Annex 2 to advice 07-2013 (dossier 2012/07): Presence of substances with an endogenous origin known or suspected to a certain level through the metabolism and/or feed in different matrices of animal species producing food

1. Introduction

The following substances have been classified with an endogenous origin or may be considered as suspected: 17β-nortestosterone, 17α-nortestosterone, 17β-boldenone, 17α-boldenone, progesterone, 17β-testosterone, 17α-testosterone, 17β-estradiol, 17α-estradiol, zeranol, taleranol, cortisol (hydrocortisone), cortisone, prednisone, prednisolone and thiouracil.

The animal species studied are bovine, porcine, poultry, equine, ovine, caprine, cervine and fish.

According to Nielen *et al*. (2007), urine is the most important sample matrix in residue analysis. The choice of the matrix depends on the substances measured. Thyreostats may be measured in thyroid gland. Exogenous administration of natural hormones may be demonstrated in hair.
The following matrices are considered: urine, feces, liver, meat, fat, plasma, hair and thyroid gland.

Natural hormone levels **in cattle** liver are similar to those in muscle tissue, whereas fat tissue accumulates lipophilic hormones.
As reviewed by Hartmann *et al.* (1998) concentrations of natural hormones in milk depend on the fat content, which correlates with the concentration level of progesterone. Furthermore, the occurrence of native steroid hormones is higher in plasma and tissues from non-excretory organs in comparison with excreta fluids such as urine and feces (e.g. sulfo- and glucurono-conjugates) (Noppe *et al.,* 2008).

In pig tissue, a similar steroid pattern as in ruminants was observed, with a predominance of the metabolic intermediates and lower concentrations of hormonally active steroids. In contrast to cattle, no accumulation of hormones in fat was found. Between gilts (female pigs) and barrows (castrated males) no remarkable differences were found (Noppe *et al.,* 2008).

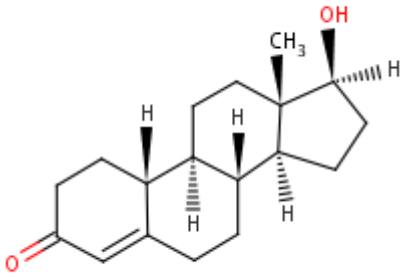
Reports about the contents of steroid hormones in **poultry** are rare.
The genetic selection that developed today's broiler has resulted in an animal that multiplies its hatch weight by 65 times within a seven-week period. The genetic selection has resulted in an animal, which grows to its physiological limit[1].

In the past, diethylstilbestrol (DES) was used for chemical castration of poultry to obtain capon. This use is forbidden now.

[1] http://www.ces.ncsu.edu/depts/poulsci/newsletter/newsletter_nov04.pdf

2. Presence of substances in different matrices of food producing animal species

2.1. Nortestosterone (nandrolone)



Chemical structure of nortestosterone (CAS N°434-22-0)

Nortestosterone (nandrolone or 17-β-hydroxy-estr-4-ene-3-one) is a C18 steroid with androgenic and anabolic properties. It is generally prepared from alkyl ethers of estradiol to resemble testosterone, but with one carbon less at the 19 position.

Bovine

Vandenbroecke *et al.* (1991) first suggested the endogenous presence of 17β-nortestosterone (17β-NT) (but not 17α-nortestosterone (17α-NT)) in the urine of pregnant bovines. Meyer *et al*. (1992) reported the presence of 17α-NT in relatively high concentrations in the urine of a cow in the period of calving and in the newborn calf itself.
The 17α-nortestosterone isomer might be endogenous in urines from pregnant cows and newborn calves (De Brabander *et al*., 1994).
During 2006, the presence of 17α-NT and, on occasion, 17β-NT was confirmed in male cattle (bulls and steers) slaughtered in Northern Ireland on welfare grounds, as a result of acute injury (Kennedy *et al.*, 2009).
Nielen *et al*. (2007) did not detect 17β-NT and 17α-NT in male and female calf urine samples collected from weeks 8 to weeks 26.
Scarth *et al.* (2011) measured concentrations of 17α-NT in urine of steer ranging from <LOD to 0.519 ng/ml (mean of 0.090 ng/ml), in urine of non-pregnant heifer ranging from <LOD to 2.124 ng/ml (mean of 0.081 pg/ml) and in urine of pregnant heifer ranging from 0.138 to 5.124 ng/ml (mean of 1.676 ng/ml) in the United Kingdom.

Porcine

Belgian and Dutch investigators found that 17β-NT occurs in the urine and in edible parts of boar (Maghuin-Rogister *et al.* (1988), Van Ginkel *et al.* (1989) and Debruyckere *et al.* (1990)).
Hereby, the absence of an isomerase in the pig should be mentioned, i.e. 17β-NT cannot be converted to 17α-NT in pigs (Poelmans *et al.,* 2005).
The data of Poelmans *et al.* (2005) illustrate that uncastrated 'old' boars contain 17β-NT in meat, liver, kidney, urine and testicle (table 1). In cryptorchids analogous results were obtained, however at lower concentrations. Concentrations ranged from 50 to 200 µg/kg.
Nandrolone and 9-Nor-4-androstenedione were detected in urine and some other matrices of animals of all sexes (including inter-sex animals) at different concentrations (Scarth *et al*., 2009).
17α-NT is not naturally present in pigs because pigs lack the isomerase enzyme, it is 17β-NT that is as a result naturally prevalent in pigs.

Table 1: 17β-nortestosterone concentration range (number of analysed samples) in meat, liver, kidney, urine and testicles of 'old' boar, cryptorchid, intersex, barrow, gilt and sow (source: Poelmans *et al.,* 2005).

17β-NT (µg kg^{-1})	'Old' boar	Cryptorchid	Intersex	Barrow	Gilt	Sow
Meat	0.7–13.4 (11)	0.1–2.4 (11)	<(1)	0.7–11.8 (11)	<(11)	0.4–0.5 (11)
Liver	1–63 (11)	0.2–12.3 (14)	<(1)	<(11)	0.1–0.9 (11)	<(11)
Kidney	2.5–232 (11)	1.3–78 (14)	1.6 (1)	0.1 (10)	0.2–0.5 (11)	0.2–1.5 (11)
Urine (µg l^{-1})	51–344 (11)	8.6–343 (14)	27 (1)	0.5–16.3 (11)	1.3–2.8 (11)	1.3–1.9 (9)
Testicle	24–144 (5)	2.2–101 (11)	5.3 (1)	–	–	–

–, no material available. <, < CC α: decision limit (Decision 2002/657/EC) (European Commission 2002).

Equine

Houghton *et al.* (1984) demonstrated that 17β-NT was naturally present in the urine of stallions.
Sterk *et al*. (1998) have shown that pregnant mares may excrete 17α-nandrolone naturally in urine at concentrations ranging from less than their LOD of 1 ng/ml up to 26 ng/ml, highlighting the need to consider the gestation status in mares.
Nandrolone in the equine is now known to originate predominantly from an analytical artifact of the degradation of a 19-carboxylic acid precursor that is endogenous to this species (Houghton *et al*., 2007).
In summary, endogenous presence of nandrolone and related 19-norandrogens were detected in urine of pregnant mares and at high concentrations in stallions (probably as a byproduct of the high concentration of aromatisation in the testes) but not in geldings or fillies (Scarth, 2011).

Ovine

Vandenbroecke *et al.* (1991) reported that the urine of eleven rams/lambs and ten pregnant/non-pregnant ewes was positive for nandrolone around the concentrations of 2.5 ng/ml when analyzed by RIA, but that the samples could not be confirmed by GC-MS (LOD not reported).

Van Hende (1995) analyzed the urine of four ewes at different stages of pregnancy and the amniotic fluid of one ewe for the presence of 17α-nandrolone. The urine of four pregnant animals was found to contain 17α-nandrolone at concentrations ranging from below the LOD to above 2 ng/ml.

Research by Clouet *et al.* (1997) demonstrated that 17α-NT could be found in the urine of pregnant sheep. 17α-nortestosterone was excreted in small amounts (less than 0.5 µg/L) during the first 4 months, and then increased to a maximum of 3.4 µg/L before parturition.

According to Casson *et al.* (2006), nortestosterone seems endemic in British sheep primarily as the 17α-isomer, but also with some 17β- present. There does not seems to be much correlation with age or sex of the animal, although the majority of the population tested was 6–12 months and some of the other categories contained very few samples (e.g. all 5 males of over 12-months contained the 17α-isomer at 0.4 ng/ml or greater). In light of this, it seems unwise to extrapolate the '17α- in male animals indicates abuse' rules from cattle to sheep. An exercise to test the urine of a controlled population will validate these conclusions, and demonstrate any link to other physiological factors such as breed or feedings regime.

Caprine

17α-nandrolone, but not nandrolone, was detected in urine during pregnancy, while neither analyte detected in non-pregnant females. Studies on endogenous concentrations in males are lacking (Scarth *et al.,* 2009).

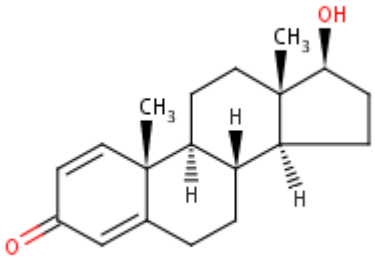
Cervine

Urinary 17α-nandrolone was detected in a pregnant red deer, but no other animals were studied (Van Hende, 1995). One of 35 urines from an Australian NMP (National Monitoring Programme) contained 17α-nandrolone but not nandrolone (Scarth *et al.,* 2009).

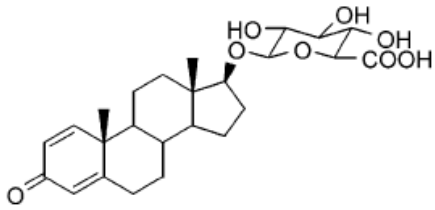
Other species

No information was found for poultry and fish.

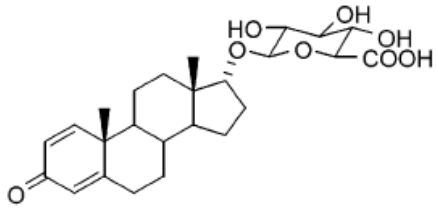
2.2. Boldenone (Bol)



Chemical structure of boldenone (CAS N°846-48-0)



Chemical structure of boldenone glucuronide



Chemical structure of 17α-boldenone glucuronide

17β-Boldenone (17β-Bol), also called 1-dehydrotestosterone or androsta- 1,4-diene-17β-ol-3-one is a steroid with androgenic activity that differs from 17β-testosterone (17β-T) by only one double bond at the 1-position. 17β-Boldenone is obtained by dehydrogenation of the male hormone testosterone.

Androgenic steroid hormones are excreted in urine together with a large variety of compounds like salts, endogenous steroids and related products of biotransformation; the conjugation with glucuronic or sulfate acid is a common route of metabolism for steroids, because they become readily excreted from the body, and may make up to 90% of the excreted metabolites.

Boldenone (α or β) has been detected in untreated animals of several animal species (De Brabander *et al*., 2004). The presence of endogenous boldenone (α or β) is mostly observed in male animals.
It was demonstrated that boldenone could be formed from phytosterols present in vegetable fat (Poelmans *et al.* 2003; De Brabander *et al*., 2004).

Poelmans *et al*. (2003) demonstrated using an invertebrate model that phytosterols can be converted to androstenedione. Androstenedione can be converted to boldenone and testosterone.

It has recently been demonstrated that some types of wooden crate in which veal calves are housed may contain precursors to boldenone (Scarth *et al*., 2012; Verheyden *et al*., 2010).

Bovine

Arts *et al.* (1996) reported the presence of 17α-Bol in concentrations varying from less than 0.1 to 2.7 µg/kg in urine samples of untreated calves. In the same samples, only traces of 17β-Bol were found (0.01–0.1 µg/kg). In regulatory controls, concentrations in the range 0.2–0.7 µg/kg were found. From those data, it can be concluded that 17α-Bol can be of natural origin in calves and cattle even when the animal has not been treated. The origin of the substance is not known.

The presence of 17α- and 17β-Bol in dried faeces, present on the animals fur, from untreated male and female veal calves has been demonstrated (De Brabander *et al*., 2004). 17β-boldenone might occur in dried faeces due to microbial conversion (De Brabander *et al*., 2004; Nielen *et al*., 2004; Rossi *et al*., 2004; Nielen *et al*., 2007).

Faecal contamination of urine collected in cattle (especially female) may be a source of boldenone since it has been demonstrated that precursors of 17β-boldenone can be detected in the faeces of rats fed with phytosterols (Blokland *et al*., 2007; Nielen *et al*., 2007).

The results for 17α-Bol and 17β-Bol in pooled urine samples from calves (SKV - Stichting Kwaliteitsgarantie Vleeskalversector, or the Foundation for the Quality Guarantee of the Dutch Veal Calf Sector) sampled between May 2000 and February 2002 in The Nederlands were analysed by gender, age and date of sampling. There was an indication that an effect existed of the sampling period because in certain periods (spring and autumn) the occurrence of 17α- and/or 17β-Bol was lower compared with other periods (summer and winter) (De Brabander *et al*., 2004).

The residue profiles of conjugated 17α-/17β-boldenone (17α/β-Bol) and androstadienedione (ADD) were investigated by Draisci *et al*. (2007) in urine of male veal calves fed two commercial milk replacers, with different contents of cholesterol and phytosterols. The urine samples were collected within 4h after feeding and later from all the animals. Detectable amounts of conjugated 17α-Bol were measured in urine collected from all calves, but the concentrations of 17α-Bol were higher in urine from calves receiving the milk replacer with the greater amount of phytosterols. During the whole experiment, 17β-Bol and ADD were never detected in the collected urine samples.

Boldenone and related compounds have been detected in urine and faeces, possibly secondary to their formation by gut bacteria. Some phase I and II metabolites were only detected upon boldenone administration.

One potential explanation for the presence of 17β-boldenone in calf urine is contamination of the sample with faeces containing 17β-boldenone (Blokland *et al.,* 2007).

According to Blokland *et al*. (2007), the origin of 17α-boldenone-conjugate can be endogenous in bovine.

Porcine

Boldenone was detected in urine and some other matrices of boars, cryptorchids, gilts and barrows at different concentrations, but not above the LOD in sow or inter-sex animal (Scarth *et al*., 2009).
In accordance with the absence of an isomerase in pigs, the 17 α-forms were not detected (Poelmans *et al*., 2005).
The data of Poelmans *et al*. (2005) illustrate that the uncastrated 'old' boars contain 17β-Bol in meat, liver, kidney, urine and testicle. In cryptorchids analogous, but lower results were obtained (table 2).

17β-Boldenone is also endogenous in male entire pigs, but it seems to be more sex dependent than 17β-nortestosterone. Surprisingly, 17β-Bol was also detected in the urine of some barrows and gilts at very low concentrations. However, care should be taken with the interpretation of these results since the mechanism of formation of 17β-Bol in urine contaminated with faeces during sampling is still unknown.

Table 2: 17β-Boldenone concentration range (number of analysed samples) in meat, liver, kidney, urine and testicles of 'old' boar, cryptorchid, intersex, barrow, gilt and sow (source: Poelmans *et al*., 2005)

17β-Bol ($\mu g\,kg^{-1}$)	'Old' boar	Cryptorchid	Intersex	Barrow	Gilt	Sow
Meat	0.5–2.5 (11)	0.7 (11)	< (1)	< (11)	< (11)	< (11)
Liver	1.3–4.9 (11)	0.5–2.3 (11)	< (1)	< (11)	< (11)	< (11)
Kidney	0.8–9.2 (11)	0.3–8.1 (14)	< (1)	< (10)	< (11)	< (11)
Urine ($\mu g\,l^{-1}$)	5.1–120.5 (11)	0.9–57.6 (14)	< (1)	1.1 (11)	0.5–0.6 (11)	< (11)
Testicle	2.1–16 (5)	0.6–15.1 (11)	< (1)	–	–	–

–, No material available. <, < CCα: decision limit (Decision 2002/657/EC) (European Commission 2002).

Poultry
No information was found for poultry.

Equine
It was demonstrated that 17β-Bol is present in entire male horses (Ho E. N. M., unpublished data, 2002). Boldenone has been shown to be naturally occurring as a sulfo-conjugate in the urine of intact males (Ho *et al.,* 2004). However, to date it has not been reported to be endogenous in geldings or fillies/mares (Scarth *et al*., 2009; Scarth, 2011). The identification of intact boldenone sulphoconjugate has provided a direct evidence for the endogenous nature of boldenone in entire male horses. The mean of free and conjugated boldenone in entire urine samples (n = 63, from 37 horses) was found to be 1.27±1.03 ng/ml, with a range of 0.1–4.34 ng/ml. The endogenous origin of boldenone was also supported by sustained and similar levels of boldenone found in urine samples collected on different occasions from the same horses (Ho *et al*., 2004).

Ovine
In two out of 961 urines from Australian NMP, low concentrations of 17α-boldenone were found (Scarth *et al*., 2009). However, these data are insufficient to draw any conclusions.

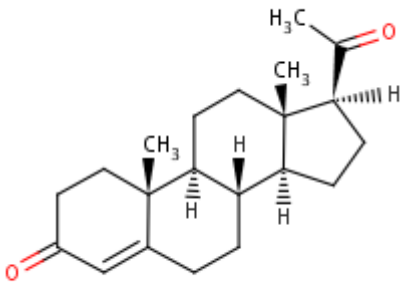
Caprine
There are insufficient data to draw any conclusions (Scarth *et al*., 2009).

Cervine
In none out of 35 urines from Australina NMP boldenone or 17α-boldenone was found (Scarth *et al*., 2009). However, these data are insufficient to draw any conclusions.

Fish
Boldenone has never been identified in invertebrates. As it was not detected in unexposed organisms, it can be hypothesized that it is a biotransformation product of testosterone (Verslycke *et al*., 2002).
It has also been demonstrated that insects and prawns are able to synthesize steroid hormones out of phytosterols, with cholesterol as an intermediary product (Verheyden *et al*., 2007).

Verheyden *et al*. (2007) evaluated the metabolic capacity of 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 0.10–0.14% (p < 0.001, significance compared to endogenous excretion of maggots) but not to boldenone itself. Furthermore, 17β-testosterone, an endogenous hormone, was transformed into androst-4-ene-3,17-dione (AED), ADD and 17β-boldenone at a significant (p < 0.001, significance compared to endogenous excretion of maggots) yield of circa 13%, 0.80% and 2.2%, respectively.

2. 3. Progesterone



Chemical structure of progesterone (CAS N° 57-83-0)

Progesterone is the major progestational steroid that is secreted primarily by the corpus luteum and, during pregnancy, by the placenta. Progesterone acts on the uterus, the mammary glands and the brain. It is required in embryo implantation, pregnancy maintenance, and the development of mammary tissue for milk production. Progesterone, converted from pregnenolone, also serves as an intermediate in the biosynthesis of gonadal steroid hormones and adrenal corticosteroids[2].
In most mammalian species, progesterone is essential for the maintenance of pregnancy. It is produced by the ovary and/or placenta and controls the duration of pregnancy by maintaining myometrial quiescence.

Simerský *et al*. (2009) reported the presence of progesterone and EAD in plant species (*Digitalis purpurea, Nicotiana tabacum, Inula helenium*) (seen annex 1). The microbial conversion of progesterone to ADD and AED however, has been previously described (Carson *et al*., 2008), indicating that progesterone may function as immediate precursor for androgen biosynthesis. This occurrence of progesterone has so far been attributed to microbial conversion of plant-sterols, that are natural constituents of wood (Jenkins *et al*., 2003; 2004).

Bovine

Progesterone is an endogenous hormone naturally present. Table 3 presents concentrations of progesterone in non-treated cattle. The progesterone production during pregnancy expresses itself in the relatively high levels measurable in pregnant cattle. The accumulation of progesterone in fatty tissue of all types of cattle is explained by the lipophilic properties of this steroid hormone (Heitzman, 1992). Levels of the steroid hormone progesterone measured in peripheral plasma of cow during the estrous cycle range from <0.2 to 8 ng/ml and during gestation range from <8 to 12 ng/ml (Hoffman, 1983).

[2]http://chem.sis.nlm.nih.gov/chemidplus/ProxyServlet?objectHandle=Search&actionHandle=getAll3DMViewFiles&nextPage=jsp%2Fcommon%2FChemFull.jsp%3FcalledFrom%3Dlite&chemid=0000057830&formatType=_3D

Table 3: Concentrations (mean values) of residues of progesterone present in edible tissues from non-treated animals expressed in µg/kg (EFSA, 2007) (compilation of data reported by various authors; taken from Paris *et al*., 2006 with permission).

	Liver	**Kidneys**	**Muscle**	**Adipose tissue**
veal calf heifers	0.16-0.75	0.03-4.07	0.0-0.9	0.87-1.60
Pregnant cows	3.4	6.2	10.1	239.0

Arts *et al. (*1991) propose reference values of <0.1-0.3 µg/L in blood plasma and <0.6-10 µg/L in urine for the concentration of progesterone in male and female calves.

Verheyden *et al*. (2010) have shown the presence of androstadienedione (ADD), androstenedione (AED), alpha-testosterone and progesterone in wooden crates used for housing veal calves. concentrations of these substances ranged from 20±4 µg/kg to 32±4 µg/kg for ADD, from 19±5 µg/kg to 44±17 µg/kg for AED, from 11±6 µg/kg to 30±2 µg/kg for alpha-testosterone and from 14±1 µg/kg to 42±27 µg/kg for progesterone, depending on the sample type. According to the authors, 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.

Porcine

In pigs, progesterone is mainly transformed into pregnanediol, which is found as a glucuronide in urine. Levels of progesterone measured in peripheral plasma of sow during the estrous cycle range from <0.2 to 25 ng/ml and during gestation range from <10 to 12 ng/ml (Hoffman, 1983).

Poultry

Levels of progesterone measured in peripheral plasma of hen during the estrus cycle range from <1.0 to 4 ng/ml (Hoffman, 1983). The avian follicle wall cells produce androgens, estrogens and progesterone which are incorporated into the yolk (Quillfeldt *et al.*, 2011). Ovulability is gained as the ability of the follicle to produce androgens, while estrogens declines, and the ability to produce progesterone increases during the final 24 h of follicular maturation. This pattern leads to a high concentration of progesterone in the outer yolk layers (median concentration of 2265 nmol/kg). Some evidence suggests that progesterone can be down-regulated, thus preventing ovulation, by stress.

Equine

Generally, progesterone is present in maternal plasma throughout gestation although its actual concentration varies amongst animal species and with gestational age. Levels of progesterone in peripheral plasma of mare measured during the estrous cycle range from <0.3 to 22 ng/ml and during the gestation range from <7 to 25 ng/ml (Hoffman, 1983). The concentration of progesterone measured by Ousey *et al.* (2005) in plasma of pregnant mare with compromised pregnancies (concentration between 1 and 9 ng/ml) were higher than in normal pregnant mare.
Haffner *et al*. (2010) have measured the concentration of progesterone in blood of Mongolian horses.
Median progesterone concentrations of 0.07 ng/ml were highest in mares followed by stallions, geldings, colts and fillies. Progesterone concentrations in serum of domestic mongalian horses ranged from <0.03 to 18.34 ng/ml.

Progesterone is a substrate for the synthesis of most other steroids including cortisol; however, it is not the most abundant steroid.

Ovine

Levels of progesterone measured in peripheral plasma of sheep during the estrous cycle range from <0.2 to 8 ng/ml and during the gestation range from <8 to 19 ng/ml (Hoffman, 1983).

Caprine

Levels of progesterone measured in peripheral plasma of goat during the estrous cycle range from <0.2 to 6 ng/ml and during the gestation range from <2 to 10 ng/ml (Hoffman, 1983).

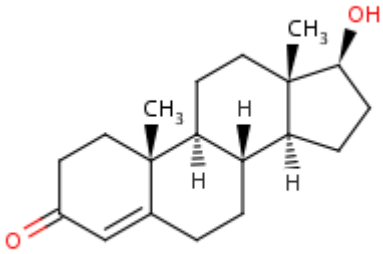
Cervine

Progesterone concentrations up to 0.9 ng/ml were measured in blood of roe deer during the rutting season by Schams *et al*. (1980). Concentrations of progesterone were measured by Kelly *et al.* (1982) in plasma of female red deer. The results of the hormone analyses suggested that the amount of progesterone in plasma correlates with the number of corpora lutea (CL) present. In pregnant animals, progesterone concentrations were high for the first 200 days of gestation. In animals with 1-2 CL the mean concentrations of progesterone remained more or less constant at 4 ng/ml until 200 days of gestation, and then declined gradually to basal levels after parturition. In animals with >3 CL, the progesterone concentrations fluctuated widely from ~8 to ~32 ng/ml over most of pregnancy but the peaks in mean progesterone values appeared to coincide with those in hinds with 1 corpus luteum.

Fish

Progesterone has been described mainly as a precursor steroid in fish and no clear role by itself has been reported (Atteke *et al.,* 2003).

2. 4. Testosterone



Chemical structure of testosterone (CAS N° 58-22-0)

Testosterone (17β-hydroxyandrost-4-en-3-one or α-4- androsten-17β-ol-3-one) is a 19-carbon steroid hormone with potent androgenic properties, including the maintenance of testicular function and the development of secondary male sex characteristics and endogenous steroid hormones. It also exerts strong anabolic effects, which initiate increased protein synthesis in muscle and bone (SCV, 2004). In the 1950s, the recognition of the growth promoting properties of such hormones led to their introduction as a tool to increase meat production. This has led to attempts to increase the weight gain and feed conversion efficiency of animals reared for meat, by supplementing their endogenous hormones with extra amounts of either steroid hormones or synthetic equivalents. The effect of these anabolic steroids is to increase lean tissue growth and to reduce fat deposition. Since fat is so energy dense, food conversion efficiency is increased (Conneely *et al.,* 2007).

Testosterone and related steroids such as 17α-testosterone, 4-androstenedione and DHEA (dehydroepiandrosterone) are ubiquitous among male and female animals of all mammalian species so differences among various groups and types are purely quantitative (Scarth *et al*., 2009).

In the circulatory system of animals, exogenous testosterone derived from an ear implant, is indistinguishable from endogenous testosterone. Excretion is predominantly via the biliary route, and to a lesser extent via the urine. Generally, the fraction of the hormone eliminated via the urine is prevalent in a conjugated form, while the fraction found in the faeces is in the free form of the hormone (SCV, 2004). Endogenous hormone levels in tissues vary with sex and breed of the animal and also depend on whether the animals have been castrated or are pregnant. Fat content also affects the level of hormones in meat; concentrations of some hormones are significantly higher in liver and kidney than in muscle tissue (Conneely *et al*., 2007).

Bovine

In ruminants, the primary metabolite of testosterone is 17α-testosterone, which is mainly excreted in feces (Rico, 1983). Levels of testosterone measured in peripheral plasma of cows during the estrous cycle range from < 0.05 to 2 ng/ml (Hoffman, 1983).

Nielen *et al*. (2007) measured natural steroids and their metabolites in calf urine samples collected from week 8 to week 26 in a controlled feeding and housing experiment using GC-MS/MS. Concentrations of 17β-Testosterone (17β-T) in male calf urine ranged from <1.0 to 10 ng/ml whereas Arts *et al*. (1991) measured 17β-T up to 28 ng/ml with RIA. Nielen *et al.* (2007) did not detect 17β-T in urine of female calves in contrast to Arts *et al.* (1991) who measured concentrations from <0.5 to 2.2 ng/ml. The most abundant natural hormone residue was 17α-Testosterone (17α-T). In female calf, urine concentrations ranged from <1.0 to 6.2 ng/ml whereas in males calf urine 17α-T concentrations ranged from <1.0 to 1000 ng/ml.
Testosterone is ubiquitous in males and females at varying concentrations in bovine (Scarth, 2011). Information on the specific metabolic routes and elimination rates for testosterone in cattle is somewhat limited (Samuels *et al*., 1998). The levels of testosterone in cattle vary in the literature, generally as a result of the analytical technique used (SCV, 2004). In bulls, the levels of testosterone

depend largely on the breed and age of the animal: for example, at 15-months the concentration of testosterone in plasma of a Simmental bull is 5.9 mg/L, of a Charolais 2.6 mg/L, of a Hereford 1.4 mg/L and of an Angus 2.5 mg/L; whereas for the same breeds of bulls at 12 months, levels range from 7mg/L to 10 mg/L (Conneely *et al*., 2007). Table 4 present concentration of testosterone in edible tissues from non-treated animals.

Table 4 : Concentrations (µg/kg) of residues of testosterone present in edible tissues from non- treated animals (EFSA, 2007) (compilation of data reported by various authors; taken from Paris *et al.,* 2006).

	Liver	**Kidneys**	**Muscle**	**Adipose tissue**
Veal calf heifers	0.021-0.126	0.043-0.356	0.006-0.029	0.021-0.296
Pregnant cows				
- 120 days	0.05	1.51	0.27	0.59
- 240 days	0.27	4.01	0.42	0.69
Bull	0.75	2.78	0.54	10.95

Scippo *et al.* (1994) propose a decision limit of 0.125 ng/ml for testosterone in plasma of heifers and of 1.5 ng/ml for testosterone in plasma of bulls. Scarth (2011) has established an approximate rank order for testosterone concentrations in the bovine as follows: hair> urine ~ fat ~ faeces ~ kidney > plasma > liver ~ muscle.

Porcine

Testosterone is ubiquitous in males and females at varying concentrations.

Poultry

The major circulating androgen in birds is testosterone. Testosterone acting via conversion to 5α-dehydro-testosterone (DHT) depresses growth in chickens (Fennell and Scanes, 1992a) but stimulates that of turkeys (Fennell and Scanes, 1992b). Androgens in combination with estrogens induce the formation of medullary bone at the time of sexual maturation (Chen *et al*., 2010). Testosterone reached the highest concentrations in interior yolk layers with a median concentration of 99 nmol/kg, whereas in the centre of the yolk the concentration was low (Hackl *et al*., 2003).

Equine

Testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations (Scarth, 2011).

Haffner *et al*. (2010) have measured the concentration of testosterone in Mongolian horses. Median testosterone concentrations for mares, stallions, and fillies were below the assay's level of detection and were reported as 0.040 ng/ml. Median testosterone concentrations were higher in geldings and colts compared with mares, stallions, and fillies. Testosterone concentrations in serum of domestic mongalian horses ranged from < 0.04 to 0.290 ng/ml.

The median serum concentration of testosterone in Mongolian stallions was 0.040 ng/ml. A possible explanation for the relatively low concentrations of testosterone in stallions was that most stallions in the herd may have been in bachelor status. Bachelor stallions are heterosexually inactive males that do not have a harem of mares. It has been reported that bachelor stallions exhibit low testosterone concentrations compared to harem stallions. When stallions move from bachelor status to harem status, there is a sharp increase in testosterone concentration (from approximately 0.9 ng/ml to 2.5 ng/ml), which is reversed when they are displaced back to bachelor status (Haffner *et al*., 2010).

Ovine

Testosterone is ubiquitous in males and females at varying concentrations in ovine (Scarth, 2011).

Concentration of plasma testosterone increases with decrease of photoperiod (Scarth *et al*., 2009). Season and housing have an effect on testosterone plasma concentrations.

Caprine

Testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations (Ahmad *et al*., 1996; Flint and Burrow, 1979).

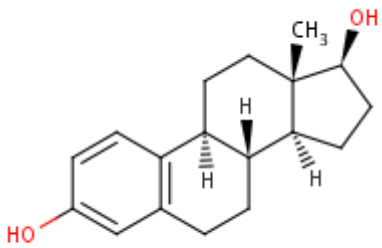
Cervine

Testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations (Hamasaki *et al*., 2000; source Scarth, 2011). During the breeding season, testosterone levels increased until the end of October (approximately 1,2 weeks before the peak of the rut) and decreased during November. Levels of serum testosterone during the breeding season generally increased with age from 48.2 (1.5 years old) to 187.9 ng/ml (5.5 years old), but decreased thereafter (Ditchkoff *et al*., 2001).

Fish

No information was found for fish.

2.5. Estradiol



Chemical structure of estradiol (CAS N° 50-28-2)

Estradiol generally refers to the 17-β-isomer of estradiol, an aromatized C18 steroid with hydroxyl group at 3-beta- and 17-beta-position. 17β-Estradiol is the most potent form of mammalian estrogenic steroids. The 17α-isomer of estradiol binds weakly to estrogen receptors and exhibits little estrogenic activity in estrogen-responsive tissues. Various isomers can be synthesized.

Bovine

Ruminants transform 17β-estradiol into estrone, and then into 17α-estradiol with a very low estrogenic activity. This form is mainly excreted in a free form in the feces (Velle, 1976). Table 5 presents the concentrations of residues of estrogens present in edible tissues from non-treated animals. Lowest levels have been determined in steers, followed by veal calves and heifers; highest levels were found in pregnant cattle (as a result of placental estrogen production). The concentrations determined in pregnant cattle increase with the length of pregnancy and are highest in liver followed by kidney, fat and muscle (Heitzman, 1992). The concentrations of 17β-estradiol reported by Hoffman (1983) in muscle are between 0.37 and 0.780 ng/ml in pregnant cow, 14.4 ng/ml in steers and 12 ng/ml in heifers. Concentrations of 17β-estradiol in liver and kidney of steers and heifers are 12, 12600, 38.3 and 39.8 ng/ml, respectively.

Levels of 17β-estradiol measured in peripheral plasma of cow during the estrous cycle range from <0.008 to 0.017 ng/ml (Hoffman,1983). Levels of free and conjugated 17β-estradiol and 17α-estradiol measured in peripheral plasma of cow during the gestation range from 0.026 to 0.060, 0.11 to 0.42, 0.09 to 0.01 and 3.0 to 49 ng/ml. Concentrations of 17β-estradiol measured by Arts *et al.* (1991) in male and female calves were <0.01 ng/ml in blood plasma and <0.2 ng/ml in urine.

Scippo *et al*. (1994) proposed a decision limit of 0.020 ng/ml for 17β-estradiol in plasma of heifers.

Nielen *et al*. (2007) did not detect 17β-estradiol (cc <1.0 ng/ml) in male and female calf urine samples collected from weeks 8 to 26. 17α-estradiol in male calf urine samples ranged from <1.0 ng/ml to 13 ng/ml while in female calve urine samples it was less abundant (<1.0 ng/ml to 1.6 ng/ml).

Table 5: Concentrations (ng/kg) of residues of estrogens present in edible tissues from non-treated animals (EFSA, 2007) (compilation of data reported by various authors; taken from Paris *et al.,* 2006 with permission).

	Main Residues	**Liver**	**Kidneys**	**Muscle**	**Adipose tissue**
Control veal calves, heifers or steers[1]	Estradiol 17α-estradiol Estradiol-esters Estrone	11 (5-53)[2] 0-5 15 (11-198)	7 (2-70) 5-10 (23-166)	5 (3-35) ND ND 6	(5-50) ND ND 23
Cows: - follicular phase - luteal phase	 Estradiol Estrone Estradiol Estrone	 23 14.3	 13.5 15.1	 30.8 18.9	 11 28.4 8 25.9
Pregnant cows: - 120 days - - 240 days	 Estradiol Estrone Estradiol Estrone	 13.3 18.2 32.7 145	 82.5 85.3 1027 142	 118 156 274 523	 48.1 1283 67.5 2786

[1] Sum of free and conjugated oestrogens
[2] In brackets, minimal and maximal values

Porcine

Pork and poultry probably contain similar amounts of estrogens as untreated cattle (Daxenberger *et al*., 2001). Levels of the steroid hormone 17β-estradiol measured in peripheral plasma of sows during the estrous cycle range from <0.018 to 0.082 ng/ml and levels of free 17β-estradiol during the gestation range from <0.015 to 0.4 ng/ml (Hoffman,1983).

Poultry

Pork and poultry probably contain similar amounts of estrogens as untreated cattle (Daxenberger *et al*., 2001). Stephany (2010) reported concentrations of 17β-estradiol in eggs from Dutch hens between 0.06 µg/kg and 0.38 µg/kg with a mean concentration of 0.14 µg/kg. According to Stefany (2010) hen's eggs are the major source of 17α- and 17β-estradiol in the consumer's daily "normal" diet. Levels of 17β-estradiol measured in peripheral plasma of hens during the estrous cycle range from <0.06 to 0.18 ng/ml (Hoffman,1983).

Equine

Haffner *et al*. (2010) measured the concentration of estradiol in blood of Mongolian horses. Median concentrations of estradiol were highest in fillies followed by colts, mares, geldings, and stallions. Estradiol concentrations in serum of domestic mongalian horses ranged from 0.03 to 0.47 ng/ml. Levels of free 17β-estradiol measured in peripheral plasma of mare during the gestation range from <0.015 to 0.071 ng/ml (Hoffman,1983).

Ovine

Levels of 17β-estradiol measured in peripheral plasma of sheep during the estrous cycle range from <0.005 to 0.022 ng/ml (Hoffman,1983). Levels of free 17β-estradiol and free 17α-estradiol during the gestation range from <0.02 to 0.214 ng/ml and <0.02 to 0.22 ng/ml, respectively (Hoffman,1983).

Caprine

Levels of free 17α-estradiol measured in peripheral plasma of goat during the gestation range from 1.1 to 2.4 ng/ml (Hoffman,1983).

Cervine

Concentrations of estradiol were measured by Kelly *et al*. (1982) in plasma of female red deer. In pregnant and non-pregnant hinds, the estradiol concentrations declined from the start of sampling to reach basal values of 0.005-0.01 ng/ml around 160-190 days of gestation or during November. After this, the values for pregnant hinds increased significantly to peak values (mean 0.35 ng/ml) immediately before parturition.

Fish

In fish species, many studies demonstrated the crucial role of estradiol in the development of the reproductive axis (ovary and especially in vitellogenesis). An increase in plasma estradiol levels occurs during vitellogenisis and estradiol acts on the liver to stimulate biosynthesis and release of vitellogenin (Atteke *et al*., 2003).

2. 6. Zeranol and taleranol

Zeranol [2,4-dihydroxy-6-(6α,10-dihydroxyundecyl) benzoic acid μ-lactone] (α-zearalanol) is a resorcyclic acid lactone and a semi-synthetic product derived from the naturally occurring mycotoxin zearalenone, which is produced by Fusarium moulds.

It is a weak estrogen and is used in the US to improve feed conversion efficiency and promote growth rates in livestock production. The major metabolites of zeranol are zearalanone and β-zearalanol (also called taleranol). β-Zearalanol probably results from the enzymatic reduction of zearalanone. Zeranol and its metabolites all had endocrine-related biological activity, zearalanone and β-zearalanol being less biologically active than zeranol. Zeranol and its metabolites were all stable under different storage conditions (Van der Merwe and Pieterse, 1994). Zeranol might arise from the metabolism of α-zearalenol and zearalenone.

Taleranol is the *β* isomeric metabolite of zeranol.

It is possible that taleranol could arise via zeranol from α-zearalenol, as well as from the metabolism of administered zeranol. Zeranol is metabolized by cattle into taleranol (β-zearalanol) and zearalanone and excreted in urine (van Bennekom *et al*., 2002).

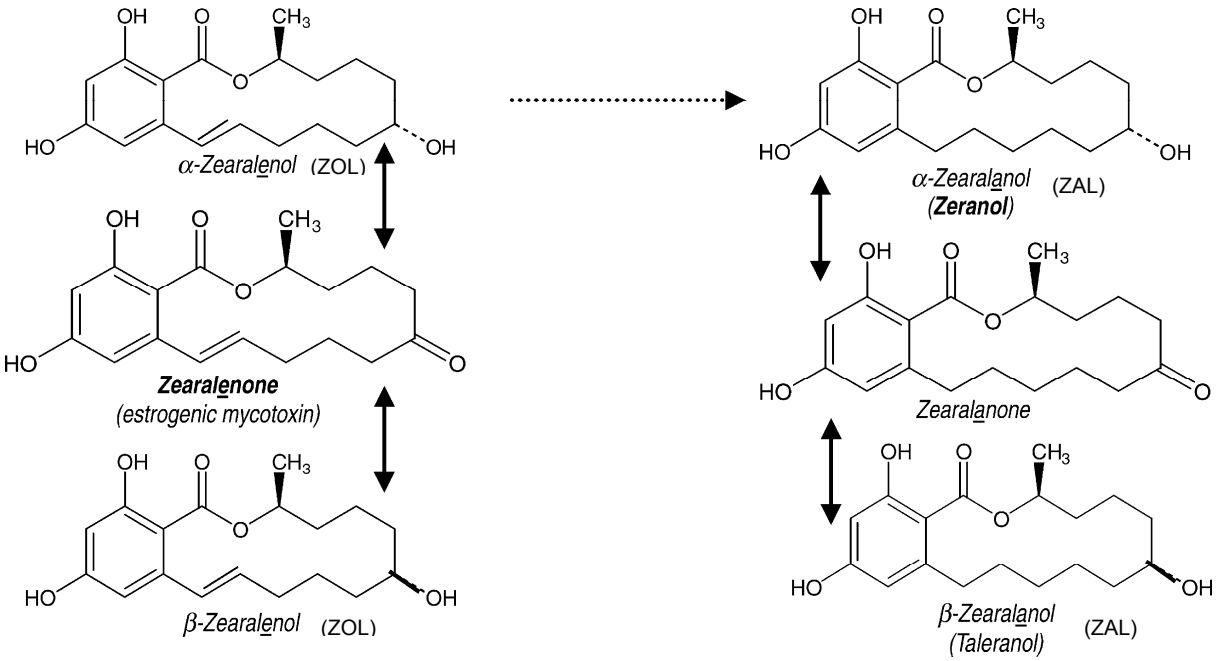


Fig. 1. Zeranol, its metabolites and the structurally- and metabolically-related mycotoxins (van Bennekom *et al.*, 2002)

It was shown in reports from New Zealand (Erasmuson *et al*., 1994; Miles *et al*., 1996) and Northern Ireland (Kennedy *et al*., 1995; 1998) that zeranol might occur naturally in urine and bile from sheep and in cattle following metabolism of the mycotoxins zearalenone and α-zearalenol, which can contaminate animal feedstuffs.

Available data in Europe indicate that maize is the most prominent cereal at risk, in terms of a high incidence and occasional high levels of contamination with zearalenone. In addition, wheat and oats as well as soybean products have been found to be contaminated occasionally. Data on the occurrence of zearalenone in straw, hay, grass and silage are less well documented, but there are indications that this exposure route needs to be considered (EFSA, 2004).

Bovine

Reduction of α- and *β*-zearalenol may occur in cattle and sheep, resulting in significant concentrations of zeranol and taleranol in urine (Kennedy *et al*., 1995; 1998; Erasmuson *et al.,* 1994; Miles *et al*., 1996; Kleinova *et al*., 2002)

The zeranol mean concentration (±SEM, n = 28) found in urine by Kennedy *et al*. (1998) was 2.25 ± 0.32 ng/ml.
As reported by Erasmuson *et al*.(1994), high amounts of α-zearalanol (α-ZAL) + β-zearalanol (β-ZAL) (up to 12.3 ng/ml) and α-zearalenol (α- ZOL) + β-zearalenol (β-ZOL) (up to 163 ng/ml) were found in urine samples (68%) of pasture-fed cattle (Minervini and Dell'Aquila, 2008).

Investigation of urine samples of heifers fed with zearalenone-contaminated oats (at dose of 2740 µg) by Kleinova *et al*. (2002) revealed that ~80% of zearalenone analyzed as the sum of the mother compound and its metabolites was transformed *in vivo* to α-zearalenol (3-5 *µ*g/L) and to its epimer *β*-zearalenol (20-65 *µ*g/L) in a ratio of 1:8, whereas zeranol and taleranol as further metabolites could be detected at lower concentration levels between 2 and 3 *µ*g/L with a ratio of 1:1. Zearalanone, which has not been reported before as a metabolite of zearalenone, was identified in traces, although not in all samples.
β-zearalenol (5.0-11.5 *µ*g/kg) along with smaller amounts of α-zearalenol (<1.0-2.4 *µ*g/kg) and zearalenone (<1.0-2.1 *µ*g/kg) have been identified in liver with analyte concentrations being distinctly lower than those observed in urine samples (Kleinova *et al*., 2002).

The analyte ratio of zearalenone/*β*-zearalenol (1:5) was found to be comparable to the values in urine, whereas zearalenone/α-zearalenol (1:1) and α-zearalenol/*β*-zearalenol ratios (1:5) were slightly higher. Zeranol and taleranol could not be detected in any of the investigated samples, which is somewhat surprising as these metabolites were found in urine samples. A possible explanation might be that they are present at concentration levels below the limit of detection of the applied analytical method. Parallel analysis of outer and inner parts of a liver revealed that zearalenone and α/*β*-zearalenol seem to be homogeneously distributed in this organ.

Concentrations of zearalenone (ZEA) and metabolites in tissues (muscle, liver, kidney, back fat) of growing bull (approximately 460 kg bw) fed with 0.1 mg ZEA/kg at a dry matter content of 88% of the total daily ration were <1 µg/kg for ZEA, < 0.5 µg/kg for α-ZOL, < 5 µg/kg for β-ZOL, < 100 µg/kg for ZAN, < 50 µg/kg for α-ZAL and < 200 µg/kg for β-ZAL. Concentrations in bile were 7-24 µg/kg for ZEA, 2-11 µg/kg for α-ZOL, 23-53 µg/kg for β-ZOL, < 100 µg/kg for ZAN, < 50 µg/kg for α-ZAL, < 200 µg/kg for β-ZAL (Dänicke *et al*., 2002a).

Porcine

A feeding study has demonstrated that zeranol is also a zearalenone metabolite in pigs, where it could unambiguously be detected in urine and muscle tissue (Zöllner *et al*., 2002).

Traces of zeranol and taleranol (<1 µg/L) were detected in urine of pig fed zearalenone contaminated oat (Zöllner *et al.,* 2002). No traces of zeranol and taleranol were found in liver. Zeranol (0.5 – 13.3 µg/kg) and taleranol (0.5 – 1 µg/kg) were found in muscle tissue (Zöllner *et al*., 2002).

Poultry

Only limited information is available with respect to ZEA metabolism in poultry species, although exposure is likely to occur via feedstuffs.

An experiment of long-term exposure of laying hens fed with 1.58 mg ZEA/kg contaminated maize for 16 weeks did not allow the detection of residues of zearalenone or its metabolites in egg yolk, albumen, abdominal fat, breast meat, follicles, ovaries and serum. Zearalenone, β and α-zearalenol were detected in the bile fluid. ZEA and α-zearalenol (α-ZOL) were detected in livers of hens at mean concentrations of 2.1 and 3.7 µg/kg, respectively (Dänicke *et al*., 2002b)
After an application of 0.04 mg ZEA/kg feed in a 5-week feeding trial, plasma, liver and breast meat samples of male turkeys did not show any detectable residual levels regarding zearalenone and its major metabolites (Dänicke *et al*., 2007). The transfer rates to bile increased linearly dependent to the dietary ZEA concentration. No data of ZEA carry-over in commercially produced eggs was found (Zinedine *et al*., 2007).

A study of Kolf-Clauw *et al.* (2008) shows that avian species produced α-zearalenol (α-ZOL) as the major metabolite *in vitro*. Geese differed from all other poultry (quail, hen, guinea-fowl, duck and chicken) by a lower production of α-ZOL. Neither zearalanone nor zearalanols were found. Findings of Kolf-Clauw *et al*. (2008) are consistent with those of previous studies in other species and in hens, showing that hepatic phase I hydroxylations result in the formation of α-ZOL and β-ZOL, and demonstrate the absence of zearalanol formation.

Ovine

Although based on data from only two animals, Miles *et al*. (1996) showed that sheep (and possibly other livestock, including non-ruminants) are capable of metabolizing zearalenone, a common contaminant of both pasture and grain-based feeds, to zearalenols and on to zearalanols.
The ovine metabolism of zearalenone (ZEA) includes the synthesis of five metabolites, such as zearalanone (ZAN), α- and β- ZOL, α- and β-ZAL and high levels of some of these forms may be excreted in the urine as glucuronides by grazing sheep (Minervini and Dell'Aquila, 2008).
Erasmuson *et al*. (1994) reported amounts of α-zearalanol (α-ZAL) + β-zearalanol (β-ZAL) up to 0.77 ng/ml and α-zearalenol (α- ZOL) + β-zearalenol (β-ZOL) up to 34 ng/ml in urine of lambs and concentrations of α-zearalanol (α-ZAL) + β-zearalanol (β-ZAL) up to 2.1 ng/ml and α-zearalenol (α-ZOL) + β-zearalenol (β-ZOL) up to 86 ng/ml in urine of sheep.

Caprine

Erasmuson *et al*. (1994) reported amounts of α-zearalanol (α-ZAL) + β-zearalanol (β-ZAL) up to 0.56 ng/ml and α-zearalenol (α- ZOL) + β-zearalenol (β-ZOL) up to 19 ng/ml in urine.

Equine

The main excretion of ZEA in the horse occurs via faeces, followed by urine (Songsermsakul *et al.,* 2006). The main metabolites detected in equine faeces are ZEA (73 ng/ml), α-ZOL (50 ng/ml) and β-ZOL (45 ng/ml). α -ZAL (27 ng/ml), β-ZAL (41 ng/mL) and ZAN (29 ng/ml) were found at lower levels. Zearalenone urinary levels of 3 ± 2 (mean ± standard deviation) and 43 ± 57 ng/ml were found in Italian and Romanian horses, respectively, as natural exposure to ZEA. Erasmuson *et al*. (1994) reported very high levels of α - + β-ZOL (up to 19 ng/ml) and α -+ β-ZAL (2157 ng/ml) in urine samples probably related to the grazing practices of those species (Minervini and Dell'Aquila, 2008).

Other species

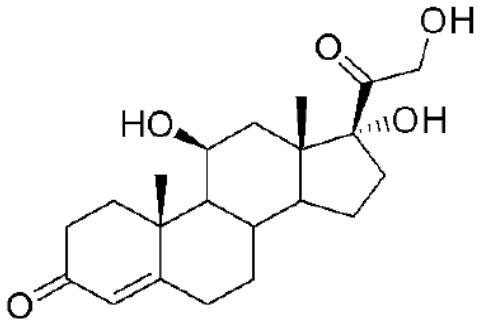
It was demonstrated that a further reduction of α-zearalenol and *β*-zearalenol may occur in deer, goats, sheep, cattle, and horses resulting in significant concentrations of zeranol (α-zearalanol, Figure 1) and taleranol (*β*-zearalanol, Figure 1) in urine (Erasmuson *et al*., 1994).

Research undertaken by MAF, and subsequently published by Erasmuson *et al*. (1994), provided compelling evidence that urinary zeranol from pasture-fed cattle, sheep, deer, goats, and horses in New Zealand originated from a dietary source (Miles *et al*., 1996).

Further reduction of the C11-C12 double bond leading to α- and β-zearalanol has been demonstrated in sheep. It was suggested that the failure to detect zearalanols in other species might be due to the use of high-performance liquid chromatography with fluorescence detection in those studies. Reduction of the C11 - C12 double bond of zearalenone leads to loss of fluorescence and the method is therefore much less sensitivity for zearalanols than for the fluorescent zearalenols (Miles *et al*., 1996).

No information was found for fish.

2.7. Cortisol



Chemical structure of cortisol (CAS N° 50-23-7)

Cortisol (11β,17α,21-trihydroxy-pregn-4-en-3,20-dione) or hydrocortisone is the main glucocorticoid secreted by the adrenal cortex. It agonizes the glucocorticoid receptor and acts to regulate inflammation and immunity as well as fat, protein and carbohydrate metabolism.

Glucocorticoids (GCs) are routinely measured to assess adrenocortical activity in free-ranging vertebrates and can be detected in blood, urine, feces, saliva, and hair. GCs can increase in response to emotional and nutritional stressors, as well as increased thermoregulatory demands. Assessment of stress hormones in the circulation can be confounded by disturbance-induced stress of capture, handling, and immobilization since GCs rise rapidly in the circulation (within minutes). In some species, rapid restraint and blood sampling before elevation of GCs is impossible or impractical. Assays that measure GC metabolites in feces or other biomaterials that accumulate steroids, such as hair, permit the non-invasive monitoring of adrenocortical activity in free-living vertebrates. Importantly, these types of samples represent a cumulative measure of GC release over a longer period of time, rather than a ‘‘snapshot’’ or point sample that is reflected in plasma samples. Furthermore, plasma GC concentrations are pulsatile and exhibit a circadian rhythm. In contrast, fecal concentrations represent a pooled fraction of conjugated steroid that mixes with digesta passing through the intestines over several hours or days (Ashley *et al*., 2011).

Bovine

Serum cortisol concentrations are often used to evaluate stress, but exhibit marked variability, for example due to circadian rhythms, so faecal corticosterone has been used to evaluate stress in cattle (Morrow *et al*., 2002; Möstl and Palme, 2002).

Dusi *et al*. (2012) have measured variable concentrations of cortisol in urine of cows (mean concentration of 3.8 ± 3.8 and 10.9 ± 8.7 µg/L at farm and 14.9 ± 9.5 and 37.0 ± 23.8 µg/L at slaughter).

Porcine

While the serum concentration of cortisol has been used as a reliable indicator of stress levels (Carrasco *et al.,* 2009), it has also been measured in skeletal muscle (Shaw *et al*., 1995), hair (Kalra *et al*., 2007), saliva (Foury *et al*., 2005), and urine (Faucitano *et al*., 2006). Cortisol levels measured in muscle of livestock can be a useful indicator of stress (Shaw *et al*., 1995). Cortisol levels can be affected both by genetic and environmental factors, such as breed, gender, susceptibility to stress, repeated noise, handling and transportation conditions, lairage time, and stunning treatment (Brown *et al*., 1998; Kanitz *et al*., 2005; Li *et al*., 2009; Warriss *et al*.,1994).The effects of batch and sex on the concentration of cortisol in the porcine longissimus dorsi muscle are presented in Table 6 (Choi *et al*., 2012).

Table 6: The effects of animal batch and gender on the concentration of cortisol (µg/dl) in the porcine longissimus dorsi muscle (source: Choi *et al.*, 2012).

	Number	Mean	Standard error	Level of significance
Batch				
1	25	20.21	2.33	NS
2	26	20.24	3.22	
3	20	21.57	3.06	
Gender				
Female	43	20.14	2.36	NS
Castrated male	28	21.37	2.20	
Total	71	20.63	1.66	

NS = not significant.

Poultry

Glucocorticoid (GC) hormones are mainly synthetized in the adrenal glands form the precursor cholesterol. Corticosterone is the main GC formed in rodents and birds. Cortisol is the major GC produced by chicken thymus and bursa (Lechner *et al*., 2001). Rettenbacher *et al*. (2009) did not detect cortisol in eggs at the time of lay and concluded that cortisol found in the course of embryonic development is probably a product of the embryos own steroidogenesis.
Sohail *et al*. (2010) measured serum cortisol concentrations in broilers of 1.04 ±0.07 ng/ml (mean) in the thermo-neutral group and of 1.91 ± 0.09 ng/ml in the heat stress group and concluded that heat stress induces an increase in the concentration of cortisol in broilers.

Equine

Cortisol (hydrocortisone) is endogenously present in the plasma of horses.
Haffner *et al*. (2010) measured the concentration of cortisol in blood of Mongolian horses. Median cortisol concentrations were higher in adult horses (geldings, mares and stallions) compared to fillies and colts. Cortisol concentrations in serum of domestic mongalian horses ranged from 10.62 to 177.56 ng/ml.

Ovine

Cortisol is metabolized mainly in the liver and excreted not only via urine but also via the bile. Cortisol metabolites can be measured in feces.

Caprine

Maejima *et al*. (2005) studied the stress responses of goats to transportation. Plasma cortisol concentrations during and after transportation were investigated. During transportation, plasma cortisol concentrations increased ($P < 0.05$) compared to those of the controls. The mean peak value observed in transported goats (64 ng/ml) was nine times higher than the corresponding mean from control goats.

Cervine

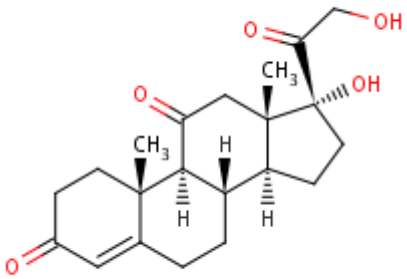
Concentrations of cortisol measured by Ashley *et al*. (2011) in feces of male and female caribou before injection of ACTH were 34.1 and 48.5 ng/g and 60.66 ng/g and 130.2 ng/g in male and female reindeer, respectively. Concentrations of circulating cortisol were 16.0 ± 13.3 ng/ml in caribou and 16.6 ± 6.6 ng/ml in reindeer.

Fish

In fish, exposure to an acute stressor stimulates the hypothalamic-pituitary-interrenal axis and results in the synthesis and release of glucocorticoid hormones into circulation. Cortisol is the primary glucocorticoid in teleost fish (Cook *et al*., 2012). Plasma cortisol concentration has been extensively used as an indicator of stress response and welfare status. Studies of Koakoski *et al*. (2012) indicate that fish at different developmental stages exhibit different time-dependent cortisolemic responses to stressor exposure.
Elevated plasma cortisol levels and a reduction in food intake are common features of the response to stress in fish (Bernier *et al*., 2004). Excess cortisol in goldfish can be associated with poor growth

despite normal food intake. Plasma cortisol concentrations of some immature and mature, male and female teleosts are reported by Milla *et al*. (2009).

2. 8. Cortisone



Chemical structure of cortisone (CAS N° 53-06-5)

Cortisone is a naturally occurring glucocorticoid. It has been used in replacement therapy for adrenal insufficiency and as an anti-inflammatory agent. Cortisone itself is inactive. It is converted in the liver to the active metabolite hydrocortisone (From Martindale, The Extra Pharmacopoeia, 30th ed, p726)[3]. 11β-Hydroxysteroid dehydrogenase (11β-HSD) is an enzyme which catalyzes the interconversion of the active glucocorticoid (GC) – cortisol to biologically inactive cortisone (Franciszek *et al*., 2010).

Bovine

Dusi *et al*. (2012) have measured variable concentrations of cortisone in urine of cow (mean concentration of 2.5 ± 1.9 and 8.4 ± 6.8 µg/L at farm and 10.1 ± 5.5 and 17.9 ± 7.4 µg/L at slaughter).

Equine

Cortisone is endogenous present in plasma of horse.

Fish

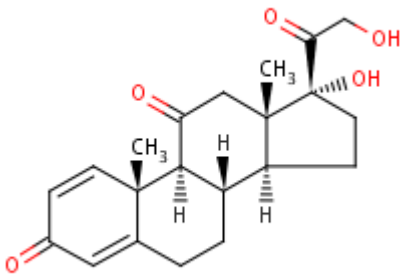
The main corticosteroids isolated from fish blood are cortisol, cortisone,11-deoxycortisol and corticosterone. But, their concentrations depend on the species, sex and reproductive status (see point 2.7. Cortisol) (Milla *et al*., 2009).

Other species

Cortisone in naturally present in ovine, caprine and cervine.
No information was found for poultry.

[3] http://chem.sis.nlm.nih.gov/chemidplus/ProxyServlet?objectHandle=Search&actionHandle=getAll3DMViewFiles&nextPage=jsp%2Fcommon%2FChemFull.jsp%3FcalledFrom%3Dlite&chemid=0000053065&formatType=_3D

2.9. Prednisone



Chemical structure of prednisone

Prednisone is a synthetic anti-inflammatory glucocorticoid derived from cortisone. It is biologically inert and converted to prednisolone in the liver[4].

According to Noppe *et al*. (2008) prednisone is susceptible to be endogenous.

Bovine

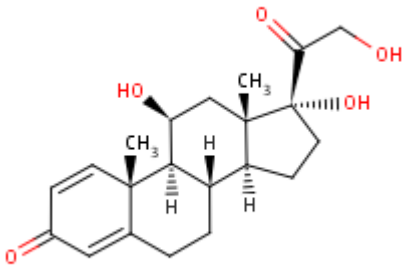
The transformation of cortisol and cortisone into prednisolone and prednisone in cattle faeces was evaluated by Arioli *et al*. (2010). The results demonstrate the hydrolysis of the conjugated form and the dehydrogenation in ring A in diluted feces. It is therefore predicted that urine contaminated with faeces may be positive for prednisone and prednisolone in the same way as they are positive for boldenone, i.e. as a result of microbiological dehydrogenase activity on cortisol and cortisone.

Dusi *et al*. (2012) have analyzed cortisol, cortisone, prednisone, prednisolone in urine of 52 lactating cows after sampling at farm and at slaughter. Prednisone was never detected.

Other species

No information was found for porcine, poultry, equine, ovine, caprine, cervine and fish.

2.10. Prednisolone



Chemical structure of prednisolone (CAS N° 50-24-8)

Prednisolone is a glucocorticoid with the general properties of the corticosteroids. It is the drug of choice for all conditions, in which routine systemic corticosteroid therapy is indicated, except adrenal deficiency states[5].

[4] http://chem.sis.nlm.nih.gov/chemidplus/ProxyServlet?objectHandle=Search&actionHandle=getAll3DMViewFiles&nextPage=jsp%2Fcommon%2FChemFull.jsp%3FcalledFrom%3Dlite&chemid=0000053032&formatType=_3D

According to Noppe *et al*. (2008) prednisolone is susceptible to be endogenous.

Schriks *et al.* (2010) have identified glucocorticoid compounds in various waste waters in The Netherlands. The highest concentration (2 *µ*g/L) was observed for prednisolone in hospital wastewater, while this compound was also found at lower concentrations in the extracts of industry (0.247 µg/L) and hospital wastewater (0.315 µg/L) from a previous study. Others glucocorticoids identified in hospital wastewater were cortisol (0.275-0.301 µg/L, cortisone 0.381-0.472 µg/L, prednisone 0.117-0.454 µg/L and triamcinolone acetonide 0.014-0.041 µg/L.
Considering the fact that glucocorticoids such as cortisol, cortisone, prednisone, and dexamethasone are relatively well removed during sewage treatment (% removal >98%) (Chang *et al*., 2007), only low concentrations of these compounds in surface waters are expected.

Bovine
The results of a study of Pompa *et al*. (2011) suggested that prednisolone could be produced endogenously. The results observed for prednisolone in the study of Pompa *et al*. (2011) suggested that it is very likely to find this corticosteroid in the urine of stressed animals. Data, however, did not clarify whether prednisolone was a metabolite of cortisol or a byproduct of its metabolic pathway. Moreover, prednisolone could also be detected under supposed non-stressful conditions.

The transformation of cortisol and cortisone into prednisolone and prednisone in cattle faeces was evaluated by Arioli *et al*. (2010). The results show the hydrolysis of the conjugated form and the dehydrogenation in ring A in diluted faeces. It is therefore predicted that urine contaminated with faeces may be positive for prednisone and prednisolone in the same way as they are positive for boldenone, i.e. as a result of microbiological dehydrogenase activity on cortisol and cortisone.

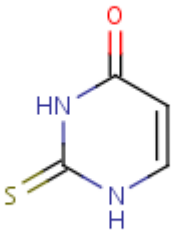
Andersen *et al*. (2008) found residues of prednisolone of 13, 5.9 and 4.2 µg/L in urine sample.
Dusi *et al*. (2012) have detected prednisolone in urine of lactating cow at farm and at slaughter. The detection frequency at slaughter was higher than at farm. Mean concentrations at farm and at slaughter were about the same (0.83 ± 0.44 ng/ml at farm and 0.83 ± 0.43 ng/ml at slaughter). When prednisolone was detected, cortisol and cortisone concentrations were on average higher. The presence of endogenous prednisolone in bovine urine seems to be strongly related to a state of stress of the animals.
Vincenti *et al.* (2012) have found traces of supposedly endogenous prednisolone (approximately between 0.1 and 0.3 µg/L) together with relatively high cortisol concentration in seven urine samples from free housing cows allegedly stressed by the urine sampling operations.

Equine
Fidani *et al.* (2012), have studied the accuracy of the analytical protocol used for the detection of low concentrations of prednisolone in racehorse urine samples and the possible endogenous origin of the detected prednisolone. They concluded that the very high frequency of prednisolone detection in the samples (78.5%), the low concentration of this steroid (about 1 ng/ml) and, importantly, the narrow range of the 95% confidence limits (0.97–1.05 ng/ml in MS^2 mode and 0.88–1.04 ng/ml in MS^3 mode), could represent evidence that its presence is endogenous. In the light of these results, this hypothesis seems the most probable, even if further studies are required to confirm it. Furthermore, a microbiological origin (i.e. fermentation of cortisol after sample collection) could not be disregarded.

Other species
No information was found for porcine, poultry, ovine, caprine, cervine and fish.

[5] http://chem.sis.nlm.nih.gov/chemidplus/ProxyServlet?objectHandle=Search&actionHandle=getAll3DMViewFiles&nextPage=jsp%2Fcommon%2FChemFull.jsp%3FcalledFrom%3Dlite&chemid=0000050248&formatType=_3D

2. 11. Thiouracil



Chemical structure of thiouracil (CAS N°141-90-2)

Thiouracil (CAS No. 141-90-2) belongs to the group of thyrostatic drugs, a complex group of substances that interfere with thyroid function, resulting in decreased production of hormones thriiodothyronine (T3) and thyroxine (T4) (De Brabander, 1984; Courtheyn *et al.*, 2002). The 2-thiouracil is a compound which has a strong inhibitory effect on the thyroid (Le Bizec *et al.*, 2011).

Thiouracil has been used as antithyroid, coronary vasodilator, and in congestive heart failure although its use has been largely replaced by other drugs. It is known to cause blood dyscrasias and suspected of terato- and carcinogenesis[6]. IARC (2001) has classified thiouracil in group 2B (Possibly carcinogenic to humans).

Besides the therapeutic use of some of thyreostatic drugs in human and feline medicine, the xenobiotic thyreostats have also been illegally exploited for fattening purposes in animal husbandry (De Brabander, 1984; Courtheyn *et al.*, 2002). The use of the most powerful thyreostatic agents results in a weight gain caused by the increased filling of the gastro-intestinal tract as well as the retention of water in edible tissues, by inhibiting the thyroid hormone production (Pinel *et al.*, 2006; Vanden Bussche *et al.*, 2011). This leads to the production of meat of lower quality and is considered as an abuse, because water is sold for the price of meat (Vanden Bussche, 2011).

The use of thyreostatic drugs for animal fatting purposes has been prohibited in Belgium since 1974 (AR du 12.04.19742).

In the framework of the national control plan of Belgium and Norway, the laboratory of Ghent University (Belgium) frequently received urines of livestock for the routine analysis of thyreostats. These urines, subsequently analyzed rarely exceeded the recommended concentration (RC) for TU of 10 µg/L. Nevertheless, 61.3% of the bovine urines obtained levels of TU below the RC, for porcine urine this was 96.3%, and for ovine urine 57.9% of the samples. The clinical background of these animals was unknown. Illegal administration for growth-promoting purposes however, seemed highly unlikely at these low concentrations, and the possibility of a natural origin more plausible (Vanden Bussche, 2011).

Some vegetables from the Cruciferae (Brassicaceae) family are known to contain substances called goitrogens that impair iodine uptake by the thyroid or impair its incorporation into thyroxine. These substances are called goitrogens and include thioglucosides (glucosinolates). Glucosinolates constitute a well-defined group of secondary plant metabolites in cruciferous plants. Glucosinolates undergo hydrolysis with the endogenous plant enzyme myrosinase. Pinel *et al.* (2006) studied the influence of a cruciferous-based feed on the occurrence of residues of thiouracil in urine of cattle.

[6] http://chem.sis.nlm.nih.gov/chemidplus/ProxyServlet?objectHandle=Search&actionHandle=getAll3DMViewFiles&nextPage=jsp%2Fcommon%2FChemFull.jsp%3FcalledFrom%3Dlite&chemid=0000141902&formatType=_3D

Bovine

Pinel *et al*. (2006) reported a correlation between the supplementation of a Brassicaceae diet to cattle and the presence of TU in bovine urine. This was considered as a first indication that TU might have a natural origin.

Vanden Bussche *et al*. (2010) have detected the presence of TU in urine of livestock in concentrations below the recommended concentration of 10 µg/L.
Le Bizec *et al*. (2011) reported concentrations of TU in bovine urine ranging from decision limit (CCα) to 22.5 µg/L and mean and median values equal to 1.4 and 0.3 µg/L, respectively.

TU was detected in concentrations ranging from 3.5 to 31 µg/kg in *in vitro* bovine static digestion simulation in colonic suspension with traditional rapeseed, coarse colza "00" meal, cauliflower, and broccoli (Kiebooms *et al*., 2012). Results of this bovine and porcine *in vitro* study (Kiebooms *et al*., 2012) confirm the active involvement of intestinal microbiota in TU formation during gastro-intestinal digestion of Brassicaceae derived feed, thus demonstrating plant-myrosinase is not the only possible mediator.

Porcine

A total of 221 porcine urine samples were investigated by Le Bizec *et al*. (2011) for TU concentrations. TU was identified in more than 80% of the samples, with concentrations ranging from decision limit (CCα) to 7.0 µg/L and mean and median values of 0.9 and 0.5 µg/L, respectively.

TU was detected in concentrations ranging from 3.5 to 26 µg/kg in *in vitro* porcine static digestion simulation in colonic suspension with traditional rapeseed, coarse colza "00" meal, cauliflower, and broccoli (Kiebooms *et al*., 2012).

Ovines

Twenty-six ovine urine samples were investigated by Le Bizec *et al*. (2011), with TU concentrations ranging from CCα to 14 µg/L and mean and median values of 3.3 and 2.2 µg/L, respectively. Concentration of TU below 10 µg/L in urine of ovine were measured by Van Den Busshe (2011) (thesis)

Other species

No information was found for poultry, equine, caprine, cervine and fish.

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