Formation of boldenone in relation to metabolism of phytosterols

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Elke productontwikkeling gaat gepaard met veel denkwerk, creativiteit, experimenteren,… Een werk van lange adem waaraan met een heel team wordt gewerkt. En dan nog slaagt niet elk idee…
Ik ben blij hier een voorstelling te mogen geven van een brouwsel dat na jaren behoort tot de geslaagde projecten.

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- ◊ Die extra toets, Peter.

Een nieuw functioneel voedingsbrouwsel, om niet aan te weerstaan ;-)

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List of abbreviations

AAS	anabolic androgenic steroids
aBol	α-boldenone
ADD	androstadienedione
AED	androstenedione
And	androsterone
APCI	athmospheric pressure chemical ionisation
AR(1)	first order autocorrelation structure
ASE	accelerated solvent extraction
aT	α-testosterone
bBol	β-boldenone
BOF	Special Research Fund
Bol	boldenone
BSE	Bovine Spongiform Encephalopathy
bT	β-testosterone
bZ	β-zearalanol
CE	collision energy
CYP	cytochromes P450 superfamily of enzymes
DE	diatomaceous earth
DHT	dihydrotestosterone
E	epitestosterone
EI	electron ionisation
EQ	equilenin
ESI	electrospray ionisation
EU	European Union
FC43	perfluorotributylamine
FOD	Federal Government Service
FT-ICR	Fourrier Transform ion cyclotron resonance
FWHM	full width at half maximum
GC	gas chromatography
GC-C-IRMS	gas chromatography combustion isotope rtio mass spectrometry
GC-EI-MS-MS	gas chromatography electron ionisation coupled to mass spectrometry
GC-MS	gas chromatography coupled to mass spectrometry
GC-MS-MS	gas chromatography coupled to tandem mass spectrometry

GC-MSn	multiple gas chromatography coupled to mass spectrometry
HESI-II	heated electrospray ionisation probe
HPLC	high performance liquid chromatography
ILVO	Institute for Agricultural and Fisheries Research
IPH	Scientific Institute of Public Health
IS	internal standard
IWT	Agency for Innovation by Science and Technology
LC	liquid chromatography
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MSn	multiple liquid chromatography coupled to mass spectrometry
LC-QqQ-MS	liquid chromatography triple quadrupole mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m	mass
MeNT	methylnortestosterone
MeT	methyltestosterone
MRL	maximum residue limit
MS	mass spectrometry
MSn	multiple mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoracetamid
MSTFA++	N-methyl-N-trimethylsilyl-trifluoracetamid, amonium iodide and ethanethiol
NP	normal phase
OHP	hydroxyprogesterone
OHT	hydroxytestosterone
P	progesterone
PBS	phosphate buffered saline
PCB	polychlorobiphenyl
PS	phytosterols / plant sterols
Q-Q	quantile-quantile
QqQ-MS/MS	triple quadrupole tandem mass spectrometry
QSAR	Quantitative Structure Activity Relationship
QSM	Quantitative Software Management
R2	correlation coefficient
REML	restricted maximum likelihood

RF	radio frequency
RIVM	The National Institute for Public Health and the Environment
RP	reversed phase
RPA	reference point of action
Rs	replacement, reduction and refinement
Si	silica
SPE	solid phase extraction
SRM	selected reaction monitoring
T	testosterone
TLC	thin layer chromatography
TOF	time of flight
U-HPLC	ultra-high performance liquid chromatography
U-HPLC-MS	ultra-high performance liquid chromatography coupled to mass spectrometry
U-HPLC-QqQ-MS-MS	ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry
UPLC	ultra performance liquid chromatography
V	volume

CHAPTER

1

General introduction and conceptual framework

In recent years, food safety problems have become a frequently recurring phenomenon. Expressions such as bacterial contamination, mad cows disease and food contamination with veterinary drugs or growth hormone residues are no longer unknown to the general public. In the European Union (EU), however, consumer protection ranks extremely high. This is expressed in the precautionary principle based on the Treaty of Amsterdam [1,2]. To reach the required level of protection, reliable data have to be made available, to enable adequate risk evaluation and subsequent action. In other words, knowledge on the presence and metabolism of a wide variety of residues should be extended and sophisticated and robust analytical methods need to be developed for the detection of these residual contaminants. This doctoral thesis addresses the occurrence of steroidal compounds,also described as growth hormones in literature, in particular boldenone and its potential precursors in mammals.

1. Steroidal compounds

1.1 Cholesterol

Cholesterol is a soft, waxy, fat-like substance present in all animal fat as well as in some vegetable fats (Fig. 1). It is found in the cell membranes of all body tissues and is transported in the blood plasma of every animal.

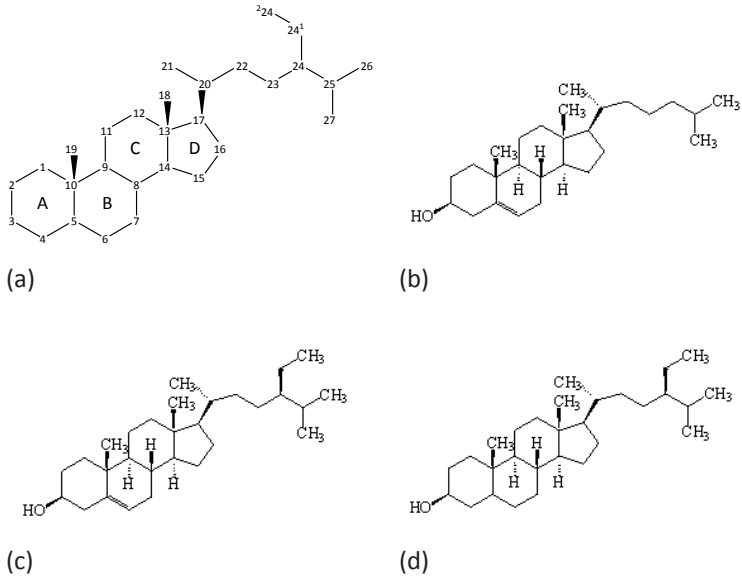

Fig. 1 The numbering of the carbon atoms in the C19 androstane nucleus (also showing some of the additional carbons extending from position 17 that are found in some of the phytosterols based on this nucleus) (a), and the chemical structures of (b) cholesterol, (c) ß-sitosterol and (d) stigmastanol.

Cholesterol can be present in the free or esterified form and has been assigned many activities, i.e. it has an important role in the structure and function of cell membranes and it has a role in the immune system and in the production of bile. Furthermore, cholesterol is also important as being the precursor of many (natural) hormones, which are synthesized through a complicated multi step pathway (Fig. 2) [3,4,5].

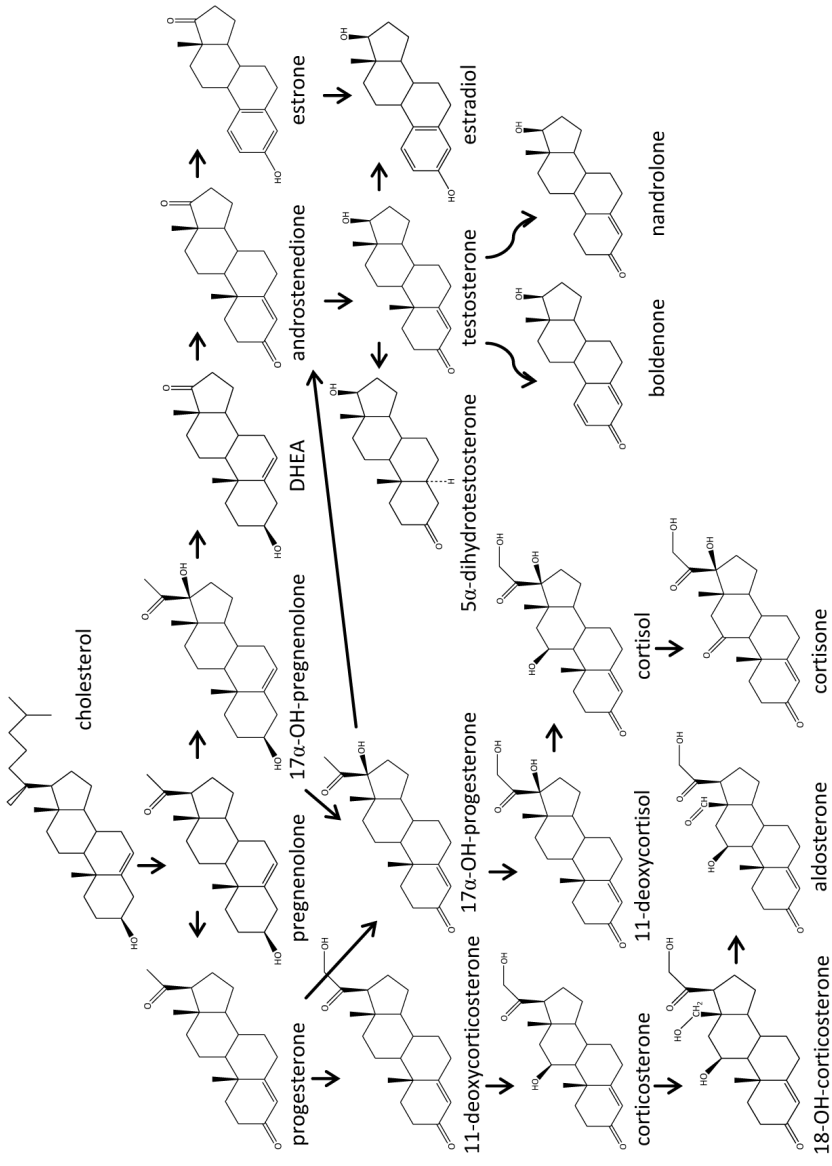

Fig. 2 Schematic of the biosynthetic pathways for endogenous steroids in mammalian species. Wavy arrows indicate putative pathway only [5]

The first step in this pathway is the oxidation of the cholesterol side chain, which leads to the formation of pregnenolone. Next, pregnenolone is converted to progesterone through a delta-4 pathway and progesterone is oxidized to finally form testosterone. In man there are two main sources of cholesterol: less than 0.5 g a day has a nutritional origin and approximately 1 g a day is synthesized endogenously, mostly by the liver, but also by the intestines, adrenal glands and reproductive organs [3,4,5]. Since the elimination of animal fat in animal nutrition, dietary cholesterol has become limited in cattle. In contrast to cholesterol, phytosterols and phytostanols are not endogenously produced by the human and animal body. Phytosterols are, however, naturally present in animal feed of vegetable origin while phytostanols always need to be added exogenously [6].

1.2 Phytosterols

Going back in history, or actually only about a decade ago, talking about phytosterols was merely reserved to scientific environments. Nowadays, people are confronted almost daily with the term phytosterols. Whether it's on TV or in journals, the functional food industry tries to create awareness on the cholesterol lowering characteristics of phytosterols.

Plant sterols or phytosterols (PS) are plant derived compounds analogous to animal cholesterol, fulfilling crucial functions in plant plasma membranes [7,8]. Phytosterols only differ from cholesterol by their side chain configuration at position 17, whereas they are both characterised by an androstane nucleus consisting of four fused ring structures, three hexane rings and one pentane ring (Fig. 1) [4,6,7,8]. An additional double bound can be present at position 22 and at position 24, substitution of a methyl or ethyl group is possible. Over 250 sterols and related compounds have been reported to occur in plants. β-sitosterol has the structure of cholesterol with an additional ethyl group on position 24 (Fig. 1). Stigmasterol has an ethyl group on position 24 and a double bound at position 22. Campesterol has a methyl group on position 24 and brassicasterol has a methyl group on position 24 and a double bound at position 22. The principal sterol in plant materials is β-sitosterol, usually accompanied by its 22-dehydro analogue, stigmasterol. The next most abundant sterol is campesterol. Brassicasterol and avenasterol occur in some plant sources only.

1.2.1 Cholesterol lowering properties

Although cholesterol and phytosterols have a similar chemical structure, their health effects strongly differ (atherosclerosis) [9]. Based on the chemical structures, phytosterols are as atherogenous as cholesterol (phytosterolemia). However, the low absorption efficiency of phytosterols in the small intestine limits this hazardous effect. Phytosterols are only absorbed

in very small amounts in the small intestine while cholesterol is absorbed for more than 33-50 % [6,10]. Moreover, the excretion rate of phytosterols is much higher than that of cholesterol. Finally, the presence of phytosterols limits the absorption of cholesterol in the small intestine, as there exists a competition between both. It appears to be clear that a high baseline dietary intake of plant sterols significantly reduces cholesterol absorption and, despite simultaneous stimulation of cholesterol synthesis, a small but significant reduction of serum cholesterol level can be observed. This explains why the use of vegetable fat, which is rich in phytosterols, is considered an important dietary component for improving blood lipid profiles and reducing the risk of coronary heart disease [7,8,11,12].
In animal feed, all animal fat has been banned and has been replaced by vegetable material since the Bovine Spongiform Encephalopathy (BSE), commonly known as mad-cows disease, and the dioxin crisis. The main kind of sterols in animal feed, specifically for cattle, are phytosterols instead of cholesterol. As it has been demonstrated that phytosterols, ingested by food, nearly pass unabsorbed the intestines, they consequently spend a longer time in the gastrointestinal tract [6]. This would possibly allow their microbial conversion into androgenic products.

1.2.2 Endocrine disrupting characteristics

The androstane structure identifying phytosterols is also typical for anabolic steroids [5]. As a result of the similarities in structure between steroids and sterols, the endocrine disrupting potential of phytosterols has been suggested by several authors [13-16]. β-sitosterol is a weakly estrogenic phytosterol which has been reported to cause reproductive disturbances in fish and to lower the sperm count of rodents although, in a preliminary study on the brown American mink indications of enhanced reproduction were demonstrated [16,17].
The estrogenic or androgenic characteristics of phytosterols have been suggested to depend on their metabolism into steroids. Phytosterols may serve as precursors of several steroids, consequently exerting a potentially negative impact on the initial hormone balance [18-21].

1.3 Phytostanols

The reduced forms of phytosterols, named phytostanols, have an intestinal absorption efficiency which is even lower, nearly zero, than that of their unsaturated derivatives [4,10,22]. Phytostanols are also characterised by higher excretion efficiencies than cholesterol molecules and inhibit absorption of cholesterol and plant sterols in the small intestine. Therefore, phytostanols have the same, or an even higher, cholesterol lowering effect than phytosterols and are added in large amounts (e.g. 2 g/100 g) to some food, e.g. dressing, margarine, cheese and yoghurt. It has been shown that consumption of these preparations

as normal dietary ingredients is acceptable and that lowering of the serum cholesterol can be up to 20 %. A long term reduction of serum cholesterol level to this extent has been calculated to reduce incidence of heart attacks by about 40 % [10]. Examples of phytostanols are stigmastanol and β-sitostanol (Fig. 1).

As mentioned earlier, cholesterol is of importance for the production of other endogenous hormones [5]. Decreasing the cholesterol content in blood plasma by consuming a phytostanol/sterol rich diet could therefore influence the steroidal hormone balance.

1.4 Steroids

Compared to phytosterols, steroids are generally well known substances, specifically as sex hormones. In mammals, this group of compounds consists of the male sex hormones, androgens, as well as the female sex hormones, estrogens, and gestagens [5]. Different steroids in living organisms are characterised by the position and types of functional groups attached to the androstane nucleus (Fig. 1,2). They differ from each other in the position, number and structure of the functional groups (hydroxyl and ketone groups, degree of unsaturation, length of side chain and so forth).

A distinction can be made between endogenous steroids, which are produced naturally in the body and exogenous or xenobiotic steroids, foreign compounds which are produced synthetically or naturally by other organisms, possessing the capability to mimick steroidal effects [23-25].
In mammals, endogenous steroid sex hormones have vital functions in biological systems. In addition to an essential role in maintaining the integrity of biomembranes, steroids typically serve as regulatory molecules in a wide range of physiologic processes [5,26,27]. In contrast, exogenous steroids have been used, since the beginning of recorded history, to enhance performance and appearance in humans, and to promote growth and improve carcass quality in livestock [28,29].

Well known examples of anabolic androgenic steroids (AAS) used in animal fattening are 19-nortestosterone, 17α-methyltestosterone, boldenone and trenbolone [23]. These molecules are derivatives of the testosterone molecule, the main natural androgenic and anabolic steroid formed in the interstitial cells of the testes [30].

1.4.1 Boldenone

Boldenone (Bol), also called 1-dehydrotestosterone or androsta-1,4-diene-17β-ol-3-one, is a steroid which only differs from testosterone (T) by one double bond at the 1-position (Fig.2) [6,24,31]. Both steroids exist as α- and β-epimers. Other steroids, closely related to boldenone and testosterone are androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED) (Fig. 2). The latter di-keto substances are precursors of respectively, 17β-Bol and 17β-T, in different vertebrate animal species. Reduction of the 17-keto group of ADD results in formation of Bol, and dehydrogenase in position 1,2 (delta 1) of the steroid structure of AED leads to ADD.
Boldenone is mainly used as undecylenate ester by bodybuilders and is illicitly administered to racing horses [25,32]. In addition, it is applied as a growth promoter to meat producing cattle. With its low androgenic characteristics but strong anabolic characteristics, boldenone allows to improve anabolic processes like growth and development of muscle mass without any undesired side-effects.

Whereas boldenone used to be a marker of illegal steroid administration in bovine, its endogenous formation has recently been demonstrated in these vertebrates [33,34], complicating decision making on growth promoting abuse.

1.5 Legislative framework

In the European Union, the use of growth promotors, or more generally, the use of pharmacologically active substances is regulated through Council Regulation 470/2009/EC [35]. This regulation describes the procedure for establishing maximum residue limits (MRLs) for pharmacologically active substances in foodstuffs of animal origin. In this regulation, reference is made to Council Directive 96/22/EC [36], laying down the prohibition of the use of substances having hormonal action in stockfarming.
In addition, Council Directive 96/23/EC regulates measures to monitor certain substances, and residues thereof, in live animals and products of animal origin [37]. The latter Directive divides all residues into Group A compounds, which comprises prohibited substances, and Group B compounds, which comprises all registered veterinary drugs. Steroids are classified as Group A substances and consequently measures are required to assure the absence of any abuse. Technical guidelines and performance criteria for residue control, more specifically for analytical residue methods, in the framework of Directive 96/23/EC are described in Decision 657/2002/EC [38].

Although this legislative framework seems to give a clear description on the regulation of hormonal substances, decision making with regard to steroids is complicated because of their endogenous formation by several vertebrates. Boldenone and related 1-dehydro androgens are examples of steroids which were once thought not to be endogenous in bovine [23,39]. Their use as growth promotors was prohibited according to the above regulations, moreover the presence of boldenone in cattles' urine has long been considered proof of illegal steroid adminstration.
Recent literature has, however, addressed the endogenous formation of steroid analytes, boldenone in particular, by livestock under certain circumstances (stress, diet) [23,25,34,39,40]. In different matrices originating from several untreated species, like microbes, crustaceans, rats, pigs, horses and cattle, boldenone has been detected [25]. However, the exact pathway leading to this steroid remains little investigated. In bovine, the most likely origin of boldenone is suggested to be through faecal conversion of precursors such as phytosterols or other steroids by gut microbes [23-25,40]. As a consequence, drug residue control mainly focusses on urine analysis, avoiding faecal contamination during sampling [39,41,42].

Although Council Regulation 470/2009/EC [35] lays down rules and procedures to establish reference points of action (RPAs) for these substances for which no MRL has been described, e.g. for the Group A compounds to which steroids belong, more insight knowledge on the occurrence of (semi)-endogenous substances remains desirable. The above described contradictions highlight the challenge of distinguishing between natural substances, or those suspected or proved to be endogenous (semi-endogenous in some species and under certain circumstances), and exogenous substances. In this context, knowledge on the presence and metabolism of endogenous steroid hormones in meat-producing animals is of utmost importance.

2. Metabolism

2.1 Metabolism approaches

Predictive drug metabolism is known to be based on models (such as Quantitative Software Management (QSM) or Quantitative Structure Activity Relationship (QSAR)), molecular modelling of enzymes and protein-ligand docking, whereas rule-based expert systems and databases also exist. In this context, Fragkaki et al. (2009) demonstrated that specific metabolism schemes can be established for common metabolic alterations which are derived from structurally similar parent molecules [30]. *In vivo* and *in vitro* experiments, however, allow for a more direct and realistic prediction of drug metabolism.

Nevertheless, the use of *in vivo* animal experiments for scientific purposes remains disputable. Not only from an ethical point of view, but also from an economical perspective, execution of *in vivo* vertebrate experiments is not encouraged. Moreover, such experiments are very time-consuming. As a consequence, alternatives for *in vivo* vertebrate experiments are searched for, taking into consideration the so-called "three Rs" in animal science, namely replacement, reduction and refinement [43]. Replacement is defined as the use of any scientific method employing non-sentient material which may replace methods using conscious, living vertebrates. Reduction concerns lowering the number of animals needed to obtain information of a given accuracy and precision. Refinement means any development that leads to a decrease in the incidence or severity of inhumane procedures applied to those animals which have to be used.

2.2 Metabolism organisms

In the past, scientific research has mainly focussed on vertebrates, and it's only in the last decades that the so called "three Rs" have become an issue. Consequently, general endocrinology has been investigated more in vertebrates than in invertebrates.

In vertebrate organisms, steroids are normally secreted into the blood-stream from the adrenal cortex, gonads and other steroid producing tissues. At the same time, these AAS are extensively metabolised, notably in the liver and the kidney, as conversion to the inactive form is required before excretion in the urine. Essentially, the effect of liver and kidney enzymes is to reduce double bounds in the steroid nucleus and introduce hydroxyl functions serving for further introduction of hydrophilic groups. Whereas there exists a bewildering number of different steroid metabolites, there are only a few enzymic conversions involved in their production. These may be simplified as: reduction of the 4-5 double bound and/or reduction of the 3-ketone function to produce a 3-hydroxyl function; reduction of the 20-ketone function to a 20α- or 20β-hydroxyl group; oxidation of the 17-hydroxyl group in C_{19} steroids, or cleavage of the C-21,20 side-chain of C_{21} steroids containing a 17α-hydroxyl group to generate a ketone function at the C-17 position [5]. The specific enzymes involved in this metabolism mainly belong to the CYP P450 family (oxidoreductases and glucuronosyl and sulfotransferases) playing important roles in hormone synthesis and breakdown. Moreover, these enzymes act towards endogenous as well as exogenous drugs, resulting in a metabolic pathway similar to that for testosterone when similar groups and configurations are present (Fig. 2) [5,44]. These enzymatically catalysed alterations, oxidations and reductions, which convert steroids into more polar compounds in order to inactivate the drug and to facilitate its elimination from the body, are categorised as Phase I reactions. In contrast, Phase II reactions conjugate the steroids or their metabolites with glucuronic acid or sulphate, also in

order to facilitate elimination of the steroids from the body [30].

Some degree of similarity between the vertebrate and invertebrate enzyme system has been shown by *in vivo* investigating the testosterone metabolism of the invertebrate mysid shrimp, *Neomysis integer* [45,46]. Also the use of the silkworm, *Bombyx mori*, and the mussel, *Mytillus edulis*, as invertebrate model organisms to replace vertebrates has been reported [47,48].
The ease of maintenance of invertebrate species under laboratory circumstances and consequently the low financial costs, as well as the ethical acceptance to use these organisms in scientific experiments, promote their use as alternatives to vertebrate animals.
In addition to *in vivo* invertebrate models, the *in vitro* use of enzyme systems, and microsomal preparations in biotransformation studies also appears a specifically useful tool to investigate drug metabolism [49-52].
Catfish hepatic and intestinal microsomes for example have been shown to convert testosterone in a regioselective and stereospecific manner resulting in 6β-hydroxytestosterone, and androstenedione among others. In addition to being oxidised, testosterone was reduced at the 3-keto position to form androstenedial [44]. The flexibility of enzyme systems to chemically interact with all four of the steroidal rings and the C17-side chain, utilising hydroxylation and reductive pathways, was demonstrated using the thermophilic ascomycete *Myceliophthora thermophila*, having a remarkable range of secretary and cell-associated enzymes. Furthermore, incubation of testosterone demonstrated these enzymes' ability to acetylate and oxidize, resulting in androstenedione [53]. It is important to point out, however, that while the qualitative profile of metabolites compares favourably between *in vitro* and *in vivo*, the quantitative profile of metabolites can vary between the two conditions.

Based on the similarities in enzymatic activity between vertebrates and invertebrates, it is suggested to perform alternative organism experiments to extend the knowledge on the metabolism profiles of specific substances. A first indication on the metabolism of phytosterols as well as steroid hormones will allow for the creation of a better understanding of the biological characteristics exerted by these substances. Nevertheless, sophisticated analytical extraction and detection techniques are required to allow an evaluation of metabolism in a wide variety of matrices at very low concentrations.

3. Analytics

Mass spectrometry (MS) as an analytical technique for determination of the structure of a molecule, was only developed in the beginning of the 20th century. The concept of MS is to ionise chemical compounds to generate charged molecules or molecule fragments, to separate these ions based on their mass-to-charge ratio and to measure the abundance of the ions [54].

Typically, MS instruments consist of an ion source, a mass analyser and a detector [54]. A schematical overview of the analytical chain in residue analysis from sample to an analytical result, is provided in Fig. 3. A wide range of possibilities for each technology exists nowadays, therefore only the principle of the technologies used in the context of this doctoral thesis are discussed in more detail.

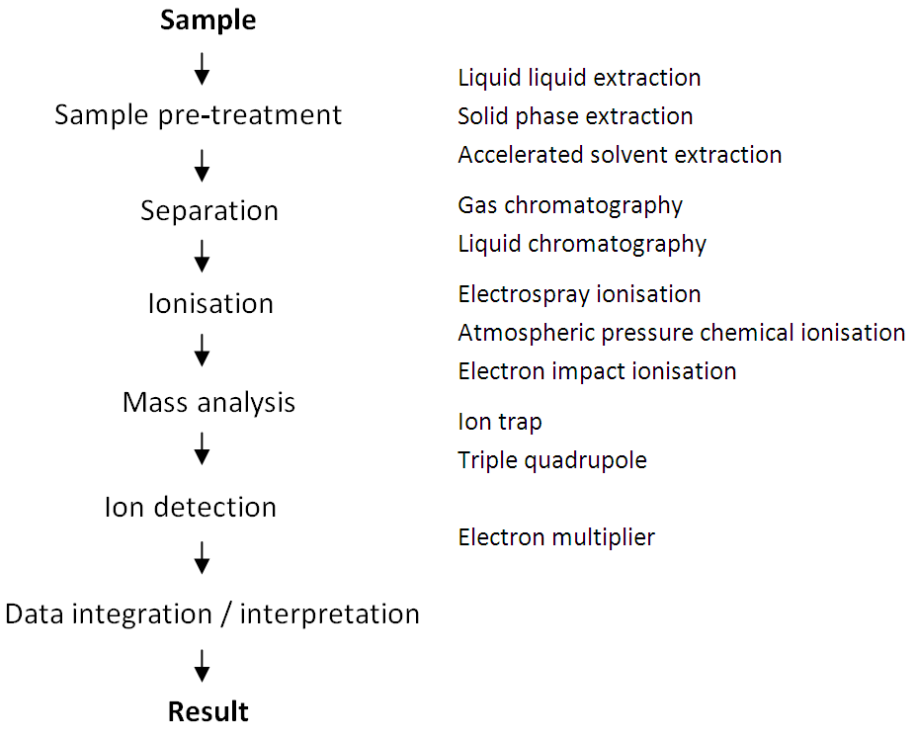

Fig. 3 The general analytical chain of techniques for residue analysis

3.1 Ionisation systems

Analysis by mass spectrometry begins with the conversion of dissolved analytes eluting from a separation system into gas-phase ions at reduced pressure. The type of analytes to be analysed by the mass spectrometer determines the ionisation system.

3.1.1 Electron ionisation

Electron ionisation (EI, formerly known as electron impact) is the most commonly used method for the analysis of volatile compounds (Fig. 4). Ionisation occurs in the gas phase by collision of the neutral sample molecules with energetic electrons, emitted from a filament by thermionic emission. This interaction causes large fluctuations in the electric field around the neutral molecules and induces ionisation and fragmentation [54].

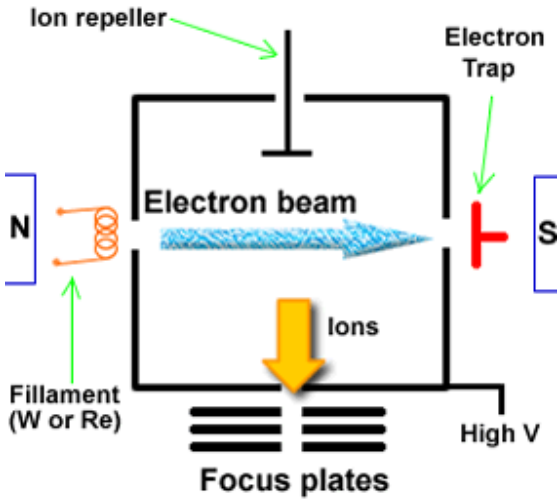

Fig. 4 Schematical process description of electron ionisation (EI)

3.1.2 Chemical ionisation

Chemical ionisation is a lower energy process than electron ionisation, resulting from gas-phase collision between sample molecules and ions of a reagent gas, obtained by electron bombardment. This ionisation by reaction of gaseous reagent is a mild ionisation procedure, producing both positive and negative ions but yielding less fragmentation.

3.1.2.1 Atmospheric pressure chemical ionisation

Atmospheric pressure chemical ionisation (APCI) is an ionisation technique which uses gas-phase ion-molecule reactions at atmospheric pressure (Fig. 5). The analyte is a gas or liquid spray which is nebulised by a high-speed nitrogen beam to be further evaporated. The APCI source contains a heated vaporiser which facilitates rapid desolvation of the spray droplets. The solvent spray is then subjected to a corona discharge creating primary ions. Generally the evaporated mobile phase acts as ionising gas. Positive ions are formed through proton transfer and negative ions are formed through electron transfer or proton loss [54-56].

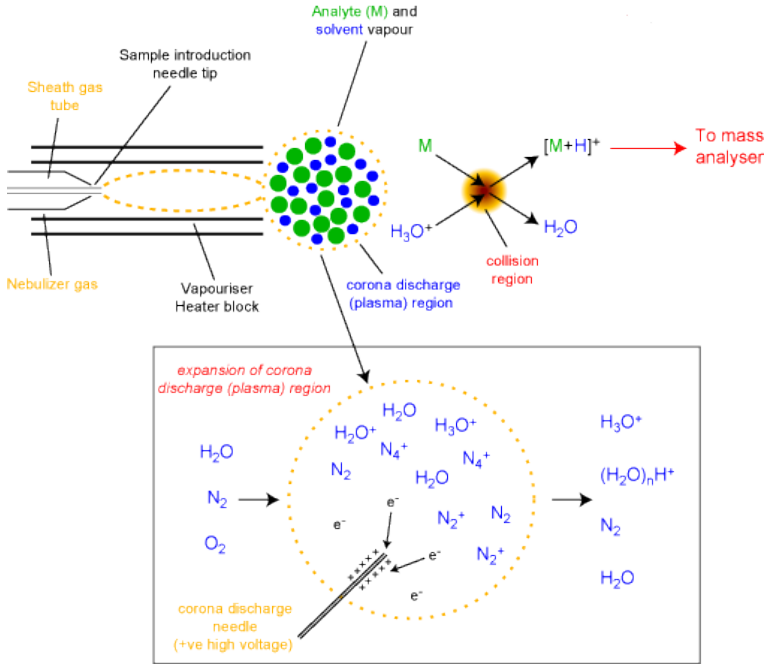

Fig. 5 Schematical process description of atmospheric pressure chemical ionisation (APCI)

3.1.2.2 Electrospray ionisation

Unlike most ionisation processes which occur in the gas-phase, electrospray ionisation (ESI) is the transfer to the gas phase of ions present in the liquid phase (Fig. 6). A prerequisite is that the analyte exists in solution as an ion. Charged droplets are formed of the solvent containing analyte, at the end of a fine capillary. Evaporation of the solvent results in the formation of small but highly charged droplets. Finally non-fragmented, protonated or cationic multi-charged gas phase ions of the analyte are produced [54-56].

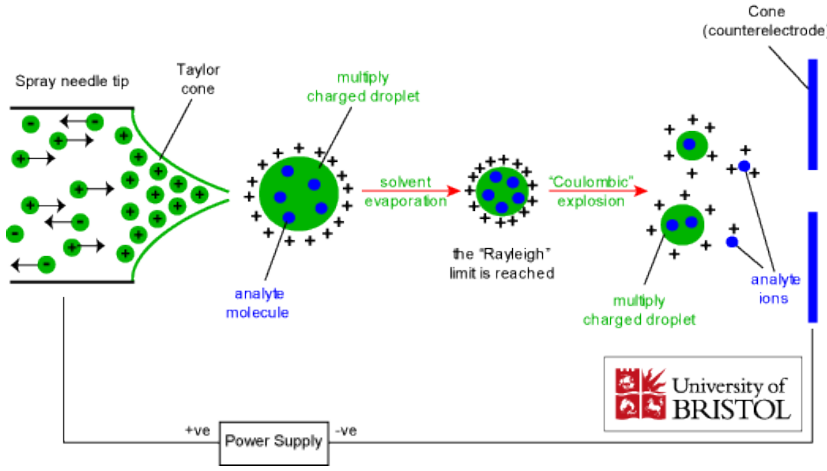

Fig. 6 Schematical process description of electrospray ionisation (ESI)

3.2 Mass analysis

Mass analysis is based on the separation, in time or space, of ions according to their mass-to-charge (m/z) ratios. In this context, it is of crucial importance to control the motion of the charged particles, ions. This can be achieved by applying a vacuum, as well as a magnetic or electric field, whereas uncontrolled collisions would produce a deviation in the trajectory of the ion, resulting in loss of its charge against the wall of the instrument [54,55].

The most commonly used detection techniques in residue analysis are ion trap (Fig. 7) and quadrupole (Fig. 8) mass spectrometers, allowing for the satisfactorily detection, identification and quantification of all major classes of veterinary drugs and growth promoting agents [57]. Based on the extended experience of the Laboratory of Chemical Analysis herewith, these techniques were chosen for the analytical analysis within the framework of this doctoral thesis.

3.2.1 Ion trap

In an ion trap, ions are trapped and sequentially ejected by separation based on their mass-to-charge ratios [54]. Trapping of ions occurs through action of three hyperbolic electrodes, a central ring electrode and two end-cap electrodes (Fig. 7). Ions are subjected to a three dimensional electric field in the space between these three electrodes, resulting in the formation of a cavity in which ions are trapped. During isolation, the ring electrode is driven at an initial imposed voltage so that all ions in a given mass-to-charge range are trapped within the imposed field. Mass selective ion ejection is induced by altering the applied voltage to destabilise the ions. Consequently, ions of increasing mass-to-charge ratio will exit the trap to be detected.

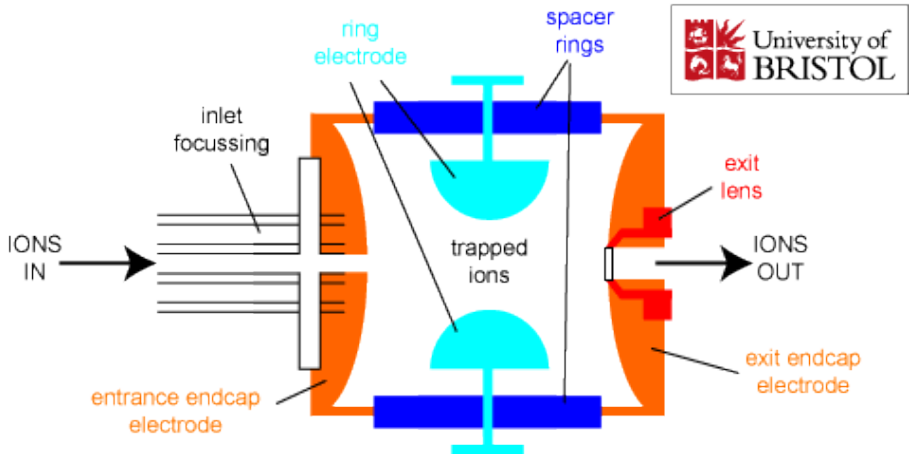

Fig. 7 Ion trap mass analyser

3.2.2 Quadrupole

A quadrupole mass analyzer acts as a mass-selective filter and is closely related to the ion trap except that it is designed to pass the untrapped ions rather than collect the trapped ones (Fig. 8). A quadrupole mass analyser consists of four parallel rods having fixed and alternating potentials applied to them. Ions produced in the source of the instrument are focussed and passed along the middle of the quadrupoles. Their motion depends on the oscillating electric fields, selectively stabilising or destabilising the paths of the ions so that only ions of a particular mass-to-charge ratio will have a stable trajectory and thus pass through to the detector. Varying the electric field allows to bring ions of different mass-to-charge ratios through the quadrupole to the detector [54,55].

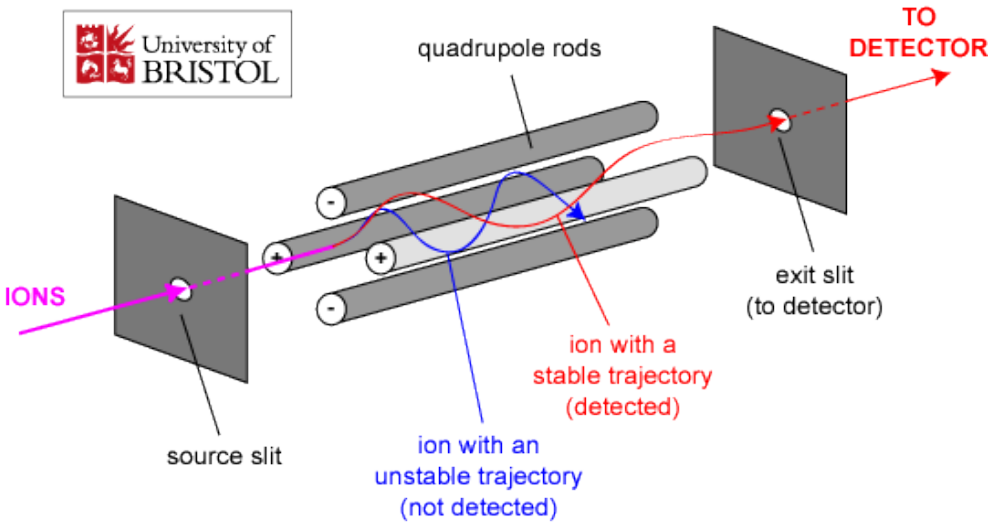

Fig. 8 Quadrupole mass analyser

Mass spectrometers have been developed with two or three mass analysers in a series to study ions fragmentation. Induced fragmentation allows for structural characterisation in addition of mass measurement. A common variation of the quadrupole is the triple quadrupole. Triple quadrupole mass spectrometers have three quadrupoles arranged parallel to incoming ions. The first quadrupole acts as a mass filter. The second quadrupole acts as a collision cell where selected ions are broken into fragments. And these resulting fragments are scanned by the third quadrupole [54]. The majority of current LC-MS based hormone and veterinary drug residue analyses relies on the high sensitivity and selectivity of the triple quadrupole tandem mass spectrometry (QqQ-MS/MS) technology [58].

3.3 Detection

Detection in mass spectrometry is based upon the measurement of the electric charges of ions. For this reason, transformation of ions coming from the mass analyser into a measurable signal is required [54]. Electron multipliers allow for the induction of a cascade of secondary emissions of electrons, finally resulting in a measurable current.

3.4 Separation techniques

An important enhancement to mass resolving and mass determining capabilities of MS is using it in tandem with chromatographic separation techniques. Chromatography is a physical separation method in which the components are selectively distributed between two immis-

cible phases, a mobile phase flowing through a stationary phase bed.
The separation technique in which the mobile phase is a gas, is gas chromatography (GC). Only during the 90s, a shift from thin layer chromatography (TLC) to GC-MS took place for residue analysis in complex matrices [23,59,60]. This method provides good sensitivity and sufficient selectivity but has the disadvantage that it requires a laborious derivatisation step. Silylation, acylation or oxime/silylation is required due to the poor thermal stability and volatility of a wide range of analytes [57,61]. Derivatisation, however, leads to structural changes of the analytes, making the interpretation of the mass spectra not always unambiguous [23,62]. As a consequence, liquid chromatography (LC), the separation technique in which the mobile phase is a liquid, has increasingly replaced GC-MS [23,57]. Today, more than 80 % of the analytical techniques for the determination of growth promoting agents are LC-MS based, usually involving a gradient run on an alkyl-bound silica column [57,63-65]. Nevertheless, it should be noted that the sensitive detection of some specific steroids still requires the use of GC-MS [66].

To further improve analysis times, resolution and sensitivity, laboratories currently consider moving their high performance liquid chromatography (HPLC) methods to new particle sizes and column geometries in order to achieve ultra-high performance liquid chromatography (U-HPLC) [67,68,69]. Initially based on the theories of van Deemter *et al.* [70], the use of small particles is one of the best solutions in the quest to improve chromatographic performances. When reducing the particle size, optimal separations are achieved at higher linear velocities and over a wider range of linear velocities. Consequently, relatively short columns packed with sub-2 µm particles allow shorter analysis time and better resolution. As a result of such column technology, high-pressure drops are generated, requiring the development of ultrahigh or very high pressure pump systems [68]. This innovative ultrahigh pressure or U-HPLC has made it possible to achieve five- to ten-fold faster separations than conventional LC systems, while maintaining or increasing resolution.
Since biological samples can be complex and sample numbers are usually large, fast separations with high resolution and high sensitivity are often required [67]. Specifically with regard to steroid analyses, the use of U-HPLC has been reported by several authors [26,40]. It is expected that commercial pump systems with higher pressure limits will further extend the scope of U-HPLC.

Besides all advantages of LC-MS as an analytical detection technique, a problem still causing concern is ion suppression, also called matrix effect. To prevent this from happening, adequate sample preparation is required.

3.5 Extraction

A range of purification and concentration approaches has been published for the extraction of steroid analytes from multiple matrices, including protein precipitation, immunoaffinity column chromatography, supercritical fluid extraction, molecular imprinted polymers, liquid-liquid extraction, solid phase extraction, accelerated solvent extraction and some very elaborate, but mostly effective HPLC fractionation processes [23,25,57,71].

Often a combination of the above extraction methods is used to achieve isolation and subsequently concentration of the analytes of interest from matrix compounds. Also for the applications discussed in this doctoral thesis, different techniques were combined. A detailed description and discussion hereof is provided in each separate chapter.

4. Conceptual framework of the study

The overall aim of the present doctoral thesis is to investigate the origin of steroids, in particular boldenone, in relation with the presence of phytosterols in vertebrate species. More specifically the research goals of this study are:

- evaluation of the use of invertebrates as alternative model for vertebrate animal experiments to establish the pathway leading to boldenone
- elucidation of the origin of a specific steroid, boldenone, in livestock
- investigation of the metabolism of phytosterols to steroids in humans

This doctoral thesis consists of four research chapters followed by a general conclusion completed with future research perspectives.

In **chapter II**, several invertebrate species, *Neomysis integer*, *Artemia franciscana* and *Lucilia sericata*, are compared for their metabolic capacity. In particular, the testosterone elimination profile of these invertebrates is quantified using liquid chromatography coupled to mass spectrometry. Their use as alternative model organism for vertebrate animal experiments is discussed.

In **chapter III**, the metabolic capacity and steroid metabolism of the maggot *L. sericata* are investigated. The conversion of testosterone as well as of several phytosterols is evaluated with particular interest for the formation of boldenone. The metabolic capacity of this invertebrate maggot is discussed with regard to its preference for cattle's dung.

In **chapter IV**, the origin of boldenone in livestock is investigated with regard to their housing facilities. Protocols are developed to extract and quantify steroids of interest in wooden matrices using sophisticated extraction and analytical techniques.

In **chapter V**, the excretion of steroids is evaluated in humans upon the oral intake of a phytosterol containing yoghurt drink. A validated analytical method using gas chromatography is used for quantification of the steroid profile.

In **chapter VI**, general conclusions are drawn and future research recommendations are formulated.

5. References

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CHAPTER

II

Alternative to vertebrate experiments

After:

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1. Abstract

As long as considered of exogenous origin, β-boldenone (bBol) used to be a marker of illegal steroid administration in calves, whereas its endogenous formation has recently been demonstrated in these vertebrates. However, research on the pathway leading to bBol remains scarce. This study shows the usefulness of *in vivo* invertebrate models as alternatives to vertebrate animal experiments, using *Neomysis integer* and *Lucilia sericata*. In accordance with vertebrates, androstenedione (AED) was the main metabolite of β-testosterone (bT) produced by these invertebrates, and bBol was also frequently detected. Moreover, *in vitro* experiments using feed-borne fungi and microsomes were useful to perform the pathway from bT to bBol. Even the conversion of phytosterols into steroids was shown *in vitro*. Both *in vivo* and *in vitro*, the conversion of bT into bBol could be demonstrated in this study. Metabolism of phytosterols by feed-borne fungi may be of particular importance to explain the endogenous bBol-formation by cattle. To the best of our knowledge, it is the first time the latter pathway is described in literature.

2. Introduction

Because of their muscle-building and growth-enhancing properties [1,2,3], steroid hormones are still illegally administered to food-producing animals. Nevertheless, the possible risks to human health from residues of growth-promoting hormones in food products of animal origin resulted in the ban of hormonal substances in animal production [4]. This European Union legislation applies equally to novel steroids, not existing in nature, and synthetically produced versions of naturally occurring steroid hormones, i.e. exogenous and synthetic endogenous hormones respectively.

As an anabolic steroid with low androgenic activity, boldenone (Bol) has frequently been subject of discussion in residue analysis. β-boldenone (bBol), also called 1-dehydrotestosterone, only differs from the major circulating androgen β-testosterone (bT) by one double bond at the 1-position (Fig. 1). Important steroids closely related to bBol and bT are the bBol epimer α-boldenone (aBol), androstadienedione (boldione, ADD) and androstenedione (AED) (Fig. 1) [5]. Especially because of the structure-similarity of Bol and T, Bol is known to be administered to cattle to promote growth [2]. However, this analyte has been shown to be a metabolite of other steroid hormones, such as testosterone (T) [6]. The incidence of Bol in biological samples has long been considered of exogenous origin. An increase in the amount of Bol-positive samples in the last decades has suggested the origin of Bol in cattle to be endogenous [7-10].

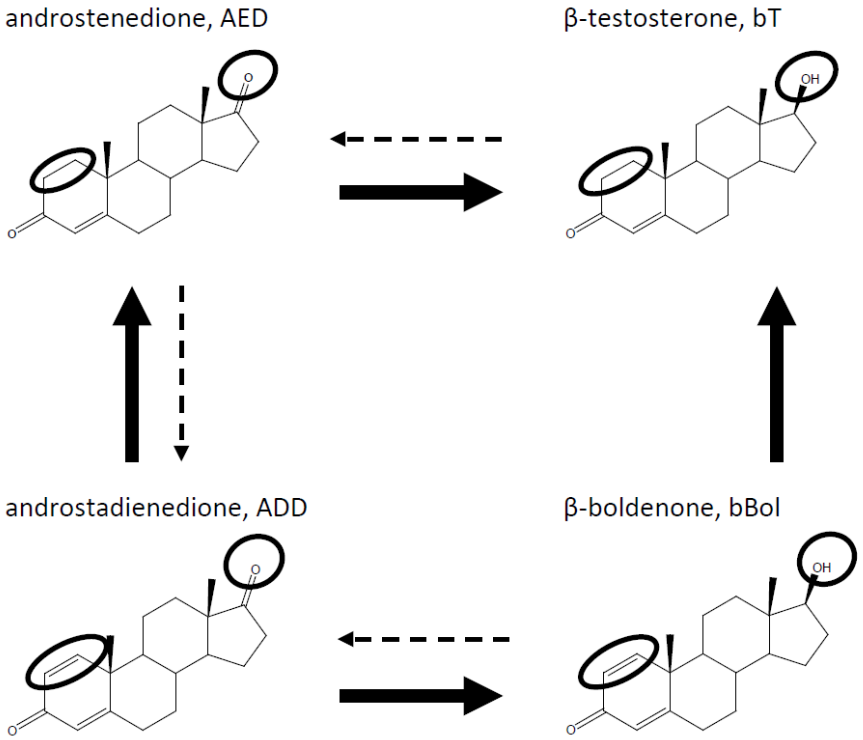


Fig.1 Mutual reductive (bold arrows) and oxidative (dotted arrows) conversions of androstenedione, ß-testosterone, androstadienedione and ß-boldenone, as has been demonstrated in the exposure medium of Neomysis integer (adapted from Poelmans et al. [31]).

For some time now it has been possible to prove the abuse of exogenous steroid hormones in livestock using sophisticated analytical GC-MS^n or LC-MS^n techniques [3,9,10,11-19]. Unfortunately, these techniques do not allow to distinguish between synthetically manufactured endogenous steroids and the chemically identical endogenous hormones which occur naturally in the animal's body. Recently many laboratories have been focussing on the development of new analytical strategies to distinguish between synthetically endogenous and naturally endogenous Bol to prove or disprove the illegal use of this steroid [20-23].

The origin of endogenous Bol and related substances in cattles' urine and faeces has often been linked to the conversion of phytosterols [8,20,21,24-26]. Animal feed is a natural source of phytosterols, such as ß-sitosterol, especially since vegetable fat has been used commonly as an additive to animal feed in response to the crises caused by bovine spongiform encephalopathy, better known as mad cow disease, and polychlorobiphenyls (PCBs) food contamination [8,27]. Phytosterols only differ from steroid hormones by their side chain [28,29], and

their conversion to steroids has been frequently reported [30-32].

However, the pathway leading to endogenously formed Bol in cattle has not been studied extensively. Vertebrate animal trials are time consuming, have serious cost implications, and are often associated with ethical constraints [33,34]. For these reasons *in vivo* invertebrate models have been proposed to simulate vertebrate pathways. In common with Bol, ADD, AED and T have also been identified in several invertebrate species [32,33,35]. In some invertebrates these steroid hormones even seem to exert functions analogous to these of their vertebrate equivalents [36]. Moreover, the metabolic reactions involved in Bol-formation, have been shown in invertebrate as well as in vertebrate animals [33,34]. Over the last decade, our laboratory has built up a solid expertise in the use of the mysid shrimp *Neomysis integer* (*Mysidaceae*; *Crustacea*) as invertebrate model partly replacing vertebrate animals in metabolism studies. Based on this experience, experiments using *N. integer* are reported in this publication [8,31-33,36]. In addition, larvae of the greenbottle fly *Lucilia sericata* (*Arthropoda*; *Diptera*) are considered of potential use in metabolism experiments because of their value as reliable substrates for qualitative analysis of drugs in human tissues, in order to provide post-mortem data for badly decomposed bodies [37]. In addition to *in vivo* invertebrate models, *in vitro* biotransformation studies using microsomal preparations also appear a specifically useful tool to characterise metabolites of particular analytes [38-42].

The aim of this study was to provide evidence for the natural endogenous formation of Bol by cattle based on both alternative *in vivo* and *in vitro* experiments. Firstly, the feasibility of using invertebrates as models for vertebrate metabolism studies was evaluated by examining the metabolic pathway of testosterone *in vivo* in two invertebrate species: the mysid shrimp Neomysis integer (*Mysidaceae*; *Crustacea*) and larvae of the greenbottle fly *Lucilia sericata* (*Arthropoda*; *Diptera*). Secondly, the enzymatic activity to convert testosterone into steroid metabolites of feed-borne fungal species, and more specifically microsomes of *Pleurotus sapidus* (*Basidiomycota*; *Agaricales*), was evaluated *in vitro*.

3. Materials and Methods

3.1 Reagents and chemicals

β-Testosterone (androst-4-ene-17β-ol-3-one, bT, purity ≥98%), methyltestosterone (17α-methyl-4-androstene-17β-ol-3-one, MeT, purity ≥97%), androstadienedione (androsta-1,4-diene-3,17-dione, ADD, purity ≥98%), stigmastanol (24α-ethyl-5α-cholestan-3β-ol, purity ≥95%), β-sitosterol (5-stigmasten-3β-ol, purity ≥70%, residual campesterol and β-sitostanol) and α-pinene (purity ≥98%) were obtained from Sigma-Aldrich Corp. (St-Louis, MO, USA). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥96%), β-boldenone (androsta-1,4-diene-17β-ol-3-one, bBol, purity ≥98%), α-testosterone (androst-4-ene-17α-ol-3-one, aT, purity ≥98%), dihydrotestosterone (5α-androstan-17β-ol-3-one, DHT, purity ≥98%) and different testosterone metabolites (4-androstene-[2α-, 6α-, 7α-, 11α-, 15α-, 16α-, 11β-],17β-diol-3-one, purity ≥98%) were purchased from Steraloids (Newport, USA). Formic acid, ethyl acetate and ethanol were of analytical grade, while methanol and water were of HPLC grade quality. All solvents and reagents were purchased from VWR International (Merck, Darmstadt, Germany). Standard stock solutions were prepared in absolute ethanol at a concentration of 200 ng μL^{-1}. Working standard solutions were prepared by appropriate dilution of the stock solutions in ethanol. All standard solutions were stored at 4°C following the quality assurance instructions of Belac accreditation (EN17025).

3.2 Sampling

Neomysis integer populations were initially collected from the brackish lake Galgenweel (Antwerp, Belgium). After a 24h acclimation period to the maintenance temperature of 15 ± 1°C, the organisms were transferred to 200-L glass aquaria filled with artificial seawater (Instant Ocean®, Aquarium Systems, France) diluted with deionised carbon-filtered tap water to a final salinity of 5‰. Cultures were fed daily with 24–48h old *Artemia nauplii ad libitum* [36].

Maggots of *Lucilia sericata* were obtained through a generous gift from PRO fishing (Oetingen, Belgium).

Fungal species were isolated from grinded corn and inoculated on Sabouraud Dextrose agar in Petri dishes as performed by The Department of Pathology, Bacteriology and Poultry Diseases (Faculty of Veterinary Medicine, Ghent University, Belgium). Subsequently the plates were incubated at 26°C for 4-7 days until harvesting.

Precultures of *Pleurotus sapidus* (8266 DSM) were prepared by The Technical Biochemistry Workgroup (Faculty of Biochemical and Chemical Engineering, Dortmund University, Germany)

as described by [43] and made available for the purpose of this article. As an up-regulation of several enzymes was shown when α-pinene was present in the growing medium, both α-pinene-induced (by addition of 1 mM α-pinene daily) and non-induced cultures were grown.

3.3 Experimental setup

3.3.1 Neomysis integer

Juvenile *N. integer* (Mysidaceae; *Crustacea*) were individually exposed to an excess of 3.5 µM of β-testosterone for 6 hours in 2 mL of medium (water with a salinity of 5 ‰, artificial sea water (Instant Ocean®) diluted with deionised carbon-filtered tap water). During exposure, test organisms were placed in 5-mL glass tubes in a temperature-controlled chamber (15°C, Liebher®, Laborimpex, Brussels) [44-46].

3.3.2 Lucilia sericata

Maggots of *L. sericata* (*Arthropoda*; *Diptera*) were individually placed into 5-mL glass tubes containing 2 mL of medium (water with a salinity of 5 ‰, artificial sea water (Instant Ocean®) diluted with deionised carbon-filtered tap water) at room temperature. Test species were exposed to an excess of 3.5 µM of β-testosterone or 2.4 µM of a phytosterol (β-sitosterol or stigmastanol) for 4 hours [32].

3.3.3 Unidentified fungal species

All media employed in fungal experiments were autoclaved prior to use and standard sterile techniques were applied throughout the procedure in accordance with the specific laboratory guidelines. Samples of fungal mycelium were transferred into 15-mL plastic screw-cap bottles. Subsequently samples were homogenised in HPLC-grade water and the mycelium was separated from the extracellular enzyme containing supernatant by centrifugation. Phosphate buffered saline, pH 7, was prepared (8 g NaCl, 0.134 g KH2PO4, 1.12 g K2HPO4) and 0.5 mL PBS buffer was added to 0.5 mL of supernatant and incubated with an excess of 3.5 µM of an analyte at 37°C for 1 hour.

3.3.4 Pleurotus sapidus

As described by Zorn *et al*. (2003) [43], mycelia of the edible basidiomycete *P. sapidus* were collected by centrifugation and the culture supernatants were discarded. Mycelia were washed once with distilled water and frozen in liquid nitrogen. Frozen mycelia were grinded

under liquid nitrogen, resuspended in phosphate buffer (pH 6.0) and centrifuged. The microsomal fraction was pelleted by high speed centrifugation, resuspended in phosphate buffer (pH 6.0) containing 10% (v/v) glycerol, and diluted in Hepes buffer (200 mM, pH 5.5) to reach a final protein-concentration of 0.2 mg mL^{-1} as determined by means of the Popov assay. In the case of freezing samples before analyses, storage occurred at -20°C. To perform metabolism experiments, a 1 mL aliquot of the microsomal fraction was incubated with 20 µM of an analyte for 22 hours at 24°C while shaking at 150 rpm.

3.4 Extraction

N. integer and maggots of *L. sericata* were isolated from their exposure medium prior to extraction of analytes from these media. Analytes were extracted from exposure media of fungal species and *P. sapidus* without removal of any residual organic material. Methyltestosterone (MeT) at a concentration of 19.8 nM was added to the medium as internal standard before extraction, except for samples of *P. sapidus* to which a 10-fold of this concentration was added. Extracts were analysed for the following Phase I metabolites: 2α, 6α, 7α, 11α, 15α, 16α, 11β-hydroxytestosterone (OHT), α-testosterone (aT), androstenedione (AED), androstadienedione (ADD), β-boldenone (bBol) and dihydrotestosterone (DHT). Extraction and clean-up are based on the method described by De Wasch *et al*. (2002) for *N. integer* [33]. In short, metabolites were extracted from the medium using 4 mL ethyl acetate and after centrifugation (5 min, 14000 x g, 4°C) the organic phase was withdrawn. Ethyl acetate fractions were combined and vacuum evaporated to dryness (Speedvac Plus SC210A, Savant Instruments, Inc., Farmingdale, USA).

3.5 Liquid chromatography – mass spectrometry

The HPLC apparatus comprised of a HP 1100 series pump, an AS3000 autosampler and a HP vacuum degasser (Agilent, Palo Alto, USA). Chromatographic separation was achieved using a Symmetry C_{18} column (5 µm, 150 x 2.1 mm, Waters, Milford, USA), which is illustrated in Fig. 2. For separation of the different compounds, a linear gradient was used starting with a mixture of 60% 0.02 M aqueous HCOOH and 40% MeOH. The methanol percentage increased from 40 to 80% in 25 minutes. The flow rate was set at 0.300 mL min^{-1}. Between each sample the column was allowed to equilibrate at initial conditions (8 minutes). Analysis was carried out using an LCQ^{DECA} Ion Trap Mass Analyser (Thermo Electron, San Jose, USA) equipped with an atmospheric pressure chemical ionisation (APCI) interface (Thermo Electron).

Table 1 Optimised working parameters of the LCQ Ion Trap Source for ionisation of specific steroids.

Ionisation source	APCI
Detection mode	positive
Capillary temperature (°C)	200
Source heater temperature (°C)	450
Sheath gas flow (au)	80
Auxillary gas flow (au)	3

In Table 1 the optimised working parameters for ionisation of the steroids under investigation are presented. The compounds were detected in positive ion mode MS full scan and MS-MS scan. For each specified steroid, Table 2 shows precursor and product ions as well as the optimal MS-MS conditions. The residue was reconstituted in 30 µL methanol and 90 µL 0.02 M aqueous formic acid, except for residues originating from *P. sapidus* which were reconstituted in 300 µL methanol and 900 µL 0.02M aqueous formic acid. A volume of 50 µL was injected on the HPLC-system. This method was originally developed by Verslycke *et al.* (2002) [36] and modified as described by De Wasch *et al.* (2002) [33]. Data processing was performed using Xcalibur® 2.0 software (Thermo Electron).

Table 2 Precursor and product ions of several steroids and their optimal APCI(+) MS-MS conditions expressed by the relative retention time (t_r, min) and collision energy (eV).

Analyte	Relative retention time, tr (min)	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)
hydroxytestosterone	0.38-0.49-0.59-0.65-0.70-0.72	305.2	251 269 287	27
androstadienedione	0.68	285.2	121 147 267	25
β-boldenone	0.80	287.2	121 135 147 173	27
androstenedione	0.82	287.2	109 251	27
β-testosterone	0.92	289.2	253 271	28
methyltestosterone*	1.00	303.0	267 285	27
α-testosterone	1.01	289.2	253 271	28
dihydrotestosterone	1.06	291.2	255 273	30

*internal standard (IS)

3.6 Quality assurance

Prior to sample analysis, standard mixtures of the targeted metabolites were injected to check the operation conditions of the chromatographic devices (Fig. 2). Different metabolites were identified based on their relative retention time, relative to the internal standard methyltestosterone, and on the ion ratio of their product ions as described in the performance criteria for analytical residue methods defined in Commission Decision 2002/657/EC [47]. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio of at least 3, was 0.1 ng on column for OHT, AED, bT, aT, MeT and 0.2 ng on column for ADD, bBol and DHT. All specified product ions were used for peak integration for quantification purposes (Table 2). Quantification occurred by fitting the metabolites' area ratio in a calibration curve established either in standard solution or in spiked matrix.

To account for the presence of any of the targeted metabolites in the exposure medium, extracts as such were run (Fig. 3). Extracts of media in which the analyte of exposure was added were run to account for microbial transformation or impurity of the standard solution in the absence of the test species. The influence of the dilution solvent, ethanol, on the metabolism pattern of the test species was accounted for by integrating a solvent control. Extracts of media in which the test species were not exposed to an analyte were analysed to determine the endogenous excretion of metabolites by the test species.

Fig.2 Chromatograms and spectra of a standard solution of steroids. A concentration of 2 ng was brought on column.

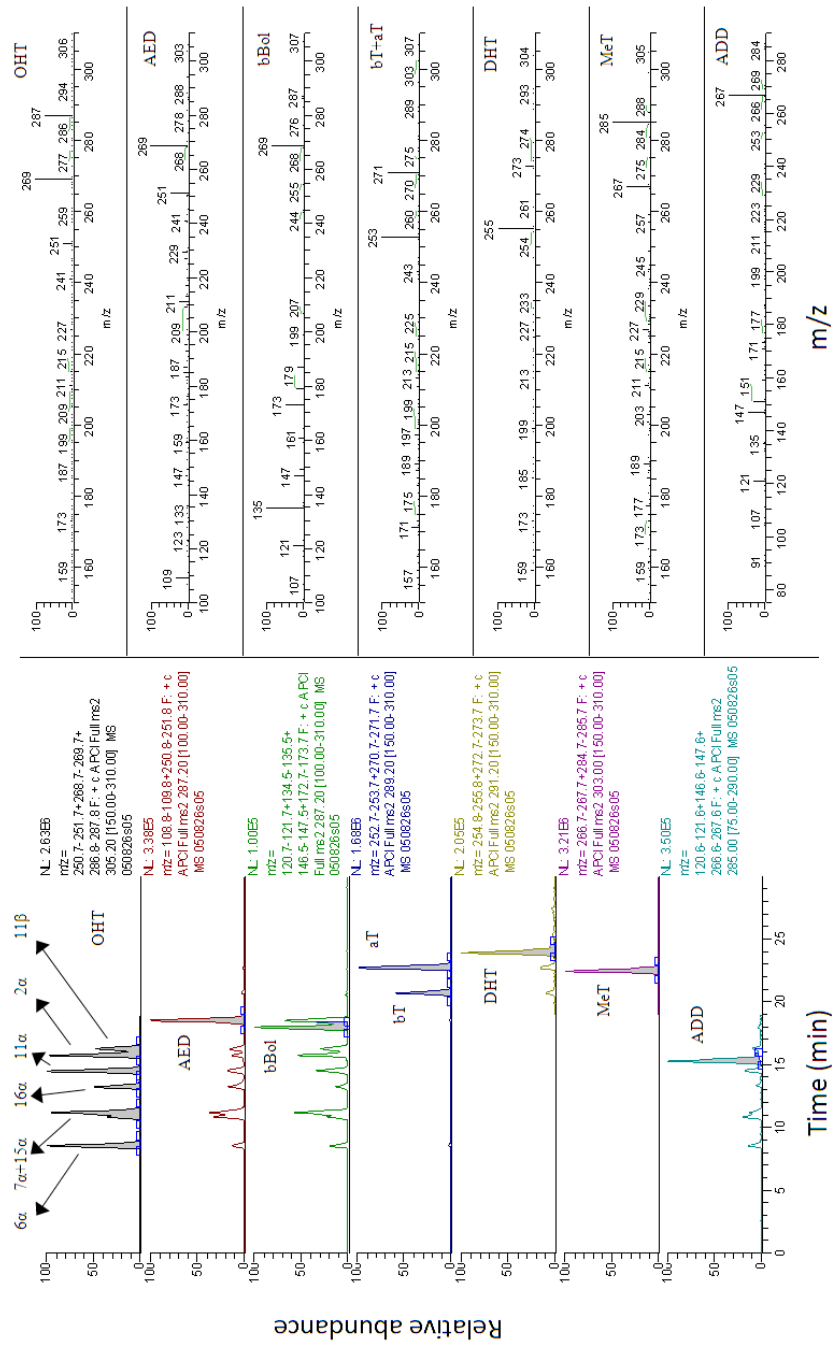
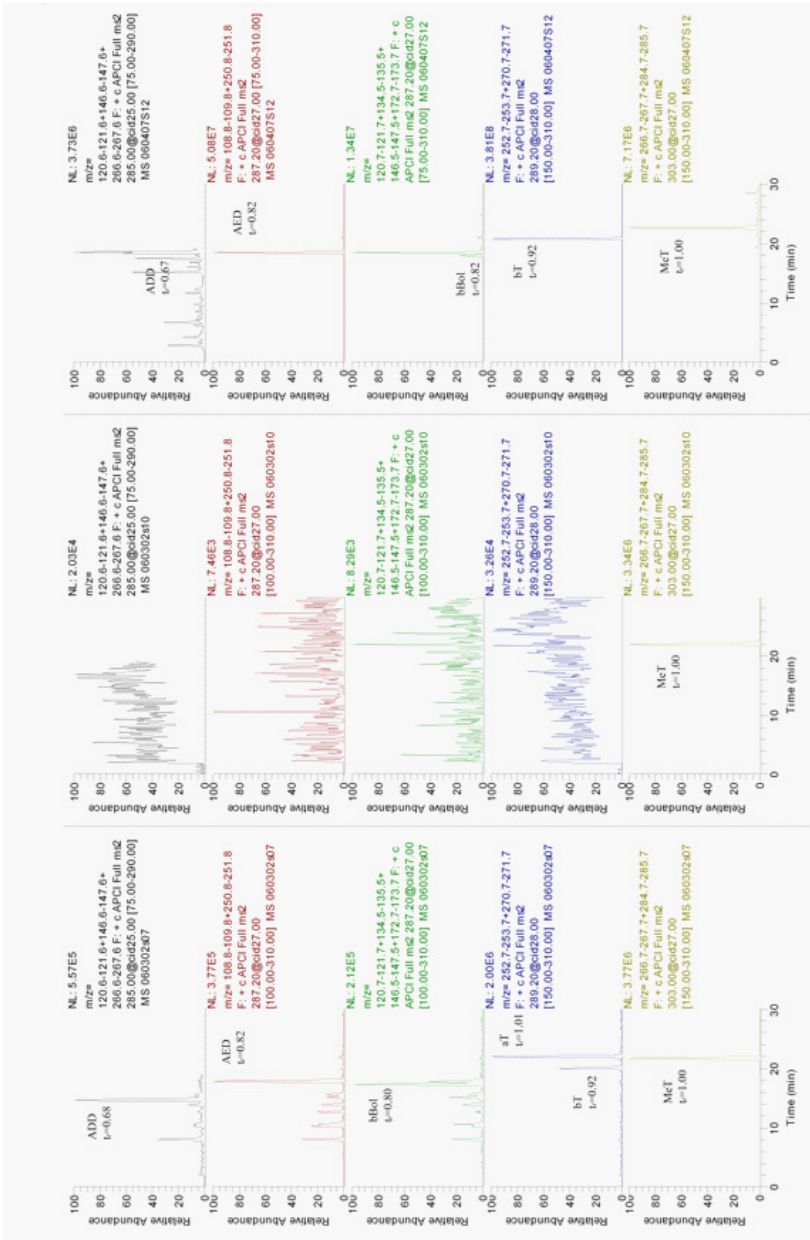


Fig. 3 Chromatograms of (left) a standard solution, (center) a blank exposure medium, and (right) a sample. All were spiked with the internal standard at a concentration of 5 ng on column. A standard solution of steroids was brought on column at a concentration of 2 ng. The sample was an extract of β-testosterone-metabolism by L. sericata.



4. Results

4.1 Method development

For the metabolism experiments using *N. integer* and several fungal species, calibration curves were set up by injecting standard solutions of all targeted metabolites several times at relevant concentrations. For experiments using *L. sericata* and microsomal preparations of *P. sapidus*, extracts of standard solutions spiked in the appropriate exposure medium, were injected several times to set up the calibration curves. In all cases, linearity was achieved in the range of 0.3 – 25.0 ng mL^{-1} for all analytes. The limit of quantification, determined by a signal-to-noise ratio of at least 6, was set at the lowest concentration of the calibration curve (0.3 ng mL^{-1}).

4.2 *Neomysis integer*

In vivo testosterone-metabolism by *N. integer* collected from the Scheldt estuary revealed that, according to mammalian experiments [48,49], AED was an important metabolite, even the major metabolite of T in *N. integer* of these considered in this experiment (Table 3). Testosterone was also converted into several hydroxy-metabolites and DHT (Table 3). Even bBol was detected as metabolite, albeit in low concentrations up to 23 ± 11 ng g^{-1} ww (Table 3). Nevertheless, the variability in excreted concentrations was high between organisms, for AED ranging from 151 ± 74 ng g^{-1} ww to 11,511 ± 3,321 ng g^{-1} ww. This batch dependency was confirmed by experiments performed in the laboratory. Although organisms were acclimatised under controlled environmental conditions (§ 2.3), hormone levels between different replica of this species upon testosterone exposure remained highly variable, marked by AED-concentrations from 479 ± 218 ng g^{-1} ww up to 15,000 ± 10,500 ng g^{-1} ww (Table 4).

Table 3 Metabolite excretion by Neomysis integer, sampled at different locations in the Scheldt estuary, after exposure to β-testosterone (mean ± S.D., ng.g^{-1} ww) (adapted from Verslycke et al., 2004 [58]).

	OHT	aT	bT	AED	ADD	bBol	DHT
sampling point 1	50 ± 14	na	a	2258 ± 719	na	6 ± 6	693 ± 585
sampling point 2	98 ± 25	na	a	11511 ± 3321	na	23 ± 11	-
sampling point 3	13 ± 10	na	a	368 ± 252	na	22 ± 21	-
sampling point 4	120 ± 35	na	a	2515 ± 1358	na	7 ± 7	663 ± 576
sampling point 5	63 ± 20	na	a	924 ± 288	na	10 ± 4	-
sampling point 6	29 ± 9	na	a	151 ± 74	na	-	-
sampling point 7	139 ± 27	na	a	2524 ± 1512	na	7 ± 9	18 ± 17
sampling point 8	81 ± 17	na	a	1180 ± 153	na	22 ± 9	-
sampling point 9	4 ± 4	na	a	33 ± 28	na	y[b]	-

[a] analyte added in excess to exposure medium, value not quantified

[b] y: metabolite was only detected in one replicate sample

na: no data available

Table 4 Overview of metabolite excretion by Neomysis integer after exposure to β-testosterone (mean ± S.D., ng.g^{-1} ww).

	OHT	aT	bT	AED	ADD	bBol	DHT
bT (n=10)[b], [44]	45 ± 14	na	a	2,005 ± 1,447	na	25 ± 12	358 ± 453
bT (n=10)[b], [45]	39 ± 14	na	a	1,137 ± 933	na	-	-
bT (n=10)[b], [45]	51 ± 23	na	a	1,027 ± 298	na	-	-
bT (n=10)[b]	143 ± 54	na	a	479 ± 218	-	-	-
bT (n=7)[b], [46]	61 ± 25	na	a	12,109 ± 6,774	5,171 ± 2,329	1,113 ± 810	na

[a] analyte added in excess to exposure medium, value not quantified

[b] n: number of replicates analysed

na: no data available

4.3 *Lucilia sericata*

Upon bT-metabolism, maggots of the greenbottle *L. sericata* mainly excreted AED in the medium, at mean concentrations of 2,831.3 ± 1,206.0 ng g^{-1} ww (Table 5). ADD and bBol were also detected in the medium, at concentrations of 170.5 ± 96.3 ng g^{-1} ww and 452.2 ± 120.1 ng g^{-1} ww, respectively (Table 5). This indicates the metabolic capability of *L. sericata* maggots (Fig. 3).

4.4 Fungal species

Several unidentified fungal species, grown on corn, converted bT into several hydroxy-metabolites at minor concentrations, and aT and AED at a mean concentration of 72.0 ± 24.9 ng g ww^{-1} and 64.1 ± 25.4 ng g ww^{-1} respectively (Table 6).

Upon exposure to β-sitosterol, AED was mainly produced at a mean concentration of 136.6 ± 67.6 ng g ww^{-1} (Table 6). No ADD, OHT, aT, bT, bBol or DHT could be detected upon β-sitosterol-exposure of fungi in our study (Table 6). Nevertheless, exposure to AED resulted in the production of ADD at a concentration of 247.5 ± 163.1 ng g ww^{-1} (Table 6). Moreover, exposure to ADD led to the production of bBol at a concentration of 87.9 ± 20.1 ng g ww^{-1} (Table 6).

4.5 *Pleurotus sapidus*

Results of these experiments are presented in Table 7. Irrespective of the experimental circumstances (fresh or frozen, induced or non-induced), AED was the main metabolite of bT with concentrations ranging from 20.6 ± 3.1 to 583.1 ng mg^{-1} protein solution. The only hydroxy-metabolite in the medium, detected at a maximum concentration of 113.3 ng mg^{-1} protein solution, was 6α-OHT.

Metabolism of β-sitosterol or stigmastanol was evaluated as well for fresh as for frozen non-induced microsomal preparations isolated from *P. sapidus*. However, none of the investigated metabolites (AED, ADD, bBol, aT, bT) could be detected.

Table 5 Metabolite excretion by maggots of Lucilia sericata after exposure to β-testosterone , β-sitosterol or stigmastanol (mean ± S.D., ng.g^{-1} ww).

	OHT	aT	bT	AED	ADD	bBol	DHT
endogenous	na	na	4.6 ± 2.0 (10/10)[b]	5.8 ± 2.2 (10/10)	- (0/10)	- (0/10)	na
bT	na	na	[a]	2831.3 ± 1206.0 (9/10)	170.5 ± 96.3 (9/10)	452.2 ± 120.1 (10/10)	na
β-sitosterol	na	na	- (0/10)	- (0/10)	31.7 ± 8.3 (8/10)	- (0/10)	na
stigmastanol	na	na	3.2 ± 1.8 (10/10)	- (0/10)	19.4 ± 5.8 (10/10)	- (0/10)	na

[a] analyte added in excess to exposure medium, value not quantified

[b] (x/n) with x: number of replicates in which the metabolite was detected, n: total number of replicates analysed

na: no data available

Table 6 Metabolite excretion by fungal species after incubation with ß-testosterone, androstenedione, androstadienedione, ß-sitosterol or stigmastanol (mean ± S.D., ng.g^{-1} ww).

	OHT	aT	bT	AED	ADD	bBol	DHT
endogenous	-	-	-	-	-	-	-
	(0/5)[b]	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)
bT	9.9 ± 14.4	72.0 ± 24.9	[a]	64.1 ± 25.4	-	-	-
	(13/30)	(5/5)		(4/5)	(0/5)	(0/5)	(0/5)
AED	3.0 ± 0.7	-	691.6 ± 330.0	a	247.5 ± 163.1	-	-
	(3/30)	(0/5)	(5/5)		(5/5)	(0/5)	(0/5)
ADD	-	-	288.6 ± 289.4	678.7 ± 259.6	a	87.9 ± 20.1	-
	(0/30)	(0/5)	(5/5)	(5/5)		(5/5)	(0/5)
β-sitosterol	-	-	-	136.6 ± 67.6	-	-	-
	(0/30)	(0/5)	(0/5)	(5/5)	(0/5)	(0/5)	(0/5)
stigmastanol	-	-	-	-	-	-	-
	(0/30)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)

[a] analyte added in excess in incubation medium, value not quantified

[b] (x/n) with x: number of replicates in which the metabolite was detected, n: total number of replicates analysed

Table 7 Metabolite production by microsomes isolated from Pleurotus sapidus activated with β-testosterone (mean ± S.D., ng.mg^{-1} protein solution).

		6α-OHT	aT	bT	AED	ADD	bBol	DHT
frozen, non-induced	non-activated	1.3 ± 0.2 (2/2)[b]	- (0/2)	- (0/2)	41.6 ± 1.3 (2/2)	- (0/2)	- (0/2)	- (0/2)
	bT-activated	12.5 ± 1.5 (2/2)	- (0/2)	a	65.7 ± 5.8 (2/2)	- (0/2)	- (0/2)	- (0/2)
frozen, induced	non-activated	0.9 ± 0.1 (2/2)	- (0/2)	- (0/2)	22.5 ± 5.5 (2/2)	- (0/2)	- (0/2)	- (0/2)
	bT-activated	9.0 ± 1.5 (2/2)	- (0/2)	a	89.3 ± 29.6 (2/2)	- (0/2)	- (0/2)	- (0/2)
fresh, non-induced	non-activated	1.1 ± 0.2 (2/2)	- (0/2)	- (0/2)	20.6 ± 3.1 (2/2)	- (0/2)	- (0/2)	- (0/2)
	bT-activated	21.1 ± 3.7 (2/2)	- (0/2)	a	159.1 ± 15.0 (2/2)	- (0/2)	- (0/2)	- (0/2)
fresh, induced	non-activated	1.2 ± 0.0 (2/2)	- (0/2)	- (0/2)	- (0/2)	- (0/2)	- (0/2)	- (0/2)
	bT-activated	113.3 (1/1)	- (0/1)	a	583.1 (1/1)	- (0/1)	- (0/1)	- (0/1)

[a] analyte added in excess in incubation medium, value not quantified

[b] (x/n) with x: number of replicates in which the metabolite was detected, n: total number of replicates analysed

5. Discussion

Research by several laboratories in Europe on the prevalence of Bol in untreated cattle indicates the frequent detection of aBol in urine samples up to a concentration of 80.0 ng mL^{-1} (Table 8). Besides aBol, bBol has even been detected in urine samples of untreated animals but not as frequently and in concentrations up to 7.0 ng mL^{-1}. Both Bol isomers have also been reported to be present in the faeces of untreated cattle (Table 8). Although bBol-positive urine samples have been used to indicate illegal animal treatment until the late 90's, Bol may now be considered as an endogenous analyte as well [7,8]. In this study, the pathway leading to endogenous Bol in cattle was simulated by *in vivo* experiments with invertebrate species as alternative models for vertebrates. Moreover, feed-borne fungal species were shown to convert the phytosterol β-sitosterol into AED, potentially contributing to an increase of steroid hormones in the animal feed hence increasing the chance of endogenous Bol-formation.

Table 8 Literature overview of α-boldenone and ß-boldenone findings in urine (ng.mL^{-1}) and faeces (ng.g^{-1}) of untreated cattle.

	Urine (ng.mL-1)		Faeces (ng.g-1)	
	aBol	**bBol**	**aBol**	**bBol**
Arts et al., 1996 [7]	<0.1 – 2.6	<0.1	na	na
De Brabander et al., 2004 [8]	nd – >80.0	nd – ≤7.0	1.0 – 10.0 *	0.1 – 2.0 *
Rossi et al., 2004 [50]	≤7.9	≤0.2 °	na	na
Nielen et al., 2004 [9]	nd	nd	0.1 – 2.0 *	0.1 – 2.0 *
Sangiorgi et al., 2005 [10]	nd	nd	-	-
Arioli et al., 2005 [51]	na	na	nd – 10.0	nd
Arioli et al., 2005 [51]			168.7 – 124664.0 *	331.5 *
Draisci et al., 2006 [52]	nd – 0.9	nd	na	na
Pompa et al., 2006 [53]	nd	nd	nd – 5.9	27.6 – 89.0
Pompa et al., 2006 [53]			nd – 3090.0 *	nd – 482.0 *
Nielen et al., 2007 [25]	nd – 2.7	nd	100.0 *	4.0 *
Gallina et al., 2007 [54]	<1.0	nd	na	na

nd: not detected; na: no data available

* : analyte was only detected in dried faeces, not in fresh rectal faeces

° : analyte was only detected in urine after faecal contamination

Several authors have suggested that a combination of high performance liquid chromatography (HPLC) and mass spectrometry (MS) is the most powerful analytical technique to study drug-metabolism [17,55]. In this study, identification and quantification of known metabolites was required. For this purpose, an ion-trap mass spectrometer with a high detection sensitivity, offering real MS^n capability, was used in atmospheric pressure chemical ionization (APCI) mode.

Most publications dealing with *in vivo* trials on Bol-formation focused on the metabolism- and excretion-profiles upon oral or intramuscular steroid administration to vertebrate animals [10,17,22,41,56]. However, to downscale expensive and time-consuming vertebrate animal experiments, intensive research on the use of alternatives has been conducted during the last decade [33,34,57].

Experiments with the mysid *N. integer,* performed at our laboratory, suggest similarity between the enzymatic steroid metabolism pathway of these crustaceans and mammals [33,36]. The vertebrate type steroid β-testosterone was used to study metabolic pathways dependent on cytochrome P450 enzymes, the latter having a major role in the oxidative metabolism of drugs in mammals [57]. Moreover, it was demonstrated by Poelmans *et al.* (2003) [31] that exposure of *N. integer* to bT primarily leads to AED-production, subsequently followed by the formation of bBol, with ADD as intermediary product (Fig. 1). Field as well as laboratory experiments proved that AED was the main metabolite (Table 3,4). These experiments, however, indicated both spatial (Table 3) and seasonal (Table 4) variability in excreted concentrations [58], forming a serious drawback in using *N. integer* as an invertebrate model organism to investigate hormonal pathways. Especially with regard to bBol, reproducibility of *in vivo* exposure experiments with *N. integer* has been reported by De Wasch *et al.* (2002) [33] to be poor. In contrast, the use of *N. integer*'s testosterone-metabolism has proven its use in eco-toxicological studies [44-46,59]. For the purpose of simulating vertebrate metabolic pathways however, another invertebrate *L. sericata*, was evaluated as model organism [32].

The study of drugs in insects, entomo-toxicology, has become an established approach in toxicological studies since 1980 [60]. In fly larvae, detection of controlled substances and drugs has been carried out by different research groups [60,61]. It should be noted, however, that in these experiments analyses were done on whole organism extracts whereas in our study metabolites were determined upon excretion in the medium, as initial experiments with *N. integer*, revealed that testosterone metabolites are preferably excreted in the medium rather than maintained in the organism's body [36]. Nevertheless, future experiments investigating extraction of metabolites from fly larval bodies might provide additional information

on Bol-formation. bT-metabolism by maggots of the greenbottle *L. sericata* mainly resulted in the excretion of AED in the medium. This reaction, comprising the common oxidation of the enol- to the keto-form, has been described as the most important conversion of bT for *N. integer* as well. The similarity of its metabolic pathways to these described for *N. integer*, suggests the potential value of *L. sericata* as invertebrate model in animal trials (Table 3,4,5). Nevertheless, this observation requires further confirmation to assure the reproducibility of the conversion of bT into bBol and consequently the value of this organism for *in vivo* metabolism experiments. Until now, excreted steroid-concentrations upon bT-exposure were highly variable for fly larvae of different origin, e.g. Belgium, Great-Britain, The Netherlands and Italy (data not shown). It should be noted that the conversion of bT into ADD and bBol was only observed in maggots originating from Italy, as shown in Table 5. As for *N. integer*, this variability in invertebrate metabolism of bT might be explained by the intestinal system of the invertebrates. Within the purpose of this article no distinction was made however between the enzymatic and the intestinal metabolism of invertebrates. In calves' faeces, the most likely origin of 1-dehydro compounds such as Bol is believed to be through conversion of specific precursors, such as phytosterols or other steroids, by gut bacteria [17,53]. However, the present results indicate the potential relevance of the presence of maggots living on faecal material to the origin of Bol-positive faeces samples. Moreover, this mechanism could possibly explain the observed increase in Bol-detection in dried out faeces (Table 8), being more exposed to flies and consequently maggots than fresh faeces.

Even though the role of phytosterols in the formation of Bol and other steroids has often been suggested, it has only been described in a few studies [52,54,62,63]. In this study, the ability of edible fungi - potentially growing on animal feed - to convert phytosterols into steroids was hypothesised. Evidence is existing on the occurrence of fungal species in corn [64]. However, this has mainly been linked to contamination of corn, and consequently animal feed, with mycotoxins [65-67]. To the best of our knowledge, interference of feed-borne fungi in steroid-formation has so far not been considered. The hormonal activity of animal feed ingredients, was taken into account in one study analysing steroid hormone excretion in veal calves' urine and faeces [25]. Unfortunately, only the estrogenic activity of animal feed was evaluated during this study.

Exposure of several fungal species to β-sitosterol mainly resulted in AED detection (Table 6). This is consistent with a study performed by [30] in which AED was the main transformation product of phytosterols in corn flour (mainly containing β-sitosterol) produced by an isolated *Fusarium* strain. The production of ADD could not be proven during their study. Accordingly, no ADD could be detected upon β-sitosterol-exposure in our experiment. Nevertheless, exposure to AED was shown to result in ADD-production, and ADD-exposure led to the

production of bBol by several fungi. These data support the assumption that isolated fungal species are able to transform phytosterols into bBol (Fig. 1), which strengthens the hypothesis that animal feed rich in phytosterols may be a potential source of bBol or its precursors. Important in this context is the fact that during this study fungal species were exposed to micrograms of phytosterols only. A more realistic approach would be exposure to grams of phytosterols. Indeed, cattle at an age of 8 to 12 months old can be fed approximately 15 kg corn-rich animal feed [68]. Depending on the specific type of animal feed and/or the addition of vegetable oils, this equals to about 20 grams of β-sitosterol [69]. Further research should therefore focus on growing fungal strains under laboratory conditions on real animal feed samples to establish steroid hormone production on feed.

To further investigate the P450 enzymatic activity of fungal species, *in vitro* evaluation of their testosterone-metabolism was performed. This resulted in the detection of several hydroxyl-metabolites, aT and AED (Table 6) and is in accordance with literature on testosterone biotransformation by fungi [70,71]. Furthermore, T-conversion by specific microsomal preparations isolated from the edible fungus *P. sapidus* was evaluated *in vitro*. In accordance with the above mentioned experiments, AED was the main metabolite of bT. The only hydroxy-metabolite of bT detected was 6α-OHT. According to Van Puymbroeck *et al.* (1998) [41], hydroxylation in position 6 of steroid hormones appears to be a common metabolic pathway by microsomal preparations. Additionally, these authors reported some interesting advantages for the use of microsomes as an *in vitro* tool for metabolism experiments. The easy preparation of microsomes combined with the relatively low costs and the possibility to preserve microsomal material for years are clear advantages. This is in contradiction with our results, since the AED-concentration of fresh induced bT-activated microsomes was about 6-fold the concentration of their frozen counterparts: i.e. 583.1 and 89.3 ± 29.6 ng mg^{-1} protein solution respectively (Table 7). Possibly this could have been prevented by cryo-preserving the microsomal preparations which appears to be a recognised way of conserving [57]. Since the applicability of fungal microsomes to predict the metabolic pathway of bT was demonstrated in this study, the metabolism of β-sitosterol or stigmastanol was also evaluated using fresh and frozen non-induced microsomal preparations isolated from *P. sapidus*. However, none of the investigated metabolites (AED, ADD, bBol, aT, bT) could be detected. Considering only the fresh microsomal preparations, induction with α-pinene resulted in a 3-fold increase of AED, 583.1 compared to 159.1 ± 15.0 ng mg^{-1} protein solution (Table 7). Consequently, these results clearly indicate the importance of a cofactor, e.g. α-pinene, to up-regulate enzyme activity. Although the enzymatic activity of fresh induced microsomes isolated from the edible fungus *P. sapidus* was demonstrated, these results are preliminary and further confirmation is needed.

6. Conclusion

While many laboratories are focussing on developing novel analytical methods to distinguish between exogenous and endogenous Bol in cattle, this study examined the potential pathways of endogenous Bol-formation.

Vertebrate enzymatic activity was simulated *in vivo* using the invertebrates *N. integer* and *L. sericata*. However, model organism variability was too high to consider these invertebrate species as alternative biotransformation models. Besides cattles' natural enzymatic metabolic pathway, this study also proposes a novel mechanism of endogenous Bol-formation. Feed-borne fungi, potentially living on animal feed, were shown capable to convert phytosterols into steroids. Consequently, increased amounts of steroid hormones, probable precursors of Bol, may unintentionally be offered to cattle through their feed.

To consider the prevalence and the amount of Bol residues in cattle's urine or faeces as proof of illegal animal treatment, a more integrated approach is required, taking into account e.g. the phytosterol-concentration in animal feed, the environmental circumstances and the ecology of gut microbiota.

7. References

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CHAPTER

III

Formation of boldenone and analogues by maggots

After:

K. Verheyden, H. Noppe, V. Mortier, J. Vercruysse, E. Claerebout, F. Van Immerseel, C.R. Janssen and H.F. De Brabander. Formation of boldenone and boldenone-analogues by maggots of *Lucilia sericata*. Anal. Chim Acta 586 (2007) 163-170.

1. Abstract

Current evidence suggests that neo formation of the anabolic steroid boldenone (androsta-1,4-diene-17-ol-3-one) occurs in calves' faecal material, making it difficult to distinguish between illegally administered boldenone and its potential endogenous presence. This strengthens the urgent need to elucidate the pathway leading to boldenone formation. In our laboratory, the invertebrate *Neomysis integer* (*Crustacea*, *Mysidacea*) was used since 2004 as an alternative model for the partial replacement of vertebrate animals in metabolism studies with illegal growth promotors and veterinary drugs, e.g. boldenone. The present study evaluates the metabolic capacity of other invertebrates, the brine shrimp *Artemia franciscana* and maggots of the greenbottle fly *Lucilia sericata*. The first results indicate that maggots of *L. sericata* are able to convert phytosterols and –stanols, nowadays in substantial amounts added to animal feed, into androsta-1,4-diene-3,17-dione (ADD), the precursor of boldenone, at a yield of 0.10 to 0.14 % ($p < 0.001$, significance compared to endogenous excretion of maggots) but not to boldenone itself. Furthermore, ß-testosterone, an endogenous hormone, was transformed into androst-4-ene-3,17-dione (AED), ADD and ß-boldenone at a significant ($p < 0.001$, significance compared to endogenous excretion of maggots) yield of circa 13 %, 0.80 % and 2.2%, respectively. In future studies these results are of value to further evaluate the use of maggots of *L. sericata* as an invertebrate model in metabolism studies.

2. Introduction

In recent years different European Union Member States have focused a lot of research on the presence and metabolism of boldenone in a number of biological samples [1]. The discussion on the origin of boldenone remains a hot topic in those laboratories that determine residues of illegally used anabolic steroids. According to the review by De Brabander *et al.* (2004), an increased number of boldenone findings, especially in bovine faeces and urine samples, should be linked to endogenous formation of boldenone besides of illegal treatment of animals and improved analytical capabilities [1].

The metabolic pathway leading to the formation of boldenone in various animal species remains unknown. In order to elucidate this pathway, animal trials are asked for. Nevertheless, the use of vertebrate animal experiments for scientific purposes still has a serious economic, ethical and ecological impact [2,3]. Because of this ongoing debate, scientists are looking for alternative methods to answer their questions.

In our laboratory, experience has been build up using the mysid shrimp *Neomysis integer* (*Crustacea*, *Mysidacea*) as an invertebrate model partly replacing vertebrate animals in metabolic studies. An indication of the similarity between the metabolism of bovine species and the invertebrate *N. integer* could be given based on the metabolism of several exogenous and endogenous anabolic steroids [2,4,5]. Results were promising, even leading to the consideration of introducing *N. integer* as suitable standard test organism for ecotoxicological studies [6,7]. Nevertheless, results were not always reproducible. According to literature, Verslycke *et al.* (2002) were the first to detect the anabolic steroid ß-boldenone in invertebrates after exposure of *N. integer* to ß-testosterone [4]. However, this conversion could not be repeated.

The weakness of the reproducibility of metabolism experiments with *N. integer* and on the other hand, the strength of invertebrate models encouraged the consideration to perform *in vivo* studies with other invertebrate species. In this paper, nauplii of the brine shrimp *Artemia franciscana* (*Crustacea*, *Anostraca*) and maggots of the greenbottle fly *Lucilia sericata* (*Arthropoda*, *Diptera*) were used to set up metabolism experiments.

The small crustacean *A. franciscana*, better known as brine shrimp, is a typical inhabitant of hypersaline environments that have low species diversity and simple trophic structures [8]. Brine shrimps' eggs are encapsulated in cysts giving them the characteristic to withstand extreme environmental conditions. Cysts can be reactivated by exposing them to more favorable conditions, allowing embryonic development to be completed. Within twenty-four to forty-eight hours, depending on temperature, hatching of nauplii occurs. It takes about three weeks from hatching, over successive developmental stages or instars, to reach the adult stage [9].
The usefulness of this invertebrate species for ecotoxicological studies results from the fact that they are cheap, commercially available and easy to rear under laboratory circumstances [10].

In the past the insect *L. sericata*, or greenbottle fly, has been known especially for its parasitic properties. Larvae, or greenbottle maggots, are facultative ectoparasites. However, these maggots are now widely used in medicine as a fast and effective means of treating necrotic chronic wounds, both in humans and animals [11,12]. In addition, they serve as reliable substrates for qualitative analysis of drugs in human tissues, in order to provide data concerning postmortem interval for badly decomposed bodies [13]. Their value for toxicological analyses remains limited since maggots metabolise and eliminate ingested substances during their development [14,15]. Nevertheless, this fact strengthens their potential use in metabolism experiments.

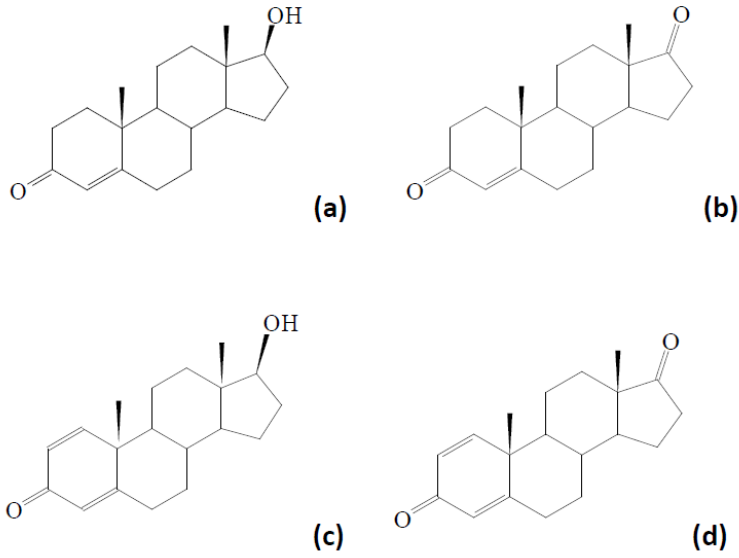


Fig. 1 Chemical structure of the most important steroids involved in this study, (a) testosterone (T), (b) androstenedione (AED), (c) boldenone (Bol) and (d) androstadienedione (ADD).

Our objective was to obtain initial data on the metabolic capacity of nauplii of *A. franciscana* and maggots of *L. sericata* to determine the endogenous origin of boldenone or its formation after exposure to the steroid hormone ß-testosterone, the phytosterol ß-sitosterol and the phytostanol stigmastanol.

17ß-boldenone (bBol), also called 1-dehydrotestosterone, is a steroid with androgenic activity. It only differs from the main steroid hormone 17ß-testosterone (bT) by a double bound at the 1-position. Other important steroids closely related to bBol and bT are their precursors androstadienedione (ADD or boldione) and androstenedione (AED) respectively. The chemical structures of these compounds are given in Fig. 1. bBol, esters of bBol and ADD are for sale as anabolic preparations. bBol improves the growth and feed conversion of cattle and might therefore be abused to achieve more efficient meat production. Transformation of bT to bBol has previously been reported in several invertebrate and vertebrate species [2]. It has also been demonstrated that insects and prawns are able to synthesise steroid hormones out of phytosterols, with cholesterol as an intermediary product [16,17]. Phytosterols or plantsterols and cholesterol are 4-desmethylsterols that share identical ring structures. The various sterols only differ in their side chain configuration. Hydrogenation in the 5α-position results in their saturated forms, the phytostanols. The most common representatives are ß-sitosterol and stigmastanol, a phytosterol and –stanol respectively, illustrated in Fig. 2 [16,18,19,20,21].

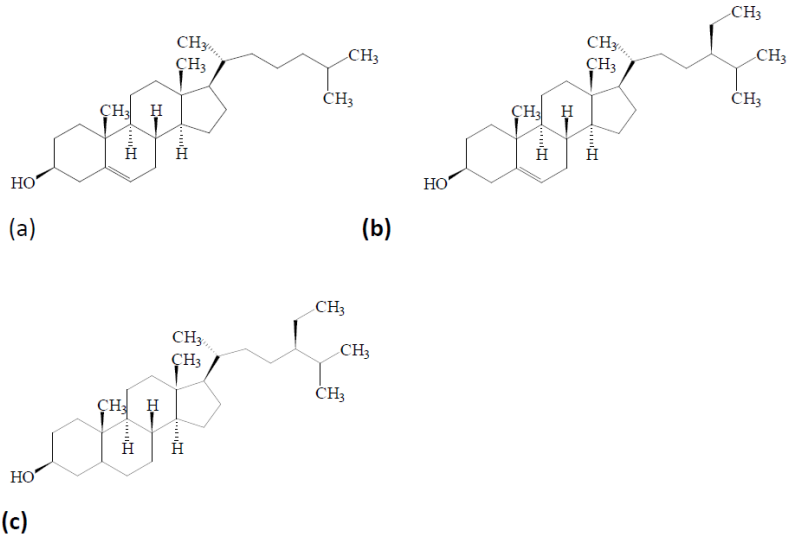


Fig. 2 Chemical structure of (a) cholesterol, (b) ß-sitosterol and (c) stigmastanol.

3. Experimental

3.1 Reagents and chemicals

ß-testosterone (androst-4-ene-17ß-ol-3-one, bT), methyltestosterone (androst-4-ene-17α-methyl-17ß-ol-3-one, MeT), androstadienedione (androsta-1,4-diene-3,17-dione, ADD), stigmastanol ((3ß,5)-stigmastan-3-ol) and ß-sitosterol (3ß-stigmast-5-en-3-ol) (contains 20% campesterol, 21% stigmasterol and 59% sitosterol) were obtained from Sigma-Aldrich (St-Louis, USA). Androstenedione (androst-4-ene-3,17-dione, AED) and ß-boldenone (androsta-1,4-diene-17ß-ol-3-one, bBol) were purchased from Steraloids (Newport, USA). Solvents and reagents were of analytical grade and purchased from VWR (Merck, Darmstadt, Germany).

For each component, a primary standard stock solution was prepared in ethanol (EtOH) at a concentration of 200 ng.µL^{-1}. Working solutions for spiking of the exposure medium were made by appropriate dilution of these stock solutions in EtOH. All standard solutions were stored at 4°C following the quality assurance instructions of Belac accreditation (EN17025).

3.2 Animal collection and maintenance

Cysts of *Artemia franciscana* were purchased from INVE (Dendermonde, Belgium). Hatching was performed by dissolving 5 grams of *Artemia* cysts in 900 mL of artificial sea water (Instant Ocean®, Aquarium Systems, Sarrebourg, France) with a salinity of 30 $g.L^{-1}$ in a 1-L cylinder-conical vessel. Vigorous aeration and continuous illumination was maintained for 24 to 48 h at 25 °C.

Maggots of *Lucilia sericata* were a generous gift from PRO fishing (Oetingen, Belgium).

3.3 Animal experiments

For experiments with *A. franciscana*, 5 mL of nauplii in artificial sea water were diluted in 1 mL of medium (water with a salinity of 5 $g.L^{-1}$ diluted from artificial sea water with deionised carbon-filtered tap water). From this dilution 100 µL was added to 1900 µL of medium in a 5-mL glass tube. Maggots of *L. sericata* were individually placed into 5-mL glass tubes containing 2 mL of medium (water with a salinity of 5 $g.L^{-1}$ diluted from artificial sea water with deionised carbon-filtered tap water).

Test species were exposed to 2 µg of the different analytes (ß-testosterone, ß-sitosterol, stigmastanol) in 2 mL of medium. A 4 h exposure period was applied at room temperature (21 ± 3 °C). These test conditions were based on similar metabolism experiments with the mysid shrimp *Neomysis integer* [22].

Modifications on above mentioned exposure conditions were evaluated in order to optimise metabolism conditions for *A. franciscana*. The influence of the medium composition (tap water / water with a salinity of 5 g.L-1), the temperature (15 °C / room temperature of 21 ± 3 °C) and the exposure period (1 h / 4 h / 6 h) was investigated. All the exposure experiments with this test species were performed in 3 replicas. For *L. sericata*, exposures were performed in 10 replicas.

Different controls were incorporated in the experiments. These are described in more detail in section ‘Quality Assurance’.

3.4 Sample extraction and clean-up

After exposure and before extraction, methyltestosterone was added to the medium at a concentration of 12 ng as internal standard. Metabolites were extracted twice from the medium using 2 mL ethyl acetate and the organic phase was withdrawn after centrifugation (5 min - 14000 g – 4 °C). The two ethyl acetate fractions were combined and vacuum evaporated to dryness (Speedvac Plus SC210A, Savant Instruments, Inc., Farmingdale, USA).

The extract was reconstituted in 30 µL of methanol (MeOH) and 90 µL of 0.02 M aqueous formic acid (HCOOH). Fifty microliters were injected on column [22].

3.5 LC-MS^2 analysis

The HPLC apparatus comprised of a HP 1100 series pump, an AS3000 autosampler (TSP, San Jose, USA) and vacuum degasser (Agilent, Palo Alto, USA). Chromatographic separation was achieved using a Symmetry C_{18} column (5 µm, 150 x 2.1 mm, Waters, Milford, USA). For separation of the different compounds, a linear gradient was used starting with a mixture of 60 % 0.02 M aqueous HCOOH and 40 % MeOH. The methanol percentage increased from 40 to 80 % in 25 minutes. The flow rate was set at 0.3 mL.min^{-1}. Between each sample the column was allowed to equilibrate at initial conditions (8 minutes). Analysis was carried out using an LCQ^{DECA} Ion Trap Mass Analyser (Thermo Electron, San José, USA) equipped with an atmospheric pressure chemical ionisation (APCI) interface and Xcalibur 2.0 software (Batavia, USA). The compounds were detected in positive ion mode MS full scan and MS^2 scan.

3.6 Quality assurance

Prior to sample analysis, standard mixtures of the targeted metabolites were injected in order to check the operation conditions of the chromatographic devices. Different metabolites were identified based on their relative retention time and on the ion ratio of their product ions based on the performance criteria for analytical residue methods defined in Commission Decision 2002/657/EC [23]. Quantification occurred by fitting metabolites' area ratio in a calibration curve. For all targeted metabolites extracts of standard solutions spiked in medium at a concentration of 0.25 to 25 ng.mL^{-1} were injected once to four times. For every single metabolite a calibration curve was constructed. The limit of detection for each component was set at the lowest detectable calibration point. Analyte recoveries were determined by adding known concentrations of the standard solutions to blank medium. The quality assurance data for AED, ADD, bBol and bT are summarised in Table 1.

Table 1 Quality assurance data (limit of detection (LOD), recovery ± standard deviation (stdev) and calibration coefficient (R^2)) for the analysis of AED, ADD, bBol and bT spiked in medium at a range of 2.5E-01 – 2.5E+01 ng.mL^{-1}.

	AED	ADD	bBol	bT
LOD(ng.mL^{-1})	2.5E-01	1.0E-01	1.0E+00	1.0E-01
Recovery ± S.D. (%)	108 ± 18.7	106 ± 20.4	104 ± 17.5	114 ± 21.9
R^2	0.93	0.92	0.99	0.97

Extracts of the medium as such were run to account for the presence of any of the targeted metabolites in the exposure medium. Extracts of medium in which the analytes, ß-testosterone, ß-sitosterol and stigmastanol, were added individualy were run to account for microbial transformation or impurity of the standard solutions in the absence of test species. The influence of the dilution solvent, EtOH, on the metabolism pattern of the test species was accounted for by integrating a solvent control. Extracts of medium in which the test species were not exposed to an analyte were analysed to determine the endogenous excretion of metabolites by the test species. All tests were performed in 10 replicas.

3.7 Statistics

All statistical analyses were assessed with the software package Statistica^{TM} (Statsoft, Tulsa, USA). An outlier analysis was performed and data were checked for normality and homogeneity of variance respectively using Kolmogorov-Smirnov and Levene's tests, both with an α-error of 0.05. If these assumptions were met, the effect of the treatment was tested for significance by one-way analysis of variance (Dunnett's test). If the assumptions were not met, data were logarithmically transformed or the non-parametric Mann-Whitney U-test was used. All box plots were created with Statistica^{TM} and show mean (small square), standard error (box) and the standard deviation (whisker).

4. Results

4.1 *Artemia franciscana*

In a preliminary experiment *A. franciscana* was exposed to a 1 mg.L^{-1} concentration of bT to assess its metabolic capacity. Several exposure conditions (temperature, exposure period, exposure medium) were evaluated. However, under none of the tested conditions metabolites could be detected in the medium.

4.2 *Lucilia sericata*

In this study extracts of the exposure medium of the maggots were specifically examined for the presence of AED, ADD and bBol. For exposure to ß-sitosterol and stigmastanol, the presence of bT as a metabolite was considered as well. The identification of the metabolites was based on what has been described previously for the mysid *N. integer* [2,4,7]. From our experience with *N. integer* [22], it was observed that excreted concentrations of metabolites in the medium were higher than those retained in the organism. Furthermore, in a preliminary experiment with *L. sericata* in which maggots were exposed to bT, AED, ADD as well as bBol were detected in the medium while only AED was detected in the organism and in minor concentrations. Based on these facts and the fact that the method for extraction of the metabolites from maggots is not satisfactory yet, extraction of the metabolites of interest was, within the scope of this article, only performed on the medium, not on the organisms.

After exposure of larvae of *L. sericata* to bT, each of the targeted metabolites AED ($p < 0.001$), ADD ($p < 0.001$) and bBol ($p < 0.001$) was detected in the medium. Mean concentrations of 1.3E+02 ± 4.3E+01 ng.mL^{-1}, 8.0E+00 ± 3.8E+00 ng.mL^{-1} and 2.2E+01 ± 4.8E+00 ng.mL^{-1} were calculated for AED, ADD and bBol respectively (Fig. 3a,b,c). These concentrations were significantly higher than what was excreted by unexposed organisms. Indeed, endogenous metabolite excretion by maggots of *L. sericata* did not contain any ADD or bBol. AED and bT, however, were detected in the medium of unexposed organisms at concentrations of 2.6E-01 ± 1.0E-01 ng.mL^{-1} for AED and 2.1E-01 ± 1.0E-01 ng.mL^{-1} for bT, indicating their endogenous origin (Fig. 3a,b,c,d). In Table 2 an overview is given of the mean concentrations of this metabolite excretion by maggots, relative to their wet body weight.

*Fig. 3 Metabolite excretion in the medium by maggots of L. sericata after exposure for 4 hours to ß-testosterone, ß-sitosterol and stigmastanol compared to the endogenous excretion (*** p < 0.001, significance compared to endogenous excretion). (a) androstenedione (AED), (b) androstadienedione (ADD), (c) ß-boldenone (bBol) and (d) ß-testosterone (bT).*

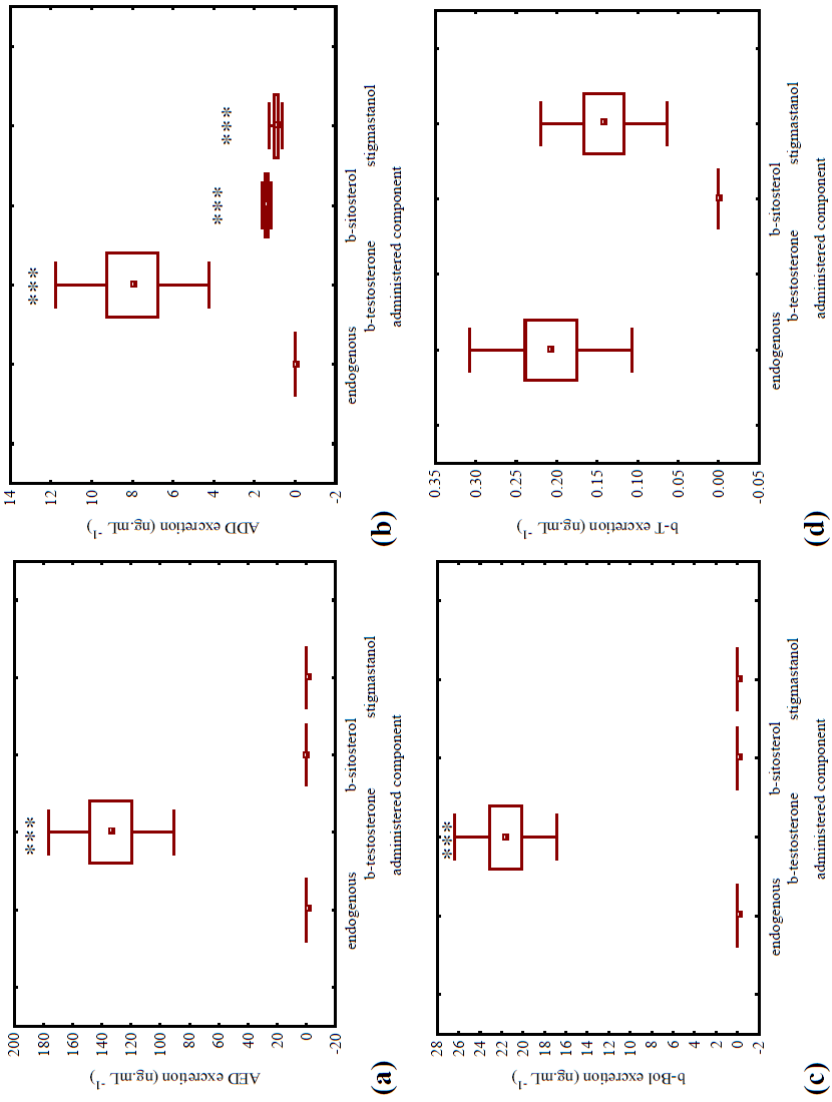


Table 2 Metabolite excretion in the medium by maggots of L. sericata, expressed as mean concentration ± standard deviation, relative to the wet body weight (ng.g ww^{-1}).

administered (2.1E+04 ± 2.4E+03)(ng.g ww-^{1})	Excretion concentration (ng.g ww^{-1})			
	AED	ADD	ß-Bol	bT
endogenous	5.8E+00 ± 2.2E+00	ND[b]	ND[b]	4.6E+00 ± 2.0E+00
β-testosterone	2.8E+03 ± 1.2E+03***	1.7E+02 ± 9.6E+01***	4.5E+02 ± 1.2E+02***	NC[a]
β-sitosterol	0.0E+00	3.1E+01 ± 8.2E+00***	ND[b]	0.0E+00
stigmastanol	0.0E+00	1.9E+01 ± 5.8E+00***	ND[b]	3.2E+00 ± 1.8E+00

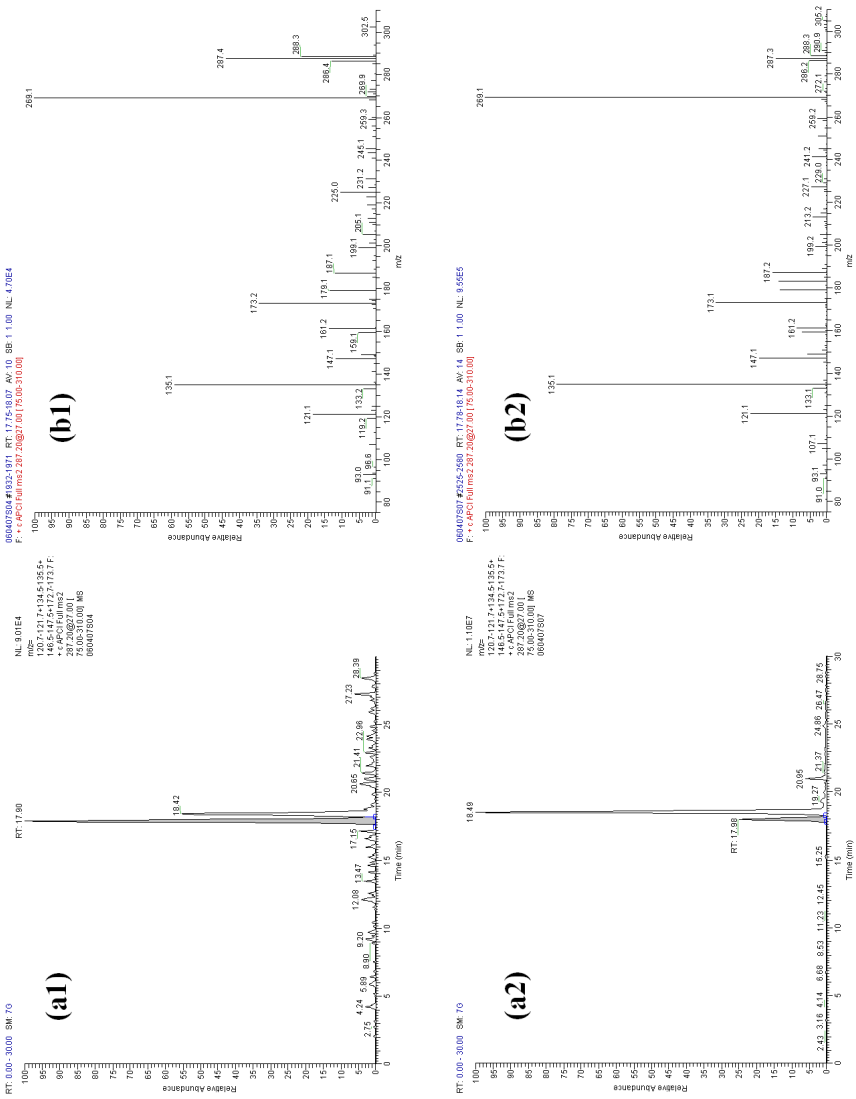
Concentrations were corrected for impurities in the standard solutions. Significant differences of metabolite excretion to endogenous excretion are indicated by *** ($p < 0.001$).

[a] : Not Considered a metabolite

[b] : Not Detected

In Fig. 4 a chromatogram and MS^2 spectrum of bBol, as detected in extracts of the medium in which an individual maggot of *L. sericata* had been exposed to bT for 4 hours, is compared to that of bBol spiked in medium. From the chromatograms it is clear that based on the retention time a peak of interest, highlighted in grey, shows up after 17.9 minutes. The peak at 18.4 minutes corresponds to AED. Confirmation for the identification of the peak at 17.9 minutes, corresponding to bBol, was achieved from comparison of the MS^2 product ions with m/z 121, 135, 147 and 173 of the precursor ion with m/z 287 of sample and spiked sample.

Fig. 4 LC-MS^2 (a) chromatograms and (b) spectra of ß-boldenone (ß-Bol) in medium (1) spiked at a concentration of 5 ng.mL^{-1} and (2) excreted after exposure of L. sericata to ß-testosterone (bT).



Exposure to ß-sitosterol of maggots of *L. sericata* resulted in AED-concentrations in the medium with a mean of 3.8E-01 ± 1.0E-01 ng.mL^{-1}, not significantly different from the concentrations in the medium of unexposed maggots, being 2.6E-01 ± 1.0E-01 ng.mL^{-1}. Significantly higher concentrations of ADD, 1.4E+00 ± 2.3E-01 ng.mL^{-1} ($p < 0.001$), were detected in the medium compared to unexposed organisms in which no endogenous excretion of ADD could be observed. In Fig. 3a and Fig. 3b these results are graphically represented. Mean concentrations of metabolite excretion expressed relative to the wet body weight of the maggots are summarised in Table 2. The excretion of bT was negligible after correction for impurities in the standard solution of ß-sitosterol as described in section 'Quality Assurance'. Because the ß-sitosterol standard consisted of a mixture of three phytosterols (campesterol, stigmasterol and sitosterol) it was not clear which phytosterol was responsible for the formation of AED and ADD by *L. sericata*.

Maggots of *L. sericata* were able to convert stigmastanol into ADD and bT after a 4 hour exposure period. Excreted concentrations of ADD in the medium of exposed organisms, with a mean of 9.6E-01 ± 3.1E-01 ng.mL^{-1}, were significantly higher ($p < 0.001$) than those of unexposed organisms, being absent or below the limit of detection (Fig. 3b). Concentrations of bT, 1.4E-01 ± 7.8E-02 ng.mL^{-1}, were not significantly different from what was excreted by unexposed test species, 2.1E-01 ± 1.0E-01 ng.mL^{-1}, as shown in Fig. 3d. An overview of this metabolism expressed as mean concentrations of excreted metabolites relative to the wet body weight of the maggots is given in Table 2. Since AED was only encountered at concentrations equalling the endogenous excretion of *L. sericata*, it was not considered a metabolite of stigmastanol.

5. Discussion

The aim of the present study was to gain information on the metabolic capacity of two invertebrates, the brine shrimp *A. franciscana* and maggots of the greenbottle fly *L. sericata*. Furthermore, results from this study provide information on their use as a model for the partial replacement of vertebrate animals in metabolism studies. Finally, the potential importance of these results in unravelling the pathway leading to boldenone formation and thus elucidating its origin is discussed.

Because of the simplicity of invertebrate animal experiments in comparison to vertebrate animal experiments, a lot of valuable time and money can be saved and in the end, less vertebrate animals have to be sacrified. In this context, we performed *in vivo* metabolism studies with *A. franciscana* nauplii. The knowledge already available on testosterone metabolism in

invertebrates encouraged the use of bT as steroid substrate for exposure to provide initial data on the metabolic capacity [2,4,22]. The present studies indicate that *A. franciscana* were not able to metabolise bT into detectable concentrations of the targeted metabolites. Although different exposure conditions were assessed, AED, ADD and bBol could not be detected in the medium as possible metabolism products of bT. This might be explained by the fact that the currently used biomass – water ratio was too low to become detectable concentrations of the metabolites. Future studies are required to assess the metabolic capacity of *A. franciscana* at different biomass – water ratios. Based on the present data, this test species was not considered a valuable invertebrate model for metabolism experiments. However, there is a need for more research to confirm or reject the present findings.

Exposure of another invertebrate species, maggots of *L. sericata*, to an excess of bT significantly increased the metabolite concentrations of AED, ADD and bBol compared to unexposed species. In Table 3 the conversion percentages of bT into the targeted metabolites are presented. AED, the direct precursor of bT, was the main metabolite excreted in the medium. This oxidative reaction comprises the oxidation of the enol- to the keto-form, a conversion that has also been described for *N. integer* [22]. ADD and bBol were detected in minor concentrations in the medium, though still significantly different from the endogenous excretion. This observation requires further confirmation to assure the reproducibility of the conversion of bT into bBol, nevertheless these results clearly indicate the metabolic capacity of *L. sericata* maggots and consequently their value for metabolism experiments. Furthermore, the metabolic pathway of bT as observed for *L. sericata* in our experiments resembles what has been described earlier for *N. integer* [22]. This suggests the potential value of maggots of *L. sericata* as invertebrate model in animal trials. However, more studies are required to elucidate the similarity to vertebrate animals' metabolism.

Table 3 Metabolite excretion by maggots of L. sericata, expressed as mean conversion percentages ± standard deviation of the exogenous administered compounds ß-testosterone, ß-sitosterol and stigmastanol.

	Conversion percentages (%)			
administered component	**AED**	**ADD**	**bBol**	**bT**
β-testosterone	13 ± 4,2	0,80 ± 0,38	2.2 ± 0.48	NC[a]
β-sitosterol	0.0	0.14 ± 0,022	ND[b]	0.0
stigmastanol	0.0	0.10 ± 0.031	ND[b]	0.0

Percentages were corrected for endogenous excretion and for impurities in the standard solutions.

[a] : Not Considered a metabolite

[b] : Not Detected

Based on experiments with *N. integer* performed in our laboratory, it has been suggested that boldenone can be formed after exposure of this mysid to several steroid hormones and their precursors [22]. This theory is based on the biosynthesis pathway of bT. *N. integer* produced bT after exposure to its precursor AED. The latter can be formed from ADD by dehydrogenation in the 1,2-position. On the other hand, reduction of the keto-group of ADD to an enol-group would result in the formation of bBol. When exposing *N. integer* to ADD, the formation of both AED and bBol was observed. Moreover, dehydrogenation in position 1,2 of bT might even directly lead to the formation of bBol (Fig. 1). Poelmans *et al.* (2006) hypothesised that exposure to a high concentration of an anabolic steroid, leads to a shift in the equilibrium between bT and AED towards AED production, subsequently followed by the formation of bBol, with ADD as intermediary product [22]. Our results, indicate the existence of a similar pathway in *L. sericata* for the formation of bBol out of bT, when administered in excess. Testosterone metabolism activity or at least the presence of androgenic compounds as a prerequisite for boldenone formation has also been suggested by De Brabander *et al.* (2004) [1]. Their hypothesis was based on the fact that boldenone is mostly reported together with substantial amounts of testosterone and in male animals.

Another theory on the origin of boldenone focuses on the transformation of phytosterols –and stanols. Since the mid 1990s there has been considerable interest in and commercial marketing of phytosterol enriched products, which are suggested to lower human cholesterol levels [19]. Furthermore, the importance of phytosterols as a substitute for fat from animal origin in animal feed has increased due to the crisis from bovine spongiform encephalopathy, as in Belgium in the late 1990's. The popularity of these components has caused

the medical and biochemical community to focus on phytosterols. Consequently, research activity on phytosterols has increased dramatically. Sidechain cleavage of plantsterols with the formation of steroid hormones or their precursors has been described in literature by several authors [5,24,25]. If ADD and other androgens, such as AED and T, are generated after degradation of phytosterols and –stanols to steroidal precursors, other effects may need to be considered. This was the incentive for investigating boldenone formation out of phytosterols and –stanols. Exposure of *L. sericata* maggots to high concentrations of ß-sitosterol and stigmastanol resulted in the excretion of ADD, the precursor of Bol, in the medium. However, direct formation of bBol out of ß-sitosterol or stigmastanol could not be proven based on the present data.

In this study, initial data on the metabolic capacity of *L. sericata* were obtained after exposure of these organisms to unrealistically high concentrations of bT, ß-sitosterol and stigmastanol. The results are of value in future exposure experiments to evaluate the metabolism of these components at environmentally and biologically relevant concentrations and in different matrices. In faeces of veal calves for example, neo formation of the anabolic steroids ADD, aBol and bBol was hypothesised by microbial conversion of steroidal feed components, such as phytosterols, present in the faeces [26]. In literature the detection of Bol in faeces is described and discussed by several authors [1,27,28,29]. Apart from the screening of faeces for boldenone, current studies also investigated urine samples [26,29,30,31]. Consequently, metabolism of phytosterols and anabolic steroids by maggots of *L. sericata* in a matrix such as faeces might provide novel insights into our understanding of boldenone formation. Furthermore, the interference of faecal contamination with boldenone formation in urine requires further investigation.

Finally, it should also be noted that the ability of maggots of *L. sericata* to metabolise ß-testosterone and also phytosterols and phytostanols, makes it interesting to extract metabolites from the organisms. In this respect, the method for androgenic metabolite extraction from the organism as developed for *N. integer* should be optimised.

6. Conclusion

The use of maggots of *L. sericata* as an invertebrate model for metabolism studies has shown to be promising. Compared to experiments with other invertebrate species, such as *N. integer* and *A. franciscana*, the yield as well as the reproducibility of the metabolic reactions have considerably been improved.

It was demonstrated that *L. sericata* is able to transform ß-testosterone into AED, ADD and bBol with a substantial yield of 13 %, 0.80 % and 2.2 % respectively. Lower conversion percentages of 0.14 % and 0.10 % were reported for the transformation of ß-sitosterol and stigmastanol into ADD.

To better understand the origin of boldenone, its precursors, such as ADD, and its metabolites, more research is required. The results presented here can hopefully further advance the current knowledge on the metabolic pathway and the origin of boldenone. More studies regarding the usefulness of *L. sericata* and related species in metabolism experiments with phytosterols and anabolic steroids under more realistic circumstances are currently ongoing.

7. References

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CHAPTER

IV

Influence of livestock housing on boldenone production

After:

K. Verheyden, H. Noppe, J. Vandenbussche, K. Wille, K. Bekaert, L. De Boever, J. Van Acker, C.R. Janssen, H.F. De Brabander, L. Vanhaecke. Characterisation of steroids in wooden crates of veal calves by accellerated solvent extraction and ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry. Anal. Bioanal. Chem. 397 (2010) 345-355.

1. Abstract

Illegal steroid administration to enhance growth performance in veal calves has long been, and still is, a serious issue facing regulatory agencies. Over the last years, stating undisputable markers of illegal treatment has become complex because of the endogenous origin of several anabolic steroids. Knowledge on the origin of an analyte is therefore of paramount importance. The present study shows the presence of steroid analytes in wooden crates used for housing veal calves. For this purpose, an analytical procedure using accelerated solvent extraction (ASE®), solid phase extraction (SPE) and ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-QqQ-MS-MS) is developed for the characterisation of androstadienedione (ADD), boldenone (bBol), androstenedione (AED), β-testosterone (bT), α-testosterone (aT), progesterone (P) and 17α-hydroxy-progesterone (OHP) in wood samples. In samples of wooden crates, used for housing veal calves, ADD, AED, aT and P could be identified. Using the standard addition approach concentrations of these analytes were determined ranging from 20 ± 4 ppb to 32 ± 4 ppb for ADD, from 19 ± 5 ppb to 44 ± 17 ppb for AED, from 11 ± 6 ppb to 30 ± 2 ppb for aT and from 14 ± 1 ppb to 42 ± 27 ppb for P, depending on the sample type. As exposure of veal calves to steroid hormones in their housing facilities might complicate decision-making on illegal hormone administration, inequitable slaughter of animals remains possible. Therefore complete prohibition of wooden calf accommodation should be considered.

2. Introduction

Substances exerting a hormonal action, are prohibited for use in animals intended for meat production in the European Union (EU) since 1981 [1]. Currently, Council Directive 96/23/EC regulates the residue control of veterinary drugs, growth promoting agents and specific contaminants in live animals and animal products [2].

Steroid hormones (estrogens, androgens and gestagens) may be illegally administered to meat producing animals because of their growth promoting characteristics [3-5]. Although the regulation on steroid abuse in livestock has long been believed to be clearly described [2], the endogenous production of specific steroid analytes by livestock under certain circumstances (stress, diet) has complicated evaluation of the results and consequently decision making [6-10]. Therefore, sophisticated analytical techniques for the detection of steroid hormones and their metabolites, are of main importance to decision makers. Whereas gas chromatography coupled to mass spectrometry ($GC\text{-}MS^n$) has long been accepted as the most powerful technique for screening and confirmatory analysis of steroids in a diversity of

matrices [11-13], liquid chromatography coupled to mass spectrometry (LC-MS^n) is nowadays the most important technique of analysis [3,5,13-19]. As such, tedious derivatisation steps can be omitted. Moreover, the introduction of ultra-high performance liquid chromatography (U-HPLC) further allows to scale down analysis time [20].

Cattle, and in particular veal calves, have often been cited with regard to illegal administration of growth promoters such as steroid analytes [5,13,21]. Compared to beef, pork, lamb and poultry production and consumption, veal is a relatively minor player in the global meat industry. Nevertheless, veal is of important value to the United States and several countries of the EU (France, Italy, the Netherlands) [22-24]. To ensure a certain animal welfare, mandatory standards were enforced on the veal industry in the EU [25,26]. As a result of these directives, a trend exists from individual housing of calves in crates towards collective housing [22,24,27-29]. Although materials used for the lateral partitions of the housing facilities mainly changed from wood to steel, wooden slatted floors are often maintained since they are more thermally comfortable [27,30].

Since calves are known to spend most of their active time nibbling non-nutritive objects such as walls or bars [27,29,31,32], the microbial conversion of plantsterols or steroid precursors to steroid hormones in the wood of their housing facilities was suggested. Moreover, the policy of feeding veal calves a liquid diet has long induced nibbling on parts of their crates, whereas the availability of solid foods decreased the time spent nibbling non-nutritive objects [31]. Nowadays, as a consequence of the aforementioned directives [25,26], the provision of a small amount of roughage in addition to a liquid diet is implied [22,30].

Plantsterols, structurally related to steroid hormones, are natural constituents of wood [33,34]. A large number of studies have been devoted to their microbial conversion into steroid analytes [35-39]. In many cases this conversion was investigated with regard to the presence of paper mill industry, processing wooden products [40,41]. Whenever mentioned related to veal calves though, plantsterols are suggested to be added to animal feed [5,9,21]. In contrast, veal calves housed in wooden crates, have always been exposed to plantsterols. But to the best of our knowledge, no scientific literature has so far related incidences of illegal steroid administration to calves being housed in wooden crates. Nevertheless, anonymous sources reported a higher incidence of boldenone positive samples in calves housed in wooden crates versus other calves [42].

This study was conducted to investigate the presence of steroid analytes in wooden crates housing veal calves since this may be a potential source of excess steroid hormones to veal.

The complexity of wood as well as the structural similarity of steroids and their low concentrations in wood, made analysis of steroids in this matrix a challenging task. Firstly, a method for fast and efficient extraction of steroid analytes from wood was developed using accelerated solvent extraction (ASE®). Secondly, fast characterisation of the steroid analytes was achieved using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-QqQ-MS-MS). Finally, these innovative techniques were applied to identify and quantify specific steroid analytes in several wood samples originating from veal calves' housing facilities.

3. Experimental

3.1 Chemicals and reagents

β-Testosterone (androst-4-ene-17β-ol-3-one, bT, purity ≥98%), methyltestosterone (17α-methyl-4-androstene-17β-ol-3-one, MeT, purity ≥97%), androstadienedione (androsta-1,4-diene-3,17-dione, ADD, purity ≥98%) and pregnolone (17α-hydroxy-progesterone, OHP) were obtained from Sigma-Aldrich (St-Louis, USA). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥96%), β-boldenone (androsta-1,4-diene-17β-ol-3-one, bBol, purity ≥98%) and α-testosterone (androst-4-ene-17α-ol-3-one, aT, purity ≥98%) were purchased from Steraloids (Newport, USA). Progesterone (P) was generally gifted by the pharmacy of the Faculty of Veterinary Medicine of Ghent University. All solvents and reagents were of analytical and HPLC grade quality and were purchased from VWR (Merck, Darmstadt, Germany).
For each component, a primary standard stock solution was prepared in ethanol at a concentration of 200 ng μL^{-1}. Working standard solutions were prepared by appropriate dilution of these stock solutions in ethanol. All standard solutions were stored at 4°C following the quality assurance instructions of Belac accreditation (EN17025).

3.2 Sample preparation

As tabulated in Table 1, a selection was made of wood species of different origin. In all the selected wood species a clear distinction between sapwood and heartwood was shown. Of all wood species only the heartwood was used to prepare the samples for this study. Prior to preparing small wood shavings, the massive batons of wood were planed. As such, only fresh heartwood was taken for this experiment, excluding influences of dust particles or chemical components due to oxidative aging of the wood surface.
In addition to these samples, wood originating from calves' crates, identified as *Lophira alata*, was examined (Samples A, C and D). One of these samples originated from a Dutch veal farm,

the other from a Belgian. Contamination of the planks with hair and faeces was considered as a separate sample, referred to as the top-wood sample (Sample B). The same analytical procedure was applied for analysis of this matrix as the one developed for analysis of wood samples.

Table 1 Overview of the trivial names, respective botanical names, families and natural growth area of selected wood species

Wood name	Botanical name	Family	Natural growth area
Angelim vermelho	*Dinizia excelsa*	*Mimosaceae*	South America
Azobe	*Lophira alata*	*Ochnaceae*	Western and Central Africa
Douglas	*Pseudotsuga menziesii*	*Pinaceae*	Europe
Massaranduba	*Manilkara bidentata*	*Sapotaceae*	South America
Oaken	*Quercus sp.*	*Fagaceae*	Europe
Oregon	*Pseudotsuga menziesii*	*Pinaceae*	West of North America
Pine	*Pinus sp.*	*Pinaceae*	Europe
Western Red Cedar	*Thuya plicata*	*Cupressaceae*	West of North America

A plane (C2500/3L DX T.400, Sicar) was used to obtain small wood shavings from the prepared batons. Rotation speed was set at 5200 tours per minute with a feeding speed of 10 meters per minute. The wood dust collector of the plane was diverted to capture the shavings in a separate dust bag. Between the sampling of the selected wood species, the entire instrument, including the dust suction collector, was cleaned carefully by high pressured air.

3.3 Extraction and clean-up

Pressurised liquid extractions were performed on a Dionex ASE® 350 Accelerated Extractor with Solvent Controller (Dionex corp., Sunnyvale, CA, USA). One gram of ground wood was dispersed in one gram of diatomaceous earth (DE, ASE® Prep Diatomaceous Earth, Dionex corp.). A cellulose filter (27 mm, Dionex corp.) was placed on the bottom of a 22-mL stainless steel extraction cell. Each cell was filled with 5 grams of aluminium oxide 90 aktiv neutral (Dionex corp.) before addition of the sample mixture. Extraction was carried out at 100°C using acetone/methanol (2/1) as extraction solvent. After filling of the cell with solvent, a pressurised static extraction phase lasting 15 minutes was performed, followed by a flow of fresh solvent. The extract was evaporated under nitrogen at 60 ± 2°C prior to solid phase extraction (SPE). Based on the method described by Impens *et al.* (2002) [11], a combination of silica (Si) cartridges (Isolute® 500 mg 10 mL SPE colums, Biotage AB, Uppsala, Sweden) and

aminopropyl functionalised silica (NH_2) cartridges (Isolute® 100 mg 1 mL SPE colums, Biotage AB) was used for optimal SPE-procedure. All analytes were eluted using chloroform/acetone (4/1). Prior to U-HPLC-MS-analysis, elutes were evaporated once again under nitrogen at 60 ± 2°C to be subsequently reconstituted in methanol diluted with aquaeous formic acid and centrifugated at 9000 tpm for 10 minutes at 4°C.

3.4 U-HPLC-MS analysis

The ultra-high performance liquid chromatography (U-HPLC) apparatus comprised of an Accela™ High Speed LC and an Accela™ autosampler (Thermo Electron Corporation, San Jose, USA). To achieve chromatographic separation several columns were tested, a Hypersil Gold™ column (1.9 µm, 50 x 2.1 mm, Thermo Electron), a Nucleodur C18 Isis (1.8 µm, 50 x 2.1 mm, Macherey-Nagel, Düren, Germany), a Nucleodur C18 Gravity (1.8 µm, 50 x 2.1 mm, Macherey-Nagel), a Nucleodur Sphinx RP (1.8 µm, 50 x 2.1 mm, Macherey-Nagel) and an Acquity HSS T3 (1.8 µm, 50 x 2.1 mm, Waters, Milford, USA). An Acquity UPLC™ in-line filter (2.1 mm, 0.2 µm, Waters) was used to improve analytical column lifetime. The mobile phase constituted of 0.1% formic acid in methanol and 0.1% aqueous formic acid. Optimised separation of all analytes was obtained on a Nucleodur Sphinx RP (1.8 µm, 50 x 2.1 mm, Macherey-Nagel) using a linear gradient starting with a mixture of 35% aqueous formic acid and 65% formic acid in methanol. The methanol percentage increased from 65 to 95 % in 2 minutes. Between samples the column was allowed to equilibrate at initial conditions for 1 minute. All analytes could be separated in a total runtime of only 6 minutes.

Detection was carried out using a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Electron) equipped with a heated electrospray ionisation probe (HESI-II). The parameters as presented in Table 2 were found to be the optimal ionisation source working parameters for the respective analytes.

Table 2 Default and optimised HESI-II working parameters for ionisation of specific steroid analytes

	Default	Optimised
Spray voltage (V)	3000	3000
Capillary temperature (°C)	250	310
Sheath gas pressure (psi)	30	45
Auxiliary gas pressure (arbitrary units, au)	30	15
Heater temperature (°C)	325	370

All analytes were detected in positive ion mode selected reaction monitoring (SRM). An overview of the transition specifics for all analytes is provided in Table 3. The mass resolution at the first (Q1) and third (Q3) quadrupole was set to 0.7 Da at full width at half maximum (FWHM). The cycle time was adjusted to 1.7 seconds, the collision gas pressure was set at 1.5 mTorr and the chrom filter peak width at 5 seconds.

Table 3 Selected reaction monitoring specifics for all analytes of interest: protonated molecules at appropriate S-Lens RF-amplitude, and the corresponding collision energy (CE) necessary for fragmentation into specific fragments

Analyte	Molecular molecule (m/z)	Product ion (m/z)	CE (V)	S-Lens (V)
ADD	285.15	77.16	51	54
		91.15	39	
		121.16	22	
		147.19	15	
bBol	287.17	77.16	51	56
		121.17	26	
		135.24	17	
AED	287.19	79.17	36	70
		81.18	37	
		97.18	21	
		109.17	25	
bT	289.20	79.15	39	70
		97.16	22	
		109.15	27	
		253.32	15	
P	315.21	79.17	38	75
		97.15	23	
		109.15	28	
		297.33	13	
aT	289.20	79.17	40	70
		97.16	23	
		109.16	27	
		253.30	16	
MeT	303.22	97.17	30	73
		109.16	28	
		285.35	15	
OHP	331.15	79.15	45	77
		97.16	28	
		109.14	30	
		295.32	17	

3.5 Quality assurance

Prior to sample analysis, standard mixtures of the targeted analytes were injected in order to check the operation conditions of the chromatographic devices. To every sample, a procedure internal standard (IS), MeT, was added prior to extraction. Different analytes were identified by comparison of their retention time relative to the IS, with that of standards. In addition, the ion ratios of the product ions were compared with those of standards. All investigated analytes were characterised by a unique relative retention time and specific ion ratio of the product ions, showing good selectivity of this analytical method. The specifity of the method was demonstrated by analysis of non-fortified *Quercus sp*. samples and samples fortified with the analytes of interest at a concentration of 100 ppb. For each analyte spiked, the chromatogram showed a significant increase in peak area and intensity at its specific retention time compared to blanks, taking a signal-to-noise ratio of at least 3 into account. Repetitive injection of a mixture of all analytes in standard solution (n=10), at a concentration of 0.1 ng on column, showed good repeatability of the method with a standard deviation in the range of 0.2% – 1.0% and 2.5% – 7.0%, respectively on the relative retention time and the area ratio. After identification, the analyte's concentration was estimated by fitting its area ratio in a eight-point calibration curve established in standard solution. 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 instrument's limit of detection, determined by standard injection with a signal-to-noise ratio of at least 3, was 5.0 pg on column for all analytes of interest. For every single analyte, correlation coefficients obtained for the calibration curves were higher than 0.97, showing good linearity in the range of 0.1 – 10.0 ng on column, and the IS at 5.0 ng on column. To quantify analytes, the standard addition approach was the method of preference.

3.6 Standard addition approach

The standard addition approach used in this study was based on the approach mentioned in Commission Decision 2002/657/EC [43]. After extraction and clean up, dried residues were divided into two aliquots of analogous mass (m) and volume (V). One aliquot, the unknown, was reconstituted in methanol/aqueous formic acid (35/65) for injection on the U-HPLC-MS-MS system. After analysis of the unknown, the concentration of each identified analyte was estimated by fitting its area ratio, $x_{unknown}$, in a calibration curve set-up in standard solution. Based on this estimation, the other aliquot was spiked with a similar known concentration of the identified analyte (A). Final reconstitution of this aliquot was also performed in methanol/aqueous formic acid (35/65). U-HPLC-MS-MS analysis of this aliquot resulted in

an area ratio of x_{known}. Using the following formula, the unknown concentration ($C_{unknown}$) was calculated:

$$C_{unknown} = x_{unknown}\, V_{unknown}\, \rho_A\, V_A / (x_{known}\, V_{known}\, m_{unknown} - x_{unknown}\, V_{unknown}\, m_{known})$$

with $V_{unknown} = V_{known}$ and $m_{unknown} = m_{known}$

$$C_{unknown} = x_{unknown}\, \rho_A\, V_A / (x_{known} - x_{unknown})$$

C = concentration
x = area ratio
V = volume
ρ = concentration
m = mass
A = identified analyte

3.7 Data analysis

Data were interpreted using Xcalibur 2.07 software (Thermo Electron, San José, USA). Statistical analyses were performed with the software package Statistica™ (Statsoft, Tulsa, USA). Data were checked for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's test respectively, both with an α-error of 0.05. The effect of a parameter was tested for significance using a one-way analysis of variance (ANOVA, Dunnett's test).

4. Results

4.1 ASE optimisation

Several parameters of the ASE procedure were optimised by analysis of *Quercus sp*. samples spiked at 200 ppb of ADD, AED, bBol, bT, P, aT and OHP while the internal standard, MeT, was spiked at 500 ppb. Spiking was done on the cellulose filter, resulting in higher recoveries with better reproducibility than spiking on top of the sample (Fig. 1). Apparently the absorbance characteristic of diatomaceous earth has a potential negative effect on spiking.

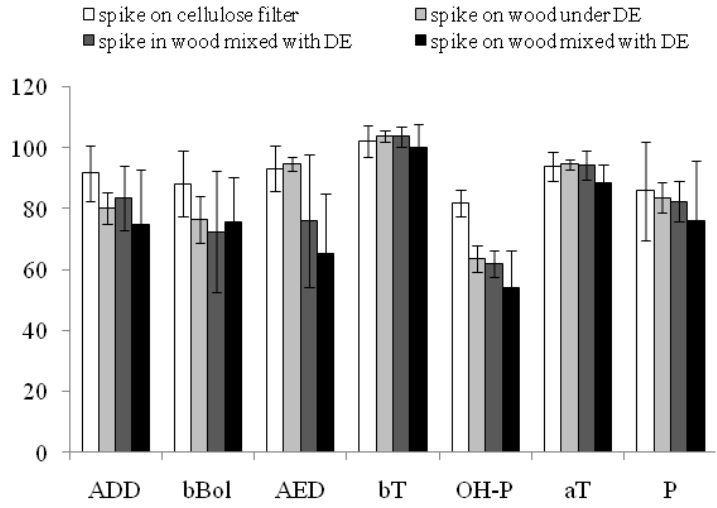

Fig.1 Mean recoveries (%) of steroid analytes (ADD, bBol, AED, bT, OHP, aT, P) spiked at 200 ppb in Quercus sp. Error bars correspond to standard deviations on the mean, with n = 5. Different colored bars represent different ways of spiking.

First, extraction solvents with different polarity characteristics were tested (n-hexane, diethyl ether, ethyl acetate, methanol, acetone/methanol (2/1)). Second, temperature (60 – 100 – 140 – 180 °C), static time (5 – 10 – 15 – 20 minutes), flush volume (20 – 40 – 60 – 80 %) and the number of extraction cycles (3 times 5 min – 2 times 10 min – 1 time 15 min) were evaluated. The average area of all targeted analytes was used as a marker to evaluate the extraction efficiency. This way the default ASE extraction procedure as provided by the manufacturer was optimised for the extraction of steroid analytes from wood by evaluating one individual parameter at a time (Table 4).

Table 4 Default and optimised working parameters for ASE extraction of steroid analytes from wood.

	Default procedure	Optimised procedure
Temperature (°C)	120	100
Static time (minutes)	15	15
Flush volume (%)	60	60
Number of cycli	1	1

The most optimal extraction solvent was selected based on analytical characteristics such as peak area and signal-to-noise ratio, but also on visual characteristics such as lightness and contamination of the obtained extract. According to these criteria, extraction with methanol or acetone/methanol (2/1) led to the best results, giving significantly higher peak areas than with n-hexane ($p<0.01$), diethyl ether ($p<0.01$) or ethyl acetate ($p<0.05$). Since peak areas were slightly higher and samples clearly less contaminated using acetone/methanol (2/1), this solvent was selected for further experiments.

Extraction efficiency at a temperature of 180°C was significantly lower compared to extraction at a temperature of 140°C ($p<0.01$), 100°C ($p<0.05$) or 60°C ($p<0.01$). No significant differences could be observed between 140°C, 100°C or 60°C. A static time of 5 minutes showed significantly lower extraction efficiency than a 20 minutes static time ($p<0.05$). Average peak areas of all analytes extracted using static times of 10 or 15 minutes were higher than in the case of a 5 minutes static time and lower than in the case of a 20 minutes static time, although not significantly. Whether this static time of 15 minutes was executed using 1 cycle of 15 minutes, 2 cycles of 10 minutes or 3 cycles of 5 minutes did not result in significant differences. An increase of the flush volume on the other hand from 40 % to 60 % significantly increased peak areas. No significant differences were shown between a flush volume percentage of 20, 60 or 80, but peak areas were slightly higher at 60 %.

As a result of these experiments, optimised working conditions for extraction were one extraction cycle of 15 minutes at 100 °C with a flush volume of 60 % (Table 4).

4.2 Clean-up optimisation

As both reversed phase (RP) SPE and normal phase (NP) SPE have been described in literature for the clean-up of steroids extracted from solid matrices [5,11,44], the optimal SPE retention mechanism (RP or NP) for this particular application was identified by evaluating both mechanisms. For this purpose, RP extraction using octadecyl end-capped functionalised silica (C18) cartridges was compared to NP extraction using silica (Si) cartridges (Table 5). For all analytes, peak areas were higher when applying the NP mechanism, using Si as specific sorbent, than when performing RP extraction. To provide an additional clean-up, these cartridges were combined with aminopropyl functionalised silica (NH_2) cartridges as described by Impens *et al.* (2002) [11].

Table 5 Different steps of SPE procedure following reversed phase (RP) retention or normal phase (NP) retention.

	RP (C18)	**NP (Si)**
Sample pre-treatment	water	optional
Sorbent solvation	methanol	n-hexane
Sorbent equilibration	water	n-hexane
Sorbent wash	water	n-hexane
Elution	methanol	chloroform/acetone (4/1)

4.3 Chromatography

Chromatography of all steroid analytes was optimised comparing different columns, column temperatures, flow rates and injection volumes. For this purpose a mixture of all analytes dissolved in the initial gradient solution was injected so that a final amount of 100 pg was brought onto the column. The choice of the column was mainly determined by the peak efficiency, measured as peak width at the baseline, and the separation efficiency for structurally related steroid analytes.

Peaks were clearly smaller using a Nucleodur Sphinx RP column under the same operating conditions. Mean peak widths for ADD using a Nucleodur Sphinx RP column were 0.43 ± 0.02 min, while widths ranged from 0.47 ± 0.07 min to 0.59 ± 0.05 min using other columns. Base peak separation of bBol and AED, and aT and bT within a runtime of 6 minutes could also best be achieved using the Nucleodur Sphinx RP column. For these reasons this column was selected for further optimisation.

The influence of column temperature (30 – 60 – 90 °C), flow rate (200 – 300 – 400 – 500 – 600 µL min^{-1}) and injection volume (2 – 5 – 10 µl) on chromatography was further evaluated. Based on the peak width, peak height and signal-to-noise, best peak performance was achieved at a column temperature of 30°C, a flow rate of 200 µL min^{-1} and an injection volume of 5 µL. In Fig. 2 chromatograms are displayed of all analytes analysed under these conditions.

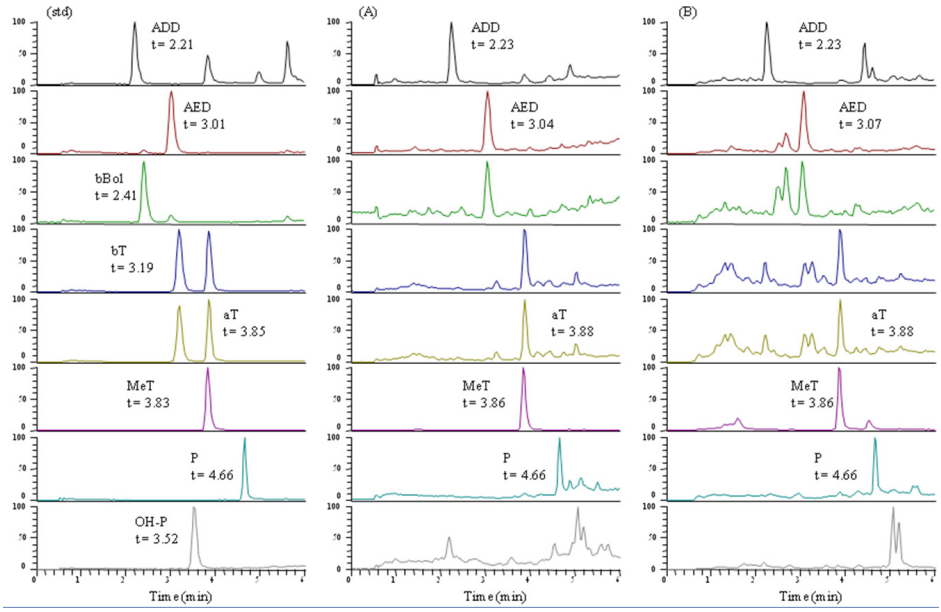

Fig.2 Chromatogram of ADD, AED, bBol, bT, aT, MeT, P and OHP for a standard mixture (std, 100 pg on column), a Lophira alata sample from a calves' crate (A) and the respective top-wood sample (urine, faeces etc.) of sample A (B).

4.4 Mass spectrometry

Starting from the default parameters for the HESI-II probe as provided by the manufacturer, capillary temperature, sheath gas pressure, auxiliary gas pressure and heater temperature were further optimised by infusion of the analytes' mixture. An overview of the optimised parameters for the HESI-II probe is provided in Table 2.

In order to establish appropriate selected reaction monitoring for the individual steroid analytes, standards diluted in the mobile phase were infused into the mass spectrometer. Collision energy (CE) of each protonated molecule was adjusted for the most abundant product ions. For each analyte at least 3 transitions were retained for identification. S-lens RF amplitude voltage was evaluated for each specific parent ion and collision energy was evaluated for each transition independently. Results are summarised in Table 3.

4.5 Characterisation of steroids in wood samples

This analytical method was originally developed to identify steroids in wood samples. The limit of detection (LOD) was determined in *Quercus sp.* by spiking the analytes of interest. Signal-to-noise ratio resulted to be at least 3 for ADD, AED, bBol, aT and P at a concentration of 5 ppb. For bT and OHP the LOD was 10 ppb. Nevertheless, attempts were made to quantify the identified steroids as well. Recoveries for steroid extraction were determined in a single wood species *Quercus sp*. by comparing the measured area ratio of a known concentration of an extracted analyte with the area ratio of a standard solution corresponding to that concentration (200 ppb). As presented in Fig. 1, recoveries using the optimised analytical procedure were 92 ± 9 % for ADD, 88 ± 11 % for bBol, 93 ± 7 % for AED, 102 ± 5 % for bT, 86 ± 16 % for P, 94 ± 5 % for aT and 82 ± 4% for OHP. For all analytes, good recoveries were achieved when extracting *Quercus sp*. samples. However, analysis of different wood species revealed a non-negligible influence of the matrix on the recoveries. For this reason the standard addition approach, compensating for differences in the wood matrix, was chosen for quantification of analytes identified in wood samples.

From the different species of wood investigated in this study (*Dinizia excelsa*, *Lophira alata*, *Pseudotsuga menziesii* (Europe), *Manilkara bidentata*, *Quercus sp.*, P*seudotsuga menziesii* (West of North America), *Pinus sp.* and *Thuya plicata*), only *Quercus sp*. resulted to contain a naturally occurring steroid, progesterone (Fig. 3). The concentration was estimated to be 14 ± 1 ppb (n = 6). According to the standard addition approach this concentration was calculated to be 19 ± 6 ppb (n = 6).

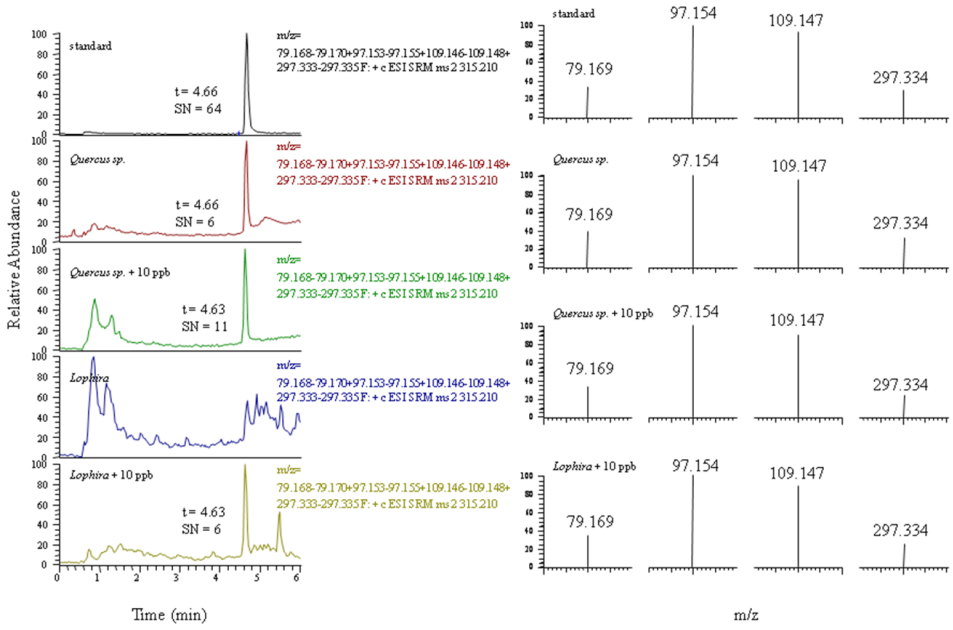

Fig.3 Chromatograms and spectra of progesterone in a 100 pg standard solution, a non-fortified Quercus sp. sample, a 10 ppb-fortified Quercus sp. sample, a non-fortified Lophira alata sample and a Lophira alata sample from a wooden crate.

Whereas none of the analytes of interest could be detected in *Lophira alata* heartwood, analysis of *Lophira alata* samples originating from wooden crates in which veal calves had been housed, showed the presence of ADD, AED, aT and P independent on the farm at which they were collected (Table 6). In Fig. 2 identification of these analytes in a wood sample (A) and a top-wood sample (B) is shown in comparison with a standard solution.

*Table 6 Concentrations of steroids (mean ± standard deviation) (ppb) in Lophira alata originating from wooden crates of a Dutch and Belgian farm, quantified using the standard addition approach (**bold**), and estimated using standard calibration curves (Italic).*

Origin	Sample Type	ADD	AED	aT	P
The Netherlands	*Wood[A] (n = 4)*	**24 ± 4**	**33 ± 8**	**11 ± 6**	**14 ± 1**
		41 ± 12	*54 ± 16*	*11 ± 5*	*13 ± 2*
The Netherlands	Top-wood[B] (n = 1)	**60**	**68**	**35**	**-**
		127	*104*	*28*	*68*
Belgium	Wood[A] (n = 4)	**20 ± 4**	**19 ± 5**	**16 ± 6**	**14 ± 3**
		35 ± 4	*33 ± 7*	*14 ± 0*	*10 ± 2*
Belgium	Top-wood[B] (n = 2)	**26 ± 3**	**35 ± 9**	**30 ± 2**	**42 ± 27**
		61 ± 2	*45 ± 2*	*20 ± 2*	*18 ± 1*
Belgium	Wood[C] (n = 3)	**22 ± 2**	**44 ± 17**	**13 ± 1**	**35 ± 19**
		38 ± 1	*78 ± 42*	*15 ± 3*	*18 ± 5*
Belgium	Wood[D] (n = 3)	**32 ± 4**	**< 10**	**-**	**< 10**
		50[S]	*< 10[S]*		*< 10[S]*

[A]: first plus second millimetre top layer of wooden plank

[B]: contamination (hair, faeces, etc) eliminated from plank

[C]: first millimetre top layer of wooden plank

[D]: third plus fourth millimetre top layer of wooden plank

n: number of replicates

[S]: only one replicate

Wood analysis resulted in similar concentrations for all analytes for the Dutch and the Belgian wood samples (A). In the case of the top-wood samples (B), concentrations of ADD, AED and aT were about twice the concentrations detected in wood samples (A). Nevertheless, progesterone could not be detected in the top-wood sample of the crate originating from the Dutch farm, while its concentration was variable in the analogous sample derived from the Belgian farm. It could be expected that the analyte concentrations decreased from the outer to the inner layer of the wooden planks. For AED and P, however, the outer concentrations (C) were higher than the inner concentrations (D). In contrast, this trend could not be observed for ADD and aT.

A comparison was made between the concentrations obtained using the standard addition approach and those estimated using appropriate calibration curves in standard solution (Table 6). For ADD and AED, an overestimation could be observed for the concentrations

estimated using calibration curves. This lies within the expectations since matrix effects are not considered using the latter approach. The difference in quantification approach was less prominent for the concentrations of aT and P. Except that, based on the calibration curves, a P-concentration of 68 ppb was established in the top-wood Dutch sample (B) while this concentration could not be quantified using the standard addition approach.

5. Discussion

The results obtaied in this study indicate the presence of steroids in wooden crates, which may lead to an excess steroid exposure to veal calves. Hence, this study is of important value to the discussion on the endogenous production of steroid analytes by veal. These results could only be achieved however, based on an analytical method allowing fast and efficient analysis of steroids in wood samples.

Accelerated solvent extraction (ASE®) has already been described by several authors as a means to extract a diversity of analytes from wood samples [45-48]. However, in unravelling the composition of wood, these studies consider analytes of interest other than steroids.
Steroids cover, dependent on the functional groups on their steroid nucleus, a rather wide range of polarity. For this reason, selection of an appropriate extraction solvent is of crucial importance. ASE® allows conventional extraction solvents to be used. In accordance with Impens *et al.* (2002) [11], extraction efficiency of steroids was significantly better using methanol or a mixture of methanol and acetone compared to less polar solvents. Whereas a large amount of organic solvent is required when using traditional methods to extract steroids from wood, elevated solvent temperatures used during ASE result in greater sample penetration capabilities and better extraction efficiencies and consequently lower solvent usage. Moreover, the combination of elevated extraction temperatures and pressures increases the kinetics of the extraction process, as such decreasing the extraction time [46,49]. For this application, extraction times above 15 minutes did not significantly alter the extraction efficiency.
To avoid interferences with low steroid contents detected in complex wood samples, a clean-up procedure was required. The most favourable results were obtained using Si cartridges in combination with NH_2 cartridges. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. As such, the unbounded silica surface of the Si cartridge allows for hydrogen bonding and Π-Π interactions among others, which gives strong retention for relatively polar compounds similar in structure such as steroids [50]. Final detection of steroids was performed using a fast and effective U-HPLC-QqQ-MS-MS method.

Over the last decades the analysis of steroids underwent an evolution from laborious GC analysis towards fast LC analysis [3,5,11-19]. Recently, the introduction of U-HPLC and UPLC even further decreased the analysis time required for steroid determination [51-53]. The latter techniques allow optimised performance and improved chromatography provided by the sub 2 µm particle columns, allowing increased separation efficiency, high sensitivity and

decreased analysis time [53].

With regard to retention and selectivity, the choice of an appropriate column for steroid analysis is besides steroids' polarity also dependent on the molecular shape. Steroids are structurally very similar so that enhanced shape selectivity may be an important consideration when choosing a suitable system for their separation. Although Hypersil Gold^{TM} columns allowed acceptable separation efficiency, columns with different reversed-phase packings were tested in order to improve the separation of bBol and AED, and bT and aT in particular. The unique concept of the Nucleodur Sphinx RP incorporates steric selectivity, in other words the ability to separate analytes in relation to their molecular structure and geometry. The unique selectivity surface existing of a bi-functional phase of octadecyl (C18)- and phenylpropylsilanes, combines both hydrophic and Π-Π interactions retaining the aromatic moieties of steroid molecules.

A methanol gradient was applied to cover the range of required solvent strength necessary to elute all steroid analytes of interest. The elution of steroids is dependent on the number and the nature of their polar functional groups and non-polar residues. Because of the absence of polar hydroxyl (OH)-groups in ADD- and AED-molecules for instance, minor retention was shown and low amounts of methanol in the mobile phase were sufficient for elution of these analytes. The incorporation of U-HPLC enabled the elution of 8 structurally closely related steroids in a runtime of only 6 minutes.

The described analytical method allowed the identification of ADD, AED, aT and P in wood samples. Interestingly, the analysis of wood samples of trees of diverse origin only showed the presence of P, and only in one species of wood *Quercus sp.* (Fig. 3). Nevertheless, naturally occurring progesterone has been previously reported in wood [40,54]. Although Carson *et al*. (2008) [40] mentioned *Pinus taeda* to be a rich source of naturally occurring progesterone, concentrations in the leafs of *Pinus taeda* were about 1000 times higher than the concentrations obtained in our study for *Quercus sp*. This might be explained however by the change in steroid content during plant development and its dependence on the species and cultivar [55]. In addition, these authors reported that, besides P, also testosterone and AED have been detected in many plants and frequently their involvement in the physiology of plants has been evidenced. For three plant species (*Digitalis purpurea*, *Nicotiana tabacum*, *Inula helenium*) Simersky *et al.* (2009) also reported the presence of P and AED, as well as OHP and 16-dehydroprogesterone [56].

In accordance with previous results, we also demonstrated the presence of P, AED and aT in wood samples originating from veal calves' crates. To the best of our knowledge, this study is the first to report the characterisation of ADD in wood samples. The microbial conversion of progesterone to ADD and AED however, has been previously described [37,40], indica-

ting that progesterone may function as immediate precursor for androgen biosynthesis. This occurrence of P has so far been attributed to microbial conversion of plantsterols, natural constituents of wood [41,57]. Nevertheless, up till now the conversion of P into ADD had not been established in wood. Indeed, it is a striking finding that only in wood samples from veal calves' housing facilities, ADD could be characterised. Concentrations of ADD in these wood samples were in the low ppb-range (< 100 ppb).

Explanations on the conversion of P or other steroid precursors to ADD in wood, can possibly be found in the crucial role specific enzymes play. As veal spread urine and faeces in their crates, the necessary microorganisms for that conversion might be available. Another source of metabolic activity might be provided by the veal calves by nibbling parts of their housing. About 22 % of the daytime of veal calves is spent on nibbling [40]. Furthermore, this oral activity is also a potential route of exposure of veal calves to an excess of steroid analytes, serving as precursors or intermediary products for bioconversion to active androgens. As a result endogenous androgen production by veal calves is likely to occur. As this might have implications for regulatory affairs on illegal administration of steroids as growth promoters, the prohibition of wooden crates as housing facilities for calves should be recommended.

6. Conclusion

In this study an analytical procedure was developed for the characterisation of selected steroids in a wood matrix. Extraction of steroids from a complex matrix such as wood in the low ppb range was achieved by a combination of accelerated solvent extraction and appropriate solid phase extraction. Sensitive detection in a total runtime of only 6 minutes was obtained by U-HPLC-QqQ-MS-MS analysis.

Using this procedure the characterisation of ADD in *Lophira alata* wood, originating from wooden crates used for housing veal calves, is reported. Moreover, several other steroids, in particular AED, aT and P, could be characterised in these samples.

Evidence on the presence of steroids in wooden crates might be of important value to decision makers, since exposure of veal calves to these analytes might induce endogenous production of active androgens. To prevent animals from inequitably being slaughtered because of the suspicion of illegal hormone administration, complete prohibition of wooden calf accommodation should be considered.

7. References

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CHAPTER

V

Excretion of boldione by humans

After:

K. Verheyden, H. Noppe, L. Vanhaecke, K. Wille, J. Vandenbussche, K. Bekaert, O. Thas, C.R. Janssen, and H.F. De Brabander. Excretion of endogenous boldione in human urine: influence of phytosterol consumption. J. Steroid Biochem. Mol. Biol. 117 (2007) 8-14.

1. Abstract

Boldenone (17-hydroxy-androsta-1,4-diene-3-one, Bol) and boldione (androst-1,4-diene-3,17-dione, ADD), are currently listed as exogenous anabolic steroids by the World Anti-Doping Agency. However, it has been reported that these analytes can be produced endogenously. Interestingly, only for Bol a comment is included in the list on its potential endogenous origin. In this study, the endogenous origin of ADD in human urine was investigated, and the potential influence of phytosterol consumption was evaluated.

We carried out a 5-week *in vivo* trial with both men (n=6) and women (n=6) and measured α-boldenone, ß-boldenone, boldione, androstenedione, ß-testosterone and α-testosterone in their urine using gas chromatography coupled to multiple mass spectrometry (GC-MS-MS). The results demonstrate that endogenous ADD is sporadically produced at concentrations ranging from 0.751 ng mL^{-1} to 1.73 ng mL^{-1}, whereas endogenous Bol could not be proven. We also tested the effect of the daily consumption of a commercially available phytosterol-enriched yogurt drink on the presence of these analytes in human urine. Results from this study could not indicate a relation of ADD-excretion with the consumption of phytosterols at the recommended dose. The correlations between ADD and other steroids were consistently stronger for volunteers consuming phytosterols (test) than for those refraining from phytosterol consumption (control). Excretion of AED, bT and aT did not appear to be dependent on the consumption of phytosterols.

This preliminary *in vivo* trial indicates the endogenous origin of boldione or ADD in human urine, independent on the presence of any structuraly related analytes such as phytosterols.

2. Introduction

In 2006, 2% of all human urine samples analysed worldwide by World Anti-Doping Agency (WADA)-accredited laboratories tested positive for prohibited substances. Forty five percent of all these adverse findings were due to the presence of anabolic steroids [1]. This class of drugs contains the male hormone testosterone (T) and several substances structurally related to it, such as boldione (androst-1,4-diene-3,17-dione, ADD) and boldenone (17-hydroxy-androsta-1,4-diene-3-one, Bol) [2] (Fig. 1). Their capability of improving muscle mass and strength has increased the use of anabolic steriods as performance enhancers in human sports [1,3,4]. However, this doping has led to a strict ban on exogenous as well as endogenous anabolic steroids in human sports [2]. Although sophisticated analytical techniques to distinguish between exogenous and endogenous targeted analytes are available [4,5], doping with endogenous steroids remains a serious issue facing doping control agencies [6].

In this context, knowledge on the origin of an analyte is of paramount importance. Boldenone,

for example, has long been considered a marker of illegal treatment when detected in urine. However, in extremely rare individual cases, boldenone of endogenous origin can consistently be found at very low nanograms per milliliter levels in urine [2,7]. In contrast, to the best of our knowledge no such evidence exists on the endogenous origin of boldione, the precursor of boldenone [8].

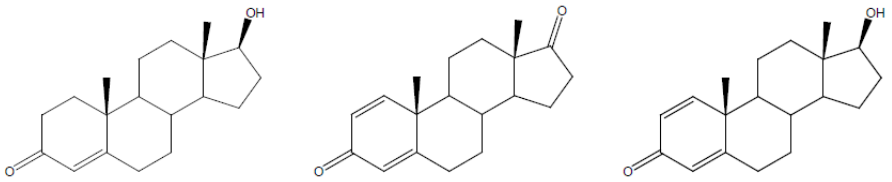

Fig. 1 Chemical structure of different steroid hormones: left: ß-testosterone (bT); center: boldione (ADD); right: ß-boldenone (bBol).

These and other anabolic steroid hormones are characterised by an androstane nucleus consisting of four fused ring structures, three hexane rings and one pentane ring [7,9]. This cyclopentanophenanthrene is also typical of sterols, such as phytosterols as well as cholesterol, which is the precursor molecule of several anabolic steroids [10-12] (Fig. 2). Phytosterols only differ from cholesterol by their side chain configuration [13-15], allowing them to compete with cholesterol for absorption from the gastro-intestinal tract [15-17]. This characteristic of competition forms the basis for the cholesterol-lowering properties of phytosterols. At a daily concentration of 2 to 3 grams, phytosterols have been shown to reduce plasma cholesterol levels in humans, improving blood lipid profiles and reducing the risk of coronary heart disease [13-15,18,19]. As a result phytosterols, natural constituents of several food products, are also supplemented to food items which are termed functional food [20,21].

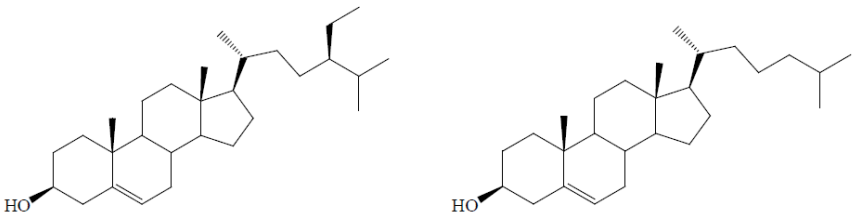

Fig. 2 Chemical structure of the phytosterol ß-sitosterol (left) and of cholesterol (right).

In the food industry functional food constitutes the single fastest growing segment [21], but consumers seem to have become more aware of potential food risks such as genetical modification, bacterial contamination, incidence of mad cow disease and the presence of growth hormone residues [22]. Tucker *et al.* [23] also reported that, already in 2001, the majority of the population in 19 out of 34 countries studied felt that their food is less safe than ten years earlier. This statement was confirmed in a recent study on the consumers' uncertainty about the safety and quality of their food [24]. Specifically, the potential presence of hormone and veterinary drug residues in food remains one of the major causes of concern to both American [23] and European consumers [24].
Biotransformation of phytosterols to steroid hormones has thus far not been demonstrated in humans while microbial conversion of phytosterols to steroids has been frequently reported [25-31]. In addition, a number of studies has been devoted to the ability of invertebrate organisms to convert phytosterols into anabolic steroids [32-34]. Recently Ros *et al.* [35] have performed a human study on the excretion of boldenone upon consumption of phytosterol-enriched margarine, however, none of the urine samples tested positive for this anabolic steroid.

The purpose of our investigation was to determine the endogenous origin of several anabolic steroids in human urine, especially boldenone and its precursor boldione. Furthermore, we hypothesized that urinary excretion of these anabolic steroids but also of the main anabolic steroid ß-testosterone (17ß-hydroxy-androst-4-ene-3-one, bT), its isomer α-testosterone (17α-hydroxy-androst-4-ene-3-one, aT) and its precursor androstenedione (androst-4-ene-3,17-dione, AED) could be associated with phytosterol consumption. In the present study volunteers were asked to consume a phytosterol-enriched yogurt drink at the recommended dose equalling 2 grams a day. Subsequently, the influence of the phytosterols on the steroidic excretion was evaluated using gas chromatographic-multiple mass spectrometric ($GC\text{-}MS^n$) analyses of the urine samples.

3. Materials and Methods

3.1 Experimental setup

A small-scale 5-week study was performed with healthy female (n=6) and male volunteers (n=6), aged 22 to 48 years old. Volunteers were randomly divided in a control and test group so that both groups consisted of 3 women and 3 men each. It was established that in daily life, these volunteers did not use phytosterol-enriched food products. During the study no restrictions on the normal diet were imposed. But, the control group was asked to keep

on refraining from using any phytosterol-enriched food products during the study. The test group was asked to consume a selected commercially available yogurt drink every morning during the first three weeks of the study. This drink contained 2 grams of phytosterols which is the daily intake recommended by the manufacturer. The last two weeks of the study the test group continued their normal diet and refrained from eating or drinking any additional phytosterol-enriched food products.

3.2 Reagents and chemicals

All solvents and reagents were of analytical grade and were purchased from VWR International (Merck, Darmstadt, Germany). ß-Zearalanol (3,4,5,6,7,8,9,10,11,12-decahydro-7,14,16-trihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1-one, bZ, purity > 98%), boldione (androst-1,4-diene-3,17-dione, ADD, purity ≥ 98%) and ß-testosterone (17ß-hydroxy-androst-4-ene-3-one, bT, purity ≥ 98%) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥ 96%), ß-boldenone (17ß-hydroxy-androsta-1,4-diene-3-one, bBol, purity ≥ 98%), α-testosterone (17α-hydroxy-androst-4-ene-3-one, aT, purity ≥ 98%) and equilenin (3-hydroxy-estra-1,3,5,7,9-pentaene-17-one, EQ, purity ≥ 98%) were purchased from Steraloids (Newport, USA). α-Boldenone (17α-hydroxy-androsta-1,4-diene-3-one, aBol, purity > 95%) was provided by The National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). Methyl-19-nortestosterone (4-estren-17α-methyl-17ß-ol-3-one, MeNT, purity > 98%) and androsterone ((3α,5α)-3-hydroxy-androstan-17-one, And, purity ≥ 98%) were obtained from the Scientific Institute of Public Health (IPH, Brussels, Belgium).

Standard stock solutions were prepared in absolute ethanol at a concentration of 200 ng μL^{-1}. Working standard solutions for spiking were prepared by appropriate dilution of the stock solutions in ethanol. All standard solutions were stored at 4°C following the quality assurance instructions of Belac accreditation (EN17025).

ß-Glucuronidase, Type HP-2, from *Helix pomatia* was purchased from Sigma-Aldrich Corp. and is stored at 4°C in the laboratory. This preparation contains glucuronidase (≥ 100 000 units mL^{-1}) as well as sulfatase activity (≤ 7500 units mL^{-1}).

The derivatisation reagent, $MSTFA^{++}$, was prepared using N-methyl-N-trimethylsilyl-trifluoracetamid (MSTFA, FilterService, Eupen, Belgium), ammonium iodide (Sigma-Aldrich Corp.) and ethanethiol (Acros Organics, Fairlawn, NU, USA) as described by Impens *et al.* [36].

3.3 Sampling

Volunteers were asked to collect 50 mL urine samples once a day, preferably in the morning before any consumption of drinks or food. Samples were taken at 11 sampling times, one prior to the imposed consumption of phytosterols (period 0) followed by two samples each week during the subsequent 5 weeks. Week 1 through week 3 corresponded to the phytosterol consumption period (period 1), and week 4 through week 5 represented the wash out period (period 2). Immediately after collection, samples were stored at -20°C until analysis.

3.4 Extraction and clean-up

Extraction was based on the method described by De Brabander *et al.* [37]. In short, urine pH was adjusted to 4 ± 0.5 before analysis. Analytes were extracted from 25 mL of urine with diethyl ether after enzymatic hydrolysis with β-glucuronidase from *Helix pomatia*. Extracts were dried and resolved in ethanol before filtration and subsequent separation with HPLC. Final analysis was performed with GC-MS-MS after derivatisation with $MSTFA^{++}$.

3.5 GC-MS-MS analysis

All chromatographic and spectrometric analyses were performed using a Trace Gas Chromatograph 2000 fitted with a Polaris ion trap mass spectrometer (Thermo Fisher, Austin, TX, USA) with a Carlo Erba AS2000 Autosampler (Thermo Fisher). Helium (99.99% purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 mL min^{-1} and perfluorotributylamine (FC43) was used as calibration gas. A sample volume of 1 µL was injected (split flow 60 mL min^{-1}, splitless time 1 min).

Gas chromatographic parameters are based on the method described by Impens *et al.* [36]. Chromatographic separation of the targeted analytes was performed on a BPX5 capillary column (25 m x 0.22 mm ID) with 5% phenyl-polysilphenylene-siloxane phase (0.25 µm film) (SGE Analytical Science Pty. Ltd., Victoria, Australia). Injector, ion source and transfer line temperature were 250, 200 and 275°C respectively. The temperature program applied started at an initial temperature of 100°C. Temperature was increased to 250°C applying a ramp of 17°C min^{-1}. Subsequently, an increase to 270°C was assessed using a ramp of 2°C min^{-1}. A final ramp of 30°C min^{-1} was applied to reach 300°C, holding this temperature for 1.30 minutes.

Spectra were obtained in positive electron impact ionisation (EI) mode MS full scan and MS-MS scan. Mass range depended on the selected precursor ion, and the collision energy ranged from 0.80 to 1.20 V.

3.6 Quality assurance

Prior to sample analysis, standard mixtures of the targeted analytes were injected to check the operation conditions of the GC-EI-MS-MS aparatus. Prior to extraction, EQ, MeNT and bZ were added to every sample as procedure internal standards (IS) at a concentration of 5.00 ng mL^{-1}. Different internal standards were used so that in each HPLC-fraction one IS was present. Different targeted analytes were identified by their retention time, relative to the appropiate IS, and by the ion ratio of their product ions based on the performance criteria for analytical residue methods as defined in Commission Decision 2002/657/EC [38]. After extraction, And was added to every sample to check the derivatisation efficiency. For quantitative analysis, analyte/internal standard peak area ratios versus spiked concentrations of the analyte were fitted in a calibration curve. Eight point calibration curves were constructed for every single targeted analyte by spiking standard solutions in ultrapure water at a concentration of 0.500-10.0 ng mL^{-1} for ADD, AED, aBol and bBol, and of 0.500-50.0 ng mL^{-1} for aT and bT. For both testosterone isomers (T) extrapolation was allowed for quantification in case this range was exceeded. The limit of quantification (LOQ) was set at the lowest calibration point, 0.500 ng mL^{-1}. Correlation coefficients (R^2) were higher than 0.90 for all targeted analytes.

Since this study entailed the intake of a commercially available yogurt drink at doses recommended by the manufacturer, no authorization from the Ughent Ethics Committee was required to carry out this experiment (as described in section 'Experimental Setup').

3.7 Data analysis

Data processing was performed using Xcalibur^{TM} 2.0 software (Thermo Fisher). All data were statistically interpreted using the statistical software package R [39]. To fulfil criteria of normality, concentration data of AED, bT and aT were log transformed. To overcome any problems with missing or zero values, a constant of 1 was added to the concentrations before log transformation. For each analyte, normality of the log transformed data was verified by generating normal quantile-quantile (Q-Q) plots of the residuals.

Correlations between all targeted analytes (AED, bT, aT and ADD) present in urine were assessed computing Spearman's rank correlation coefficients. These correlations were computed for the total dataset, but also for data of the female group, the male group, the control group and the test group separately. Consequently, conclusions at group level could be formulated. The null hypothesis that the Spearman correlation is zero was verified using a permutation test in which permutations were only considered within sample times. All hypothesis tests were performed at the 5% level of significance so that at a p-value less than the

significance level of 0.05, the null hypothesis was rejected. This procedure is consistent with the design of the study.

To examine any effects of: (1) phytosterol consumption (person-type: control or test persons), (2) different consumption periods (period: period 0, period 1 or period 2) and (3) gender (male or female) on urinary excretion of AED, bT and aT, a linear mixed model with a first order autocorrelation (AR(1)) structure in time and with person as random effect was used. Analysis started with the most complex model one is willing to consider, including the main effects, person-type, period and gender, and their two-factor interactions. The final model was selected using a backward-elimination model selection procedure. Since the main research questions on the influence of phytosterol consumption are related to differences in hormone excretion of control and test persons, and between the three periods, the factors person-type and period were never eliminated from the model. All hypothesis tests were performed at the 5% level of significance using Wald tests. The restricted maximum likelihood (REML) method was chosen for parameter estimation. The most parsimonious models for log AED, log bT and log aT are reported and further discussed.

To statistically interpret data of ADD, their conversion into binary-model-type data, representing either the absence (0) or the presence (1) of ADD in urine, was required. As indicator variable ADD was analysed similarly as log AED, log bT and log aT, except that a generalised linear mixed model was used. This model was further characterised by the binomal distribution, the logit link and also a random effect for person. The Laplace approximation to the likelihood was considered for parameter estimation. Since the logit link is used, the effect sizes are expressed in terms of odds ratios, which are the ratios of the odds of the presence of ADD in one group (e.g. gender, period, …) as compared to the odds in another group. The odds of the presence of ADD is defined as the probability that ADD is present divided by the probability that ADD is absent. All hypothesis tests were performed at the 5% level of significance using Wald tests.

4. Results

Urine samples were screened for aBol, bBol, ADD, AED, bT and aT. As presented in Table 1, boldenone (Bol) was not detected in any of the urine samples. The presence of boldione (ADD), the direct precursor of Bol, was demonstrated sporadically in period 0, 1 and 2 in some male and female volunteers of both the control and test group (Table 1). In Fig. 3 chromatogram and spectrum of ADD detected in urine of a male volunteer are compared with those of a standard solution of this analyte. ADD could be detected in urine samples in concentrations ranging from 0.751 ng mL^{-1} to 1.73 ng mL^{-1}. It should be noted that the detected ADD concentrations were close to the limit of quantification (0.500 ng mL^{-1}). All other targeted analytes were frequently present in the urine of both male and female volunteers (Table 1).

Table 1 Urinary concentrations of ADD, AED, bT and aT (mean ± SD) for female and male control and test persons in period 0, period 1 and period 2.

		female			male		
		period 0	period 1	period 2	period 0	period 1	period 2
ADD (ng mL-1)	control persons	nd[1] (0 / 2)[2]	nd (0 / 17)	nd (0 / 12)	1.33 (1 / 2)	nd (0 / 17)	nd (0 / 11)
	test persons	nd (0 / 1)	nd (0 / 17)	0.909 (1 / 12)	0.769 ± 0.0254 (2 / 3)	1.34 ± 0.557 (2 / 18)	nd (0 / 11)
AED ($ng\ mL^{-1}$)	control persons	1.28 ± 1.00 (2 / 2)	5.69 ± 5.50 (15 / 17)	6.81 ± 5.34 (12 / 12)	1.35 (1 / 1)	5.87 ± 3.94 (17 / 17)	7.96 ± 4.31 (11 / 11)
	test persons	0.729 (1 / 2)	5.05 ± 4.62 (15 / 17)	12.4 ± 22.9 (11 / 12)	1.30 ± 0.235 (3 / 3)	2.77 ± 1.72 (17 / 18)	9.79 ± 14.0 (10 / 11)
bT ($ng\ mL^{-1}$)	control persons	1.67 ± 0.175 (2 / 2)	2.14 ± 0.328 (7 / 17)	1.91 ± 0.463 (7 / 12)	13.7 (1 / 1)	12.1 ± 10.1 (16 / 17)	14.4 ± 20.4 (10 / 11)
	test persons	3.02 ± 0.941 (2 / 2)	3.96 ± 2.63 (14 / 17)	2.47 ± 0.855 (10 / 12)	2.86 ± 2.11 (3 / 3)	10.1 ± 6.83 (16 / 18)	10.7 ± 11.6 (11 / 11)
aT ($ng\ mL^{-1}$)	control persons	1.71 ± 0.244 (2 / 2)	4.52 ± 4.88 (14 / 17)	3.65 ± 2.10 (11 /12)	36.8 (1 / 1)	40.7 ± 44.9 (16 / 17)	74.7 ± 108 (10 / 11)
	test persons	3.70 ± 1.39 (2 / 2)	5.33 ± 4.33 (16 / 17)	3.17 ± 1.66 (12 / 12)	3.53 ± 2.74 (2 / 3)	10.3 ± 7.51 (15 / 18)	12.0 ± 12.0 (11 / 11)

[1] nd, not detected

[2] (x / n) with x = number of samples in which the analyte was detected, n = total number of samples analysed over that period

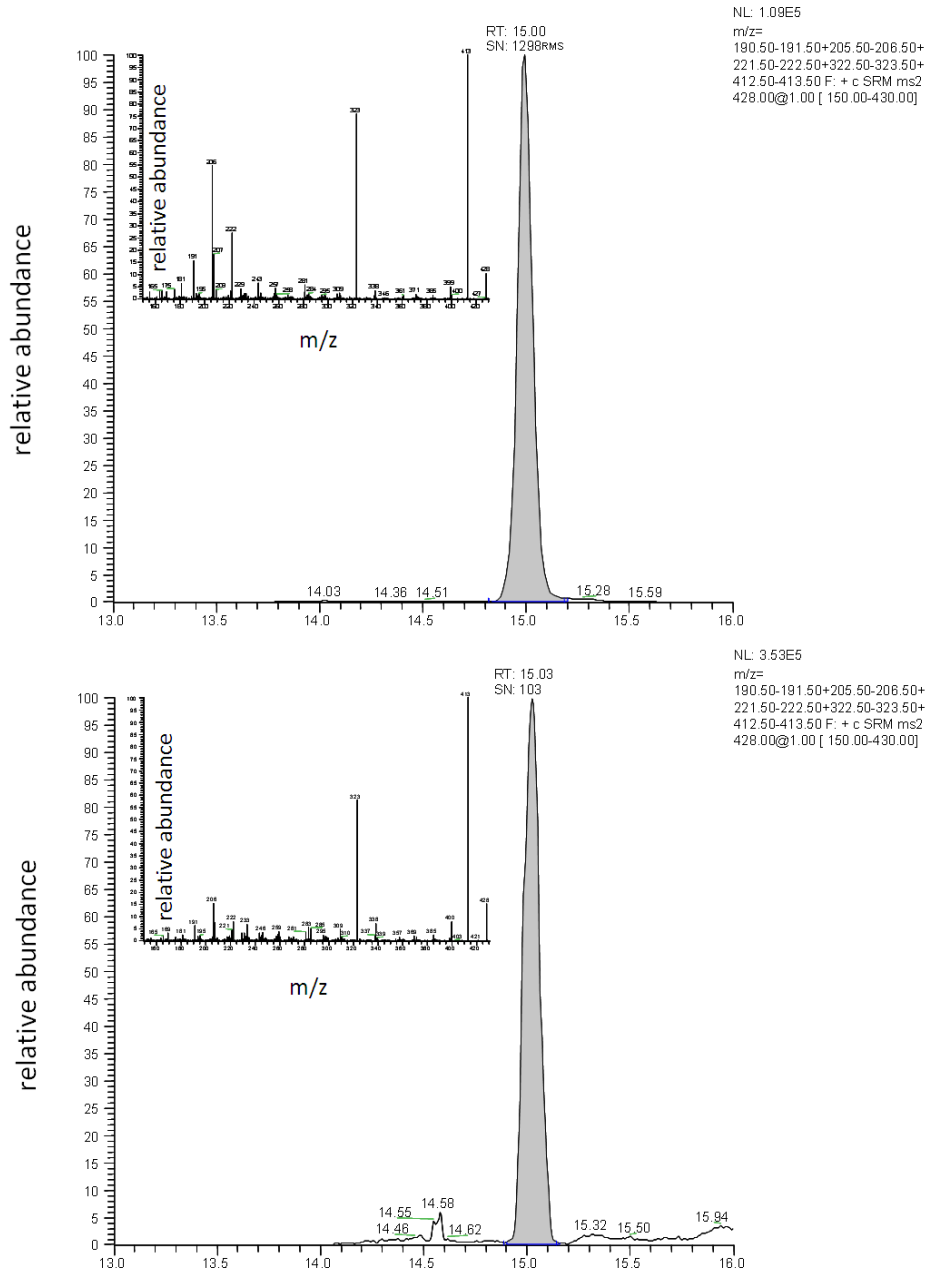

Fig. 3 Chromatogram and spectrum (inset) of boldione (ADD): (up) standard solution (2.00 ng on column) and (under) urine sample of a male test person (1.33 ng on column).

Table 2 presents the correlations between the urinary analytes established using the non-parametric Spearman's rho correlation coefficient. A positive correlation indicates a tendency that both variables decrease or increase simultaneously, whereas a negative correlation indicates that when one variable increases, the other tends to decrease, and vice versa.

Between bT and its precursor AED, a significantly positive correlation could be established ($p=0.0006$) and this correlation could be totally attributed to men ($p_{male}=0.0000$ versus $p_{female}=0.2580$). Also aT was significantly positively correlated to AED ($p=0.0000$). But this correlation was not gender dependent, although it was stronger in male than in female volunteers ($p_{male}=0.0000$ versus $p_{female}=0.0080$). bT and aT appeared to be positively correlated ($p=0.0000$). In contrast, the significant negative correlation found between ADD and aT ($p=0.0066$) could be only attributed to male volunteers ($p_{male}=0.0100$ versus $p_{female}=0.3340$). A slightly negative but non-significant relation could be established between ADD and AED and between ADD and bT. Moreover, these correlations were stronger in male than in female volunteers (ADD – AED: $p_{male}=0.3380$ versus $p_{female}=1.0220$, and ADD – bT: $p_{male}=0.01600$ versus $p_{female}=1.0400$).

No influence of phytosterol consumption could be attributed to the observed positive correlations between bT and AED, aT and AED and bT and aT, ($p_{control}=0.0000$ versus $p_{test}=0.0580$, $p_{control}=0.0020$ versus $p_{test}=0.0080$, and $p_{control}=0.0000$ versus $p_{test}=0.0000$, respectively). Remarkably, all correlations in which ADD is considered, were stronger in the test group than in the control group (ADD – AED: $p_{control}=0.9780$ versus $p_{test}=0.1540$, ADD – bT: $p_{control}=0.9480$ versus $p_{test}=0.3260$, and ADD – aT: $p_{control}=0.9400$ versus $p_{test}=0.0300$). Based on these results, it can not be excluded that the latter correlations experienced an influence of phytosterol consumption.

Table 2 Spearman's rank correlation coefficients for the total dataset between concentrations of AED, bT, aT and ADD detected in urine.

	AED	bT	aT	ADD
AED	1.00 (n = 132)	0.329* (n = 132)	0.471* (n = 132)	-0.110 (n = 132)
bT	0.329* (n = 132)	1.00 (n = 132)	0.800* (n = 132)	-0.218 (n = 132)
aT	0.471* (n = 132)	0.800* (n = 132)	1.00 (n = 132)	-0.288* (n = 132)
ADD	-0.110 (n = 132)	-0.218 (n = 132)	-0.288* (n = 132)	1.00 (n = 132)

No statistically significant effect of phytosterol consumption on the excretion of the anabolic steroids could be established.

The effect of period on the mean log AED, however, was found significant (p=0.0034), but, this was true for both the control and the test group. No significant effect of person-type on the mean log AED could be established (p=0.3555). Pair-wise comparisons of the mean log AED between the three periods revealed that the mean log AED in period 2 proved to be 0.744 larger than in period 0 (95% CI: [0.224, 1.26], p=0.0050) and 0.368 larger than in period 1 (95% CI: [0.107, 0.629], p=0.0058). However, the mean difference in log AED between periods 1 and 0, was not significant. This difference was estimated to be 0.376 (95% CI: [-0.130, 0.882], p=0.1457).

The model selection for log bT resulted in a model with a significant gender effect (p=0.0003) but a non-significant effect of period (p=0.9045) and person-type (p=0.3254). The mean difference in log bT between males and females was estimated to be 1.19 (95% CI: [0.780, 1.60]). Log aT also exhibited a significant effect of gender (p=0.0012) and of the interaction of gender and person-type (p=0.0157). The effect of period and of person-type on the mean log aT was not significant (respectively, p=0.3419 and p=0.0598). The mean log aT for males in the control group was 1.93 (95% CI: [1.26,2.61]) larger than for females in the control group. In the test group the mean difference was estimated to be as low as 0.454 (95% CI: [-0.213,1.12]). For males, the mean log aT in the test group was expected to be 1.29 (95% CI: [-1.96,-0.62]) smaller than in the control group. For females, the mean difference in log aT between test and control persons was estimated to be 0.188 (95% CI: [-0.482,0.858]).

The presence of ADD was significantly affected by the period (p=0.0060). In particular, the odds ratio of the presence of ADD in period 2 as compared to period 0 was estimated to amount 0.0219 (95% CI: [0.00124, 0.393], p=0.0094). For period 1 as compared to period 0, the estimated odds ratio equalled 0.0282 (95% CI: [0.00266, 0.298], p=0.0030). The presence of ADD in period 2 in comparison with period 1 was not significant (odds ratio equalled 0.782, p=0.8651). Also the effect of person-type on the presence of ADD was not significant (p=0.1324).

5. Discussion

In this human *in vivo* trial ADD, the direct precursor of Bol, was sporadically detected in urine samples of both male and female volunteers. The detection of this anabolic steroid in the control group as well as in the test group who had consumed phytosterols points to its endogenous origin. Influences of oral phytosterol consumption on this natural production and consequently the urinary excretion of ADD and other anabolic steroids could not be univocally demonstrated.

The most striking observation of the present study was the occurrence of ADD positive urine samples in the control group. For doping control agencies this finding may be of considerable importance because ADD is still listed as a prohibited exogenous anabolic steroid [2]. However, a comment has been included in this list indicating that in rare cases the production of these anabolic steroids listed as exogenous, may be endogenous. In case of a positive urine sample, microbial activity after sampling has frequently been mentioned as the cause of false positive results [10,40,41]. In our study however, special care has been taken not to contaminate the samples with gastro-intestinal flora during collection. Moreover, degradation of the samples owing to inaccurate storage conditions was prevented by immediately storing the samples at -20°C after collection.

As studied by Kim *et al.* [3], administration of ADD leads to Bol in humans. In their study, the urinary excretion profile of a healthy male volunteer was evaluated over 48 hours after oral administration of one tablet of ADD (100 mg tablet^{-1}). Bol was the main metabolite excreted in urine. From their experiment it was also concluded that the excreted amount of Bol reached its maximum (971 ng mL^{-1}) 3.6 hours after administration of ADD and decreased to 486 ng mL^{-1} after 48 hours. However, the exact pathway describing human ADD metabolism to Bol has not been elucidated and no long-term data on Bol excretion by humans are available.

Since ADD, the direct precursor of Bol, was occasionally detected in urine in this study, detection of Bol was also expected. However, compared to an administered ADD concentration of 100 mg [3], naturally occurring concentrations of this analyte might have been too low to allow for Bol formation. Sub-optimal enzymatic conditions in the human intestinal tract of our subjects might also explain the lack of Bol production. The importance of the environmental conditions for ADD and Bol incidence has been discussed by Mareck *et al.* [40]. In this review, a study was reported in which the human gastro-intestinal tract was simulated to establish optimal conditions for the formation of ADD, Bol and its metabolites. In simulations with enhanced oxygen conditions, the increased redox-potential in the gut led to large quantities of ADD while Bol could not be detected. This observation may explain the occasional detection of ADD as established in our study.

Next to Bol and ADD, the urinary excretion of AED and of bT and aT, two stereoisomers of testosterone (T), was also measured in our study. These anabolic steroids are relevant for human steroidogenesis and are metabolically linked. For this reason, specific interactions between these analytes were investigated. Between AED and both T isomers a positive correlation was established, which can be explained by the fact that AED is the direct precursor of T [12,18]. An increase in AED will therefore lead to increased T production and excretion. ADD is a metabolite of T [9], and this is reflected in our study by the negative correlation between both T stereoisomers and ADD. Metabolism of T, and thus a decrease in T concentration, will result in an increase of its metabolite ADD. AED can also be considered an important metabolite of T [9]. However, a positive correlation between AED and T was observed in our study. This may be explained by AED being of higher importance as precursor of T than as its metabolite. Not unexpectedly, all correlations described here were more obvious in the male than in the female volunteers and some can be totally attributed to males. An explanation for these results may be that all anabolic steroids under consideration were male sex hormones [1,7].

Urinary excretion of hydroxylated metabolites of T generated by different isoenzymes of cytochrome P450, such as AED and ADD, appears to be relatively low [7]. Although ADD concentrations were close to the limit of detection, in our study bT, aT, and AED could be detected at concentrations well above their limits of detection using GC-MS-MS. Since 1972 mass spectrometry (MS) is used to analyse urine samples for doping control purposes [4]. Combined with gas or liquid chromatography (GC or LC) and various ionisation methods, mass spectrometry enables reliable screening and confirmation of anabolic steroids in different matrices. Up till now, its specificity and sensitivity have made GC-MS^n the method of choice for detection of anabolic steroids in urine [4,42,43]. However, mainly due to reduced sample pre-treatment, particularly by the lack of derivatising, LC-MS-MS is gradually becoming more important for doping analyses purposes [5].

Within this research framework, it was attempted to relate the urinary excretion of anabolic steroids to phytosterol consumption. The success of phytosterols as functional-food ingredients is related to their cholesterol-lowering activity. The competition of phytosterols and cholesterol for absorption from the gastro-intestinal tract has been described extensively [13,14,16,17]. Moreover, it has been demonstrated that absorption of phytosterols is limited compared to that of cholesterol [15-17]. Consequently, phytosterols spend a longer time in the gastro-intestinal tract, possibly allowing their microbial conversion into androgenic products [8]. Therefore it was assumed that short-term increased intake of phytosterols by humans could alter their urinary excretion of anabolic steroids. Based on our 5-week *in vivo*

trial, no significant effect of phytosterol consumption on anabolic steroids' excretion could be demonstrated. More particularly, no relation between the incidence of ADD and phytosterol consumption could be established. ADD was, however, more related to other anabolic steroids following phytosterol consumption. The lack of a significant influence of phytosterols on ADD excretion is inconsistent with reports on the microbial transformation of phytosterols into ADD [44-46]. These *in vitro* experiments demonstrated that ADD production was positively correlated to the presence of phytosterols. Although *in vitro* experiments can give good indications, the present comparison indicates that this approach does not always reflect what may occur in the *in vivo* situation. Previous *in vivo* research conducted by Ros *et al.* [35] corroborates the results of our study. It must be emphasised that in both studies phytosterols were consumed at the doses recommended by the manufacturers.

Considerable individual variation in concentrations of urinary steroids' excretion was observed in our study (Table 1). However, the wide intra- and inter-individual variability in excreted testosterone concentrations has been previously demonstrated by several studies [10,47,48]. To accurately perform steroid profiling, the ratio of testosterone (T) to epitestosterone (E) should have been investigated [10]. Under natural circumstances, the endogenous T/E ratio is not intra-individually variable but remains constant [6]. Although this ratio is sensitive to variations between individuals, inter-individual comparison of this ratio would probably be more reliable than comparing absolute steroid concentrations. However, within the scope of our study, especially for analysis of ADD and Bol, steroid profiling was not specifically required. But it might be of particular interest for any long-term evaluation of the potential effects of phytosterol consumption on human steroidogenesis.

6. Conclusion

Particularly prominent in this study was the detection of endogenous boldione or ADD, the direct precursor of boldenone, in human urine using GC-MS-MS. Urinary boldenone excretion could not be proven, whereas other anabolic steroids such as AED, aT and bT were frequently excreted by both males and females. 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 and the small sample size, 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.

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CHAPTER

VI

General conclusions and research perspectives

In modern agricultural practice, growth promoting agents, such as hormones, may be applied to stimulate animals' growth and to consequently increase the profit per unit head for the farmer [1-4]. The European Union Council Directive 96/22/EC states however that substances with a hormonal action are prohibited for use in animals intended for meat production [5]. Consequently confirmation of the presence or absence of residues of growth promoting agents or their metabolites in a diversity of matrices, using reliable analytical techniques, is essential in order to control abuse. Besides abuse, safety implications of steroids in food have also gained importance now that consumers' awareness on food safety is growing [6].

The ambiguous origin of the steroid hormone boldenone and its metabolites in cattle has driven scientists to develop sophisticated analytical detection methods to distinguish between endogenous and exogenous boldenone [7,8,9]. Besides the analytics, less attention has been paid to the elucidation of the pathway leading to the formation of endogenous boldenone in cattle. Nevertheless, this information would help to recover consumers' confidence in foods of animal origin. In addition, the scientific debate on the origin of boldenone has, within the context of this doctoral thesis, been extended to human research. Being sensitive to the functional food industry, human individuals suffering from high cholesterol levels, have found their way to phytosterol enriched foods. Based on mammalian research with cattle, it is hypothesised that human phytosterol consumption might also lead to steroid excretion.

Addressing the research needs mentioned in the introduction to this doctoral thesis, the overall aim of the present study was to investigate the origin of steroids, boldenone in particular, in vertebrate species in relation to the presence of phytosterols. More specifically the research goals of this doctoral study were:

- evaluation of the use of invertebrates as alternative model system for vertebrate animal experiments to establish the pathway leading to boldenone
- elucidation of the origin of a specific steroid, boldenone, in livestock
- investigation of the metabolic transformation of phytosterols into steroids in humans

1. Legislative framework

As discussed in the introduction of this doctoral thesis (**Chapter I**), the legislative framework to be applied to steroid abuse in livestock isn't as transparent nowadays as it used to be in the past. Novel sophisticated analytical techniques allow the detection of steroids in a diversity of matrices at extremely low levels. Consequently, substances once thought not to be of endogenous origin, now seem to be endogenously produced by vertebrate animals under specific circumstances [4,10]. Due to their dual synthetic/endogenous nature, they have been named pseudo-endogenous or grey zone substances by Van Thuyne (2006) [11]. Whereas a total prohibition used to be in place for the presence of these pseudo-endogenous substances in food of animal origin [5], Council Regulation 470/2009/EC [12] lays down reference points of action (RPAs) for those substances for which no MRL has been described [4, 13]. Nevertheless, such a pragmatic measure does not elimate the need for understanding the origin of these pseudo-endogenous substances.

2. Alternative to vertebrate experiments

Nowadays, ethical issues associated with vertebrate animal experiments are well-known and extensively discussed [14,15,16]. As a result, intensive research on the development of suitable alternative methods has been conducted during the last decade. Living organisms, such as invertebrates, likewise allowing the assessment of metabolism profiles of different compounds as vertebrates, have been considered as an appropriate substitute to evaluate metabolism.

In vertebrate organisms, steroids secreted into the bloodstream from the adrenal cortex, gonads and other steroid producing tissues, are extensively metabolised in the liver and the kidney, as conversion to the inactive form is required before excretion in the urine. The specific enzymes involved in this metabolism mainly belong to the CYP P450 family (oxidoreductases and glucuronosyl- and sulfotransferases) and are known to play important roles in hormone synthesis and breakdown [17,18]. Using the invertebrate *Neomysis integer,* the cytochrome P450 enzyme activity has been investigated by means of the testosterone metabolism [15]. Based on current evidence, testosterone and progesterone, being physiologically important compounds, have often been used as model substrates in metabolism experiments examining cytochrome P450 enzymes [15,18,19]. *In vivo* biotransformation experiments with *N. integer* demonstrated the presence of a complex steroid hydroxylase system consisting of different P450 isozymes, similar to vertebrates [15]. In this doctoral thesis, the testosterone metabolism of several invertebrate organisms was assessed to obtain data on their metabolic capacity (**Chapter II and III**).

In depth research was performed and is described on the metabolic capacity of the maggot *Lucilia sericata* (**Chapter III**). A first indication on the metabolic capacity was gained by investigating testosterone metabolism in this invertebrate. The oxidation of β-testosterone to AED turned out to be the main metabolic pathway, resembling what has been described for the invertebrate mysid *N. integer* [15]. Moreover, β-boldenone was detected when exposing *L. sericata* to an excess of β-testosterone. The oxidation of β-testosterone to androstenedione, followed by dehydrogenation at the 1,2-position to androstadienedione and finally reduction of the keto-group of the latter giving β-boldenone, appears a pathway present in *L. sericata* as well as in *N. integer* [15]. These observations indicate similarity in enzymatic activity between the selected invertebrates and vertebrates.

The long-standing use of the ecological relevant invertebrate mysid shrimp *N. integer* in ecotoxicity testing has been described by Verslycke [15] among others [14]. Nevertheless, extensive organism variability does not promote this species as a suitable substitute for vertebrates in metabolism studies. Also for the invertebrate maggot *L. sericata*, reproducibility problems were encountered when performing metabolism studies using organisms of different origin. As a result, both invertebrate species, *N. integer* and *L. sericata*, were not considered fully valuable alternative biotransformation models for vertebrate models under the present circumstances. Lack of an understanding of the background levels of variability has been reported to be the major limiting factor in the use of physiological endpoints. The three main sources of variability have been recognised as organismal (e.g. sex, age, season,…), environmental (food, temperature,…) and methodological (tissue used, sampling method, …) [20]. Although, environmental variability is the hardest parameter to control, organismal variability might also cause poor reproducibility. In contrast, methodological variability can be adequately monitored and minimised through standardisation of testing procedures in the laboratory [15].

The use of the maggot *L. sericata* as model organism in this study, has been related to boldenone formation in faeces of cattle (**Chapter III**). Neo-formation in faeces of several anabolic steroids has been reported in literature to be the result of microbial conversion of steroid precursors present in faeces [21]. The present study, suggests, however, a novel organism performing such conversion of faecal steroids, namely maggots of the greenbottle fly *L. sericata*. Confirmation of this hypothesis was not within the scope of this doctoral thesis, but it may be the subject of interesting future research.
In literature, it has been mentioned that faeces is no longer considered as a reliable matrix to demonstrate illegal steroid administration in some countries, since it is hard to control

preservation conditions of faeces samples [22,23]. In addition, contamination of urine with faeces should definitely be avoided [4,22,23]. Residue analysis in livestock as well as doping analysis in humans and animals therefore focusses on the analysis of urine samples, although hair also appears a usefull matrix [4,24].

3. Phytosterols as precursors for boldenone in livestock?

3.1 Animal Feed

Apart from cattles' natural enzymatic metabolic pathway, a novel mechanism leading to boldenone formation in cattle was demonstrated in **Chapter II**. As a result of the BSE and dioxin crisis all animal fat has been banned out of animal feed, being replaced by vegetable material. Since then, the main kind of sterols in animal feed, essential for cattle, are phytosterols and no longer cholesterol [25]. In accordance with cholesterol, phytosterols can also function as potential precursors for several androgens. Microbial biotransformation of phytosterols has even been reported with regard to the detection of boldenone in livestock [26-29].

The susceptibility of animal feed to the presence of fungal species has been described in literature, mainly with regard to mycotoxins [30-32]. To the best of our knowledge, interference of feed-borne fungi in steroid-formation by cattle has so far not been considered. Conversion of phytosterols to steroid hormones in cattle's feed would, however, potentially contribute to an increase of steroid hormones in the feed, hence increasing the chance for interference with the endocrine homeostasis in pre-puberal animals [28].

In **Chapter II** of this doctoral thesis, conversion of phytosterols in animal feed by feed-borne fungi was investigated, not with respect to mycotoxines as by many research groups [33-35], but concerning the ability of these fungi to transform phytosterols to steroids. Steroid biotransformation by the fungus *Cochilobolus Lunatus* is among the earliest examples of biocatalysis to produce stereo- and site-specific alterations. Both hydroxylation and dehydrogenation activity, expressed by the cytochrome P450 enzyme system, have been demonstrated [19]. Cytochrome P450 enzyme activity has also been demonstrated by Breskvar (1994) [36] in the fungus *Rhizopus nigricans*. In relation to this fungal P450 enzyme activity, side-chain cleavage of phytosterols has been demonstrated to result in steroid formation [37-39], which was confirmed in this research using edible fungi isolated from maize. Phytosterol degradation, potentially contributing to an increase of steroid hormones in animal feed, might therefore increase the chance of endogenous boldenone formation.

An appropriate standardised *in vitro* way to perform metabolism studies consists of working with microsomal or S9 (the post-mitochondrial spin tissue fraction) preparations. Utilisation of *in vitro* preparations is moderately straightforward, fast and amenable for experienced lab technicians, and is therefore an important means to conduct metabolism studies [40]. Fungal microsomes were used in this doctoral thesis to investigate the metabolism of testosterone, as well as of several phytosterols (**Chapter II**). Freshly induced microsomal preparations, isolated from the edible fungus *Pleurotus sapidus*, were proven to contain sufficiently enzymatic activity to metabolise testosterone into its main metabolite AED and 6α-hydroxytestosterone. According to Van Puymbroeck *et al.* [40], hydroxylation in positon 6 of steroid hormones appears to be a common metabolic pathway by microsomal preparations. Exposure of fungal species to the phytosterol β-sitosterol mainly resulted in AED detection. This result is consistent with a study performed by Lin *et al.* [41] in which AED was the main tranformation product of phytosterols in corn flour produced by an isolated *Fusarium* strain. Whereas only microsomes were used within the context of this doctoral thesis, the use of S9 preparations should be considered in future research since the S9 fraction contains a wider range of drug metabolising enzymes [42].

3.2 Housing facilities

In **Chapter IV** an additional way of exposure to an increased amount of steroids is discussed. As wood is a natural source of phytosterols [43,44], veal calves growing in wooden crates may be more exposed to plantsterols. Within this context, wood used for calves' housing facilities was subjected to sophisticated extraction- and analytical techniqes. Several steroids were detected in the investigated wood samples, namely progesterone, androstenedione, α-testosterone and even androstadienedione. Whereas other authors have described progesterone to be a natural constituent of several wood species of diverse origin [45,46], our results point to the crucial role of specific microbial enzymes in these biotranformations. The presence of cattles urine, faeces and/or saliva on wood may apparently induce the microbial conversion of naturally present phytosterols and steroid precursors, such as progesterone. Nibbling of the calves on the wood may subsequently give rise to an exposure to an increased amount of intermediary products which can be further converted to active steroids. As a result, endogenous androgen production by veal calves is likely to occur independent on illegal administration of steroids as growth promotors. Evidence on the presence of steroids in wooden crates might be of important value for decision makers, since this kind of exposure might induce the endogenous production of steroids by veal calves. This may complicate decisions with respect to illegal administration of steroids as growth promotors. To prevent animals from inequitably being slaughtered because of suspicion of illegal hormone administration, prohibition of wooden calf accommodation should therefore be considered.

4. Boldenone and boldione excretion by humans

Safety concerns with regard to the widespread consumption of phytosterols are more important in humans than in mammalian livestock. Partly related to our wealthy lifestyle, many human individuals struggle with high cholesterol levels. Although a healthy diet and more exercise might be sufficient to lower cholesterol levels in many cases, phytosterol enriched food products appear more promising in this concern [31-34]. Although cholesterol and phytosterols have similar chemical structures, their health effects strongly differ [47]. Phytosterols are to the same extent atherogenous as cholesterol, but the low absorption efficiency of phytosterols in the intestine limits this hazardous effect [25,48-50]. As there exists some competition between cholesterol and phytosterols, the presence of the latter has also been reported to limit the absorption of cholesterol in the gastro-intestinal tract [48-50]. This explains how phytosterol-enriched food products decrease cholesterol levels in the blood [51-53]. The commercial availability of these functional food products certainly eases their access, whereas they should be considered as medicines [54]. Harmful clinical or chemical adverse events have not been observed, based on clinical studies [44]. Neverteheless, several studies have shown that consumption of phytosterols slightly reduces the absorption of fat-soluble vitamins [51,55]. This can however be prevented by limiting the daily dosage of phytosterols [56]. Concern has also been expressed that phytosterols might have estrogenic effects, however comprehensive studies have consistently demonstrated a lack of toxicity in animal models and humans [56]. Several animal studies have reported multiple adverse effects of phytosterols on endocrinology [57-61]. Sharpe *et al.* (2007) describe in their study the influence of phytosterols on a rate-limiting regulatory protein in steroid biosynthesis [61]. Nevertheless, literature on phytosterols' long term effects on steroidogenesis remains scarce. An interesting issue concerns the metabolism of phytosterols into structurally related steroids (**Chapter V**). Along with their characteristic to compete with cholesterol for absorption in the gastro-intestinal tract, phytosterols spend a longer time in the intestine, possibly allowing their microbial conversion into androgenic products [25].

Within the scope of this doctoral thesis, human metabolism of an orally consumed phytosterol-containing yogurt drink was investigated by evaluating urinary steroid excretion. Just like in doping analysis of athletes, urine samples are the prefered matrix to perform these analyses [36,37]. In our study no evidence of phytosterol related anabolic steroids' excretion was observed. The results demonstrated, however, that endogenous ADD, the direct precursor of boldenone, was sporadically detected in urine samples of both male and female volunteers. The detection of this anabolic steroid in the control group as well as in the test group who consumed a significant amount of phytosterols, points to its endogenous origin. For doping control agencies this finding might be of considerable importance because ADD

is still listed as a prohibited exogenous anabolic steroid [62]. Nevertheless, a comment has already been included in this document indicating that in rare cases the production of these anabolic steroids listed as exogenous, may be endogenous. Based on the results from our study, endogenous ADD excretion does however seem to occur quite frequently.
In a recent study, Piper *et al.* [63] even present data providing strong evidence of endogenous Bol production by humans. These authors developed a gas chromatography coupled to combustion isotope ratio mass spectrometry (GC-C-IRMS) method for urinary analyses of Bol allowing them to distinguish whether its source is exogenous or endogenous.

5. Future research perspectives

5.1 Analytics

For the metabolism experiments as well as for the steroid analysis in wood discussed in this doctoral thesis, a targeted LC-MS/MS approach has been followed. Nevertheless, in residue analysis there exists a trend towards non-targeted LC-MS approaches [64-66]. These full-scan MS approaches offer the advantage of retrospective evaluation of data for the detection of non a priori targeted compounds. A prerequisite for the application of such a generic screening method is the use of very sensitive full-scan accurate mass analysers e.g. time-of-flight (TOF), Fourier Transform ion cyclotron resonance (FT-ICR) or Fourier Transform Orbitrap, and the development of less selective sample preparation procedures [66]. For the purposes of this research, however, the targeted approach was prefered. Moreover, LC-QqQ-MS is still the most frequently applied technique in residue analysis [66].

The interpretation of residue analyses in the EU is based on a criteria approach, although standardisation and formal validation of this methodology have recently been recognised as major deficiencies [4,13]. These deficiencies are in most cases so fundamental that it is difficult to allow reliable comparisons between data sets. Efforts have however been performed in this context and a promising technique appears gas chromatography coupled to combustion isotope ratio mass spectrometry (GC-C-IRMS), measuring the relative composition of ^{12}C and ^{13}C atoms of steroids rather than their absolute concentrations [4,63,67]. As such a fingerprint of untreated animals could for instance be defined, allowing to draw conclusions on the deviating pattern of animals potentially illegally treated with growth promotors.

5.2 Alternative to vertebrate experiments

Some similarity between the steroid metabolism of the invertebrate *Lucilia sericata* and vertebrate species was demonstrated in this doctoral thesis (**Chapter III**). However to further investigate the potential use of this organism as alternative model organism, modifications to optimise the exposure conditions are required. Within this context, it is important to consider the variability in the gastro-intestinal system of different organisms of the same species, as this organismal variability might lay at the origin of the observed poor reproducibilty of metabolism experiments. Standardisation of the experimental organisms is therefore of utmost importance and should get sufficient attention. This aspect is crucial to be able to distinguish between mammalian and microbial intestinal metabolism and to correctly reflect metabolism results on vertebrates.

5.3 Phytosterols as precursors for boldenone in livestock?

Data were gathered in this doctoral thesis in support of the assumption that fungal species are able to transform phytosterols into boldenone, which strengthens the hypothesis that animal feed rich in phytosterols may be a potential source of boldenone or its precursors. Important in this context is the fact that during the present study fungal species were exposed to micrograms of phytosterols only. A more realistic approach would be exposure to grams of phytosterols. Future experiments should therefore evaluate phytosterol metabolism at environmentally and biologically relevant concentrations of phytosterols, by growing fungal strains under laboratory conditions on real animal feed samples for instance.

5.4 Boldenone and boldione excretion by humans

Important to consider with regard to the human *in vivo* trial performed within the framework of this doctoral thesis, was the relatively short-term duration of study. Although three weeks are described to be sufficient for phytosterol-enriched food products to exert their cholesterol lowering effects [62], this research period did not allow to draw unambiguous conclusions with regard to urinary steroid excretion. A more extensive long-term study is suggested to further explore the potential interferences of phytosterols with human steroidogenesis and excretory steroid patterns. In addition, in future studies focus would preferably lay on the determination of the ratio of testosterone to epitestosterone instead of the absolute testosterone concentration. As such, intra-individual variability in excreted testosterone concentrations would be eliminated [68].

6. References

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SUMMARY

1. Summary

Over the past 4 decades, the presence and metabolism of endogenous steroid hormones in livestock has been the subject of much research.

Whereas many laboratories have recently been focussing on the development of new analytical techniques to distinguish between synthetically endogenous and naturally endogenous boldenone, research on the pathway leading to endogenous boldenone remains scarce.
The aim of this doctoral thesis was therefore to provide evidence for the natural endogenous boldenone formation by vertebrates, as well as to elucidate potential pathways leading to this formation. Based on some degree of similarity between the vertebrate and invertabrate enzyme system, the metabolic capacity of several specific invertebrate species was assessed by evaluating their testosterone metabolism *in vivo*. As a second goal, alternative exposure routes leading to an excess exposure to steroids were considered as well.

In **chapter I**, an introduction is given to anabolic androgenic steroids, with particular reference to boldenone. Their chemical structures, functions and biological effects, particularly in relation to phytosterols, are presented. In this regard, the legislative framework to be applied to the presence of steroids in livestock is discussed. Emphasis was put on the search for alternatives of vertebrate animals for metabolism studies. Several *in vivo* alternatives are considered and discussed for their usefulness in metabolism studies. The analytical methodology was discussed with regard to the analytical techniques used in this doctoral thesis. Finally, the conceptual framework of this doctoral thesis is presented.

In **chapter II**, potential pathways of endogenous boldenone formation in cattle were investigated. Firstly, it was attempted to mimick vertebrate steroid metabolism using alternative invertebrate species. Testosterone metabolism by the maggot *Lucilia sericata* and the mysid *Neomysis integer* was assessed to obtain initial data on their metabolic capacity. The results of these experiments revealed interesting similarities in enzyme systems between invertebrate and vertebrate species. However, model organism variability was too high to consider these invertebrate species as valuable alternative biotransformation models. In addition, the role of feed-born fungi, potentially living on animal feed, in the endogenous production of boldenone by cattle was discussed. These organisms were shown capable to convert phytosterols into steroids *in vitro*. Consequently, increased amounts of steroid hormones, probable precursors of boldenone, may unintentionally be offered to cattle through their feed.

In **chapter III**, testosterone metabolism as well as phytosterol conversion are compared in more detail in several potential invertebrate model organisms, *Lucilia sericata*, *Neomysis integer* and *Artemia franciscana*. Compared to experiments with *N. integer* and *A. franciscana*, the yield as well as the reproducibility of the metabolic reactions using the maggots of *L. sericata* have been shown promising. It was demonstrated that *L. sericata* is able to transform bT into AED, ADD and bBol, while conversion of phytosterols also resulted in ADD.

In **chapter IV**, endogenous boldenone formation in calves was discussed in relation to their housing facilities. The presence of steroids in wooden crates for housing veal calves was demonstrated using a newly developed analytical procedure for extraction (accelerated solvent extraction, ASE) and detection (ultra high performance liquid chromatography coupled to triple quadrupole mass spectrometry, U-HPLC-QqQ-MS-MS) of selected steroids in a complex matrix such as wood. Evidence on the presence of steroids in wooden crates, as an additional potential source for excess exposure of steroids to veal, might induce endogenous production of active androgens.

In **chapter V**, the conversion of phytosterols to steroids was extended to humans. Urinary steroid excretion was evaluated upon oral administration of a phytosterol-containing yogurt drink. No evidence of phytosterol related anabolic steroids' excretion was observed after consumption of phytosterol-containing functional food at the recommended dose. In contrast, this study demonstrated endogenous boldione or ADD, the direct precursor of boldenone, to be present in human urine. Analysis of the urine samples was performed using a validated gas chromatography coupled to multiple mass spectrometry (GC-MS/MS) method.

In **chapter VI**, general conclusions and future research perspectives are formulated. In conclusion, this doctoral thesis demonstrates novel potential pathways leading to boldenone formation in vertebrates, based on alternative metabolism studies.

2. Samenvatting

Het voorkomen en metabolisme van endogene steroïden bij nutsdieren is de laatste 40 jaar onderwerp van intensief onderzoek geweest.

Terwijl vele laboratoria recentelijk hun aandacht richtten op de ontwikkeling van nieuwe analytische methodes om synthetisch endogeen boldenone te onderscheiden van natuurlijk endogeen boldenone, werd weinig onderzoek verricht naar het ontrafelen van de pathway die leidt tot endogeen boldenone.
Het huidig doctoraatsonderzoek heeft tot doel de vorming van natuurlijk endogeen boldenone bij vertebraten aan te tonen, alsook potentiële pathways die leiden to deze vorming te onderzoeken. Gebaseerd op een bepaalde mate van gelijkenis tussen het vertebrate en invertebrate enzymatisch systeem, werd van verschillende invertebraten de metabolismecapaciteit bepaald door het *in vivo* opvolgen van hun testosteronmetabolisme. Daarnaast werden ook alternatieve routes beschouwd die zouden kunnen leiden tot blootstelling aan een buitengewone hoeveelheid steroïden.

In **hoofdstuk 1** wordt een inleiding gegeven tot anabole androgene steroïden, met speciale aandacht voor boldenone. Hun chemische structuren, functies en biologische werking wordt besproken in relatie tot fytosterolen. De wettelijke omkadering zoals die geldt voor het gebruik van steroïden in vee wordt in parallel aangekaart. Vanuit dit standpunt wordt het zoeken naar alternatieven voor het gebruik van vertebraten in dierexperimenten benadrukt. Verschillende *in vivo* alternatieven worden beschouwd en hun nut in metabolisatie experimenten wordt besproken. Bovendien wordt de analytiek toegelicht met betrekking tot die technieken die gebruikt werden in dit doctoraatsonderzoek. Tot slot wordt het conceptueel kader van dit doctoraatsonderzoek geschetst.

In **hoofdstuk 2** worden potentiële pathways, die tot endogene boldenone vorming in rundvee zouden kunnen leiden, onderzocht. Om te beginnen wordt getracht het vertebrate steroïdale metabolisme na te bootsen met behulp van alternatieve invertebrate organismen. Het testosteronmetabolisme van de made *Lucilia sericata* en de aasgarnaal *Neomysis integer* wordt bestudeerd om initiële data te genereren van hun metabolisatiecapaciteit. Op basis van de resultaten van deze experimenten kunnen interessante gelijkenissen tussen de enzymatische activiteit van invertebrate en vertebrate species worden vastgesteld. Desalniettemin was de variabiliteit tussen verschillende organismen van eenzelfde species te groot om deze invertebrate organismen als waardig alternatief te beschouwen voor vertebraten in metabolisatie-experimenten. Daarenboven wordt de rol van voedergerelateerde schimmels besproken in

relatie tot de endogene productie van boldenone in rundvee, daar deze schimmels mogelijks diervoeder besmetten. *In vitro* wordt aangetoond dat schimmels in staat zijn fytosterolen om te zetten in steroïden. Bijgevolg zou via het voeder onopzettelijk een overmaat aan steroïdale hormonen, potentieel precursoren van boldenone, worden gegeven aan rundvee.

In **hoofdstuk 3** wordt de metabolisme van testosteron, alsook van fytosterolen, meer in detail vergeleken voor een aantal potentiële invertebrate modelorganismen, namelijk *Lucilia sericata*, *Neomysis integer* en *Artemia franciscana*. In vergelijking met experimenten met *N. integer* of *A. franciscana*, is zowel de opbrengst als de reproduceerbaarheid van de metabolische reacties bij experimenten met larven van *L. serciata* veelbelovend. Er werd aangetoond dat *L. sericata* bT omzet naar AED, ADD en bBol, terwijl omzetting van fytosterolen resulteert in de vorming van ADD.

In **hoofdstuk 4** wordt endogene boldenone vorming bij kalveren besproken met betrekking tot de stal waarin ze gehouden worden. De aanwezigheid van steroïden in houten kratten, waarin kalveren werden gehouden, kon worden aangetoond door gebruik van een nieuw ontwikkelde analytische procedure voor extractie (accelerated solvent extraction, ASE) en detectie (ultra high performance vloeistof chromatografie gekoppeld aan triple quadrupole massa spectrometrie, U-HPLC-QqQ-MS/MS) van specifieke steroïden in een houtmatrix. Deze indicatie van aanwezigheid van steroïden in houten kratten induceert mogelijks de endogene productie van actieve androgenen, gezien kalveren op deze manier blootgesteld worden aan extra steroïden.

In **hoofdstuk 5** wordt de menselijke omzetting van fytosterolen naar steroïden beschouwd. Hiertoe wordt de steroïdale excretie geëvalueerd in urine na orale inname van een met fytosterolen-aangerijkte yoghurt drink. De excretie van anabole steroïden blijkt echter niet gerelateerd aan de inname van fytosterolen, bij orale inname aan de aanbevolen hoeveelheid. Daarentegen werd endogeen boldione of ADD, de directe precursor van boldenone, teruggevonden in menselijk urine. Voor de analyse van de urine stalen werd een gevalideerde GC-MS/MS method aangewend.

In **hoofdstuk 6** worden de algemene conclusies en toekomstperspectieven van dit doctoraatonderzoek geformuleerd. Samengevat toont het onderzoek beschreven in dit proefschrift nieuwe potentiële pathways voor de vorming van boldenone in vertebraten, dit gebaseerd op metabolismestudies met alternatieve organismen.

CURRICULUM VITAE

1. Curriculum vitae

Karolien Verheyden werd geboren op 17 september 1979 te Aarschot. Na het behalen van het diploma hoger secundair onderwijs aan het Heilig Graf Instituut te Sint-Truiden (Latijn-Wiskunde) begon zij in 1998 met de studie Bio-ingenieur (Bachelor) met vervolgens de optie Milieutechnologie (Master) aan de Universiteit Gent. In juni 2003 behaalde zij het diploma Bio-ingenieur in de Milieutechnologie.

Na een verblijf in het buitenland waar zij op vrijwillige basis meewerkte aan verschillende ecologische projecten, trad zij in 2005 in dienst als wetenschappelijk medewerker aan de Universiteit Gent, Faculteit Diergeneeskunde, meer specifiek bij de vakgroep Veterinaire Volksgezondheid en Voedselveiligheid, Laboratorium voor Chemische Analyse. Zij werkte op een vierjarig onderzoeksproject met als titel “Analytiek en metabolisatiestudies van (oxy-) fytosterolen en –stanolen ter bepaling van hun biologische effecten”, financieel gesteund door het Bijzonder Onderzoeks Fonds (BOF) van de Universiteit Gent.

Daarnaast heeft zij ook actief meegewerkt aan de ontwikkeling van analytische extractie- en detectiemethodes in het kader van het FOD-project “Vroegtijdige en betrouwbare detectie van beregeur en de genetische aanleg ervan”, in samenwerking met het Instituut voor Landbouw- en Visserij Onderzoek (ILVO).

Karolien Verheyden is auteur of mede-auteur van meerdere publicaties in nationale en international tijdschriften. Zij nam actief deel aan verschillende nationale en internationale congressen.

Eind 2009 verliet zij reeds de Universiteit Gent om te starten als medewerker in de Regulatory Affairs van het farmaceutische bedrijf Ajinomoto OmniChem.

2. Personalia

Name:	Karolien Verheyden
Date of birth:	September 17, 1979
Place of birth:	Aarschot, Belgium

3. Record of education

2005 – 2009	PhD researcher in Veterinary Medicine, Laboratory of Chemical Analysis, Ghent University, Ghent, Belgium. **Project**: Analytics and metabolism of (oxy)phytosterols and –stanols for determination of their biological effects. **Promotors**: Prof. dr. Hubert F. De Brabander, Prof. dr. Colin R. Janssen.
2004	Scientific Volunteer, in preparation of a IWT-scolarship, at the Laboratory of Environmental Toxicology and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium. **Study of literature**: Internal distribution and toxicological availability of sink in the estuarine invertebrate *Neomysis integer* (Crustacea; Mysidacea). **Promotor**: Prof. dr. Colin Janssen
1998 – 2003	Master in Bioscience Engineering, Ghent University, Ghent, Belgium. Option: Environmental Technology, honours. **Thesis**: Endocrine disruption in the estuarine invertebrate *Neomysis integer* (Crustacea; Mysidacea). **Promotors**: Prof. dr. Colin R. Janssen; Co-promotor: dr. Tim Verslycke.
1997 – 1998	College (AFS-student), Sacred Heart College, Wanganui, New Zealand.
1991 – 1997	Secondary School, Heilig Graf Instituut, Sint-Truiden, Belgium. Option: Latin-Mathematics, honours.

4. Professional Employment

2009 – present	Regulatory Affairs Officer, Ajinomoto OmniChem, Wetteren, Belgium.
2005 – 2009	PhD researcher in Veterinary Medicine, Laboratory of Chemical Analysis, Ghent University, Ghent, Belgium.

5. Publications

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H. Noppe, B. Le Bizec, **K. Verheyden**, H.F. De Brabander. Review article : Novel analytical methods for the determination of steroid hormones in edible matrices. Anal. Chim. Acta 611 (2008) 1-16.

Co-author of several proceedings and publications in international journals.

6. Oral presentations

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Speaker at several small workshops to a diversity of publics.

7. Poster presentations

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Co-author of several poster presentations.

8. Foreign research visits

Training 'Metabolism experiments using fungal microsomes', Technical University of Dortmund, Department of Biochemical and Chemical Engineering, Dortmund, Germany, 2008.

Practical training and literature reviewing on the subject of *in vitro* drug metabolism at the Horseracing Forensic Laboratory (HFL), Ely, United Kingdom, 2007.