/MS, Thermo Fisher Scientific, San Jose, USA). This method was previously validated for quantitative detection of AAS in urine and *in vitro* digestion samples [9, 22].

For the analysis of glucocorticoids, the authors used the untargeted method described by De Clercq *et al.* (2014), with the use of an UHPLC system coupled to a high-resolution Orbitrap Exactive™ benchtop mass spectrometer (**UHPLC-HR-Orbitrap-MS**, Thermo Fisher Scientific, San Jose, USA) [30].

3.6 QUANTIFICATION

Prior to each analysis, the individual targeted compounds and standard mixtures were injected to check the selectivity and operational conditions of the chromatographic devices. The different metabolites were identified based on their relative retention time, relative to the internal standard. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio of at least 3 was **5 pg on column** for all analytes of interest. All specified product ions were used for peak integration for quantification purposes.

Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard. In addition, the **standard addition approach** was used for urine specific quantification. The applied formula, explained in detail in Decloedt *et al*. (2015), with C_A representing the unknown urinary concentration, is:

$$C_A = \frac{Ar_A}{(Ar_B - Ar_A)\ x\ \rho}$$

A varying **fluid intake** between sampling can result in fluctuations of the urinary flow, and hence dilution or concentration of urinary components [31]. To facilitate proper interpretation of, and comparison between, urine samples, corrections had to be made to the measured concentrations. Urine densities were measured with a Digital Hand-Held 'Pocket' Urine S.G. refractometer (PAL-USG (CAT), Atago, Tokyo, Japan). Urine samples were allowed to equilibrate to room temperature (23 °C) before measuring urine densities, as in healthy individuals a good correlation between specific gravity (SG at room temperature) and osmolality is obtained [32].

Cone *et al.* (2009) specifically described a method of correction ('normalization procedure') based on specific gravity, which the authors used to correct for excessive dilution or concentration of the urinary components:

$$\text{Concentration}_{\text{normalized}} = \frac{\text{SG}_{\text{ref}}-1}{\text{SG}_{\text{specimen}}-1} * \text{Concentration}_{\text{measured}}$$

SG_{ref} is the reference specific gravity and $SG_{specimen}$ was given by the specific gravity of the urine sample of interest, determined for each sample by measuring the degree of light refraction with the refractometer. All concentrations presented in the results section are SG normalized concentrations.

4. RESULTS

4.1 URINARY PROFILE AFTER INTRA-ARTICULAR ADMINISTRATION OF BETAMETHASONE

In the post-treatment urine samples only traces of betamethasone (<0.5 ng/mL) could be detected, and only in the first days post-treatment. The endogenous glucocorticoids, cortisone and cortisol (hydrocortisone), were present before treatment at concentrations ranging between 1 and 5 ng per mL, repressed for a few days shortly after treatment (0 to 3 ng per mL) and peaked between day 37 and day 48, up to respectively 36 ± 2 ng per mL and 33 ± 2 ng per mL. This suppression and rise of production of endogenous glucocorticoids (**Figure 3A**, p. 177) is possibly induced by the HPA axis as a part of the negative feedback system.

Simultaneously, a yet unknown effect on the excretion profile of the anabolic-androgenic steroids ADD, α-testosterone (αT) and β-testosterone (βT) was seen post-treatment (**Figure 3B**, p. 177). A temporary decrease of urinary αT and βT levels was detected shortly after treatment, whereas ADD, formerly not found in the gelding's urine, appeared in low concentrations (up to 4 ng per mL), as long as 93 days post-treatment. Additionally, αT-levels peaked between day 48 and day 77 (12 ± 2 ng per mL), short after the incline of endogenous glucocorticoids levels. These results suggest a correlation between the increase in AAS excretion and the endogenous glucocorticoid overshoot after exogenous glucocorticoid suppression.

In general, the analysis of post-treatment urine samples revealed that the influence of the treatment undoubtedly could be measured, up to several weeks after betamethasone intra-articular infiltration. The overall impression was a disruption of pre-treatment AAS concentrations, a change in mutual proportions as well as in their long-term concentration pattern. ADD or boldione for instance, was not found before treatment, but could be found for weeks after treatment, with concentrations up to 4 ± 1 ng mL.

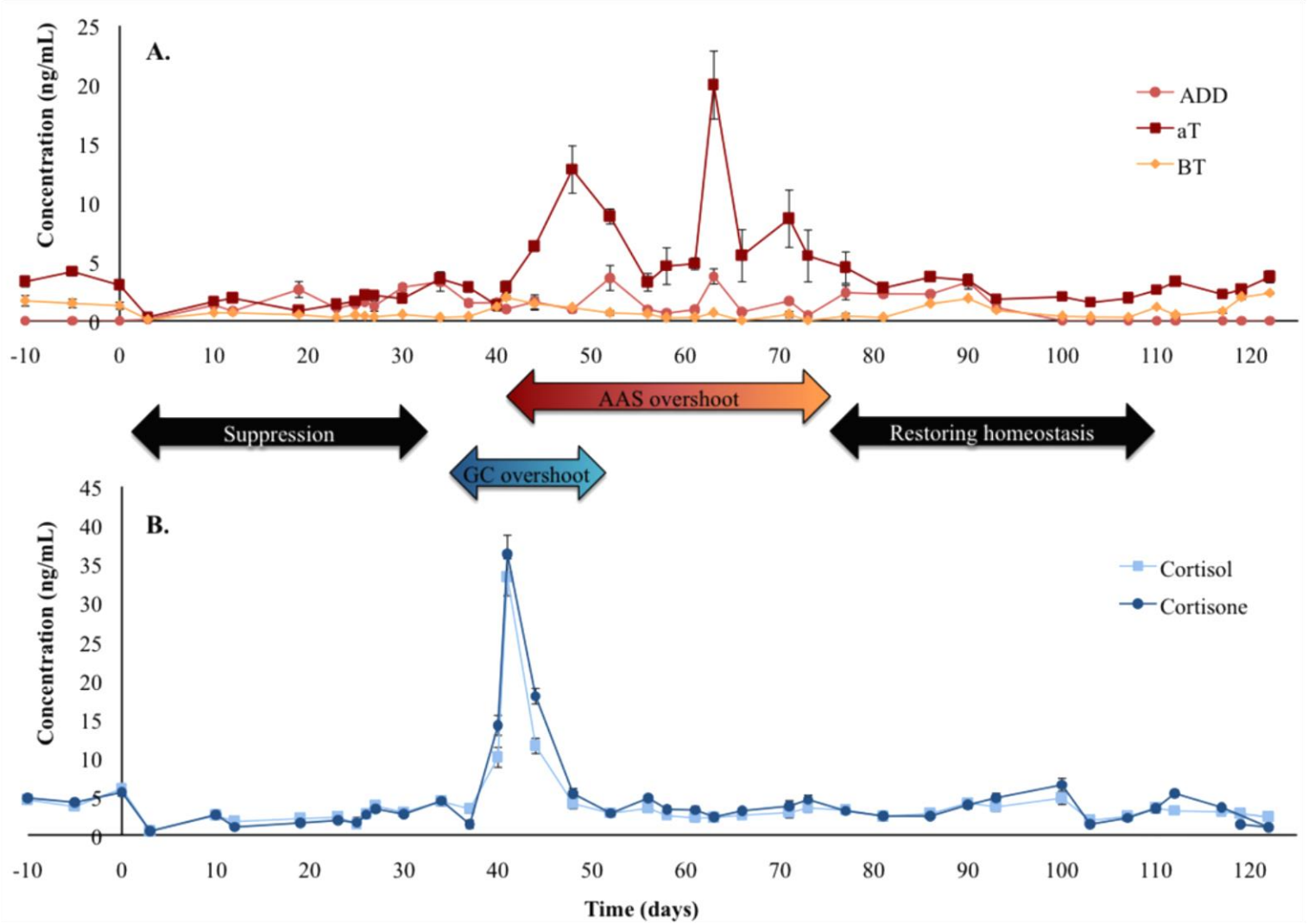

Figure 3. Long term effect of an intra-articular administration of the glucorticoid betamethasone (day 0) on the urinary excretion of (A) endogenous and semi-endogenous anabolic-androgenic steroids. and, **(B)** (endogenous) glucocorticosteroids

4.2 *IN VITRO* DIGESTION: BIOTRANSFORMATION OF GLUCOCORTICOIDS

No significant concentration of AAS could be measured after analysis, in either series of *in vitro* digestion simulations (different equine inocula) with respectively cortisol, betamethasone, cortisone or prednisolone. No direct transformation from glucocorticosteroids to AAS was shown in the hindgut.

5. DISCUSSION

Based upon the results obtained from this case study a hypothesis regarding the interconnection between glucocorticoid administration and AAS excretion can be formulated. Following the exogenous, intra-articular injection of betamethasone, pituitary ACTH-release dropped, causing the **endogenous adrenal glucocorticoid**-synthesis to stagnate (measurable as a decline in both cortisol and cortisone levels) [13]. In humans, it has already been described that glucocorticoid therapy, and especially prolonged high dose systemic therapy, can have a suppressive effect on endogenous steroid production. Therefore, dose and duration of glucocorticoid therapy should be kept as low as possible [33].

When urinary betamethasone elimination continued and betamethasone effect decreased, the regulatory (positive) feedback loop was activated, resulting in higher endogenous glucocorticoid-production and ultimately even an **overshoot** of endogenous glucocorticoids that was seen from day 37 to 48 for cortisol and cortisone. This overshoot was then quickly normalized by the regulatory system, revealing glucocorticoid urinary levels as before and restoring homeostasis. This demonstrates the adequate control of glucocorticoid concentrations by the HPA axis. Shortly after the GC overshoot emerged, a significant **rise in αT levels** was detected, implicating a possible correlation between increased (endogenous) glucocorticosteroid concentrations and raised urinary αT levels.

The horse in this study was only suffering from mild bone spavin, therefore only 2x1 mL (a total of 11.4 mg betamethasone) was injected. Consecutively, **traces of betamethasone**, the originally injected glucocorticoids, were only found during the first days post-treatment (<0.5 ng per mL). This was in line with previously obtained results by Vine *et al.* (2007), who noted that following intra-articular injection of 1 mL of Celestone Chronodose® into both radial carpal joints of both front knee joints,

betamethasone could be detected in urine and plasma for three days and one day, respectively and no betamethasone acetate could be detected in either urine or plasma [34].

Higher initial glucocorticoid doses are however quite common in veterinary medicine, glucocorticoid doses up to 0.06 mg/kg body weight are therapeutically used in horses, by which it is recommended not to exceed a total body dose of 30 mg betamethasone and respect a limitation of 15 mg per joint. In acute cases of atopic or flea allergy dermatitis, anti-inflammatory dosages (prednisolone, 0.5–1 mg/kg/day) alleviate pruritus and limit self-trauma from scratching until the underlying cause can be addressed. Similar dosages are used in the management of chronic allergic bronchitis and feline asthma [35].

When using these higher initial doses it can be expected that the effects as seen in this *in vivo* experiment will be more pronounced. When achieving higher initial glucocorticoid levels, the sensitivity of the analytical methods (UHPLC-Orbitrap-HRMS system for glucocorticoids) might be met to a better degree, enabling a more profound analysis of betamethasone and its metabolites, and the effects on endogenous AAS and glucocorticoids over a longer period of time.

Based on the results of the *in vitro* digestion experiments, the authors can yet reject the hypothesis in this concern; **no direct (intestinal) biotransformation** of glucocorticosteroids to AAS by microbial fermentation in the equine hindgut could be detected. The *in vivo* case study on the other hand supplies sufficient preliminary results to support an **indirect interconnection** of glucocorticoid treatment and the detection of AAS, probably by influencing the HPA axis. The amount of testosterone synthesized is regulated by the HPA axis. When testosterone levels are low, gonadotropin-releasing hormone (GnRH) is released by the hypothalamus, which in turn stimulates the pituitary gland to release the follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These latter two hormones respectively promote spermatogenesis in the Sertoli cells, while LH stimulates **synthesis of testosterone** in the Leydig cells of the testis (stallions), thecal cells of the ovaries, placenta (mares) as well as in the zona reticularis of the adrenal cortex (geldings, stallions and mares) (See also chapter I, **Figure 1.4**).

This case study can be regarded as a **first step** in the unravelling of the possible correlation between the administration of glucocorticoids and the excretion of anabolic-

androgenic steroids in equine urine. Elaborating on these initial findings, large scale experiments, will allow to include the effect of **additional factors** (such as stress or pain, training, diet and disease) influencing the andrenal gland activity. As in this study, such **large scale experiments** should include the analysis of both pre- and post-treatment urine samples, compared to a group of untreated control horses (both geldings and mares). Additionally, based upon these first results, experiments elucidating the effect of **other glucocorticoids** (e.g. prednisolone and cortisone) on the steroid excretion profile in horses are to be considered as well.

Overall, experiments as those described in this study conduce to the elucidation of possible **alternative origins of endogenous AAS**, such as ß-Bol, and contribute to a future where proper and honest doping policies can be applied in equine sports. Overall, the experiments indicate a significant long-term influence of glucocorticoid treatment on the AAS urinary excretion profile. Given the fact that glucocorticosteroids play an important role in worldwide anti-inflammatory therapy, especially in horses and equine sports, it stands clear that further research is needed to elucidate the exact correlation with the excretion of anabolic-androgenic steroids. to avoid possible inadvertent doping results for AAS, following glucocorticoid treatment (See also Chapter VII).

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CHAPTER VII

General Discussion and Future Perspectives

GENERAL DISCUSSION

1. POSITIONING OF THE RESEARCH

1.1 ECONOMICAL RELEVANCE OF EQUINE SPORTS AND RACING

The great economical relevance of horse-related business in **Flanders** was proven by the results of a large study published in **2008**, carried out at the request of the Flemish government. With 200.000 people involved with horses on a regular basis, 150.000 horses registered, and over 3.550 fulltime equivalents working in 1.750 different companies, horse-related business can be considered an important contribution to the Flemish economy. Together, these created an **added value** of over **219 million euros** in 2008 [1]. More recent figures of September 2013 showed that the importance only increased over the next years, reflected by the number of people involved and the number of horses registered in Belgium (over 243.000 people and over **172.000 horses** registered in Flanders).

In Belgium **horse racing** is not as important anymore as it used to be some decades ago, with only a few prestigious races left. "Waregem Koerse" at the Hippodroom surrounding the Gaverbeek (Waregem) and "Oostende Koerse" at the Wellington Hippodroom (Ostend) are the most well-known examples, attracting thousands of spectators for each race day. Six racing associations organise races throughout Belgium, generating a yearly turn-a-round of 6 million euros [1].

In other (European) countries horse racing still commands widespread public support, with ten to hundred thousands of people visiting, and betting on trot and gallop races. **France** is one of the **main players** in this field, with over 240 hippodromes organising more than 7000 races a year, with 9.9 billion euro in the game and over 70.000 employers involved directly or indirectly (2011) [2].

1.2 ANABOLIC STEROIDS ABUSE, BACK TO THE FUTURE?

As discussed in the introduction, anabolic steroids abuse has been a matter of all times. The first widespread usage of steroids in horses dates back to the early 60's and 70's. As strict **zero-tolerance** has been held for many years, anabolic-androgenic steroids might seem to be an issue of the past as new and possibly more effective **"designer" drugs**

have been developed over the years. AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, a metabolic modulator and TB-500, a synthetic peptide, containing an active binding site for thymosine β-4, stimulating muscle development in horses, are examples of those newly developed performance enhancers. However, **recent cases** of steroid abuse prove that, although AAS abuse is better under control than it was some decades ago, it will be of all times. The potential of AAS to improve performance remains too tempting to some trainers and riders.

In **racing** today use of anabolic steroids is still among the most high-profile medical issues, with recent cases of trainers Mahmood Al Zarooni (United Arab Emirates, 2013) and Gerard Butler (Britain, 2013) being suspended for the **illegal use of AAS**. Data from the FEI Tribunal website on the other hand illustrate that from January 2010 to December 2012, 41 **endurance** horses globally tested positive for anabolic steroids, and more than 80 percent of the eventual cases before the Tribunal came from FEI Group VII countries (Middle-East).

But not only in racing and endurance AAS abuse remains a major issue. If Group VII countries are excluded from the endurance records, endurance worldwide has a cleaner AAS abuse record than **show jumping**. Jumps trainer Philip Fenton in Ireland for example was recently handed a three-year ban for having 1 kg of the banned steroid Nitrotain (Ethylestrenol) on his premises (2014) (FEI Table of Suspensions, July 2015). Maxime Livio, a CIC*** French eventing rider, his horse Bingo S tested positive for testosterone in Pattaya, Thailand (2014).

These examples illustrate that, unfortunately and despite great efforts from the regulatory organs, AAS abuse is still rooted deeply into different equine sport disciplines at both the amateur and professional level.

1.3 ENDOGENOUS AAS: COMPLICATING DOPING ANALYSIS

Steroids can be classified into **three broad classes**: exogenous, designer and endogenous steroids. With the present-day AAS abuse issue in mind (**1.2**), the development of new and better techniques is needed to detect, and to distinguish between, steroids belonging to these different classes.

Anabolic steroids have been studied for over 50 years and during that time numerous compounds with a variety of functional groups have been produced and many have been

published. Only a small number have been introduced to the pharmaceutical market, the so-calles "known" **exogenous steroids**, available on the market today (e.g. stanozolol and trenbolone) [3]. They contain **synthetic structures** that do not occur in natural steroids (**Figure 1**). Confirmation of exogenous steroid abuse is relatively straightforward, as qualitative demonstration of the compound in the sample suffices.

Designer steroids are also exogenous steroids, containing synthetic structures that are thought not to occur naturally, but they have not yet been classified as a controlled substance and in many cases, like a pro-hormone, require a chemical reaction or enzymatic alteration once in the body to become active. In most cases they possess additional **minor modifications** compared to the well-known marketed exogenous steroids. These minor changes render their **detection** with targeted mass spectrometry more difficult. A worrying feature is that no data are available on the efficacy and safety of the use of these compounds [3].

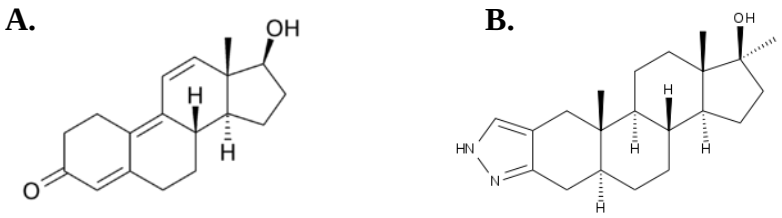

Figure 1. Chemical structures of exogenous steroids A) trenbolone (Finaplix, Tren) and, B) stanozolol (Winstrol, Winny). Stanozolol and trenbolone both contain a synthetic, conjugated system.

Endogenous steroids, such as testosterone, are steroids that are known to exist naturally, in one or more animal species [4]. Confirmation of endogenous steroids abuse is difficult, as simple quantitative detection of the compound is not sufficient. In horses only testosterone and 17β-boldenone are generally regarded as endogenous (respectively in all horses and in stallions only).

The classification of a steroid as "endogenous" is however a **grey area**. The "semi"-endogenous presence of 17β-boldenone and related compounds for example, in mares and geldings, is a complicating factor in doping control (chapter II). Proper investigation on the origin of these AAS is required to guarantee adequate doping policies in the future [5].

1.4 ANALYTICAL METHODS TO DETECT (ENDOGENOUS) STEROID ABUSE

In the introduction the most applied analytical methods used to detect steroid abuse were already mentioned, but depending on the context and the specific class of steroids (see **1.3**), the method of choice can be different. Most methods are based upon the direct detection of the steroid of interest and/or its metabolites in biological matrices, merely urine and/or blood samples. This direct detection is coupled to two different approaches. For boldenone for example, a **quantitative threshold concentration** has been set for stallions (15 ng per mL), while zero-tolerance is held for mares and geldings.

For testosterone a threshold concentration of 20 ng per mL for geldings and 55 ng per mL for mares is held. For stallions no threshold has been set, as the natural testosterone concentration is under the influence of age, seasonal variations and whether or not the stallion is a breeding stallion [6]. The idea of a threshold concentration relies on the **statistical likelihood** that a certain concentration can be detected in an **untreated horse**. The threshold must be based upon populations' studies and relevant to the concentrations measured post-treatment [7]. If the threshold concentration is set too high, concentrations measured post-treatment could be inadvertently listed as endogenous.

As an **alternative** to the direct measurement of endogenous steroids a range of assays have been developed, **measuring the biological effect** of the steroid(s) rather than the responsible compound(s). Two categories of assays can be distinguished: biosensors and biomarkers. Biosensors utilise biological techniques to detect steroidal activity in a sample *ex vivo*, whereas biomarker techniques aim to monitor perturbation of "normal" *in vivo* physiological parameters.

With the rise of high resolution non-targeted approaches, the use of **specific qualitative biomarkers** has gained popularity over the last few years. Biomarkers or biological markers are metabolites that are measurable indicators of some biological state or condition, e.g. (illegal) treatment with a certain compound. Biomarker monitoring can already be considered a new era in human anti-doping [8] and different biomarker approaches are being developed for equine purposes at the moment. As such, longitudinal monitoring of biomarkers can reveal non-physiological responses independently of the used doping technique or substance, and may cause sanctioning of illicit practices [9].

An example hereof is an efficient strategy developed to screen for **abuse of nandrolone**, a "semi"-endogenous steroid in stallions, monitoring the endogenous steroid profile

disruption in urine and blood upon nandrolone administration [9]. A panel of (endogenous) steroids of interest were extracted from equine urine and plasma samples and analysed by GC-MS/MS for quantification. Statistical processing of the collected data permitted to establish statistical models capable of discriminating control samples from those collected post-administration. These **statistical models** succeeded in predicting the compliance status of routine samples collected from racing horses.

Another example of biomarker use in equine anti-doping is the detection of **boldenone misuse**. Exogenous boldenone is known to be extensively conjugated in phase-II metabolism (see Introduction). Gomez *et al.* [10] found that after boldenone treatment, boldenone sulphate, and in some cases αBol sulphate, were present in urine samples, together with low concentrations of exogenous boldenone (the original, active drug) and BM1 (the main boldenone metabolite, 5β-androst-1-en-17β-ol-3-one). Thus, according to Gomez *et al.* [10], **βBol and αBol sulphates** may be used as markers for the exogenous administration of boldenone and they can be used to reduce the number of samples to be analysed by IRMS. In samples were boldenone and BM1 are detected at low concentrations, that thus might be of endogenous origin, only if boldenone sulphates and αBol sulphates are also present, further analysis by IRMS will be needed to confirm exogenous administration. However on the other hand, Ho *et al.* [11] identified intact boldenone sulphoconjugates as a direct evidence for the endogenous nature of boldenone in entire male horses. In addition, the limitations of the IRMS approach (see chapter I) need to be kept in mind.

This illustrates that both the quantitative threshold concentration and the qualitative biomarker biosensor/biomarker approaches have their **limitations**, including requiring large population studies for validation and the fact that statistical outliers can be present at any time. These limitations are part of the reason that **zero-tolerance** is still the preferred method to deal with "semi"-endogenous steroids, unless irrefutable evidence has been gathered proving the endogenous prevalence of a certain compound, in mares, geldings and/or stallions. Solid and sound, large-scale evaluation of the endogenous steroid profile of untreated horses on the prevalence and origin of endogenous AAS was necessary.

2. SCIENTIFIC CONTRIBUTIONS AND RESEARCH FINDINGS

Within the conceptual framework of this thesis, a number of research questions (objectives) have been postulated (See AIMS, p. 29). The accomplishment of these **different objectives** has been described in the different chapters (II to VI). As the findings from chapter II conflicted with zero-tolerance policy currently held in equine sports, additional research was needed to elucidate the **underlying biological mechanisms** behind these inadvertent findings. Different experiments were set-up to test different hypotheses. In the following section, the eliciting findings and conclusions of the different chapters will be presented and integrated in the light of the entire study (**Figure 2**).

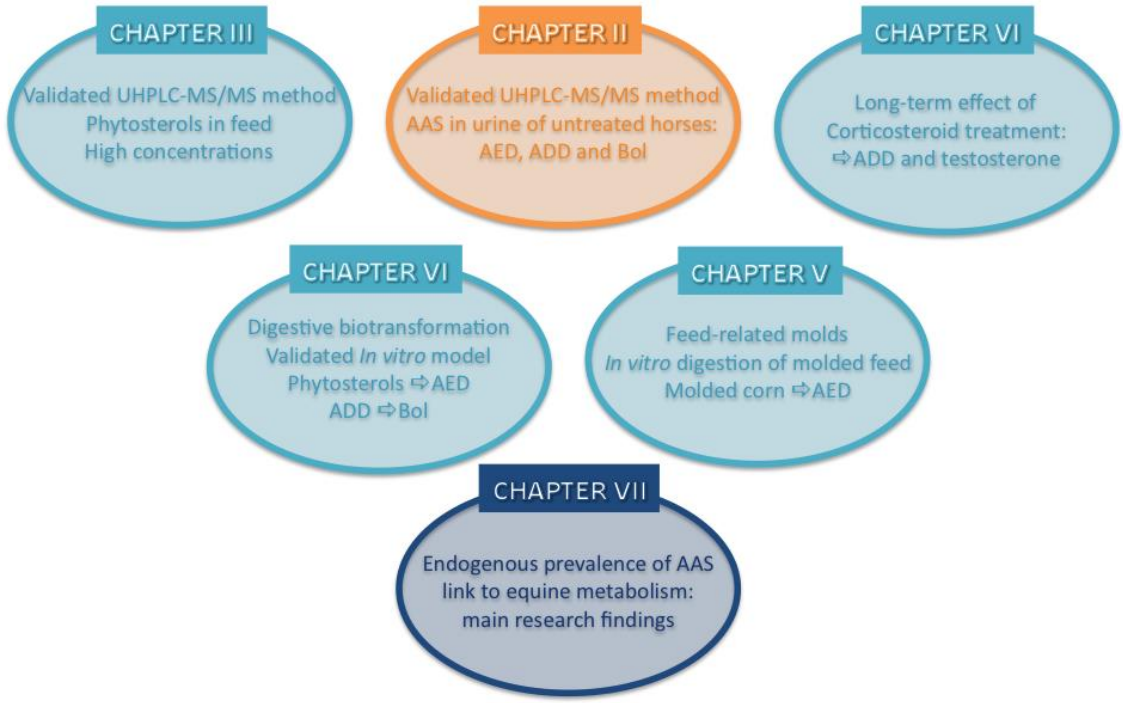

Figure 2. Schematic overview of the main accomplishments of this thesis

2.1 DEVELOPMENT AND VALIDATION OF A UHPLC-MS/MS METHOD TO QUANTIFY LOW LEVELS OF AAS NATURALLY PRESENT IN URINE OF UNTREATED HORSES

As highlighted in the introduction (Chapter I, 3. ENDOGENOUS STEROIDS) and earlier in this section (Chapter VII, **1.3**), the endogenous prevalence of AAS can seriously complicate doping analysis. In horses, **endogenous boldenone** was found in the urine and faeces of entire males [11, 12]. As such, today, IFHA and FEI (2014 Equine Prohibited

Substances List) abandoned the zero tolerance for stallions and set a threshold for free and conjugated boldenone of 15 ng/mL [13, 14]. Despite this threshold for entire males, the presence of boldenone in the urine from mares or geldings is still prohibited. For testosterone, thresholds are set as well (55 ng/mL for mares and fillies (unless in foal) and 20 ng/mL for geldings).

Although these thresholds have been set, data on the endogenous prevalence of boldenone, testosterone and other related **AAS in untreated horses** are scarce. Most studies published so far focussed on stallions and/or horses to which steroids had been administrated intramuscularly or orally [11, 12, 15-19], and not on **naturally present** endogenous AAS, with βBol as the most illicit candidate for endogenous presence.

Therefore, the first objective was to analyse urine samples from healthy, guaranteed untreated, out-of-competition horses, in an attempt to improve the knowledge on the natural, endogenous AAS present in horse urine in general. A specific extraction method, including extensive sample clean-up was combined with a new, sensitive and rapid **UHPLC-MS/MS method**. In total, urine samples of **105 guaranteed untreated horses** (47 geldings, 53 mares, and 5 stallions) were screened for βBol and five other related steroids: ADD, AED, αT, βT and P via the newly developed and validated UHPLC-MS/MS method. In contrast to what was expected, all steroids that were included in this method could be shown to be naturally present in urine of at least one horse, and **81%** of these horses were found to excrete steroids at a certain level. Nevertheless, almost all of them could be considered "negative" in the light of horse doping control.

Low concentrations of **progesterone, β-testosterone, and α-testosterone** (1–5 ng/mL) were found in the majority of the urine samples. Occasionally, other steroids (ADD, βBol, and/or AED) were also found in the urine of untreated geldings or mares at low concentrations (0.5–5.0 ng/mL). Especially the detection of **ADD and βBol** in urine form an untreated mare and gelding was intriguing, as zero-tolerance for both compounds is held under FEI and IFHA regulations. **ADD and AED**, which are chemically closely related to βBol and βT, were also found in the urine of some horses. ADD was found in the urine of a mare with an **elevated concentration of α-testosterone** (below the threshold), yet eightfold higher than the average determined for mares in this study, suggesting that the mare was in estrus and/or the presence of ovarian tumours or cysts (See also chapter I) [20].

In chapter II, all **stallions** were shown to produce high concentrations of βT, and two of them also excreted βBol and ADD. The βT concentrations were, as expected, much higher than those for geldings and mares and in line with previously published results by Popot *et al.* (2008) and Ho *et al.* (2004). The detection of **boldione (ADD)** in stallions is rather new. Popot *et al.* [12] stated that 1,4-androstadiene-3,17-dione (ADD) was undetectable in faeces collected from untreated horses and according to FEI and IFHA regulation, ADD is listed as a forbidden substance in urine (listed under its trivial name boldione) [13, 14].

As highlighted in chapter II, the 105 horses screened during this part of this research project most horses were used for **recreation only** and guaranteed to be **untreated** with AAS. Still, a limited number of horses were found to excrete AAS (Bol, ADD or AED). Other authors and personal communication (Laboratoire des Courses Hippiques, routine analysis) revealed that results of the horses reported in this study were not the only (untreated) horses with **low, yet detectable levels of endogenous AAS** (e.g. Bol and ADD) in their urine [10].

In a **separate study** 80 additional horses were screened for the excretion of 1-dehydro AAS [21]. This study included horses **in competition**, mostly racing but also some dressage and jumping horses were sampled. Consequently, for some horses from this second group it could not be guaranteed that they weren't treated with AAS (see further on in this section). In this additional screening, the focus was more on horses in competition, where **stallions and geldings** are preferred for their athletic performance and stable character. Therefore, the distribution of the horses over the different genders shifted towards geldings and stallions, compared to the group of horses analysed in chapter II. In this second study 16 stallions, 39 geldings and 25 mares were sampled.

The results obtained from this screening of, possibly treated, sport horses were merely **in line** with the results obtained in Chapter II for untreated horses, when looking at βT, **αT and P**. The average values for αT for mares and geldings were not significantly different form the respectively obtained values in chapter II. However, some outliers could be observed among the sport horses. An **increase** was seen between the average value for **βT** in urine of mares (1.4 ± 2.4 ng/mL) and geldings (1.4 ± 2.0 ng/m) from the first group (chapter II) and the average values for mares (1.8 ± 2.7 ng/mL) and especially geldings (2.8 ± 12.2 ng/mL) belonging to the **(sport) horse group**.

αBol was **not found** in any of the urine samples from geldings, mares and stallions belonging to both populations. In the sport horse group 7 out of 16 stallions excreted **β-Bol**, at an average concentration of 1.0 ± 1.4 ng/mL, in line with previous results. As in chapter II, **ADD** was found in some **stallions** (n = 2/16, at an average concentration of 2.7 ± 1.0 ng/mL).

Additionally, two mares and three geldings from the sport horse group were found to excrete **βBol at low concentrations (<2 ng/mL)**. The first gelding was found to excrete βBol (1.4 ng/mL)and his **βT** concentration was also **elevated** (40 ng/mL). This concentration is above the accepted threshold for testosterone (22 ng/mL) and high enough to form a plausible explanation for the detection of β-Bol. As this horse wasn't treated with AAS, the complementary results for β-Bol and βT point towards **cryptorchism** for this horse.

The second βBol excreting gelding from the sport horse group, a six-year old trotter, excreted 1.7 ng/mL βBol but didn't exceed the FEI/IFHA threshold for testosterone (9.4 ng/mL). Still, this testosterone level is **tenfold higher** than the average for geldings from the untreated horses screening in chapter II. Upon request, the owner of the horse revealed that the horse had received "an undefined treatment" a few weeks before sampling. Other horses from the same stable had elevated testosterone levels as well, as already touched upon earlier on in this section, enforcing the **suspicion of AAS abuse** at this specific stable.

The other βBol excreting horses from the sport horse group, a third gelding (0.6 ng/mL βBol) and two mares (0.3 and 0.2 ng/mL βBol) didn't show any abnormalities in their testosterone levels and were guaranteed to be untreated with AAS. The values obtained for these horses are most **likely true endogenous levels of β-Bol** and in line with the low levels measured in chapter II.

This screening of sport horses showed some nice examples of the **thin line** anti-doping analysis has to walk on when evaluating inadvertent results. As proven by the example from the trotting stable, owners and trainers are very inventive at circumventing doping regulations. This highlights the importance of evaluating relevant, untreated reference populations and taking into account the entire **excretory profile, medical history and behaviour of a horse** before drawing conclusions regarding the classification of

inadvertent results as of endogenous or exogenous origin. As touched upon earlier on in this manuscript, and although these techniques still have their inherent limitations (see further on and chapter I, p. 32-33), biomarker approaches, IRMS and the development of the Equine Biological Passports (EBP) (see: 3. FUTURE PERSPECTIVES), will be essential tools to aid in this decision.

An additional possibility that needs to be taken into account upon detection of AAS in urine samples is the *ex vivo* formation of steroids in urine. It is established that (bovine) urine can result positive for boldenone and ADD in consequence of faecal contamination. The simple transfer of steroids to urine is one minor aspect of faecal contamination; a secondary aspect is d*e novo* production of steroids in faeces after deposition and in faeces-contaminated urine. *De novo* production is almost certainly due to microbial activity, either from the urine or gut microbiota. Up until now, the responsible microorganisms are to be identified and the precursor compounds and transformations leading to the presence of these illegal steroids are still to be revealed.

Yet, similar results have been reported in equine urine samples. For example, laboratories may observe a low concentration of boldenone or boldenone-sulphate in a routine post-racing sample for testing and notice an increase of this compound upon storage. Furthermore, upon addition of deuterated testosterone-sulphate or methyltestosterone to these samples, the conversion to deuterated boldenone and methandienone could be observed (personal communication, AORC laboratories). Different approaches can be used to tackle this *ex vivo* formation of steroids, including strict, sterile sampling procedures to minimise microbial contamination, pre-extraction and direct storage at -20 °C of the samples.

2.2 EXTRACTION OPTIMISATION, DEVELOPMENT AND VALIDATION OF A UHPLC-MS/MS METHOD FOR THE DETEECTION AND QUANTIFICATION OF PHYTOSTEROLS IN FEED

Endogenous βBol found in stallions is thought to originate from a similar transformation of naturally present, high levels of (β-)testosterone [12] (testicular aromatisation) (**Figure 3**). For mares and geldings other **precursors**, including **phytosterols** from feed, have been put forward to explain the prevalence of low levels of steroids such as βBol and ADD in urine, as it has been proven that steroid hormone intermediates could be

produced from phytosterols through microbial transformation [22-24], and/or feed-related molds [25] (See also Chapter V).

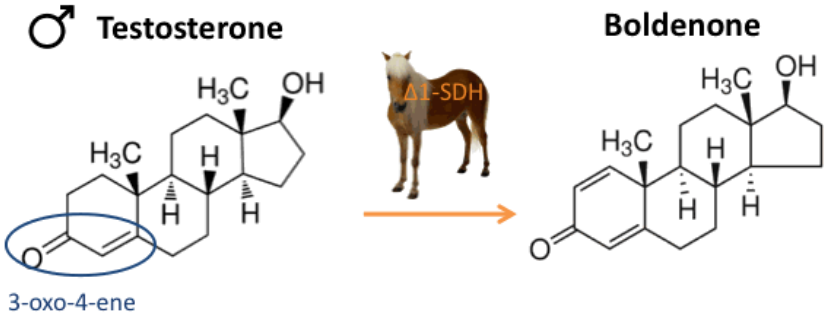

Figure 3. Illustration of the (microbial) Δ1-steroid dehydrogenase (SDH) activity held responsible for the detection of boldenone in entire male horses.

Despite the dual interest in phytosterols, both as cholesterol lowering agents and as possible steroid precursors, accurate and fully validated methods for the **quantification** of phytosterols in food and feed samples are scarce. Therefore, a new UHPLC-MS/MS method was optimized and fully validated for extraction and detection of the main phytosterols (β-sitosterol, campesterol and stigmasterol). A **fractional factorial design** was used to optimize the extraction procedure and the **applicability** of this newly optimised method was confirmed by analysing matrices with known phytosterol contents (tomato, grains). Afterwards yet unexplored solid feed samples (roughage and concentrates) and some additional food samples (liquid and solid) were analysed.

Grass (*Lolium Perenne*) was found to contain at least 130 mg phytosterols per 100 g d.w. while **hay** (mixed origin) was found to even contain up to 190 mg phytosterols per 100 g d.w., with elevated stigmasterol (ten times higher than grass). For the sampled feed **grains** (e.g. corn, oats and barley) total concentrations of phytosterols reached 25 to 85 mg (per 100 g d.w.). The highest levels were measured in corn, the lowest in oats. **Straw** (*Triticum*) was found to contain total concentrations of phytosterols up to 80 mg per 100 g d.w., comparable to the concentration measured in corn, however with a different **distribution between the different phytosterols** (straw was four times higher in stigmasterol than corn).

Dry peas (*Pisum sativum L.*) are high in fibre and protein and therefore a popular ingredient in concentrates, yet limited data were available on the phytosterol content [26]. This study showed that yellow peas contain respectively 4.7 ± 0.3 mg stigmasterol, 10 ± 0

mg campesterol and 67 ± 3 mg β-sitosterol per 100 g f.w., proving that yellow peas are indeed a relevant source of phytosterols, containing comparable levels of phytosterols as grains.

Even **apple flesh** was found to contain significant amounts of the three main phytosterols (12 ± 1 mg/100 g f.w.). In comparison, Piironen *et al.* (2003) was not able to quantify stigmasterol and found campesterol and β-sitosterol concentrations reached 16 mg/100 g f.w. [27]. This difference can be attributed to the difference between "Jonagold" and "BORKH" apple species and the fact that the peel and seeds, which are known to be phytosterol-rich, were included by Piironen *et al*. Other sources refer to **12 mg total phytosterols/100 g f.**w. as the reference value for apples, and the latter is in line with the sum of the concentrations found for apples in this study [28].

Overall, analysis of these different **feed** samples confirmed the applicability of the newly developed extraction and detection method and showed that both concentrates (dry peas and regular grains such as oats, barley and corn) as well as bedding material (straw) and roughage (hay, grass) can contain **large quantities of different phytosterols**, supporting the hypothesis of a possible involvement in endogenous steroid formation

2.3 VALIDATION OF AN *IN VITRO* DIGESTION SIMULATION OF THE EQUINE HINDGUT AS A TOOL TO STUDY THE INFLUENCE OF PHYTOSTEROL CONSUMPTION ON THE EXCRETION OF ANABOLIC–ANDROGENIC STEROIDS IN HORSES

Phytosterols, which were found to be present at very high concentrations (up to 200 mg per 100 g d.w.) in **grains and feed** (**2.2**), have been put forward as a possible explanation for the endogenous presence of low levels of AAS in urine of mares and geldings. Nevertheless, the possible **biotransformation** had thus far not been investigated in horses. *In vitro* simulations of the horse hindgut were set up, using faecal inocula obtained from different horses to study the possible digestive transformation.

***In vitro* digestion methods** have been widely used and refined for ruminants since their initial development by Tilley and Terry [29]. However up until now, no fully validated, standard equine *in vitro* digestion protocol was available. A reliable *in vitro* digestion method however can provide timely and cost-efficient evaluation of nutrient behaviour *in vivo* and even allow for quality control of processed feeds. Therefore, an **equine protocol** was developed, adapted from protocols for other monogastric organisms by Tilly *et al.* and Boisen and Fernandez (see Chapter IV) [29, 30]. In parallel, Biddle *et al.* published

the use of a very similar equine *in vitro* model to identify of lactate-utilizing bacteria that differentially respond to starch induction, in the light of laminitis (2013) [31].

The **functionality** of the *in vitro* model as a tool to study equine hindgut fermentations, was validated by assessing the microbiological growth (cell density and pH) and bioactivity of the hindgut flora, through the formation of **SCFA** and the consumption of amino acids and carbohydrates throughout the digestion process. The same parameters were monitored by Kiebooms *et al.* (2012), Biddle *et al.* (2013) (horse) and Molly *et al.* (1994) [31-33] to validate the use of similar *in vitro* digestion systems for pigs and humans. The latter Simulator of the Human Intestinal Microbial Ecosystems (**SHIME**) is the most well-known example. *In vitro* digestion samples were analysed with the previously validated UHPLC–MS/MS method, which was additionally validated for *in vitro* digestion samples (Chapter IV).

Addition of βBol to the *in vitro* digestions gave rise to the formation of ADD (androsta-1,4-diene-3,17-dione) or αT. Upon **addition of ADD** to the *in vitro* digestions, the transformation of ADD to βBol was observed and this for all horses' inocula, in line with *in vivo* treatment with ADD, an additional confirmation of the functionality of the *in vitro* model. The transformation ratio proved to be inoculum and thus horse dependent, and no α-Bol was found in any of the *in vitro* digestion samples.

The addition of pure **phytosterols (>50% β-sitosterol)** or phytosterol-rich herbal supplements on the other hand, did not induce the detection of βBol, only low concentrations of **AED**, a testosterone precursor, could be found. As such, the digestive transformation of ADD could be linked to the detection of βBol, and the consumption of phytosterols to low concentrations of AED, but there is no direct link between phytosterols and βBol. Therefore, the digestive transformation of consumed phytosterols cannot be directly linked to the detection of βBol, but, by providing the necessary precursors, they might form an intermediate step in the systemic steroid biotransformation pathway.

Previous research by other authors has shown that feed contaminants can be absorbed from the intestinal tract [34]. In the latter case, this can include steroids, steroid precursors and phytosterols. **Passage by the liver** leads to the formation of type II metabolites, steroid glucuronide- and sulphate conjugates, which are excreted and

detected in urine [35]. Other transformation reactions, the so called **type I transformations**, can also take place in the liver (see: 3. FUTURE PERSPECTIVES).

Additionally, **predigestive influences** such as UV radiation, oxidation, heat, moist and feed-related molds can also affect the phytosterol side chain stability in feed [25]. These parameters could trigger **local hotspots** for transformation in a feed batch, especially when self-heating of molded feed is involved [36]. If a horse consumes such hotspots, this could lead to the transient detection of low concentrations of AAS in the horse's urine.

2.4 MOLDY FEED, A POSSIBLE EXPLANATION FOR THE EXCRETION OF ANABOLIC-ANDROGENIC STEROIDS IN HORSES?

Due to their parasitic nature, molds are very interesting **biotransformation candidates** as they are capable of adapting to different substrates. Microorganisms capable of degrading hydrophobic hydrocarbons (such as phytosterols) generally need specific physiological properties, including active transporters, cell wall adaptations and the excretion of biosurfactants [37-39].

Figure 4. Moldy corn

Molds however developed their own efficient strategy to cope with difficult substrates, by producing a wide variety of **extracellular enzymes and biosurfactants** [40], to promote the extracellular transformation of these difficult substrates. These biosurfactants are specifically interesting in the light of phytosterol conversion, as phytosterols are difficult to solubilize, highly hydrophobic substrates that are as such difficult to access enzymatically.

As a result, it may be hypothesized that aerobic **storage of moldy feed** can lead to the formation of steroids or steroid precursors. The aerobic incubation of moldy corn indeed showed that AED can be formed and this in both PBS buffer and CPB medium, at a comparable concentration. Traces of αT and P were also detected.

On the level of the horse, the consumption of moldy or bacterially contaminated feed could lead to the detection of endogenous steroid levels. To tackle this question a full-fledged ***in vitro* approach** was set-up, following the previously validated *in vitro*

digestion protocols (See chapter IV and **2.3**). All samples were analysed with the sensitive and robust **UHPLC-MS/MS method** validated for *in vitro* samples (chapter IV).

When adding **(moldy) corn and/or phytosterols (>50% β-sitosterol)** to the *in vitro* digestion, AED was detected throughout the entire digestion period. The highest concentrations of AED were obtained for the combination of moldy feed with added phytosterols. In the latter case, the phytosterols were transformed. The combination of **mold enzymatic activity and the *in vitro* digestion fermentation** reactions showed to be crucial to power the transformation of phytosterols into AED, the main testosterone precursor.

The difference between moldy corn with and without added phytosterols indicates that the conversion ratio is higher with added phytosterols, probably due to the fact that the bioavailability of these added phytosterols exceeds the **bioavailability of the phytosterols embedded in corn**. *In vivo*, the stomach and small intestinal digestion will contribute to the bioavailability of phytosterols from the feed, by liberating phytosterols from the matrix by acidic and enzymatic hydrolysis. When adding phytosterols to the extracellular enzymes of all three corn-related mold isolates, AED peaks were detected as well, but not to a significant level. As a result, it may be deduced that the extracellular enzymes were not able to directly transform β-sitosterol into AED, but that the combination of mold extracellular enzymatical activity and fermentation (digestion) was crucial.

Still, **no ADD or Bol** was detected during the *in vitro* digestion experiments. The extracellular enzyme experiments with the *Mucor* corn isolate revealed the formation of ADD, the main boldenone precursor, from pure phytosterols (>50% β-sitosterol), but at a very low rate and with a longer incubation time (4 hours).

This study focused on biotransformations taking place in the **feed itself**, catalysed by mold enzymes, and the effect of the equine hindgut digestive processes (including microbial fermentation) on the digestion of (moldy) feed. It is important to keep in mind that the suit of processes taking place between consumption of feed and the urinary excretion and detection of steroids is very complex, involving many different contributors and side-reactions. **Other metabolic pathways** can additionally contribute to the formation of intermediate precursors (e.g. AED from moldy feed to testosterone). Recent

work by Fabregat *et al.* (2015) confirmed the formation of 1,4-androstadiene-3,17-dione (ADD), androsta-4,6-diene-3,17-dione, 17β-hydroxy-4,6-androstadiene-3-one and 17β-hydroxy-1,4-androstadiene-3-one (boldenone) upon hepatic phase I metabolism of T. Analogously to T, this pathway leading to the formation of Δ1 and Δ6 metabolites, might also prevail for other steroids, opening the possibility of targeting **additional biomarkers** [41].

Moreover, compounds and phase I metabolites may be further reduced and/or modified during phase II catabolism in the liver. The set of phase I and II products could in turn be transformed in the hindgut by the wide range of microorganisms, under aerobic or mainly anaerobic conditions. This **enterohepatic circulation** might additionally increase the yield of biochemical modifications by reprocessing the "flow-through" [42].

Yet unexplored and thus unchartered reactions and reaction products of the enterohepatic circulation should be taken into account, when considering the connection to the **urinary excretion** of steroids. This biological complexity is hard to reproduce *in vitro*. The results obtained in this are sufficient to support additional *in vivo* research is warranted to further unravel the correlation between the controlled consumption of phytosterol rich or enriched feed, albeit exposed to mold enzymes, and the urinary and faecal excretion of steroids in horses (see: 3. FUTURE PERSPECTIVES).

2.5 INFLUENCE OF GLUCOCORTICOID TREATMENT ON THE EXCRETION OF ANABOLIC-ANDROGENIC STEROIDS IN EQUINE URINE

The detection of AAS (ß-Bol, AED and/or ADD) in urine of horses that were not treated with AAS (Chapter II) often **coincided** with an anti-inflammatory, analgesic glucocorticoid **therapy** in the weeks or months before sampling. Earlier studies in veal pointed at the enteric tract as a potential source of ß-Bol and related steroids [43]. Therefore, the hypothesis was raised that GC could be **transformed** to AAS by the microbiota of the equine colon, as glucocorticosteroids (GC) are structurally related to the group of AAS.

***In vitro* digestion simulations** were set up according to the previously validated protocol (chapter IV), with different GC precursors being submitted to the digestive fermentation reactions: cortisol, betamethasone, cortisone and prednisolone. No significant concentration of AAS could be measured at any of the sampled points in time during digestion, in either series of *in vitro* digestion simulations (cortisol, betamethasone,

cortisone and prednisolone). **No direct transformation** from glucocorticosteroids to AAS was shown in the hindgut.

However, the urinary AAS and GC **excretion profile** of a gelding treated intra-articularly with betamethasone was found to be **disrupted** by this glucocorticoid treatment. At first, the pituitary ACTH-release dropped, causing the endogenous adrenal GC-synthesis to stagnate [44]. In humans, it has been described that glucocorticoid therapy, and especially prolonged high dose systemic therapy, can have a suppressive effect on endogenous steroid production [45].

When urinary betamethasone elimination continued and betamethasone levels decreased, the regulatory (positive) **feedback loop** was activated, resulting in higher endogenous GC-production and a temporary overshoot for the endogenous corticosteroids, cortisol and cortisone and αT and ADD. This **overshoot** was then quickly normalized by the regulatory system, revealing urinary levels as before and restoring homeostasis.

As the horse in this study was only suffering from mild bone spavin, a moderate **dose of betamethasone** was administered. It is expected that the effects as seen in this *in vivo* experiment will be more pronounced and long-lasting when higher initial GC doses are being administered. The sensitivity of the analytical methods (UHPLC-Orbitrap-HRMS system for GC) would be met to a better degree, enabling a more profound analysis of betamethasone and its metabolites, and the effect on endogenous GC and AAS over a longer period of time (see: 3. FUTURE PERSPECTIVES).

Based on the results of the *in vitro* digestion experiments, the authors can reject the original hypothesis in this concern; no direct (intestinal) biotransformation of glucocorticosteroids to AAS by microbial fermentation in the equine hindgut could be detected. The **interconnection** of GC treatment and the detection of AAS must be indirect, most likely by influence the **hypothalamic-pituitary-andrenal axis**. The amount of testosterone synthesized is regulated by the HPA-axis. When testosterone levels are low, GnRH is released by the hypothalamus, which in turn stimulates the pituitary gland to release FSH and LH. These latter two hormones stimulate the synthesis of testosterone in the testis (stallions), thecal cells of the ovaries, placenta (mares) as well as in the zona reticularis of the adrenal cortex (geldings, stallions and mares).

In chapter II, ADD was found in the urine of a mare that also excreted α-testosterone. What's interesting in the light of this part of the study is that this horse was suffering from navicular disease and was treated with GC two months before the sampling took place. To that concern, the results obtained for the gelding in the current study are very much in line with the, at that time unexplainable, momentary result of this mare. Additionally, the gelding that was found to excrete **β-Bol and AED** in chapter II was also **treated intra-articulary** with GC, a few weeks before sampling. Unfortunately, no data could be obtained on the treatment dosage and GC used. The other horses that were found to excrete AED, and no ADD or Bol, were not known to be treated with GC in the weeks or months before sampling.

In general, the analysis of post-treatment urine samples revealed that the influence of GC treatment undoubtedly could be measured, **up to several weeks** after betamethasone intra-articular infiltration. The overall impression was a disruption of pre-treatment AAS concentrations, a change in mutual proportions as well as in their long-term concentration pattern. ADD or boldione for instance, was not found before treatment, but could be found for weeks after treatment, with concentrations up to 5 ng per mL.

Given the fact that glucocorticosteroid treatment plays an important role in **worldwide anti-inflammatory therapy**, especially in sport horses, it stands clear that further research is needed to elucidate the exact correlation with the excretion of anabolic-androgenic steroids. Larger scale experiments, as well as experiments with other GC are to be considered to this extend, to avoid possible inadvertent doping results for AAS, following glucocorticoid treatment (see: 3. FUTURE PERSPECTIVES).

3. FUTURE PERSPECTIVES

3.1 ENDOGENOUS STEROID FORMATION

The mere focus of this research project was on the confirmation of endogenous AAS in untreated and the possible correlation with digestive or mold-related biotransformation of phytosterols (*in vitro*), focussing on reactions taking place in the hindgut (See also chapter I, IV and V). Recently however SHIME technology, mimicking the entire human digestive tract, has been expanded to hosts other than humans, such as production animals (e.g. pig) and companion animals (e.g. dog, cat). An **equine SHIME model** might be the next best thing (www.prodigest.eu). This ongoing advancement of equine *in vitro* digestion simulations might offer great opportunities to continue the unravelling of the role of phytosterols in the detection of AAS in horses.

Still, some additional transformations are to be taken into account *in vivo*. To study these type I transformation reactions, separate *in vitro* set-ups have been developed as well. Scarth *et al.* (2010) for example, used **equine liver microsomes** and S9 tissue fractions to study the metabolism of the androgenic/anabolic steroid stanozolol [46]. Using high-resolution accurate mass full scan analysis on the Orbitrap, equine liver microsome and S9 *in vitro* fractions were found to generate the same major type I metabolites observed following *in vivo* administrations. Wong *et al.* (2011) on the other hand, confirmed the use of **homogenized liver** and, in addition to the previously reported *in vitro* metabolites, some additional known *in vivo* metabolites in the equine could also be detected for testosterone (βT) and epitestosterone (αT) [47].

Unfortunately, to our current knowledge, the equine liver metabolism of AED or phytosterols has not yet been tested. Labrie *et al.* (1997) did confirm that one of the **17β-hydroxysteroid dehydrogenase isoenzymes (17β-HSD)** is able to control the last step in the formation of testosterone from AED in all rhesus monkeys and human peripheral intracrine tissues examined. Types 3 and 5 17β-HSD, respectively catalyse the formation of testosterone from AED in the testis and peripheral tissues [48]. Additional *in vitro* experiments with liver microsomes or liver homogenate might help to unravel the underlying pathways responsible for endogenous steroid formation.

3.2 DIETARY CONTAMINATION AND TRANSFORMATION

Feed intake was monitored yet not controlled nor taken into account during the population studies monitoring the urinary excretion of AAS (Chapter II and VII). An interesting approach is currently put into practice, whereby all **feeds** that were supplied to horses that test "positive" for urinary AAS excretion are being subjected to both microbiological and chemical analyses.

Chemical analysis could reveal which phytosterols are present and at what concentrations (chapter III), and if the feed already contains AAS or AAS precursors. These can in turn be transformed enzymatically, either by the horse's liver and/or intestinal microbiota, into boldenone or related AAS. The chemical, analytical methods applied today are increasingly sophisticated and sensitive, the likelihood of detecting **contaminants** increased, with limits of detection that were previously unattainable. A good example hereof is a method developed for the detection of thyreostats in feed by Kiebooms *et al.* (2015), able to detect as low as 0.5 ng TU (thiouracil) per g feed [49].

Furthermore, an increasing variety of **(herbal) supplements** is brought onto the market, introducing unusual components into the equine diet, including exotic herbs and **plant derivates or extracts** [50]. In Europe most feeds and supplements are tested by their manufacturers for potential contamination with commonly recognized, prohibited substances. These analyses are offered by routine labs such as HFL Ltd (UK) and LCH (France). However, the ongoing **globalisation of the feed industry** hampers this strategy, with feedstuff and raw materials being imported from different regions of the world, were quality control might be insufficient [51]. Additionally, novel crop infesting plants and (micro)organisms may be found in these countries. No official records are available for horse supplements, but results for human sport supplements indicated that **up to 20%** of supplements tested contained prohibited substances, including anabolic steroids such as testosterone and nandrolone [52]. **Labelling hay, haylage and silage** is even less widespread, although roughage quality is highly variable, depending on field conditions during growth and harvesting (humidity, soil, use of fertilisers/pesticides, etc). Forage analysis typically consists of Crude Protein (CP), Acid Detergent Fiber (ADF), Neutral Detergent Fiber (NDF), Calcium (Ca), Phosphorus (P), Potassium (K) and Magnesium (Mg). Other elements such as Iron (Fe), Manganese (Mn), Zinc (Zn) and Copper (Cu) are also useful in determining the final ration (e.g. www.sgs.com, hay and forage testing).

Additionally, the detection of specific mold, yeast or bacterial species not present in steroid free feed might lead to the identification of crucial contributors in the formation of AAS *in vivo*. Bacterial identification traditionally relies on **phenotypic identification** using gram staining, culture and biochemical methods. However, these methods suffer from two major drawbacks. First, they are limited to organisms that can be cultured *in vitro*, which is not the case for all organisms (the so-called **viable but non-cultivable/culturable organisms**, VBNC) [53]. Second, some strains exhibit **unique biochemical characteristics** that do not fit into patterns that have been used as a characteristic of any known genus and species [54].

Therefore, **non-culture-based**, molecular, techniques have been developed to overcome these limitations of traditional phenotypic procedures for the detection and characterization of bacterial phenotypes. Real time PCR and microarrays are the most commonly employed molecular techniques [55]. **Real time PCR** is highly sensitive and especially interesting as it allows both identification and quantitation of bacteria at species level. **Microarray** based bacterial identification relies on the hybridization of preamplified bacterial DNA sequences to arrayed species-specific oligonucleotides. Each probe is tagged with a different coloured dye which fluoresces upon hybridization.

To identify **mold and yeast** species merely culture-dependent, phenotypic and limited non-culture dependent, molecular techniques are available. ISO horizontal methods (e.g ISO 21527:2008) for the enumeration of yeasts and moulds in foods and animal feed are still the most applied, with adaptations for dried (<0.95 water activity) and non-dried feed and food. Similar standard methods have been published by other bodies (e.g. USFDA, US Food and Drugs Agency). These methods typically employ a **surface plating technique**. Selective agars for yeasts and moulds usually contain antibiotics to help suppress bacterial growth. Plates are typically incubated at 25 °C for 5 to 7 days (see chapter V) and then examined for the presence of yeast and mould colonies.

3.3 PHYTOSTEROL-ENRICHED FEED CONSUMPTION: *IN VIVO*

An interesting set-up that has not been tackled in this study, is the *in vivo* feeding of **phytosterol-enriched feed** and the influence thereof on the excretion of anabolic-steroids in horses. Thus far, similar studies have been performed in humans, veal calves and rats, with differing results. In **rats** it was shown that the consumption of 0.5 g phytosterols per kg bodyweight led to faecal excretion of ADD, AED and androstanedione [56].

In **humans** the influence of phytosterol consumption on the excretion of boldione (ADD) was measured in a small-scale 5-week study with healthy volunteers asked to consume a phytosterol-enriched yogurt drink every morning during the first three weeks of the study [57]. The results demonstrate that αT, βT and AED were frequently excreted by both males and females, while **endogenous ADD** was **sporadically** produced at concentrations ranging from 0.8 to 1.7 ng per mL urine. Endogenous Bol could not be proven. **No evidence** of phytosterol related anabolic steroids' excretion was observed after consumption of phytosterol containing functional food at the recommended dose. Considering the relatively short-term duration of this *in vivo* trial, small sample size and relatively low recommended dose used as a reference (2 g per day, 0.025 g per kg bodyweight) it is recommended to conduct more extensive long-term studies to further explore the potential interferences of phytosterols with human steroidogenesis and excretory steroid patterns.

Some feeding experiments have been performed in **veal calves** as well, fed on milk replacers with differing phytosterol contents [58, 59]. In urine from control animals, the **αBol concentration (<2 ng per mL)** was strictly related to the phytosterol content of the diet, while, in urine from animals treated with ADD and βBol, αBol levels were not modified by the production from dietary precursors. The results confirmed that a αBol level higher than 2 ng per mL should be considered as evidence of suspected illegal treatment and that the urinary excretion of βBol is due to exogenous administration of 17β-Bol. According to these results, αBol concentrations below 2 ng per mL can be considered of **endogenous origin.**

In vivo experiments with controlled feeding of **moldy feed** to horses are not possible because of ethical reasons, keeping the possible health issues related to the consumption of moldy feed in mind. Alternatively, **mold extracellular enzymes** could be isolated by centrifugation and used to **pretreat** phytosterol-rich feeds. These feeds can in turn be fed to the test individuals, to mimic the effect of moldy feed consumption, without exposing the horse to the possible health risks linked to the consumption of moldy feed such as the accumulation of mycotoxins.

3.4 SILAGE, HAYLAGE AND MASHES

Both silage, haylage and mashes are feedstuffs produced at elevated temperatures and under high-moist conditions which makes them **prone to spoilage**. With the results of

chapter V in mind, it would be very interesting to monitor the effect of silage and mash production (and spoilage) on the **stability of phytosterol sidechains**, under the different microbiological and fungal conditions, associated with the respective production process.

Mash ("*Slobber*" in Dutch) is typically fed to horses during winter and there are a variety of **bran mash** recipes commonly used. Most involve mixing warm water with (wheat) bran until the bran is saturated: the mixture clogs together when squeezed. Wheat bran is a low-density feed that is similar in nutrient content to oats, with about four times the phosphorous content of most grains and relatively high in fibre and vitamins. A number of other ingredients can be added to increase nutritional value of the mash or to make it more appealing to the horse. Providing horses with a bran mash is especially encouraged when the horses might not be drinking enough **water**: following stressful work or during long trailer transport. As mashes are usually prepared on the spot, the risk of spoilage (and mold-related biotransformations) is rather low.

Silage/haylage on the other hand is the high-moisture feedstuff resulting from the acidic preservation by **fermentation** of forage crops. In Belgium, grass and corn (and/or corn stalks and leaves) are the most used raw materials. The most common definition states that silage is moist (35-50 % DM) and in most cases stored as a horizontal, bunker silo with above-ground concrete walls. Silage is well-known in veal feeding practice. Haylage on the other hand is drier (50-75 % DM) and packed as round bales in plastic. Some haylage is so dry (70-85% DM) it is merely hay wrapped in plastic [60].

Both for silage and haylage there are two main phases in the ensiling process. The first step in the preservation of silage/haylage is the **aerobic phase**, which occurs in the presence of oxygen, until oxygen is depleted through the process of respiration of the plant material. Water-soluble carbohydrates (sugars) are oxidized with the production of carbon dioxide and heat. The first phase should be as brief as possible to maintain silage quality. Excessive aerobic fermentation reduces the energy content of the silage and may cause heat damage to proteins. Additionally, as molds need oxygen for growth, rapidly creating anaerobic conditions well cease mold growth. This is very important in the light of the results of chapter V, where moldy feed was linked to the detection of AED, the testosterone precursor. Shortening the aerobic phase can be achieved by compressing and tightly packing fermented fourage with adequate thickness of plastic to remove air. When

available oxygen is depleted **anaerobic bacteria** (merely *Lactobacillus* sp), which only grow in the absence of oxygen, take over and the fermentation process begins.

Early harvested, high quality forage usually supplies adequate starch and sugars for fermentation, but late-harvested forage (alfalfa at mid- to late flowering or grass that is headed) may not, making them more prone to spoilage and mold-related biotransformations (as discussed in chapter V). Moist conditions favour the anaerobic phase. Therefore, silage has a lower pH (pH 4.3-4.7) compared to hay and haylage, that show little or no lactic acid bacteria activity and thus higher pH (pH 6-5.6). As molds favour neutral pH environments, incomplete fermentation of silage might elevate the risk of mold hotspots, which have been linked to the detection of AED in chapter V. Adding a commercial *Lactobacillus* sp inoculum to the forage can aid fermentation. *Lactobacillus* sp are always present in the field, but lower levels might be present when the crop grows under cool conditions. To get good coverage the liquid inoculum can be applied on the chopper as the forage passes.

3.5 INFLUENCE OF CORTICOSTEROID TREATMENT ON THE EXCRETION OF AAS

The results of chapter VI revealed that (intra-articular) corticosteroid treatment can have significant influence on the endogenous steroid excretion in horses. As these results were only based upon a clinical case study combined with the results of the urinary screening (chapter II and VII), it would be interesting to repeat this experiment on a **population of horses** (mares and geldings), and compare the results to a relevant group of horses treated with a placebo. A **placebo treatment** with e.g. non-steroidal hyaluronic acid will mimic the stress caused by the "handling" of treatment (handling by the vet, local anaestheticum and injection of the drug). **Stress** is an important parameter to be controlled, as it could have a significant influence on the results, as stress influence the pituitary axis regulation of endogenous (stress) hormones such as cortisol and cortisone.

In this particular case study betamethasone was the corticosteroid of choice to treat the clinical signs of bone spavin in the horse studied. Comparing the effect of **different glucocorticoid treatments** (e.g. with prednisolone, cortisone or methylprednisolone) can deliver more information regarding the specific influence of different glucocorticoids on the excretion of both endogenous glucocorticoids and anabolic-androgenic steroids.

Additionally, the dose used is an interesting variable as well, as thus far no answers can be formulated regarding the **dosage-dependence** of the phenomena described in Chapter VI. Given the fact that glucocorticosteroids play an important role in worldwide equine sports medicine, it stands clear that additional, profound research is needed to elucidate possible alternative origins of endogenous AAS, such as ß-Bol. The possibility of measuring inadvertent doping results for AAS, following glucocorticoid treatment, could then be excluded, which will in turn contribute to a future where proper and honest doping policies can be applied in equine sports.

3.6 EQUINE BIOLOGICAL PASSPORT (EBP)

In humane anti-doping, **WADA** introduced the Athlete Biological Passport (ABP) in 2009. An APB is "*an individual, electronic record for professional athletes, in which profiles of biological markers of doping and results of doping tests are collated over a period of time*". The EBP is considered to be an effective tool in the fight against doping as **monitoring selected biological variables over time** can indirectly reveal the effects of doping, rather than attempting to detect the doping substance or method itself (See also biosensors/markers, **1.4**) [61].

The **concept** of launching a similar equine biological passport (EBP) is not new, the first discussions started back in 2010, but the tone and urgency of its discussion among veterinary and regulatory authorities is changing in the wake of 2015's recent doping scandals (e.g. cobalt scandal in Australia). This sudden public focus might spur funding that helps turn complicated concepts, such as introduction of EBPs as a means to curtail doping, into reality.

With the **metabolomic technology** invested in the equine passport, the industry can be proactive in identifying those who are engaging in doping practices, without necessarily knowing what the specific substance is, eliminating the strict reliance on targeted analysis of post-racing urine and blood samples. Every horse has a **unique metabolic signature** that can be identified by monitoring metabolites or marker. This individualized approach facilitates a greater understanding of the specific traits of each horse, providing more precise measurement of the **biological effect of training, stress and/or drugs** (controlled medication or doping) than is available with current technologies. Metabolomics is already recognized as a cutting-edge science in human medicine and anti-doping.

Additionally, the EBC can include monitoring the genes of racehorses, identifying changes throughout their careers, to combat **gene doping**. Gene doping is defined by the World Anti-Doping Agency as "*the non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to improve athletic performance*". Suspected targets for gene doping are erythropoietin (EPO), myostatin and the insulin-like growth factor (ILGF).

Leading racing authorities are currently working on the development of EBPs. Unfortunately, the use of biological passports will be a **logistic and analytical challenge** to all but the top-flight racing analytical labs around the world. The EBP is a whole-of-organisation initiative requiring high-tech equipment and the cooperation of analysts (scientists), stewards, anti-doping investigators and veterinarians to ensure all of the available information concerning prohibited practices to be integrated in real-time.

Additionally, the development and introduction of the athlete biological passport (ABP) showed that many **legal issues** can arise in the light of the case law of the Court of Arbitration for Sport (CAS) [62]. For example, a procedural particularity of the longitudinal profiling cases relates to the starting point of the **time-limit to lodge a claim** against an athlete at internal level: doping charges on the basis of longitudinal profiling entail a series of tests along with the evaluation of the results. This is why an international federation can only establish the offence and raise charges once the panel of medical experts have determined that the athlete's blood profile constitutes sufficient proof of the recourse to a prohibited method.

From the fact that the detection method of ABP is not based on the finding of a prohibited substance arise certain other well-worth mentioning legal issues as well, since the application of the ABP is **only indirectly regulated**. Art. 3.2 WADC ("Methods of Establishing Facts and Presumptions") states that "*Facts related to anti-doping rule violations may be established by any reliable means, including (...) conclusions drawn from longitudinal profiling".* As a consequence, the validity of the ABP as a reliable means to establish an anti-doping rule violation has been **contested** by certain athletes stating that it is merely a "*useful screening test*". This has been countered by the Panel, but it illustrates how difficult it is to develop and introduce a new detection method as a watertight, legally robust test.

As a final **conclusion** regarding the EBP we can state that in the past doping sinners have always been a step or two ahead of the authorities, but when this new way of handling drug abuse -not by finding the drugs but finding changes in the physiology of the horse created by the drugs- can be put into practice, the gap may be about to close. A **cheerful outlook** for the fight against doping abuse in equine sports!

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SUMMARY

SUMMARY

The higher the pressure to win, the more athletes are inclined to take steps to improve one's performance through questionable means. The same goes for today's high-performance equine sports, where doping is a hot topic. Therefore, strict anti-doping and medication rules are being enforced "*to maintain the welfare of the horse and to ensure fair play in competition, in the light of breeding and bets being placed on the outcome of the race*". All horses in-competition are regularly subjected to doping analysis, to prevent the abuse of forbidden substances from affecting the performance of the horse. Anabolic-androgenic steroids (AAS) have been part of the forbidden substances list for many years, because of their muscle building and performance enhancing capacities and possible side-effects. For most AAS zero tolerance is held. In this context, the aim of this research project was to assess the possibility of the natural presence of AAS in horses, and the underlying biomechanisms.

In **chapter I** the main steroids and their natural prevalence in humans and horses are listed. The biological synthesis of steroids in the horse and the theoretical link to phytosterols are also discussed. In the light of the link between phytosterols and AAS, both the horse's endocrine system and digestive tract were illustrated. Some important physiological disorders of the endocrine system that can influence the endogenous steroid production, such as Cushing's disease and ovarian tumours, were touched upon as well.

Only two steroids, testosterone and boldenone, are generally regarded as potentially natural steroids in horses, and can thus be present in urine. For these compounds, gender-specific thresholds have been set by the different regulatory organs (e.g. Fédération Equestre Internationale and International Federation of Horse Racing Authorities). To maintain these thresholds multiple mass spectrometric methods have been developed and used over the years. The historical evolution of these analytical methods has also been dealt with in chapter I. Nowadays increased analytical capabilities, such as better limits of detection, allow to detect continuingly lower and lower (endogenous) levels of steroids.

In **chapter II** a new and sensitive UHPLC-MS/MS method to quantify naturally present AAS in urine was developed and validated. With this method urine samples from 105 horses that were not treated with AAS were analysed. More than 80% of the mares and geldings showed to excrete low levels of steroids, in most cases testosterone and/or

progesterone. Occasionally, low concentrations of the AAS androst-4-ene-3,17-dione (AED), androsta-1,4-diene-3,17-dione (ADD) and boldenone were also found (<5 ng/mL). At the moment however, zero tolerance is held for both ADD and boldenone. In the strict sense, these samples, and thus these horses, did not comply with current anti-doping regulations, despite the fact that they were not treated with AAS.

A first hypothesis was formulated, linking these results to the transformation of phytosterols from feed, as these are chemically closely related to AAS. However, no data regarding the natural content of phytosterols in feed were available. In **Chapter III** an accurate extraction and detection method to quantify phytosterols in feed was optimized and validated. With this method we were able to quantify the main phytosterols (β-sitosterol, campesterol and stigmasterol) in concentrates (oats, barley, corn, dry peas) and roughage (hay, grass, straw). In both concentrates and roughage high concentrations of phytosterols showed to be present, varying between 25 and up to 190 mg per 100 g dry weight.

In vitro digestion simulations were used as a tool to study the hypothesized biotransformation of phytosterols from feed to AAS. Up until now, no standardized *in vitro* digestion protocol is available for horses. Therefore, in **chapter IV** an existing protocol for monogastric animals was adapted and validated to simulate the equine hindgut. To validate this model the production of short chain fatty acids, conversion of amino acids, depletion of sugars and growth and activity of the microbial flora was monitored. Transformations that take place *in vivo*, such as the transformation of ADD to boldenone could be simulated *in vitro*, supporting the functionality of the *in vitro* digestion model. No direct biotransformation of phytosterols to AAS was seen, but the main testosterone precursor AED could be formed.

Previous research (Verheyden *et al.*, 2010) indicated that feed-born molds naturally present in veal feed could also play a role in the formation of AAS or AAS precursors out of phytosterols. The experiments described in **chapter V** focused on the analysis of molded (horse) feed and the possible effect of accidental consumption of this feed on the horse. *Mucor* sp. infected corn showed to be a possible source of AED. *In vitro* digestion of this molded corn, with or without added phytosterols, led to an increased detection of AED. These results confirm the biotransformation of AED from phytosterols, the

combination of mold enzymatic activity and the *in vitro* digestion fermentation reactions showed to be crucial to power the transformation of phytosterols into AED.

Based upon these results, the endogenous detection of ADD or βBol in urine samples from some mares or geldings (chapter II) remains unexplained. Yet, the horses that were found to excrete ADD or βBol showed to be treated with glucocorticoids a few weeks or months before sampling. A two-sided experiment, as described in **chapter VI**, was set-up to unravel this possible involvement of glucocorticoid anti-inflammatory treatment in the detection of AAS. Urine from a gelding treated with glucocorticoids was monitored up to 120 days post-treatment. On the other hand, *in vitro* digestion simulations were used to test the possible direct biotransformation of different glucocorticoids (cortisol, cortisone, betamethasone and prednisolone) into AAS. The *in vivo* results revealed that both the endogenous glucocorticoid and the steroid profile were disrupted, up to 70 days post-treatment. Consecutively, endogenous concentrations of cortisol and cortisone (day 37 to 48) and testosterone (day 41 to 77) were increased. The results of the *in vitro* digestion experiments however rejected the direct intestinal transformation from glucocorticoids to AAS. The interconnection of glucocorticoid treatment and the detection of AAS must be less direct, most likely by influencing the hypothalamic-pituitary-andrenal (HPA) axis.

In **chapter VII** the eliciting findings and conclusions of the different chapters were integrated in the light of the entire study. We can conclude that it is possible to detect low concentrations of AAS (AED, ADD or Bol) in urine of mares and geldings that were never in contact with synthetic AAS. The detection of AED can be coupled to the consumption of molded corn, while the detection of ADD (and potentially βBol) could be seen in correlation with a glucocorticoid treatment, disrupting the horse's endogenous anabolic steroid profile. In this way, mere qualitative detection of these compounds does not imply AAS abuse. Additional analyses, such as Isotope-Ratio Mass Spectrometry (IRMS) or the detection of abuse-related biomarkers, are needed to reject or confirm exogenous administration. The detection of high concentrations (> 5 ng/mL) of AAS still points towards AAS abuse. These results should not be considered as a safe conduct for unbounded AAS detection in geldings and mares, nor is it a passport for impunity.

Eventually, chapter VII sums up the main research questions that are to be investigated in this area in the near future. Important next steps will be the unraveling of the role the liver plays in the endogenous formation and transformation of AAS, a profound analysis of

contaminated feed and the analysis of (spoiled) fermented feed such as haylage and silage. Additional experiments are required to better understand the possible involvement of glucocorticoid treatment on the excretion of endogenous (cortico)steroids post-treatment. Finally, chapter VII focuses on the development of "Equine Biological Passport" (EBP), which will contain both genetic and biochemical information, a means of tracking biological changes in horses over time. In the near future the EBP might allow putting a stop to doping in equine sports, by staying ahead of the cheats. These are bright prospects, leading towards clean and fair equine sports competitions worldwide.

SAMENVATTING

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Waar het in de sport om winnen, eer en geld gaat is middelenmisbruik om de sportieve prestaties te verbeteren nooit veraf. In de hedendaagse, prestatiegerichte paardensport is doping dan ook een 'hot item'. Strenge doping- en medicatieregels dienen nageleefd te worden met als doel "*het welzijn van het paard te beschermen en de veiligheid en de integriteit van de sport te garanderen, met eerlijke wedstrijden waarbij de uitslagen authentiek zijn met het oog op de fokkerij en de aan deze wedstrijden gekoppelde weddenschappen*". Paarden die uitgebracht worden op wedstrijd, in eender welke discipline, worden dan ook regelmatig gecontroleerd op het gebruik van verboden middelen. Anabole-androgene steroïden (AAS) maken sinds jaar en dag deel uit van de lijst met verboden middelen, omwille van hun spierversterkende en prestatie bevorderende eigenschappen en hun mogelijke nevenwerkingen. Voor de meeste AAS wordt bijgevolg dan ook een nultolerantie beleid gevoerd. Ondermeer door de verbetering van de analytische methoden wordt het mogelijk om steeds lagere concentraties van deze stoffen te meten. Dit onderzoek richtte zich dan ook op het mogelijk van nature voorkomen van lage concentraties van deze AAS bij paarden, en de achterliggende biomechanismen.

In **hoofdstuk I** wordt een overzicht gegeven van de verschillende steroïden en hun al dan niet natuurlijk voorkomen bij paarden en mensen. De natuurlijke synthese van steroïden door het paard alsook het theoretische verband met fytosterolen komt hier aan bod. In het kader van deze link met fytosterolen wordt in dit hoofdstuk naast het endocrien systeem ook het verteringsstelsel van het paard kort weergegeven. Enkele belangrijke klinische afwijkingen van het endocrien systeem, die een invloed kunnen hebben op de endogene productie van steroïden (zoals de ziekte van Cushing en ovariële tumoren), worden eveneens besproken.

Van slechts twee steroïden, testosteron en boldenone, wordt er algemeen aanvaard dat ze van nature geproduceerd kunnen worden door paarden en dus voorkomen in de urine. Voor deze stoffen werden dan ook geslachtsgebonden, maximale drempelwaarden vastgelegd door de verschillende regulatorische organisaties (o.a. Fédération Equestre Internationale, FEI en Internation Federation Horse Racing Authorities, IFHA). Om deze regels te kunnen handhaven werden doorheen de jaren verschillende, massa-spectrometrische, methoden ontwikkeld en op grote schaal toegepast. De evolutie van

deze analytische methoden wordt ook besproken in hoofdstuk I. Door de verbetering van deze analysemethoden wordt het mogelijk om steeds lagere (endogene) concentraties steroïden te meten.

In **hoofdstuk II** wordt de ontwikkeling en validatie van een nieuwe, gevoelige UHPLC-MS/MS methode voor de kwantificatie van AAS in urine besproken. Met behulp van deze methode werden urinestalen van 105 onbehandelde paarden geanalyseerd. Hieruit bleek dat meer dan 80% van de ruinen en merries lage concentraties van de verschillende steroïden uitscheidden, voornamelijk testosteron en/of progesteron. Bij enkele paarden werden echter ook lage concentraties AAS zoals androsteendion (AED), androstadiëendion (ADD) en boldenone teruggevonden (<5 ng/mL). Voor zowel ADD als boldenone geldt tot op vandaag de nultolerantie. Strikt genomen zouden deze paarden dus niet voldoen aan de regels, ondanks dat ze niet behandeld werden met AAS.

Als mogelijke verklaring voor deze resultaten werd de endogene omzetting van fytosterolen uit het voeder, die chemisch nauw verwant zijn aan steroïden, naar voor geschoven. Data met betrekking tot het natuurlijk voorkomen van fytosterolen in voeder waren echter niet voor handen. In **hoofdstuk III** wordt dan ook de ontwikkeling van een accurate extractie- en detectiemethode voor de kwantificatie van fytosterolen in voeder besproken. Met behulp van deze methode werd het mogelijk de gehalten aan de belangrijkste fytosterolen (β-sitosterol, campesterol en stigmasterol) te bepalen in krachtvoer (haver, gerst, maïs, erwten) en ruwvoer (hooi, gras en stro). In zowel kracht- als ruwvoer bleken hoge concentraties fytosterolen aanwezig te zijn, gaande van 25 tot 190 mg per 100 g drooggewicht.

De mogelijke endogene omzetting van fytosterolen uit het voeder naar AAS werd bestudeerd met behulp van *in vitro* verteringssimulaties. Aangezien er tot op heden geen gestandaardiseerd *in vitro* digestieprotocol beschikbaar is voor paarden, werd in **hoofdstuk IV** een bestaand protocol voor éénmagige (niet-herkauwende) dieren geadapteerd en gevalideerd voor de simulatie van de achterdarmvertering van het paard. Voor de validatie van dit model werd zowel de productie van korte keten vetzuren als de omzetting van aminozuren en depletie van suikers alsook de groei en activiteit van de microbiële flora opgevolgd. *In vivo* aangetoonde omzettingen, zoals de omzetting van ADD naar boldenone, konden *in vitro* gesimuleerd worden, wat de functionaliteit van het

in vitro model bevestigt. Er bleek geen rechtstreekse omzetting van fytosterolen naar AAS plaats te vinden, hoewel de testosteron precursor AED wel gevormd kon worden.

Voorafgaand onderzoek (Verheyden *et al.*, 2010) toonde aan dat schimmels die van nature voorkomen op het voeder van runderen ook een rol kunnen spelen bij de omzetting van fytosterolen naar AAS of AAS precursoren. Het onderzoeksluik dat in **hoofdstuk V** besproken wordt omvat daarom de analyse van beschimmelde (paarden)voederstalen alsook het mogelijke effect van accidentele consumptie van dergelijk beschimmeld voeder op het paard. Met *Mucor* sp. besmette maïs bleek een bron van AED te zijn. *In vitro* digestie van deze beschimmelde maïs, al dan niet aangerijkt met fytosterolen, gaf aanleiding tot een verhoogde vorming van AED. Deze resultaten bevestigen de vorming van AED uit fytosterolen en dit onder invloed van de gecombineerde enzymatische werking van enerzijds de schimmels en anderzijds de darmflora van het paard.

Het natuurlijk voorkomen van ADD of βBol bij ruinen en merries (hoofdstuk II) kan hierdoor echter niet verklaard worden. De paarden waarbij ADD of βBol aangetoond werd, bleken echter vaak een glucocorticosteroïde behandeling te hebben ondergaan in de weken of maanden voorafgaand aan de staalname. Een tweeledige experimentele opzet, zoals besproken in **hoofdstuk VI**, werd dan ook uitgevoerd om de mogelijke rol van glucocorticoïden in de vorming van AAS te ontrafelen. Enerzijds werd een met glucocorticoïden behandelde patiënt opgevolgd tot 120 dagen na behandeling, om de langetermijneffecten op het endogene steroïdprofiel te bestuderen. Anderzijds werden verschillende glucocorticoïden (cortisol, cortisone, prednisolone en betamethason) onderworpen aan de enzymatische activiteit van vertering (*in vitro*). *In vivo* werd vastgesteld dat zowel het endogene glucocorticoïden- als het steroïdenprofiel tot 70 dagen na de initiële glucocorticoïde behandeling verstoord was. Achtereenvolgens werden verhoogde endogene concentraties cortisol en cortisone (dag 37 tot 48) en testosterone (dag 41 tot 77) waargenomen. Voor behandeling kon geen ADD waargenomen worden, waar dit na behandeling wel het geval was (tot 100 dagen na de initiële behandeling). De resultaten van de *in vitro* digesties toonden echter aan dat deze verstoring niet het gevolg was van een rechtstreekse intestinale omzetting van glucocorticoïden naar AAS. Het verband tussen de glucocorticoïden behandeling en de detectie van AAS bleek indirect, waarschijnlijk via het beïnvloeden van de hypothalamus-hypofyse-bijnier-as.

In **hoofdstuk VII** worden de resultaten van de verschillende in het kader van dit onderzoek uitgevoerde experimenten geïntegreerd en de belangrijkste wetenschappelijke conclusies geformuleerd. We kunnen besluiten dat het mogelijk is om, weliswaar lage, concentraties AAS aan te treffen in de urine van ruinen en merries die niet behandeld werden met AAS. Voor de detectie van AED kan de consumptie van beschimmeld voeder als mogelijke verklaring naar voor geschoven worden. De detectie van ADD en/of βBol is mogelijk gelinkt aan een voorafgaande behandeling met glucocorticoïden die het endogene steroïdprofiel van het paard verstoorde. Het aantreffen van deze componenten is dan ook geen sluitend bewijs voor het aantonen van misbruik. Bijkomende analyses zoals Isotoop-Ratio Massa Spectrometrie (IRMS) of de detectie van biomerkers gerelateerd aan AAS misbruik dienen dan ook toegepast te worden op stalen waarin dergelijke AAS aangetroffen worden. Op basis van de resultaten van dit werk kunnen we wel stellen dat het aantreffen van hogere concentraties AAS (> 5 ng/mL) nog steeds wijst in de richting van misbruik van deze stoffen. Deze resultaten kunnen dan ook niet als vrijgeleide beschouwd worden voor het gelegitimeerd aantreffen van AAS bij ruinen en merries.

In hoofdstuk VII worden tenslotte tevens enkele interessante onderzoekspistes voor de nabije toekomst besproken. Belangrijke volgende stappen in dit onderzoek zijn enerzijds het ontrafelen van de rol van de lever in de endogene vorming en transformatie van AAS alsook een uitgebreide studie naar het voorkomen van gecontamineerd voeder in de praktijk en analyse van (al dan niet bedorven) gefermenteerd voeder zoals kuilvoer en slobbers. Daarnaast zijn bijkomende studies nodig om inzicht te krijgen in de effecten van behandeling met glucocorticoïden op de excretie van endogene (cortico)steroïdhormonen. Als laatste wordt in dit hoofdstuk ook de rol van het "*Equine Biological Passport*" (EBP) besproken. De ontwikkeling van een biologisch paspoort, dat zowel genetische als biochemische informatie bevat, kan toelaten om in de nabije toekomst dopingmisbruik een halt toe te roepen. Goede vooruitzichten die de sport enkel maar ten goede kunnen komen.

CURRICULUM VITAE

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Personalia

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Tel.	+32 498 79 31 95
E-mail	Anneleen.Decloedt@Ugent.be
Date of Birth	1988-11-04
Place of Birth	Ghent
Nationality	Belgian

Education

Doctoral Schools in Life Science and Medicine (Ghent University)

Specialist courses (2012-2015)

- Brewing Technology, 3 credits, 18/20
- Lab Animal Science I, 5 credits 18/20
- Lab Animal Science II, 5 credits 16/20

Transferable skills (2012-2015)

- Leadership Foundation Course
- Advanced Academic English: Conference Skills – Presentation Skills English
- Project Management
- "*Practicumtraining voor assistenten*"
- "Plunge into your bussinessplan" (in collaboration with Food2Know)

Higher education

Master in Science (2009-2011)

Biochemistry and Biotechnology with great honours

major microbiology, Ghent University

Masterthesis: "Association of the food-borne pathogen *Salmonella* with free-living *Acanthamoeba*", Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Hygiene and Technology,

Bachelor in Science (2006-2009)

Biochemistry and Biotechnology with great honours

Ghent University

Bachelorprojects: "Denitrification by *Bacillus* isolates"
"Functional genomics in *Xenopus*"

Secondary Education

General Secondary Education, Science – Math with great honours

Emmausinstituut Aalter, 2000-2006

Scientific participations

Participation in different (international) congresses, symposia and conferences:

- EuroFood Chemistry XVIII, Madrid, Spain, *October 13-16, 2015* (Poster)
- BioINNO Conference, Brussels, Belgium, *September 25, 2015*
- KVCV symposium MS in food and feed II, Ghent, Belgium, *September 15, 2015* (Poster)
- Belgian Brewing Conference, Leuven, Belgium, *September 6-8, 2015* (Oral presentation)
- 3th Saskatoon International Workshop on Validation and Regulatory Analysis focusing on the residues of veterinary drugs, pesticides and other contaminants in foods, Calgary, Canada, *June 16-19, 2015* (Oral presentation)
- 35th European Brewery Convention, Porto, Portugal (Poster)
- 7th European Equine Health & Nutrition Congress: feeding the equine athlete, Ghent, *March 26-27, 2015* (Poster and oral presentation)
- 6th Mytox Happening, Ghent, Belgium *March 11, 2015*

- Studiedag Brouwerij Fermentatio, Ghent, Belgium, *December 5, 2014*
- 2nd International Congress on Food Technology, Kusadasi, Turkey, *November 5-7, 2014* (Poster)
- 4th International Young Scientists Symposium on Malting, Brewing and Distilling, Ghent, Belgium, *October 28-30, 2014* (Poster)
- Symposium Quality Control, Ghent, Belgium, October *23, 2014*
- 7th International Symposium Hormone and Veterinary Drug Residue Analysis, Ghent, Belgium, *June 2, 2014* (Oral presentation)
- 47th European Veterinary Conference 'The Voorjaarsdagen', Amsterdam, The Netherlands, *April 17 – 19, 2014*
- 13th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology, Bruges, Belgium, *January 28 – 29, 2014*
- Symposium Quality Control, Ghent, Belgium, *October 17, 2013*
- Symposium Trends in Food Analysis VII, Ghent, Belgium, *September 19, 2013*
- Symposium EuroFood Chem XVII: Food for the future – the contribution of chemistry to improvement of food quality, Istanbul, Turkey, *May 7-10, 2013* (Poster)
- 6th European Equine Health & Nutrition Congress, Ghent, Belgium, *March 1-2, 2013*
- Symposium Quality Control, Ghent, Belgium, *October 20, 2012*
- Minisymposium on Metaproteogenomics: Functional analysis of microbial communities and consortia without cultivation, BSM, Brussels, Belgium, *September 25, 2012*
- 17th Conference on Food Microbiology, BSFM, Brussels, Belgium, *20-21 September 2012*
- Protozoa Symposium, Belgian Society of Parasitology and Protistology, Brussels, Belgium, *March 14, 2012*
- 16th Conference on Food Microbiology, BSFM, Brussels, Belgium, *September 23, 2011*
- 2nd Workshop on Bacterial and Fungal Biofilms, FWO Research Community Biology & Ecology of Bacterial & Fungal Human Biofilms, Ghent, belgium, *September 22, 2011*
- 2nd International ISEKI FOOD Conference, Milan, Italy, *August 31 - September 2, 2011*

Scientific publications

A1 **Decloedt A.I.**, Van Landschoot A., Vanhaecke L. (2015) Validated UHPLC-MS/MS method for the extraction and detection of phytosterols in food, feed and beverages. *Analytical Bioanalytical Biochemistry* (submitted)

A1 **Decloedt A.I.**, Damen S., Vanhaecke L. (2015) Effect of glucocorticoid treatment on the excretion of anabolic-androgenic steroids in equine urine. *Equine Veterinary Journal* (submitted)

A1 **Decloedt A.I.**, Bailly-Chouriberry L., Vanden Bussche J., Garcia P., Popot M.A., Bonnaire Y., Vanhaecke L. (2015) Moldy feed, a possible explanation for the detection of anabolic-androgenic steroids in horses? *Drug Testing and Analysis* (special issue, submitted)

C3 **Decloedt A.I.**, Van Landschoot A. and Vanhaecke L. (2015) Development and validation of a new extraction and UHPLC-MS/MS detection method to quantify phytosterols in food and feed samples. XVIII Euro Food Chem, Madrid, October 13-16

C3 **Decloedt A.I.**, Vanhaecke L. (2015) Optimization of phytosterol extraction and analysis from animal feeds by D-Optimal design and UHPLC-MS/MS. KVCV mass spectrometry in food and feed II, Ghent, September 15

C3 **Decloedt A.I.**, Watson H., Van Landschoot A., Vanderputten D. (2015) Technology for the production of gluten-free malt beer. 15th International Belgian Brewing Conference Chair J. De Clerck XV, Leuven, September 6-8

A1 Hemeryck L.Y., **Decloedt A.I.**, Vanden Bussche J., Geboes K.P., Vanhaecke L. (2015) High Resolution Mass Spectrometry Based Profiling of Diet-Related DeoxyriboNucleic Acid Adducts. *Analytica Chemica Acta* doi:10.1016/j.aca.2015.08.019

A1 **Decloedt A.I.,** Bailly-Chouriberry L., Vanden Bussche J., Garcia P., Popot M.A., Bonnaire Y., Vanhaecke L. (2015) *In vitro* simulation of the equine hindgut as a tool to study the influence of phytosterol consumption on the excretion of anabolic-androgenic steroids in horses. *The Journal of steroid biochemistry and molecular biology* 152:180-192. doi:10.1016/j.jsbmb.2015.06.001

P3 Van Hoorde K., **Decloedt A.I.**, Koek J., Vandamme P., Van Landschoot A. (2015) The microbiology of 37 different Belgian beers of more than 25 years old unravelled with MALDI-TOF MS. Proceedings of the 35th European Brewery Convention, Porto, May 24-28

P3 **Decloedt A.I.**, Bailly-Chouriberry L., Bonnaire Y. and Vanhaecke L. (2015) Does the consumption of moulded feed affect the excretion of anabolic-androgenic steroids in horses? Proceedings of the 7th European Equine Health and Nutrition Congress, Bruges, March 26-27

A1 **Decloedt A.I.,** Bailly-Chouriberry L, Vanden Bussche J, Garcia P, Popot MA, Bonnaire Y, Vanhaecke L (2015) A validated UHPLC-MS/MS method to quantify low levels of anabolic-androgenic steroids naturally present in urine of untreated horses. *Analytical and bioanalytical chemistry*, 407 (15):4385-4396. doi:10.1007/s00216-014-8428-x

C3 Vandoorne S., **Decloedt A.I.**, Vanderputten D., Van Landschoot A. (2014) Technology to produce gluten-free barley malt beers. Proceedings of the 2nd International Congress on Food Technology, Kusadasi, 05-07 November

C3 **Decloedt A.I.**, Jonas Schoelynck J., Eric Struyf E., Van Landschoot A. (2014) Dissolved Silicon Content in Belgian Beers. Proceedings of the 4th International Young Scientists Symposium on Malting, Brewing and Distilling 2014, Ghent, 28-30 October

A1 Vanden Bussche J, **Decloedt A.I.**, Van Meulebroek L, De Clercq N, Lock S, Stahl-Zeng J, Vanhaecke L (2014) A novel approach to the quantitative detection of anabolic steroids in bovine muscle tissue by means of a hybrid quadrupole time-of-flight-mass spectrometry instrument. *Journal of chromatography A*, 1360:229-239. doi:10.1016/j.chroma.2014.07.087

C3 **Decloedt A.I.**, Bailly-Chouriberry L., Bonnaire Y., Vanhaecke L. (2014) Unraveling the anabolic-androgenic steroid profile of untreated horses by UHPLC-MS/MS. 7th International Symposium Hormone and Veterinary Drug Residue Analysis, Ghent, 2 June, oral presentation Anneleen Decloedt and abstract in proceedings

C3 Van Landschoot A., **Decloedt A.I.**, Schoelynck J., Struyf E. (2014) Elements and beers and effect of some metals ions on colloidal stability of beer. 17th School of Fermentation Technology, Krakau, 20-23 Mars, abstract in proceedings

C3 Van Landschoot A., Schoelynck J., **Decloedt A.I.**, Struyf E. (2013) Factors related with dissolved silicon content in Belgian beers. EuroFood Chem XVII: Food for the future – the contribution of chemistry to improvement of food quality, Istanbul, 7-10 May, abstract in Proceedings, Hacettepe University, Food Engineering Department, ISBN 978-605-63935-0-1, 317

Awards

- Winner of the "7th EEHNC free communication award" for the best abstract, poster and oral presentation at the 7th European Equine Health and Nutrition Congress (*March 2015*)
- Team Manager of the Beer4Dreams team that won both the first price and the price of the audience at the Belgian Ecoptrophelia competition, organized by FEVIA (Fédération de l'Industry Alimentaire) (*29th April 2014*)
- The same team represented Belgium at the European Ecotrophelia Competition in SIAL (The World's Largest Food Innovation Marketplace) in Paris (*October 19-23, 2014*) and obtained a honourable 4th place

Publications in popular media

Radio 2: radio-interview April 29, 2014, on air April 30, 2014: "Nieuw bier"
Kanaal Z: interview April 30, 2014: "Studenten innoveren in voeding"
Ecotrophélia Europe 2014 book "The future of food, European Food Innovation" Student Awards, Genther: new beer, p 18-19

www.versele-laga.com/en/Landingspages/Feeding-the-equine-athlete
http://horsetalk.co.nz/2015/04/04/equine-athletes-horse-nutrition-congress/
http://www.anky.com/en/news/anky-shared-feeding-practices-at-7th-european-equine-health-nutrition-congress

Students

Bachelor and master projects

- ***Maxim Backaert***: "Validation of a UHPLC-MS/MS method for the detection of phytosterols in beer/ the brewing process and other solid- and liquid feed and food samples", February – June 2015, Bachelor (Bio)Chemistry, HoGent
- ***Sander Damen***: "Effect of glucocorticoid treatment on the hormonal profile of horses", summer 2014, 3th bachelor Veterinary Medicine (Honours Program for promising students)
- ***Toon Babylon***: "Studying the interaction of food-borne pathogens with free-living protozoa", 2012, 1th master Biochemistry and Biotechnology, Ghent University
- ***Jolien Van Hecke***: "Association of *Acanthamoeba* with the food-born pathogen *Salmonella"*, 2012, 1th master Biochemistry and biotechnology, Ghent University

Master thesis

- ***Anneleen Michielsen***: "Biotransformation of phytosterols in feed to AAS in horses", July 2014 – June 2015, 3th master Veterinary Medicine, Ghent University
- ***Marieke Logghe:*** "Quantification of phytosterols in grains and feed", September - December 2014, master Industrial Sciences: biochemistry, Ghent University
- ***Lindsy Marin***: "Biotransformation of phytosterols and the possible link with the endogenous formation of anabolic steroids in horses", February - June 2014, master Industrial Sciences: Biochemistry, Ghent University
- ***Tijs Vanhevel***: "*In vitro* digestion simulations as a tool to study the endogenous formation of steroids in horses", February - June 2013, master Industrial Sciences: chemistry, KaHo Sint-Lieven-KULeuven

Promotor of two literature studies in the light of the master thesis (Emilie Callaghan and Carolien Schmitt, 2nd master Veterinary Medicine, 2015) and member of the reading and examination committee of different bachelor and master theses.

Resources

September 2011 – December 2011
DeHousse-scholarship (Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Hygiene and Technology, prof. K. Houf)

January 2012 – Augustus 2012
FCWO scholarship: "Association of the food-borne pathogens *Salmonella* and *E. coli* with free-living protozoa, impact on control and virulence" (Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Hygiene and Technology, prof. dr. K. Houf)

September 2012 – Augustus 2014
Scholarship funded by the Fédération Nationale des Courses Françaises (FNCF): "biotransformation of phytosterols" (under the supervision of the Laboratoire des Courses Hippiques) and executed at the Laboratory of Chemical Analysis, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University (Prof. dr. L. Vanhaecke)

September 2014 – December 2014
Scholarship funded by the Laboratory of Chemical Analysis, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University (Prof. dr. L. Vanhaecke)

January 2015 – December 2016
Hogent PWO research project (75%, "Technology for the production of gluten-free malt beer") and Ghent University (25%, teaching assistant) (Faculty of Bioscience Engineering)

DANKWOORD

Ja, een dankwoord… hoe begin je daaraan?

Bij het begin dan maar… een dikke 4 jaar geleden zette ik mijn eerste schuchtere stapjes in de wereld van "het onderzoek", met een masterthesis aan de vakgroep Veterinaire Volksgezondheid en Voedselveiligheid, bij prof. Kurt Houf. Daar kwam ik onder de vleugels van Julie B terecht die mij gezwind over, door en tussen de eerste onderzoeksobstakels heen loodste. Tijdens deze thesis kwam ik al snel tot de vaststelling dat onderzoek weliswaar geen "9 to 5 job" is (en soms zelfs geen 9 to 9), maar wel de ideale aanvulling op mijn aangeboren nieuwsgierigheid. Uren konden we discussiëren over de resultaten en mogelijke theorieën om deze te verklaren. Julie B, bedankt voor je enthousiasme!

Tijdens deze periode maakte ik ook reeds kennis met de andere collega's binnen het Labo voor Hygiëne en Technologie, met centraal Soetkin, Martine en Sandra, Laid, het manusje-van-alles, Inge, Tomasz en later ook Glynnis, Ellen en Natascha. Het was altijd fijn om over de middag eens een andere wind door mijn hoofd te laten waaien!

De goesting om verder te gaan in onderzoek zette mij ertoe aan om mee te doen aan de, voor sommigen welgekende en alom gevreesde, IWT selectie. Hiervoor bleek ik een metgezel binnen het labo te hebben: Gerty. Na één week braaf rug aan rug in het "studentenbureautje" werd het ijs alsnog gebroken. Lijstjes en zelfs volledige webconstructies en tijdlijnen werden opgesteld. Het mocht niet baten… ;-) Het IWT bleek helaas net niet voor ons weggelegd, maar gelukkig kon ik alsnog verder onderzoek doen, met dank aan een FCWO mandaat.

Een jaartje later begon mijn "oude liefde" biochemie toch terug te kriebelen. Lynn (prof. Vanhaecke), bedankt dat je mij in september 2012 de kans gaf om op dit doctoraatsproject aan de slag te gaan. Anneleen, biochemie en paardjes: een veel betere match bestaat er niet. Dit was om verschillende redenen echter geen vanzelfsprekende keuze. We zeggen het misschien niet vaak genoeg, maar, Lynn je bent echt de spil van het labo, waar we (de eerste keren weliswaar met knikkende knietjes) altijd bij terecht kunnen met alle wetenschappelijke en minder wetenschappelijke problemen. Bedankt!! Zonder uw hulp was dit doctoraat nooit in drie jaar afgewerkt geraakt.

Er waren echter ook vele anderen binnen het Labo voor Chemische Analyse die elk op hun eigen wijze hun steentje bijgedragen hebben aan dit werk. Julie VDB

(dit wordt vooral niet complex met alle Julie's), jij was degene die mij in dit project op gang zette en de eerste uurtjes aan de TSQ met mij doorbracht. Bedankt! Jella, statistisch talent en altijd te vinden voor een praatje en goudeerlijk advies. Dat apprecieer ik in je!

Maar ook de vaste kern (Dirk, Mieke, Lucie, Beata, Vicky, Johan, Joke en Ine) en de mede-doctoraatstudenten/lotgenoten mogen niet ontbreken. Karen, mijn eerste bureaugenote, die mij de basis van die soms wel eens vervloekte, massa spectrometers bijbracht. Lieven, uw doctoraat heeft mij de laatste paar maanden verdacht veel gezelschap gehouden als "voorbeeld" (soms tot frustratie van Lynn, als ik bijgevolg teveel bladzijden schreef…). Julie K, die tweede bron van decibels, die samen met Lieselot af en toe wel eens de oorzaak was dat wij met zijn drieën, net iets te gezellig, overuren maakten. Nathalie, zonder uw methode had hoofdstuk VI er toch wel anders uitgezien. Kaat, het bureaugenootje bij wie ik in de laatste maanden regelmatig eens kon ventileren over mijn doctoraatsstress. Veel succes met jouw doctoraat! Gabriel, you might not be the most outgoing person of us all, but you are always there when we need you. We still want to come to Chili, so once you start the ranch, let us know! Carolien, wij brachten niet veel tijd samen door op het labo, maar veel succes met het IWT, zet hem op!

Daarnaast wil ik hier ook even kort de verschillende (thesis)studenten die tijdens de afgelopen drie jaar meegewerkt hebben aan dit project de revue laten passeren: Tijs, Lindsy, Marieke, Sander, Carolien, Emily, Anneleen en Maxim. Het was fijn om met jullie samen te werken! Ik hoop dat ik jullie toch een beetje heb kunnen besmetten met de liefde voor onderzoek.

Ook mijn "nieuwe" collega's van de brouwerij (Dana, Sylvie, Veerle en André) mogen niet ontbreken, omdat ze in de afgelopen maanden altijd oprecht geïnteresseerd waren in de vorderingen van mijn doctoraat, en mij waar mogelijk ook geholpen hebben zodat ik de ruimte had om dit werk tot een goed einde te brengen. Hellen, vanaf nu ben ik jou volwaardige glutensidekick. Ik ben er zeker van dat ook jij dit binnen een paar jaar tot een goed einde zal brengen. Sven, bij deze ook nog eens bedankt voor het uitwerken van de cover!

Dit doctoraat was echter ook nooit tot stand gekomen zonder de lieftallige ondersteuning van een aantal uitlaatkleppen, die bereid waren om mij te ondersteunen als het eens wat minder ging of waar nodig te temperen in mijn enthousiasme…

Eerst en vooral was er "de paarden" en alle mensen die ik zo door de jaren heen leren kennen heb. Sommige daarvan gaan ondertussen al heel wat jaren mee. Lisa, we zien elkaar niet zo vaak meer, maar ik heb onze gesprekken altijd ten zeerste geappreciëerd. Moeten we misschien toch eens terug verandering in brengen! Karen, sinds jaar en dag (ik ben de tel al lang kwijt…) mijn knotsgekke gezel voor allerhande feestjes, ritjes, crossen en wat je ook maar kan bedenken. Bedankt! Stijn, jij die me altijd terug dat duwtje gaf als ik mezelf weer eens onderschatte. Bedankt, voor alles wat ik van je geleerd heb… Je komt er wel! En Robin, wanneer kom jij nog eens "de Pieter" temmen?

Iza, veel uitleg hoeft er bij jou niet bij. Om het met iemand zijn legendarische woorden te zeggen "Jullie zijn soms echt twee dezelfde!!!". Bedankt voor de talrijke hobbelrondjes met Limit en Pieter en de steun de laatste tijd (je weet het zelf wel). Als het gesprek begon met "We nemen de grote toer…", dan wisten/weten we alletwee hoe laat het is.

Anderzijds was er ook de "Lifetime fitness" bende., met dank aan Karen om mij daar te introduceren. Nieuwe vriendschappen werden gesmeed, en elke vorm van (doctoraats)frustratie kon er met een lach en een extra portie zweet direct weggetraind of gesauna't worden. Justine, Sam, Katinka, Tijl, Maarten, Lotte, Jochen, Arno, en zovele anderen. Bedankt! Naast al het gesport en inhoudsloos gezwans was er ook plaats voor serieuze gesprekken, ritjes met de kever/mustang en fanatieke uitspattingen in de vorm van loopwedstrijden, merci daarvoor Joey! Bram, ook wij maalden vele kilometers af door de bossen. We waren soms een beetje verdwaald, maar ook dat komt wel weer goed ;-)

Conny, Sandra en Elien, ook onze vriendschap ontstond initieel via "de fitness". Maar intussen is er maar eentje meer actief en horen we elkaar niet altijd even regelmatig, maar binnenkort gaan we zeker nog eens op zwier en drinken we er eentje extra op dit doctoraat! Davy, ook jou leerde ik eigenlijk via de fitness

kennen, maar ondertussen hoor je eigenlijk al lang niet meer enkel in dat rijtje thuis. Iemand op wie ik altijd kan rekenen. Topper! Matthias, ons connectie rijkt intussen ook al zo ver terug in de tijd dat het wat moeilijk is om je aan een specifieke paragraaf toe te wijzen :-), maar weet dat jij voor mij het levende bewijs bent dat onmogelijk niet bestaat, als je er maar voor gaat.

Dit brengt mij dan ook (genadeloos) bij de finale smeltkroes van deze verschillende uitlaatkleppen: het beruchte "Dreamteam"… Ik vorm, met enige trots, de connectie tussen jullie allen. Vanaf het eerste "dreamteam" avondje was het al direct duidelijk dat, om in de context van mijn doctoraat te blijven, de chemie tussen ons onovertroffen is… Ik hoop dan ook dat we nog vele gezellige avonden, feestjes en weekendjes mogen meemaken! #ohohvossemeren #onesieparty

Annelies. Mijn grote zus en grote voorbeeld. Mensen halen ons vaak door elkaar (met dank aan de naamsverwarring en uiterlijke gelijkenissen), maar we verschillen toch ook op veel vlakken van elkaar, en dat botst(e) ook wel eens. Desondanks had ik mij geen betere zus kunnen wensen. Ik wens Xavier en jou alle geluk toe met jullie kleine wondertje in spe. De tante (ahum meter!) zal met alle plezier babysitten, de pony wordt ook alvast klaargestoomd!

Tenslotte zou ik ook heel graag mijn ouders willen bedanken voor alle steun die ik van hen doorheen de jaren mocht ontvangen. Het is niet altijd gemakkelijk geweest, met enkele behoorlijke up's en down's along the way. Ik ben heel blij dat jullie er vandaag alsnog beiden bij kunnen en mogen zijn, en deze verdediging, hopelijk met trots, kunnen bijwonen. Nooit was er iets te veel gevraagd, en die onvoorwaardelijkheid is iets om te koesteren.

Anneleen

"There is nothing like a dream to create the future,

So if you think adventure is too dangerous,

try routine…

it's lethal "

(Adapted from Paulo Coelho and Victor Hugo, Les Miserables)