EURL Reflection paper 2.0:

Natural growth promoting substances in biological samples

Presence - and formation - of hormones and other growth promoting substances in food producing animals.

Current approaches for enforcement and research needs for full implementation in residue control

Wageningen Food Safety Research

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Preface

From 29 November to 1 December 1995, Directorate-General VI, Agriculture, of the European Commission organised the "Scientific conference on growth promotion in meat production"[1]. During this conference, worldwide leading scientists discussed the existing and emerging growth-enhancing substances, the assessment of health risks, and the methods for detection and surveillance. One of the papers, presented by the group of Guy Maghuin-Rogister (University of Liège) was titled: "Methods of detection and surveillance of natural sex steroid hormones". This paper assessed the possibilities of setting thresholds based on quantitative data obtained with immunoassays. For various situations, it was concluded that discrimination between untreated and treated animals should be possible but that further research would be needed to effectively enforce the ban on the use of hormones for growth promotion.

Also during the Euroresidue conferences, held from 1990 until 2022, it has shown that even when some strategies are determined, for a number of naturally occurring compounds there is still need for an enforcement strategy.

In 2014 we evaluated the status of natural occurring growth promoters in the EURL Reflection paper. We wanted to provide enforcement bodies with practical guidelines and to identify the remaining research needs.

Now, eight years later, we updated the EURL Reflection paper. Literature of the past 8 years is incorporated, and we give an update on research and strategies, and new endogenous compounds that are identified.

We expect that with this 2.0 version, we will provide updated information to enforcement agencies and laboratories and channel our research activities for the coming years to solve the blind spots addressed in this Reflection paper.

[1] European Commission, Directorate-General VI Agriculture (1995), Proceedings of the Scientific conference on growth promotion in meat production.

Current knowledge of the presence and mass concentrations

The first version of this report was prepared as part of the 2013 EURL-work programme. Its objective was to provide the European Commission (EC), National Competent Authorities (CA), and National Reference Laboratories (NRL) with an up-to-date overview of scientific knowledge necessary to interpret the results of residue testing for a variety of compounds. Since 2013 new techniques have been implemented, and new compounds have been discovered with a (semi) endogenous origin. Therefore, the Reflection paper of 2013 is updated to a 2.0 version with the latest peer-reviewed articles, insights, research, and compounds. It is not intended to provide a complete literature review on each topic but to summarize the available information as to the date of 2022 in such a manner that guidelines for enforcement can be established. To achieve these objectives, this report;

(1) Provides guidance to enforcement bodies on the interpretation of the results of laboratory tests and addresses further actions

(2) Defines the research agenda for the next five years to supplement scientific information and technical possibilities to ensure effective enforcement of current legislation concerning the use of hormonal growth-promoting compounds in meat-producing animals.

We expect that this document will contribute to effectively controlling the abuse of "Natural growth-promoting substances in biological samples".

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Summary

The classical approach to detect the abuse of banned substances, in which biological samples are semi-quantitatively tested for the presence of a banned substance or a marker metabolite, cannot be directly applied in case a natural background level can be present. However, even when this background level is relatively low, the mere fact that the presence of a particular analyte due to general or specific physiological conditions cannot be excluded with certainty, asks for a different analytical testing strategy. This reflection paper summarizes the approaches a wide range of authors followed when trying to solve this challenge.

The classical testing model comprises two steps: a screening procedure focussing on selecting suspect samples while avoiding false negative results, followed by a confirmatory step focussing on the unambiguous confirmation of the identity of the analyte.

Screening for the abuse of (semi) natural hormones can follow a similar approach since also, in this case, the outcome of the screening procedure is either a result "compliant", no indication of treatment with a banned substance, or "suspect", treatment cannot be excluded. However, the definition of a suspect is slightly different in this case. In the classical model, suspect means that there are indications that the target analyte is present in the sample tested. When testing for natural hormones, suspect means that there are indications that the animal from which the sample was taken was treated with a banned substance. This difference has a significant impact on the confirmatory procedure to be applied. Three different types of confirmatory methods for (semi-)natural compounds can be identified.

(1) Confirmation of the identity of a compound of which no endogenous source is known. This type of confirmatory method is similar to the approach used for exogenous compounds, e.g. synthetic esters of (semi-)natural compounds or confirmation of a biomarker proven not to be present endogenously.
(2) Confirmation based on statistical solid differences between the analyte isolated from a sample and an endogenous reference compound. This situation applies in case combustion-Isotope Ratio Mass Spectrometry is used for confirmation. In selected cases, the difference in C12/C13 between endogenous and synthetic forms of a molecule can be such that discrimination is possible with a significant solid difference.
(3) Confirmation on the basis of another significant difference between data obtained for a particular animal or group of animals and the corresponding reference population. This could involve exceeding threshold levels, differences in ratio's between selected compounds and differences in profiles of metabolites and/or biomarkers.

Until now, this third type of confirmation is usually considered for screening purposes only, and this reflection paper is presented as such. Depending on the significance of the differences observed, use as a confirmatory method can be considered. Currently there are no criteria available for this type of analysis. However, for this, scientific and statistical considerations are not enough. Full agreement with Competent Authorities will be needed.

Specific recommendations are prepared and presented in this manuscript for the different groups of (semi-) natural hormones.

1. Introduction

Hormonal active compounds can be present in biological samples like edible tissues, serum or plasma, fat, hair and skin. The origin of the compounds in the samples can be the result of any (combination) of the following specific situations:

1. Endogenous production as part of the normal physiology of the animal species concerned
2. Endogenous production under specific circumstances: e.g. age, physiological status such as heat, pregnancy and puberty, injury or stress
3. Production within the sample due to the presence of specific bacteria or enzymes
4. Production in the sample through instability, heat temperature, light, moisture etc.
5. Uptake from, or formation in, the digestion track from feed compounds
6. Following treatment of the animal by injection, orally through feed or drinking water, implantation under the skin, or dermally (pour-on)
7. A combination of the above situations

The latter two circumstances represent or involve the treatment of the animal. In the European Union, hormonal growth-promoting compounds and beta-agonists are prohibited by Directive 2008/97/EC [1]. Annex I of CDR (EU) 2022/1644 lists these compounds A(1a) Stilbenes, A(1b) Antithyroid agents, A(1c) Steroids, A(1d) Resorcylic Acid Lactones including zeranol, protein and peptide hormones A(3e)and A(3f) for the corticosteroids. This Delegated Act entered into force end of 2022 [2].

The fact that for a range of compounds, multiple explanations can be given for their presence in a biological matrix, severely hampers the enforcement of the ban on their use in meat production. For enforcement, the analytical result of a residue test, expressed as a statement on the presence of a particular analyte and, if applicable, with a semi-quantitative indication of the mass concentration, must be supplemented with a statement on the interpretation. In case the presence of a compound was confirmed this statement should ideally say whether its presence could be a result of semi-natural processes (situations 1 – 5 above) or that treatment is the only explanation. In cases such a definitive answer cannot be supported scientifically, it will be necessary to provide an indication of the possible explanations.

This report summarizes the current scientific knowledge on the "semi-natural" occurrence of hormonally active compounds. It is not intended to give a full literature review but to summarize the current knowledge and define the starting point for further evaluation in terms of "options for enforcement" and "the identification of further research needs".

Based on the groups included in CDR (EU) 2022/1644 [3] the following selection was made *a priori*.

A1a. Stilbenes: Since there are no indications that the stilbenes currently included in residue control are produced (semi-)naturally, they are not included in this review.

A1b. Antithyroid agents: The presence of especially thiouracil in biological samples, mainly urine, is well documented and Chapter 3 summarizes the current knowledge. The

presence of Mercaptobenzimidazol due to environmental contamination is also discussed in Chapter 3.

A1c. Steroids: Steroids clearly form the largest and most complicated group of compounds. For this overview they are grouped as follows:

- Traditional natural hormones: 17β-estradiol, 17β-testosterone and Progesterone (Chapter 2)
- Nortestosterone (Chapter 4)
- Boldenone (Chapter 5)
- 1-Testosterone (Chapter 6)

A1d. Zeranol: The production of the *Fusarium* toxin zearalenone in animal feed is well documented, as well as the link with the presence of zeranol in samples obtained from animals which received such feed (Chapter 7).

A3e. Protein and peptide hormones, Growth hormones, bST and recombinant bST in milk and plasma is listed in Chapter 9.

IGF-1 and related growth factors are discussed in chapter 10.

A3f. Corticosteroids. Some corticosteroids, e.g. dexamethasone, have a registration as veterinary drug, others are natural compounds (cortisol, cortisone). The presence of prednisolone, however, until recently could not be explained. Current knowledge seems to indicate that this compound can be produced in biological materials under specific conditions (Chapter 8).

The classical testing model, which basically is a qualitative model with well-defined and determined performance characteristics like CCβ-screening and CCα confirmation, is based on a two-step approach: screening followed by confirmation. Although there are typical methods for both purposes, the difference between methods for screening and confirmation is based on the level of information obtained by the analysis and not by the method used. More and more high-end mass spectrometric methods are also used for large-scale screening.

This classical qualitative model cannot solve the enforcement problems discussed in this report. The qualitative confirmation of the presence of a well-defined target analyte will not provide the necessary reliability for the conclusions to be drawn. The type of information obtained from the analyses is more complex in nature and involves data on the parent compounds, biomarkers (direct metabolites or indirect markers), precursors or combinations of these compounds, up to the level of profiling. This includes quantification of multiple analytes, extensive statistical evaluation and modelling of the results.

In principle, these issues can be dealt with in a science-based manner. However, it is also necessary to think about the consequences for enforcement. For this, it will be necessary to include the opinions of policymakers and competent authorities as well.

2. The natural hormones 17β-estradiol, 17β-testosterone and progesterone

2.1. Introduction

Already for decades the analytical problem associated with the presence of natural hormones in biological samples has been an issue of concern and debate. The mere fact that these compounds are present in almost all matrices, and the complicated relations between the different steroids, have hampered the development of a definitive approach for control. Figure 2.1 shows the relation between the different natural hormones.

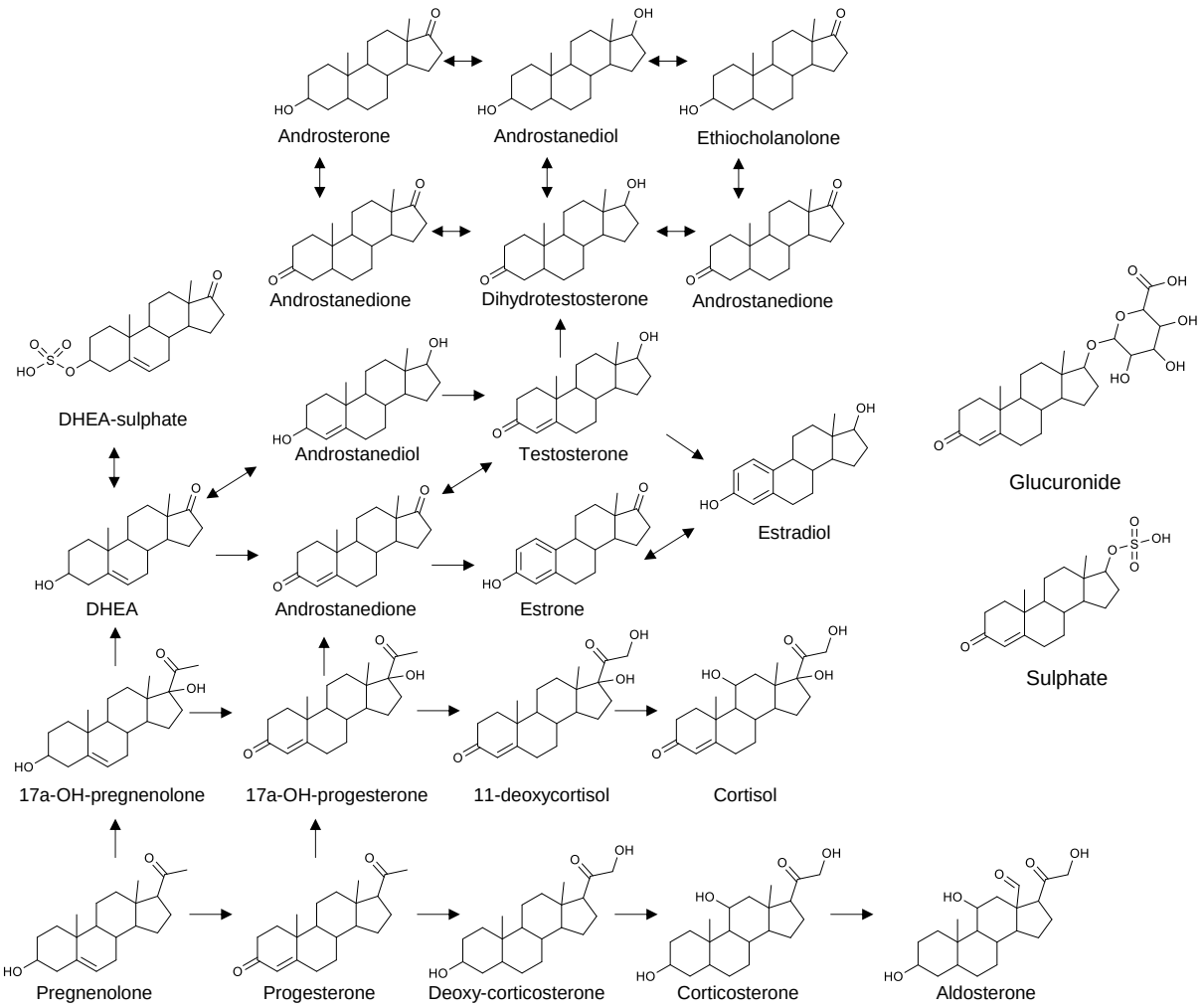


Figure 2.1. Steroidgenesis, the steroid pathway showing relations between individual steroids.

The availability of methods for analysis in principal never has been a problem since these hormones are very important in human clinical diagnostics and quantitative methods, mainly based on immunoassays, already are available since the 1970's. The first systematic listing of analytical methods for residue control was published by the EC in 1994 [4]. This report states the following on the interpretation of quantitative results: "Naturally occurring steroid residues.

- Until the Scientific Veterinary Committee makes its report on the physiological levels of "natural" sex hormones in plasma the following provisional procedure for dealing

with the illegal use of "natural" sex hormones for fattening purposes in bovine animals for the year 1991 shall be adopted.

- In the course of testing for "natural" sex hormones in accordance with Regulation (EU) 2017/625 [5] (live bovine animals at the farm or at the slaughterhouse) or in the course of any other testing, levels of estradiol or testosterone exceeding the level given in Table 2.1, are found in the blood plasma of at least one bovine animal, the measures foreseen in Article 6, paragraph 1, 2 and 3 of Regulation (EU) 2017/625 should immediately be carried out."

Table 2.1. Maximum level for estradiol and testosterone in bovine plasma [4].

	Age (months)	Maximum plasma concentrations	
		Male bovine (μg L^{-1})	Non-pregnant female bovine (μg L^{-1})
Free estradiol-17β	≤ 6	–	0.04
	≤ 18	0.04	–
Free testosterone-17β	≤6	10	–
	6 – 18	30	–
	≤ 18	–	0.5

In fact, these values remained the "officially recommended values" for decades, only slightly modified in 2007 and when the MMPR Guidance paper of 2021 [6] was published. The importance of confirmatory analyses made it necessary to increase the level of 0.04 μg $L^{-1}$to 0.1 μg L^{-1}, the lowest limit for confirmatory analyses practically achievable at that time.

Natural hormones are included as group A3 in CDR (EU) 2022/1644 and include 17β-estradiol, 17β-testosterone and progesterone. Research supporting implementing control strategies for these substances has been on-going for several decades. Due to the global differences in legislation concerning the use of 17β-estradiol as growth promoting substance, the focus has been on this compound in many studies. Notwithstanding the large number of published studies, it is concluded that there are currently only limited technical possibilities to prove the abuse of natural hormones based on analyses of biological samples for the parent compound or its metabolites. The only approach applied so far is the use of isotope ratio Mass Spectrometry in combination with Gas Chromatography (GC-CIRMS). This approach has been applied in France since 2005 but has not yet been widely implemented further. This conclusion applies to the six groups of animal species (Annex 1) and includes all relevant matrices. For screening purposes, approaches were described for 17β-estradiol and 17β-testosterone in bovines, based on serum or plasma analyses, and for 17β-estradiol for urine analysis. These approaches are based on setting threshold levels, the upper limit of natural concentrations. However, the concern is that these threshold concentrations are too high and may lead to an unacceptable number of false negative results. Notwithstanding the technical difficulties, several authors have published new approaches for both effective screening and confirmatory analyses. Some of these approaches are currently being implemented or have the potential to become part of routine official analyses in the near future.

2.2. Analytical methods

Analytical methods for screening and confirmation of natural hormones, including the major metabolites 17α-estradiol and 17α-testosterone are widely available. For screening

methods immunoassays are popular, which are frequently based on methods initially developed for diagnostic purposes in human medicine. Many methods for instrumental screening and confirmation based on various Mass Spectrometric approaches have been published. Successful combinations have been made with either LC or GC.

Screening methods

The national residue control plans as submitted by the EU Member States include a range of analytical methods for natural hormones. Most laboratories use plasma or serum for screening, either by immunoassay or MS detection. A very small number of laboratories perform urine screening analyses, and only a few analyse hair samples. These approaches result only in very few non-compliant results. Based on the lack of discriminating power of the current screening methods for serum and the absence of reliable confirmatory techniques, a reliable assessment of the degree to which natural hormones are abused cannot be made.

In general, it must be concluded that most screening methods lack the discriminating power necessary to differentiate natural occurrence and treatment-based presence of natural hormones.

The following alternative approaches, which are less widely used, are available for screening:

a. Methods based on changes in the histology of specific tissues
b. Methods based on the detection of changes in the occurrence of a specific biomarker
c. Methods using effect based screening, bioassays
d. Methods based on steroid profiling

a. Histology

Screening animals based on histological examination of selected tissues is among the oldest approaches used in screening for the use of hormones. Despite the complexity of interpretation, there is still potential for screening, mainly for male veal calves, where changes in the histology of the prostate can provide useful information. [7,8].

b. Biomarkers

Biomarkers can be used to identify specific animals, e.g. animals treated differently from corresponding control animals. Regal *et al.* [9] published a metabolomic study in which the influence of a treatment with estradiol or progesterone on the liquid chromatography-high resolution mass spectrometry (LC-HRMS) total ion chromatogram was determined [9]. This untargeted approach resulted in sets of potential biomarkers for both treatments. The structure of these biomarkers remains to be elucidated, and the discriminating power of these biomarkers is being evaluated.

Transcriptomics based biomarker approaches based on RNA-sequencing form a novel approach. In a study by Riedmaier et al. in heifers, a set of 40 selected candidates, a set of 20 was significantly regulated [10]. With principal component analyses it was possible to discriminate animals treated with a combination of trenbolone-acetate and 17β-estradiol from a control population.

c. Effect-based screening

Effect-based screening methods were developed in response to EU legislation which prohibits the use of compounds with hormonal activity without specifying the exact compounds this includes. Therefore, screening tests should be based on detecting hormonal activity. For this reason, several bioassays have been developed, including the yeast bioassays for screening for androgenic and estrogenic activity [11,12]. These bioassays are based on expressing the human androgen receptor (hAR) or the human oestrogen receptor α (hERα) and yeast-enhanced green fluorescent protein, the latter in response to androgens or oestrogens. The response of these assays is a combined result of the individual responses (in terms of relative hormonal activity) of all active compounds present. The applicability of bioassays is limited to samples of young animals (well before slaughter age) that do not yet produce hormones. From puberty onwards, the animals have too many hormones endogenously, which interfere with the reliable detection of any exogenous hormones present. Though not implemented yet for routine analysis, bioassays are used in several EU Member states within the framework of special surveys.

d. Steroid profiling

A relatively new approach for screening is the use of steroid profiling. Targeted analysis for single compounds does not provide discriminative information due to the very small changes in mass concentration resulting from treatment. But individual steroids are part of a complex physiological system balancing between the different active steroids, their precursors and metabolites (Steroidgenesis). The hypothesis for screening methods based on steroid profiling, which implies the quantitative analyses of a large number of individual compounds, is that the combined effect on all the individual compounds provides a diagnostic tool for detecting abuse. Anizan *et al.* published a study in which the excretion of 25 known conjugated compounds (phase II metabolites) before and after administration of androstenedione was followed [13]. Blokland *et al.* published a study on the effect of treatment with several natural hormones on the steroid profiles for 17 steroids, aglycons, glucuronides and sulphates determined separately, was determined [14]. Multivariate statistical analyses showed that the model could classify animals into a treated and an untreated group. Both studies show the potential of steroid profiling as a promising strategy to determine whether or not bovine animals have been treated with (natural) hormones. Kaabia *et al.* published a profiling study to detect the administration of Nandrolone in horses [15].

The above mentioned approaches are only able to give indirect prove of an abuse of natural hormones. The reason for this is that all of these approaches are indirect. None of the methods indicates the presence of a specific compound of which the mass concentration and identity can be subsequently confirmed during confirmatory analyses. And here we have the problem! What initially, and still rightfully, was considered the main advantage of "effect based screening', now becomes a challenge. There is an effect, and the screening result is "suspicious", but what specific compound is responsible remains unknown. We do not know the cause of the effect, and subsequently, we do not know what to confirm. However, they provide a good basis for further on-farm investigation.

The major objective during the validation of these screening methods is to determine the rate of false negative and false positive results. However, classical validation of these parameters only is possible if the specific compounds to confirm are known. For full

implementation, alternative validation procedures for biomarker-based tests will be necessary.

Confirmatory methods

Confirmatory methods for natural hormones must fulfil two separate conditions:

1. The compound(s) determined, the profile established or biomarker(s) detected must be sufficiently diagnostic to "prove" abuse with a natural hormone.
2. The identity of marker compounds is confirmed on the basis of criteria laid down in Commission Decision 2021/808 [16].

The following confirmatory approaches to detect abuse of synthetic natural steroids are:

a. Methods allowing the detection of steroid esters: the detection of the steroid ester provides unambiguous proof for the administration of an exogenous form of the hormone

b. Methods based on Isotope Ratio Mass Spectrometry (IRMS).

a. Steroid esters

The detection and confirmation of steroid esters in biological samples is one of the oldest analytical approaches for proving the abuse of (natural) hormones. However, its applicability was limited to the analyses of alleged injection sites. Based on the meat inspection during slaughter, veterinarians were able to detect possible locations at which substances were injected. These tissues were removed from the carcass and extracted. Only limited extract purification was necessary to detect the steroid esters as such, using techniques like HPLC combined with Diode Array detection (DAD) [17]. For decades, however, the detection of intact hormone esters remained limited to these application sites.

In recent years the analysis of intact hormone esters gained renewed interest based on new knowledge concerning the incorporation of steroid esters in hair and the increased analytical possibilities to detect and confirm very low concentrations of steroid esters in serum.

Hair. Many scientific papers have described the detection of doping agents, therapeutic compounds, drug abuse and tobacco residues in human hair samples. In 1994 several authors reported the possibility of using hair analysis for the detection of anabolics and beta-agonists in animals, followed in 2005 by a study in which the detection of steroid esters in hair was described [18,19]. Later, Gray *et al.* published a method for 18 steroid esters in equine mane hair [20]. Until now, the use of hair for the direct analysis of steroid esters has been limited. Only two Member States have included hair analysis in their routine monitoring programs, a few more use it in special national programs. Part of the reluctance to use hair as an analytical matrix is due to the risk of external contamination of the animal. A recent study Groot *et al.* showed that, at least for clenbuterol, the chances of such (accidental) external contamination are very limited [21].

Serum. In a recent paper, a new UPLC–MS/MS method was described allowing the detection of steroid esters in serum of breeding (bovine) and racing (equine) animals [15]. The time during which the steroid-esters can be detected in the blood, after intramuscular injection depends on two factors: the time needed for the steroid ester to reach the blood stream and the efficiency of the esterase activity present in the blood for the specific steroid

ester. The next critical parameter is the sensitivity of the analytical method. In order to allow the simultaneous detection of estrogenic and androgenic esters, it was necessary to derivatize the hydroxyl-group at position A3 of estradiol and related structures through dansylation. This derivatisation step has a positive influence on the detection limit, which was 0.02 µg L^{-1} for most estradiol esters, with the exception of estradiol decanoate (0.1 µg L^{-1}). For the androgens testosterone and nandrolone the LOD ranged from 0.020 to 0.050 µg L^{-1}. After intramuscular injection of 17β-estradiol-benzoate the maximum concentration in serum was reached after 9 days. The release of the esters is more rapid for shorter chain than longer or aromatic ester chains, explaining their shorter detection time window.

In an *in vivo* study on bovine animals, in which three heifers were treated with testosterone-propionate (100 mg), it was demonstrated that this substance could be detected in serum [22]. However, the period during which detection was possible was limited to 48 hours. The rapid liberation and subsequent hydrolysis of the ester in serum hampered the longer detection.

Based on these studies, it can be expected that the useful time window can be extended due to the much lower LODs achievable with modern MS techniques. An important development is a discovery that steroid esters (especially the shorter forms) are much more stable when blood is taken into sodium fluoride and potassium oxalate vacutainers rather than simple lith-heparine or EDTA tubes [22]. This allows detection for a prolonged period of time.

Though analytically still challenging and further studies to confirm the applicability under *in vivo* conditions remain to be performed, the determination of intact steroid esters in hair and blood (serum or plasma) samples seems a promising approach. The technique provides a high degree of confidence concerning the conclusion that the treatment of the animals can only explain the analytical result is particularly strong, notwithstanding the necessity of adequate precautions against external contamination. Serum and plasma have the disadvantage that sampling of blood from live animals in some Member States is considered too invasive and subsequently not feasible. Hair sampling is relatively simple although precautions must be taken to avoid external contamination, including hair washing.

b. Isotope Ratio Mass Spectrometry methods

Isotope Ratio Mass Spectrometry (IRMS) is a versatile application to determine the isotopic composition – usually expressed as a ratio – of a wide range of materials and compounds. IRMS is suitable for determining isotope ratios of the lighter elements, which include bio-elements such as carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), hydrogen ($\delta^{2}H$), oxygen ($\delta^{18}O$) and sulphur ($\delta^{34}S$). Because isotope ratios provide crucial information about fundamental processes and the patterns that emerge from these processes, therefore IRMS is used in many research fields. The Belgian Scientific Committee of the FAVV strongly encourages this approach.

Applications of IRMS within agricultural research are mainly aimed at gaining a better understanding of complex biochemical processes such as nutrient cycling, nutrient uptake and metabolic processes - for example, to optimize human and animal diets - whereas applications in food science are mostly related to food safety and authenticity. Due to innovations and improvements in the IRMS technique, the list of applications is growing steadily, now also including distinguishing natural (endogenous) and synthetic

(exogenous) hormones in sportsmen and farm animals, for which compound-specific isotope analysis is required [23-25].

Even though IRMS is considered a suitable confirmatory technique, its discriminating power has a statistical basis and depends on the observed actual differences in isotope ratios of the endogenous and synthetic forms. Dietary factors, no doubt, are an important parameter. However, it is demonstrated that this basis can be strong enough to reach the reliability needed for confirmation.

2.3. Research Summary

Testosterone

The majority of data available for residues of testosterone was specifically obtained for bovine animals. The parent compound 17β-testosterone is the major androgenic steroid in all food producing animals. However, metabolism pathways can be very different in different species resulting in different analytical targets for analyses of excreta. Excreta are the matrices with the highest levels, both for the parent compound and the major metabolite in bovine animals, 17α-testosterone.

A review showed that data published by several authors for male animals, with ages up to 32 weeks, all showed plasma levels < 10 µg L^{-1} (range 0.1 - 8 µg L^{-1}) [26]. Data for urine were very limited and too few to draw any quantitative conclusions. A study of Angeletti *et al.* showed that the ratio between testosterone and 17α-testosterone in urine cannot be used in bovine animals as an indicator for abuse as it is in humans and horses [27].

For female animals, data for both plasma and urine were very limited. The highest reported value for plasma was 0.04 µg L^{-1}, well below the recommended threshold. For pregnant animals no data are available for any matrix. Lactating animals show slightly higher values, but all below the threshold of 0.5 µg L^{-1} in plasma. The remaining question is: "Are these threshold values not too high in order to be discriminative?".

A paper of Blokland *et al.* provides a large set of data for steroids part of the steroid synthesis pathway [14]. This paper focussed on methods to discriminate control populations from populations of treated animals. Individual values for the aglycon (A), glucuronide (G) and sulphate (S) forms are provided. Control animals analysed for 17β-testosterone showed that the major part is excreted as glucuronide conjugate, for female animals the range was form 0.19 – 0.68 µg L^{-1}, for male animals from 0.07 – 3.24 µg L^{-1}.

For testosterone the overall conclusion for bovine animals was: "Ubiquitous in males and females in varying concentrations". No threshold values could be established. On the other hand, no indications were obtained that the recommended values, as mentioned by Ray Heitzmann [4] for plasma and in the MMPR Guidance paper [6] are invalid, i.e. no values were found in untreated animals exceeding these levels. At that time, however, no discrimination was made between "intact" and "castrated" male animals.

Other species

Also, for ovine animals, available data are limited. Serum levels can be very different between breeds. However, no data were published for ovine animals with values exceeding the threshold values in bovine animals. Levels in female animals are presumed to be 100-fold lower. Complicating factor is that ovines are seasonal breeders. 17α-testosterone is

the major metabolite and conjugation (glucuronides and sulphates) is extensive. The overall conclusion for ovine animals is identical to the conclusion for bovine animals.

Data for porcine animals also highlight the importance of measuring the conjugated form (sulphates) in serum. In male animals (boars), plasma levels started around 1 µg L^{-1} and reached maximum levels of 3 µg L^{-1}after 7 months for free testosterone and around 6 µg $L^{-1}$for testosterone-sulphate. After that period levels dropped again. In general, levels in barrows (castrated pigs) are between seven and 500-fold lower and levels in gilts/sows were even lower than that.

The threshold levels for bovine animals can be exceeded in boars. The remainder of the conclusions, however, is identical to those for bovine animals. For the other matrices, no threshold levels can be set at the moment. Also, for the minor species, equine, cervine and caprine too few data are available to set specific threshold values. Currently, it must be concluded that levels of 17β-testosterone in all matrices and species are highly variable and setting thresholds for individual compounds is not possible.

17β-estradiol

The use of 17β-estradiol as a growth promoter is highly debated during the past decades. The hormone dispute for the World trade Organization (WTO) in fact, completely focussed on this compound and its possible harmful properties. Despite the many papers that have been published on 17β-estradiol, the amount of useful information on possible threshold levels is very limited. A selection of the most relevant papers is summarized below.

a. Studies to determine background levels

Within the framework of the WTO dispute, a large study was performed in which samples of meat obtained from the US domestic market were compared with samples from the US hormone-free programme. This study showed a small difference between the two populations, each consisting of approximately 100 samples. The median value shifted from 0.01 µg kg^{-1}in the " hormone free" population to 0.02 µg kg^{-1}in the "US domestic market" population. The first population included one sample with 0.10 µg kg^{-1}, whereas the latter showed a 99 percentile at 0.27 µg $kg^{-1}$and a single observation around 1 µg kg^{-1}.

A study to determine control values in untreated animals was performed by Arts *et al.* [28]. In total, 280 blood plasma samples obtained from female and male veal calves were analysed. Only one sample contained a level exceeding the LOD of the assay (0.01 µg L^{-1}) with a value of 0.016 µg L^{-1}. Values for urine, sum of free and conjugated 17β-estradiol ranged from < 0.01 µg L^{-1}– 0.1 µg L^{-1}at 15 weeks and from < 0.01 µg L^{-1}– 0.2 µg L^{-1}at 28 weeks. For female animals were similar: < 0.01 µg L^{-1}– 0.06 µg L^{-1}at 15 weeks and < 0.01 µg L^{-1}– 0.1 µg L^{-1}at 28 weeks. Values for the metabolite 17α-estradiol were much higher, up to 7.1 µg L^{-1}.

A paper of Blokland *et al*. provides a large set of data for steroids in urine part of the steroid synthesis pathway [14]. This paper focussed on methods to discriminate control populations from populations of treated animals. Individual values in urine for the aglycon (A), glucuronide (G) and sulphate (S) forms are provided Control animals analysed for 17β-estradiol showed that the major part is excreted as glucuronide conjugate, for female animals the range was form 0.16–0.43 µg L^{-1}, for male animals from 0.08–0.18 µg L^{-1}.

Schilt *et al.* reviewed published data and 17β-estradiol levels in the urine of male and female veal calves [29]. Based on the analyses of more than 10.000 individual samples obtained from presumed non-treated animals, he concluded that chances of total (free plus conjugated) 17β-estradiol exceeding 2.0 μg $L^{-1}$are extremely low. He suggested to analyse multiple animals form the same heard. In non-treated animals the chances that three animals exceed this threshold value are 1: $5*10^8$. However, maintaining this threshold will inevitably reduce the changes in detecting abuse after treatment. However, for female animals the "Whitten effect", where female animals synchronise oestrous and therefore maximum values coincide at the same time and therefore could cause false positives [30].

From the papers reviewed here it is concluded that the recommended threshold value of 0.04 μg L^{-1} in serum or plasma, for female animals < 6 months and male animals < 18 months, still, can be considered an acceptable upper limit for background levels. The value of 0.1 μg L^{-1}mentioned in the MMPR Guidance paper [6], which is based on the analytical possibilities for confirmatory analyses, must be considered relatively high.

b. Dosing studies

Fritsche *et al.* described a study in which beef steers were treated with a combination of progesterone (200 mg) and estradiol benzoate (20 mg) [31]. Steers were chosen for their low endogenous sex hormone levels (low ng kg^{-1}range). This study did not reveal any increase in levels of these analytes, nor their metabolites. Only a small shift in ratio between parent and metabolite was observed in samples of urine. A study by Scippo *et al.* in which animals were treated showed values of 17β-estradiol ranging from 6.6 to 17.6 μg L^{-1} [32]. Using an action limit of 1 μg L^{-1}resulted in a 95% chance of detecting truly positive samples.

Henricks *et al.* published a paper with serum values after the treatment of a heifer with 17β-estradiol [33]. The animal was treated with Revalor H, a preparation containing 14 mg 17β-estradiol and 140 mg trenbolone-acetate. Mass concentrations within serum samples remained < 0.01 μg L^{-1} in control animals, but reached a maximum value of 0.1 μg L^{-1}after 60 days.

Progesterone

The third steroid that should be considered in this framework is Progesterone. Though frequently replaced by synthetic analogues, inclusive the US registered feed additive melengestrol-acetate, it has been found in e.g. illegal cocktails. Endogenous levels of progesterone are known to be highly variable and very few systematic studies are available. One detailed study was performed in Northern Ireland. A reference population of 94 urine samples from steers, known not to be treated with any banned substance, was analysed (Kennedy, DG, personal communication). Progesterone was present in all samples at concentrations up to 3.5 μg L^{-1}, resulting in an Upper Limit of Normality of 4.2 with 99% certainty.

Summary

Annex 1 summarises the published information on 17β-estradiol, 17β-testosterone and progesterone.

2.4. Proposed strategy for monitoring

Effective implementation of a control programme to test meat producing animals for possible abuse of natural hormones will only be possible when validated confirmatory methods are available routinely and implemented. Currently, two such approaches are available, namely the direct detection of esters of steroids in application sites, hair, serum or plasma samples and the discrimination of exogenous and endogenous forms of the target molecules in urine samples based on $\delta^{13}C$ values. Both approaches show good potential for routine control and some member states have already implemented methods to detect steroid-esters in hair or blood. Only very few member states use IRMS for detecting synthetic natural hormones.

Steroid esters in application sites

This is technically feasible with current analytical methods. Applicability is limited to special situations.

Steroid esters in hair

Currently feasible and applied. Not all parameters, inclusive of the effectiveness of inclusion for all compounds, have been completely evaluated yet, but research is on-going.

Steroid esters in serum / plasma

The direct detection of steroid esters in serum is strong proof of abuse. An important drawback is the fact that for most Member States on farm sampling of blood will not be possible. However, before implementation can be achieved, further research will be necessary.

- Can the necessary sensitivity be obtained with current instruments in use for routine monitoring?
- How long after treatment can the steroid esters be detected, and what is the influence of the specific ester (low versus high molecular weight esters)?
- How can blood samples easily be obtained at the farms?

Isotope ratio MS

- An important advantage of GC-c-IRMS is the fact that urine can be used as matrix. Urine is easily sampled both on farm and at the slaughter house. Moreover, most of the potential screening methods are also based on urine, allowing confirmatory and screening analyses on the same sample.

Remaining research questions:

- Is the sensitivity of IRMS sufficient to test residues for estradiol, testosterone and progesterone?
- How many regional differences in ratio can be expected?
- What is the effect of different feeds on the endogenous delta values?
- How many different internal reference compounds need to be tested?
- Can IRMS also be used for other synthetic natural compounds

For now, the general conclusion from the on-going studies is that sensitivities in the 10 – 20 µg kg^{-1} range can be achieved with the current generation of instruments.

Research agenda

The implementation of LC-IRMS to widen the scope of IRMS for other synthetic natural hormones.

Enhance the selectivity of sample clean-up to improve the detection capabilities and sensitivity of IRMS.

Improve current methods to detect various steroid esters in hair and blood, including blood sampling and the use of new smart sampling techniques such as dried blood sampling and coated blade extraction.

EURL Recommendations

For each Member State individually

- Implement in national programmes the determination of steroid esters in samples of hair or serum.
- Implement one of the screening methods described and make provisions for confirmatory analyses by GC-c-IRMS.

General

- Continue research on screening methods like steroid profiling or threshold levels of particular marker metabolites
    - Collect data from national programmes
- Continue research on the applicability of testing for steroid esters (EURL/NRL/OL network) in hair and serum.
- Evaluate the screening methods in combination with the confirmatory methods.

3. Thiouracil

3.1. Introduction

Thyrostats are orally active drugs that may be used to increase the weight of cattle before slaughter. The weight gain is mainly due to increased water absorption and retention within the edible tissue and filling of the gastrointestinal tract due to inhibiting thyroid hormone production. The consequence of such abuse is the production of inferior meat quality and a potential risk to public health due to the drug residues. Thyrostats have been classified by the International Agency for Research on Cancer (IARC) as belonging to group 2B, which represent "possibly carcinogenic to humans" compounds. For this reason, thyrostats have been banned in Europe since 1981 (Regulation (EU) 2017/625)[5]. When animals are treated with these compounds, approximately 5 grams per day are administered to reach weight gain, and this results in concentrations in urine of over 100 µg L^{-1}.

Currently, 2-Thiouracil (TU) (Figure 3.1) belongs to group A2 compounds stipulated in CDR (EU) 2022/1644 [3], which means their use is forbidden in food producing animals. No Minimum Required Performance Level (MRPL), Maximum Residue Limit (MRL) or Reference Point for Action (RPA) has been established so far. In the Minimum Method Performance Characteristics (MMPR) Guidance paper of 2021 a recommended concentration of 10 µg L^{-1} or kg in urine and thyroid was proposed [6]. However, this included the comment that concentrations smaller than 10 µg L^{-1}or kg could be of natural origin due to a cruciferous diet (*Brassicaceae*) [34]. For several years now, it has been assumed that low levels of thiouracil can have a (semi)natural origin.

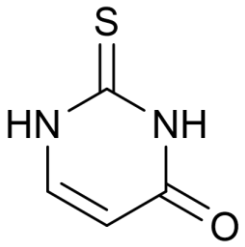


Figure 3.1. 2-Thiouracil chemical structure.
Molecular weight: 128.15 g mol^{-1}, Molecular formula: $C_4H_4N_2OS$

3.2. Analytical methods

Forty to fifty years ago, thyrostats screening was performed by weighing the thyroid gland, which increases in size and weight due to these hormones. Subsequently, newer and more sensitive methods were developed, like the HPTLC method developed by De Brabander et al. (1988). Later, Schilt *et al.* [35] and De Brabander *et al.* [36] presented a gas chromatography-mass spectrometry (GC-MS) method for thyrostat confirmation. From 2001 onwards, various liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were published [37-39], which included 3-iodobenzylbromide derivatization to the extraction procedure. Bussche *et al.* developed a method for analyzing feed and food [40]. This method uses enzymatic hydrolysis to convert glycosinolates using the plant-derived enzyme myrosinase [40]. Additionally, Bussche *et al.* presented a method for direct detection without derivatization of thyrostats in urine [40]. Afterward, other recent studies also developed LC-MS/MS methods for the detection of thyrostats in milk and cheese

[41,42], bovine urine [43,44], thyroid glands [43,45] and faeces [46] at low concentration levels achieving CCα-values ≤ 2 µg L^{-1}.

However, due to the instability of thyrostatic compounds in urine [40] the implementation of stability protocols is being proposed by the EURL. To increase the stability of the thyrostats in urine, in particular, thiouracil, decreasing its pH to 1 and adding an EDTA solution is needed to prevent its decrease. Also, the amount of freeze-thaw cycles, the exposure to room temperature, and the long-term storage in the freezers of the samples should be kept to a minimum for the same reason. Therefore, it may be clear that there is a need for stability protocols for these samples in laboratories.

Currently, reliable and sensitive methods are available for the control of residues using LC-MS/MS and are sensitive enough with regard to the recommended concentration (10 µg L^{-1}) set by the EURL in the MMPR Guidance paper of 2021 [6].

In the EURL working programme in 2020-2022 research was performed on the possibility of using LC-IRMS for establishing the origin of thiouracil.

To determine the carbon isotope ratio ($^{13}C/^{12}C$) of truly synthetic 2-thiouracil, analytical standards were purchased and analyzed using the developed LC-IRMS method. The average carbon isotope ratio (expressed as delta, in (%)) and the deviation (n=5) were determined in eight different analytical standards.

Deviation of carbon isotope ratios between suppliers was observed. This could indicate that manufacturers used different methods of synthesis or different starting products (different origins). Limited standard deviation (within a group) was observed, demonstrating a stable LC-IRMS method for 2-thiouracil standards. Based on the analytical standards, the carbon isotope ratio of synthetic 2-thiouracil ranges between -32.940 and -40.281%.

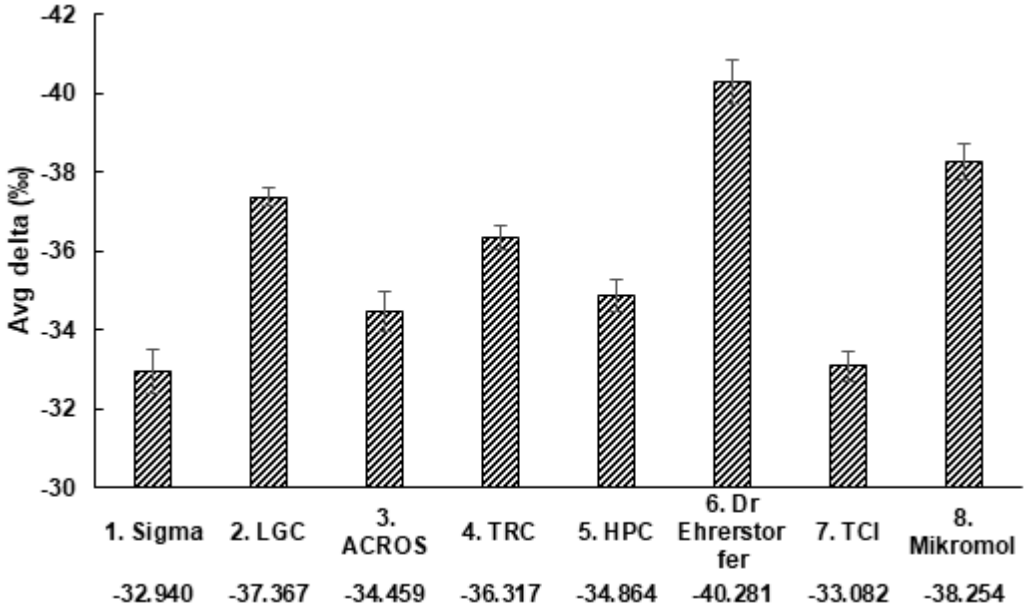


Figure 3.2 Carbon Isotope Ratio ($^{13}C/^{12}C$) of 2-thiouracil standards of 8 different suppliers. The lower row is the average delta value.

To determine the carbon isotope ratio of "natural" 2-thiouracil in urine, a specific sample clean-up to extract 2-thiouracil from bovine urine samples was developed and tested while minimizing other matrix artifacts (see figure 3.3). Unfortunately, the sample clean-up demonstrated poor recovery, and 2-thiouracil could not be detected in fortified water or urine samples.

Figure 3.3 Schematic overview of the developed clean-up for 2-thiouracil in bovine urine samples.

For this research state-of-the-art sample clean-up techniques were tested for 2-thiouracil extraction. As LC-IRMS is a highly specialized technique, it requires highly pure and clean extracts for analysis. With the current techniques and knowledge, no sample clean-up for 2-thiouracil for LC-IRMS could be developed to detect natural 2-thiouracil in bovine urine. Therefore it was decided to not put more effort into LC-IRMS for 2-thiouracil.

3.3. Research summary

Origin of Thiouracil

In 1945, Griesbach *et al.* found that cruciferous and brassicaceous vegetables contain substances called goitrogens [47], which impair iodine uptake by the thyroid and consequently inhibit the conversion of thyroxine (T4) to triiodothyronine (T3). In 2006, Pinel *et al.* showed in a single animal experiment that there was a link between the cruciferous diet and findings of thiouracil in urine [38]. Concentrations in urine did not exceed 10 µg L^{-1}. Due to the fact that more EU Member States introduced LC-MS/MS analysis in their laboratories and National Residue Control plans, more cases of thiouracil detection were reported. The recovered values sometimes even exceeded the 10 µg L^{-1} threshold (RC).

Bussche *et al.* showed that thiouracil was found in untreated animals in porcine, bovine, and ovine species but also in a dog and in human volunteers [48]. In the animal species, the concentrations did not exceed 10 µg L^{-1}. The authors also provided evidence that endogenous plant compounds can be converted by myrosinase hydrolysis into thiouracil, which can be the reason for the thiouracil findings in the investigated animal species.

The latest scientific knowledge by Kiebooms *et al.* presented *in vitro* model studies of the gastrointestinal tract (stomach/rumen, small intestine, colon) of bovines and porcines in respect to thiouracil formation upon Brassicaceae digestion [49]. This showed that traces of thiouracil were formed after adding various Brassicaceae (rapeseed, rapeseed meal, broccoli, cauliflower) to colonic fluids. Concentrations in the colonic suspensions upon rapeseed digestion ranged from 3.5-31 µg kg^{-1}in the bovine model and from 3.6-26 µg kg^{-1} thiouracil in the porcine model. Active bacterial involvement was shown to be the main reason for the formation.

Observed concentrations of Thiouracil

Le Bizec *et al.* analysed 1322 French urine samples collected from different species: bovine, ovine and porcine [50]. For ovine, only 26 samples were analysed, for porcine animals,

201 samples were analysed. Concentrations in ovine ranged from CCα – 14.3 μg L^{-1} Thiouracil. The mean for the ovine samples was 3.3 μg L^{-1} and the median was 2.2 μg L^{-1}. The authors did not observe differences between male and female animals. The 95th and 99th percentiles were, respectively 12.9 and 14.3 μg L^{-1}. All porcine samples showed concentrations of thiouracil below the recommended concentration of 10 μg L^{-1}. For bovine animals, a differentiation was made between the sex and the age (Table 3.1). Figure 3.4 shows the data distribution for calf (N=362, male and female) urine samples.

Table 3.1. Concentrations (μg L^{-1}) TU found in bovine samples [50].

	Male 6-24 months	Female 6-24 months	Female >24 months	Male calves <6 months	Female calves <6 months
N	279	213	196	195	144
Mean	1.4	0.8	0.8	2.1	2.4
Median	0.2	0.0	0.1	1.0	1.1
Min	CCα*	CCα	CCα	CCα	CCα
Max	11.9	20.8	15.7	22.5	18.7
95 perc.	5.7	3.1	3.9	6.7	9.0
99 perc.	9.1	8.1	8.8	21.4	14.1

*CCα= <0.7 μg L^{-1}

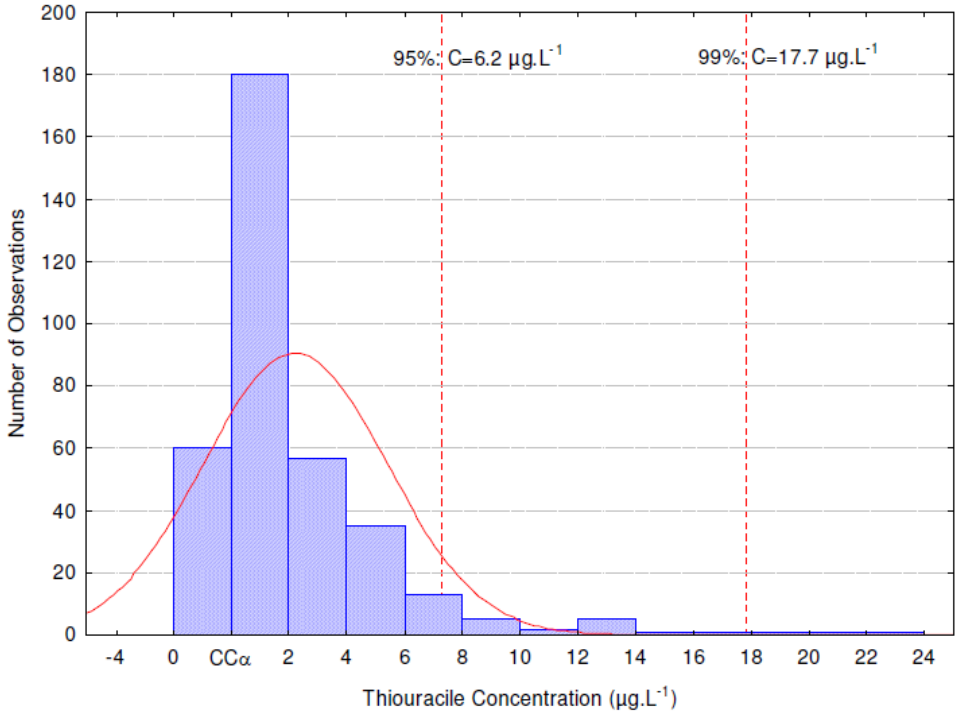


Figure 3.4. Frequency distribution of thiouracil in calf urine samples. Reproduced from [50].

In the Netherlands, a similar study with 294 samples was performed with young animals comprising calves younger than 30 weeks (Table 3.2). These samples were taken during a period of 4 weeks in May 2012 and therefore did not include seasonal effects, if present. Samples were collected from animals feeding only on milk or milk replacers (white meat production) and animals also consuming other feed (rosé meat production). The obtained results about the distribution of thiouracil concentration in urine of white and red meat and in white and red meat studies can be observed in Figure 3.5 and Table 3.3, respectively.

Table 3.2. Concentrations TU (µg L^{-1}) found in young bovine animals.

	Male (white meat) 5-20 weeks	Female (white meat) 5-20 weeks	Male (rosé meat) 15-30 weeks	Female (rosé meat) 15-30 weeks
N	109	41	144	-
Mean	4.5	3.9	9.1	
Median	3.6	2.5	8.2	
Min	0,1*	0.6	0.8	
Max	18.0	19.3	34.0	
95 perc.	11.3	10.8	18.8	
99 perc.	16.7	17.3	30.0	

*CCα= <0.2 µg L^{-1}

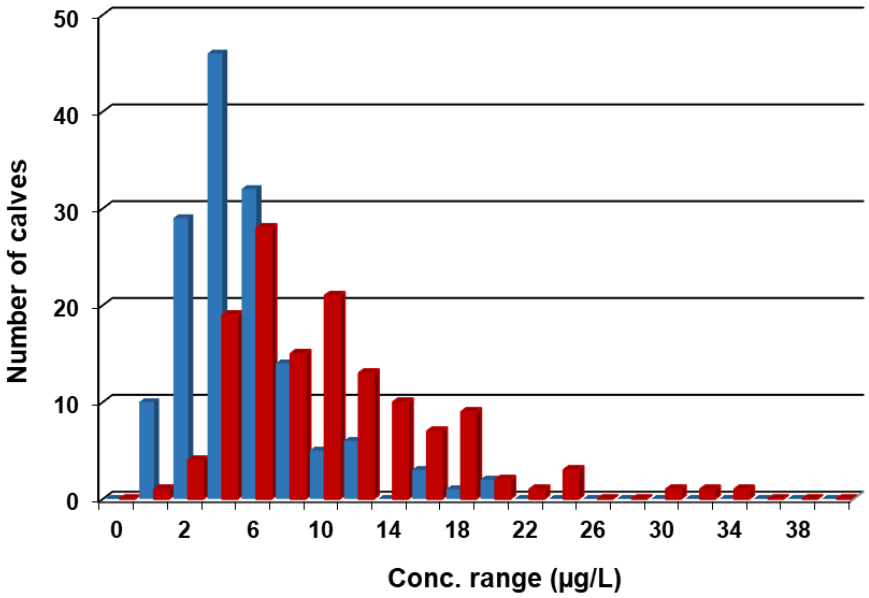


Figure 3.5. Distribution of TU concentration µg L^{-1}) in urine of white meat (blue bars) and rosé meat (red bars) in calves in the Netherlands, monitoring May 2012.

Table 3.3. Frequency distribution of TU in white meat and rose meat in the Netherlands.

CRL recommended conc. (µg L^{-1})	White (%)	Rosé (%)
≤ 10	91.9	64.7
> 10 to ≤ 20	8.1	30.1
> 20 to ≤ 30	0.0	3.7
> 30 to ≤ 40	0.0	1.5
> 40	0.0	0.0

Regarding these aforementioned studies, when comparing the French and the Dutch results for calves < 6 months, it is observed that for the animals feed only on milk, the 99th percentile values are very close to each other for male and female in the Netherlands (16.7

and 17.3 µg L^{-1}). In France, these values are 14.1 µg L^{-1} for females and 21.4 µg L^{-1} for males. For the older Dutch animals, feeding on solid feed, the 99 percentile is higher, namely 30 µg L^{-1}.

At a later stage, 28 samples were collected in the Netherlands of rosé calves older than 30 weeks (32-46 weeks old). The average TU concentration was 6.8 µg L^{-1}) and the 99th percentile was 22.3 (µg L^{-1}). The trend line in Figures 3.6a and 3.6b shows that there is no significant correlation between age and thiouracil concentration. The feed is most likely the major component that influences the height of the thiouracil concentration formed in the gastro-intestinal tract.

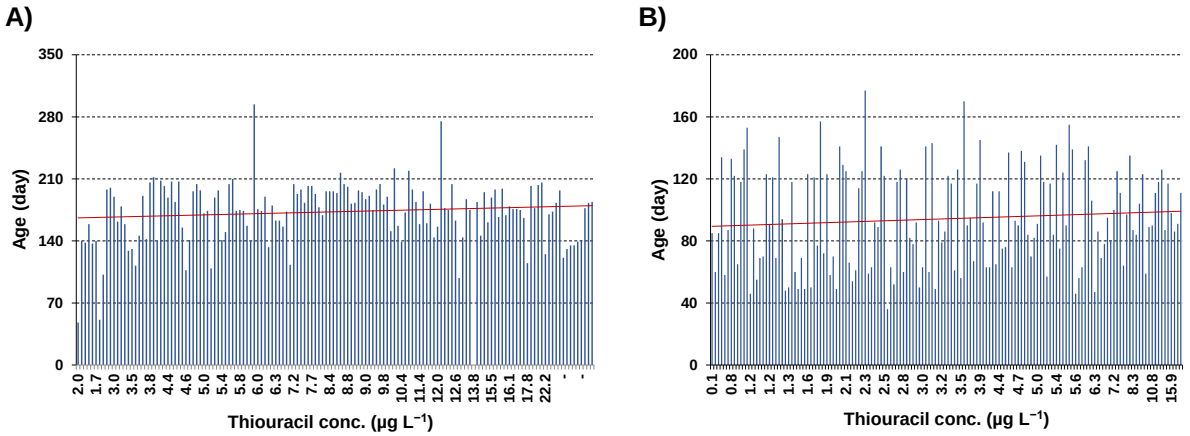


Figure 3.6. TU concentrations in (A) rosé meat animals and (B) white meat animals .

Wauters *et al.* performed a retrospective study to evaluate the applicability of the recommended concentration for monitoring thiouracil in urine samples of bovines, small livestock, and pigs, by statistically approaching sample outcomes gained in several European member states from January 2010 to December 2012 [51]. Considering the EURL reflection paper [52] and the current study results, a revision of the recommended concentration was advised. Moreover, it is important to consider influencing factors, such as the applied laboratory protocol (all species), gender (cattle), and age (cattle). Based on their study, they conclude that they agree with the proposed increase of the RC from 10 to 30 µg L^{-1}, according to the reflection paper of the EURL [52].

Thyrostats levels after treatment

There is not much information on the illegal use and the elimination kinetics of thyrostats. Heereman *et al.* describe the elimination kinetics of methylthiouracil (MTU) after single or multiple oral dosages of 5 g of MTU, showing the drug appeared rapidly in plasma, urine and milk, but was quickly eliminated after 1 day dropping levels below the limit of detection (LOD) of 1 mg L^{-1} [53].

In 2012 an animal experiment was performed in which a young heifer was treated with thiouracil. From t=0 (August 22, 2012) till t=14 (September 4, 2012) two oral capsules containing 2.5 g of thiouracil were administered daily. Urine was collected from 8 days before the experiment (t=-8 (August 14, 2012)) to 14 days after the start of the experiment (t=14). An excretion curve of TU is shown in Figure 3.7.

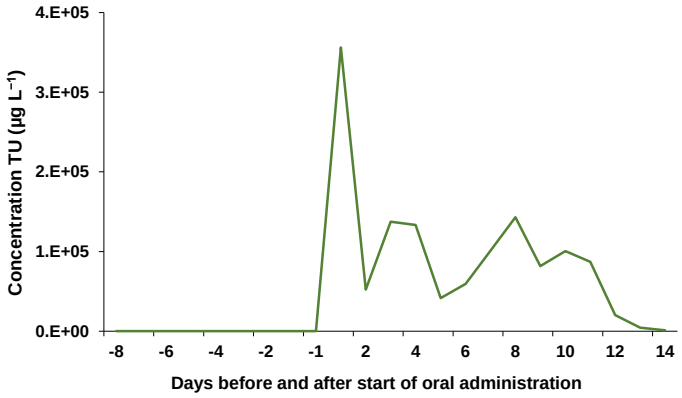


Figure 3.7. Excretion curve of Thiouracil.

The data shows that the TU levels after treatment are in the mg L^{-1} range instead of the µg L^{-1} range, which is by far exceeding the levels currently observed in presumed untreated animals.

In a later study, Wauters *et al.* [45] tried to differentiate between low dose thiouracil treated cows and calves and rapeseed enriched diet fed animals. This study clearly showed that an RC of 30 µg L^{-1}may result in the legalization of (single-dose) low-level abuse of TU. For cows and calves with urinary TU levels close to 30 µg L^{-1}, they proposed to do thyroid gland analysis for TU, a method which showed non-overlapping discriminating TU levels, with a suggested threshold of 10 µg kg^{-1} for both study groups of animals. From the same study urine samples (n=330) were assessed through metabolic fingerprinting, using liquid chromatography and Q-Exactive™ Orbitrap mass spectrometry [44]. The assessed urinary fingerprints consisted of up to 40,000 features, and with multivariate discriminant analysis significant metabolome differences between treatments ($Q^2(Y) \geq$ 0.873) were found. Twelve metabolites (including thiouracil) were assigned as marker potential, which could be combined into age-dependent biomarker panels.

Recently, Blokland *et al.* [54] carried out a study where bovines and pigs were fed with rapeseed, rapeseed with thiouracil, or regular feed with thiouracil at both low and high concentrations in order to determine possible biomarkers as a discriminant of the endogenous/exogenous presence of thiouracil. Authors observed that administration of thiouracil leads to concentrations of thiouracil and its biomarkers higher than the current threshold of 10 µg L^{-1}. During the first week after treatment, animals feed with rapeseed showed higher thiouracil concentrations than those with regular feed, usually higher than 10 µg L^{-1} and in some cases in treated pigs, higher than 30 µg L^{-1}. Under this scenario, a positive finding of thiouracil in urine (> 10 µg L^{-1}) after slaughter in cattle could come from animals feed rapeseed and/or animals in the first week after treatment with bovine thiouracil. Thereby, the authors suggested remaining 10 µg L^{-1} in bovine urine as a good indication for abuse, whereas they remark that the threshold of thiouracil should be raised to 30 µg L^{-1} in pig urine. Moreover, the authors also identified and confirmed with reference standards several biomarkers for thiouracil treatment. During treatment with 2-thiouracil, the concentration of 4-thiouracil did not show any change however low concentrations of 6-methyl-thiouracil were found, while 6-methyl-thiouracil was not found in urine samples from the Dutch routine control programs containing (endogenous) 2-thiouracil above the threshold value. In this way, 6-methyl-thiouracil was identified as indicator of thiouracil abuse, whereas 4-thiouracil was indicative for endogenous formation.

Mercaptobenzimidazol

Among the thioureylene compound family, 2-Mercaptobenzimidazole (MBI) (Fig. 3.8) is well known to be used as a corrosion inhibitor, copper-plating brightener, and rubber accelerator. However, MBI is a toxic and poorly biodegradable pollutant and therefore it can also enter the food chain via feed, water, or soil. Additionally, samples in the laboratory can be contaminated with MBI via materials used during sample clean-up.

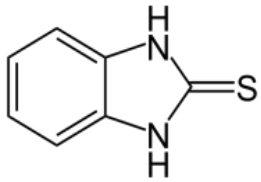


Figure 3.8. Molecular structure of 2-Mercaptobenzimidazol.

Until now few animal studies have been carried out on MBI. In rats, the inhalation toxicity of MBI revealed that its exposure resulted in increased thyroid weight, thyroid follicular cell hyperplasia, reduced triiodothyronine and thyroxine levels [55]. Additionally, major fetal malformation was observed by Yamano *et al*. (1995) when they studied the adverse effects of MBI on pregnant rats and their fetuses [56]. In cattle, in order to study the toxicology of MBI and its effect on the function protein during blood transportation process, Teng *et al*. the effect of MBI on the conformation of bovine serum albumin has been studied [57]. For this purpose, authors used spectroscopic and molecular docking methods under physiological conditions. Since the positively charged MBI can spontaneously bind with the negatively charged BSA through electrostatic forces with one binding site, the site marker and the molecular docking study revealed that MBI bound into site II (subdomain IIIA) of BSA, which further led to some secondary structure and microenvironmental changes of BSA.

Summary

Annex 1 summarises the published information on thiouracil and other hormones.

3.4. Proposed strategy for monitoring

There is no doubt that the low levels of thiouracil that are occasionally detected in samples of urine can be the result of thiouracil precursors being present in animal feed. Several reports indicate that these levels can exceed the current Recommended Concentration for Control of 10 µg L^{-1} and therefore, the EURL proposes to increase the threshold level for follow-up of Thiouracil findings to 30 µg L^{-1} in urine samples from pigs.

The diagram describing how to assess the origin of thiouracil in both pig and bovine animals is shown in Figure 3.9. As can be observed, when the concentration of 2-thiouracil is up and above 10 µg L^{-1} or 30 µg L^{-1} in bovine or pig urine samples, respectively, it is advised to determine the concentration of 4-Thiouracil and 6-methyl-thiouracil. These compounds are used as markers to indicate the exogenous or endogenous origin of 2-thiouracil in analyzed urine samples.

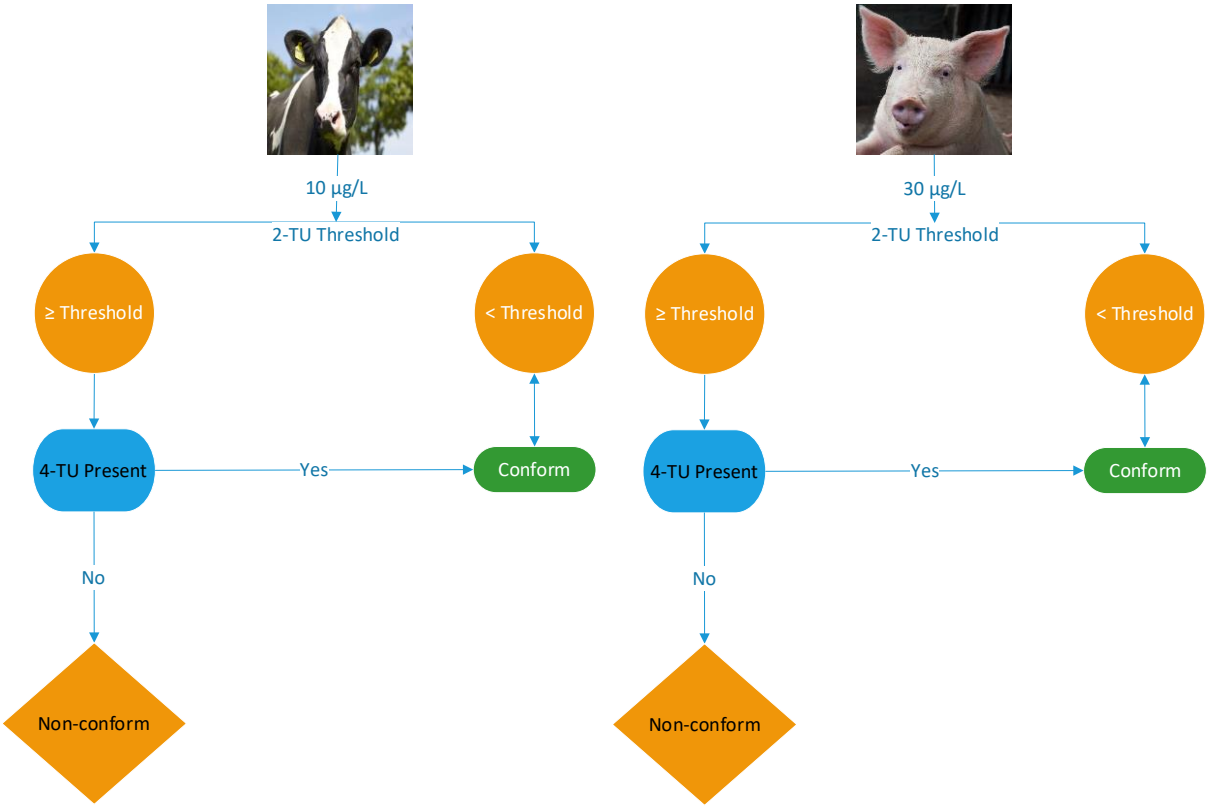


Figure 3.9. Flowchart Decision on Thiouracil finding in porcine and bovine animals.

EURL Recommendations

For the European Commission

- Change the Recommended Concentration for thiouracil control from 10 µg L^{-1} to 30 µg L^{-1} in pigs.

For the individual Member States

- Stabilize samples of urine during storage and avoid freeze-thaw cycles.
- If the threshold concentration in urine is exceeded, determine the concentration of the 4-thiouracil biomarker.
- If 2-thiouracil is found and 4-thiouracil is detected, then the origin of 2-thiouracil is most likely from natural contamination/production.
- If 6-methylthiouracil is found in a sample containing 2-thiouracil, than the sample is non-compliant.
- When mercaptobenzimidazol is found, check the used sample clean-up carefully, do a follow-up visit to the farm, and take samples from drinking water, soil and feed to exclude environmental contamination.
-

General

- Continue research on innovative broad effect-based methods and the discussions on their implementation in residue control.

- In theory, LC−IRMS could discriminate between naturally formed and synthetic thiouracil. However, due to difficulties isolating thiouracil, this topic is paused for the moment and will be researched again when there are new technical solutions
- Collect more data on the concentrations of 2-thiouracil and 4-thiouracil.

4. Nortestosterone

4.1. Introduction Nortestosterone

Among the compounds used for growth promotion, 17β-19-nortestosterone (17β-NT), also named nandrolone, which belongs to the group of androgenic anabolic steroids, has traditionally taken a prominent place. As is the case for many other anabolic steroids, it is usually administered in esterified form (Figure 4.1), e.g. via an ear implant or an intramuscular injection. Nortestosterone (NT) is not registered as a veterinary drug for food-producing animals and belongs to the A1c group of CDR (EU) 2022/1644. No Maximum Residue Limit (MRL) or Reference Point for Action (RPA) is established. However, in the MMPR Guidance paper of 2021 [6] a Recommended Concentration of 0.5 µg L^{-1} in urine, 1 µg kg^{-1} in muscle and of 2 µg kg^{-1} in liver for control was proposed.

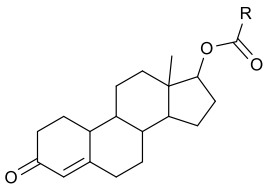


Figure 4.1. Structures of NT esters. NT-acetate: R=CH_3, NT-benzoate: R=phenyl, NT-cypionate: C_2H_4-cyclopentyl, NT-hemisuccinate: CH_2-succinyl, NT-decanoate: R=C_9H_{19}, NT-undecanoate: R=$C_{10}H_{21}$, NT-laureaet: $C_{11}H_{23}$, NT-phenylpropionate: R=C_2H_4-phenyl, NT-propionate: R=C_2H_5.

The most commonly known esters are nandrolone decanoate and nandrolone phenylpropionate. The injected nandrolone esters are quickly converted to "free" 17β-nortestosterone but how the free 17β-NT is further metabolized depends on the animal species. For instance, cattle metabolize the free 17β-NT to 17α-NT, which is detected in the highest concentration in urine. However, it is known that 17β-NT can be endogenously present in stallions and male pigs, and the main metabolite in cattle 17α-NT may also occur naturally in pregnant cows and neonatal calves. Reports on the natural occurrence in biological samples originate from the 1980's. Since then, many studies have been published on the natural occurrence of nandrolone and its metabolites in various species.

4.2. Analytical methods

Several methods are available for urine analyses of 17β-NT and its metabolites, based on GC-MS(MS) or LC-MS(MS). Most methods are multi-analyte methods that include a high number of steroids. Among them, research works have been focused on the analysis of urine [58-62], hair [63], liver [64], accessory glands [65], plasma [66,67], serum [15,68] and teeth [43]. In milk, a multi-analyte method for steroids is also published [69]. Besides, various LC-MS/MS methods are also published for the analysis of intact phase II metabolites in urine [70]. For screening of meat samples, methods with low detection limits are available using ELISA and LC−MS/MS [71].

In the human sports doping field, some methods have been published using isotope ratio mass spectrometry (IRMS) to distinguish endogenous nortestosterone and metabolites from synthetic nortestosterone [72]. In residue analysis, this method is not (yet) routinely used.

4.3. Research summary

17β-NT is known to occur naturally in the urine of boars and stallions [73-75], its main bovine metabolite, α-nortestosterone (17α-NT), occurs naturally in pregnant cows and neonatal calves [76]. Furthermore, 17β-NT and metabolites, including 17α-NT have also been detected in animal matrices from the ovine [77], caprine [78] and cervine [79] of untreated animals.
In bovine animals 17α-NT is the major metabolite. 17α-NT was also found in bile in an experiment in which Nandrosol was administered to calves, with 17α-NT levels in bile being higher than in urine. Only the intact esters were present in bovine hair after administration [80].

To date, it is only possible to determine with a chemical analysis technique in urine whether it is natural or administered nortestosterone. In certain animal species it is possible to look at certain metabolites which are only formed when nandrolone is administered. Different research groups performed studies to find new marker metabolites. For calves (both male and female) it was shown that after intramuscular administration of 17β-NT-laureate, profiles of 17β-NT metabolites in urine, the estranediol isomers 5α-estrane-3β,17β-diol (ABB), 5β-estrane-3α,17β-diol (BAB), 5α-estrane-3β,17α-diol (ABA), 5α-estrane-3α,17β-diol (AAB) and 5β-estrane-3α,17α-diol (BAA) (see Figure 4.2), are deviating from the normal urine metabolite profile in the bovine population for nortestosterone. Therefore, after intramuscular administration, ABA was determined as the main metabolite, while in non-treated cows 17α-NT was the major metabolite [34]. Thus, finding a higher concentration of ABA compared to 17α-NT in the urine of cattle may indicate a prohibited treatment. However, there is still insufficient data to link a limit value to the concentration of ABA, whereby it can be assumed with certainty that the animal has been treated with nandrolone.

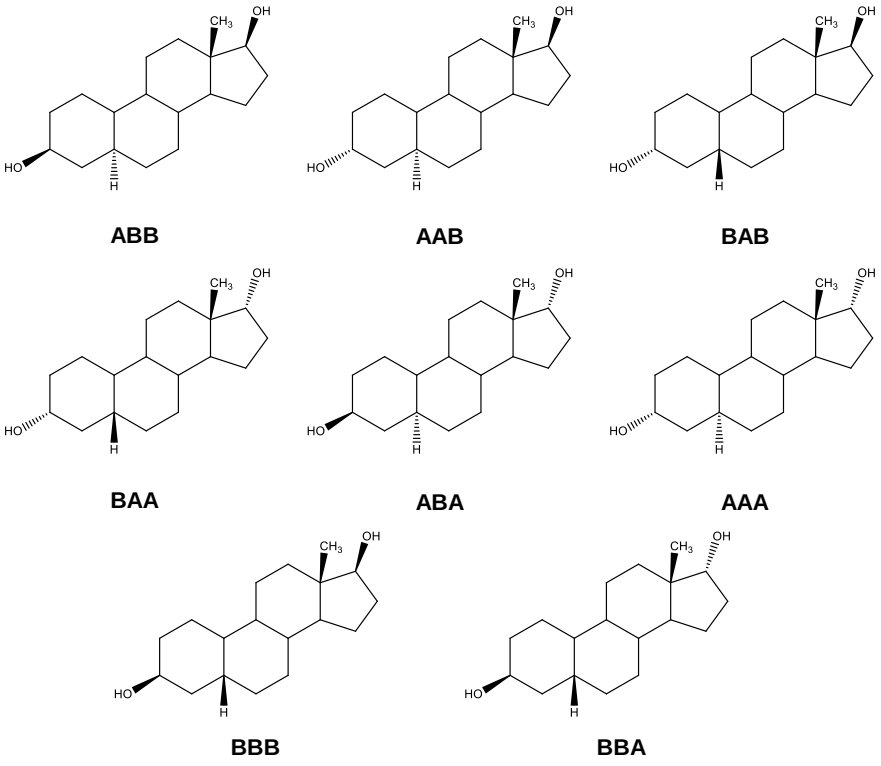


Figure 4.2. Chemical structures of the NT metabolites: estranediol isomers.

The relation between the natural presence of 17α-NT and 17β-NT and acute injury was established in slaughtered male cattle (bulls and steers;[81]). There was no evidence of abuse at any of the farms involved and the phenomenon occurred in four different regions of the EU. The relation with the release of dehydroepiandrosterone (DHEA) in response to the stress of the injury was tested by intravenous administration of DHEA to two normal steers, and 17β-NT was confirmed in the urine of one steer.

Ventura *et al.* showed in an animal experiment that after intra-molecular administration of 17β-nortestosterone-laurate to male pigs (barrows) the main metabolites were 17β-nortestosterone-sulfate, free noretiocholanolone, nor-epi-androsterone, 5β-estrane-3α,17β-diol and 5α-estrane-3β,17β-diol [82]. In untreated barrows, only 17β-norandrosterone and estrone were found Scarth *et al*. determined the biomarker noretiocholanolone in the free fraction of urine form boars and gilts [83]. A threshold value of >7.5 μg L^{-1} for boars and 19.2 μg $L^{-1}$for gilts was determined.

In pigs, high levels are observed in boars but only low levels in barrows and gilts. In practice, however, high levels of 17β-NT are occasionally found in urine from barrows. This can be due to cryptorchid animals, i.e. those having one testicle in the abdomen that produces hormones. In a small percentage of female pigs (sows) also, a low level of 17β-NT can occur [75]. An animal experiment with nandrosol has shown that administration of nandrosol indeed causes a growth-promoting effect in boars [80]. It was also shown that nandrosol was present in hair of boars treated with nandrosol. Other nortestosterone esters that might be illegally used can also be observed in the hair of the treated animals. Ventura *et al.* showed in an animal experiment that after intra-molecular administration of 17 β - nortestosterone-laurate to male pigs (barrows) the main metabolites were 17 β - nortestosterone-sulfate, free noretiocholanolone, nor-epi-androsterone, 5β-estrane-3α,17 β-diol and 5α-estrane-3β,17β-diol [82]. In untreated barrows only 17 β-norandrosterone and estrone were found. Scarth *et al.* validated the biomarker Noretiocholanolone in the free fraction of urine from boars and gilts [83]. A threshold value of > 0.008 μg $L^{-1}$for boars and 0.022 μg L^{-1} for gilts was determined. An overview of the natural occurrence of 17β-NT in urine obtained from pigs and the levels in treated pigs based on literature data is shown in Table 4.1.

Van Hende *et al.* analysed the urine of four ewes at different stages of pregnancy and the amniotic fluid of one ewe for the presence of 17α-NT; the urine of four pregnant animals was found to contain concentrations ranging from the limit of detection to above 2 ng mL^{-1} [79]. Clouet *et al.* also analysed urine samples of pregnant sheep for 17α-NT. Detection was realized at different stages of pregnancy [77]. Small concentrations (< 0.5 μg mL^{-1}) were found during the first 4 months of pregnancy and increased until parturition, particularly during the last month of pregnancy.

In a study of Sterk *et al.* urine samples of goats, mares and ewes, taken at different stages of their pregnancy, were investigated for the presence of 17α-NT and 17β-NT [78]. In 18 out of 60 urine samples of 4 pregnant mares, 17α-NT was detected ranging from 1–26 ng mL^{-1}. In goats only in a single sample 17α-NT was found exceeding the limit of detection (1 ng mL^{-1}). The urine samples from 5 pregnant ewes did not contain 17α-NT concentrations exceeding 0.5 ng mL^{-1}. 17α-NT was also detected in urine of a pregnant red deer [79] and in one out of 35 cervine urines from an Australian National Monitoring Programme [26].

Table 4.1. Literature overview showing normal (endogenous) values and values after administration of 17ß-NT(-esters) in porcine urine.

Type animal	Description	Values of 17β-NT in urine (μg/l)	17β-NT in urine after administration (μg L^{-1})	17β-NT ester in hair after administration (μg L^{-1})
Barrows	Castrated fattening pig	0.5-16.3 [75] n.d. [76] 1-35 [80]		13-600 [80]
Boars	Male fattening pig	51-344 [75] Up to 300 [76] 2-400 [80]	0.6-234 [82]	
Cryptorchids	Male fattening pig with 1 or 2 inner testicles	8.6-343 [75] Up to 110 [76]		
Gilts	Female pig that has not yet given birth	1.3-2.8 [75] < 2 [76]		
Fattening sows	Female fattening pig that has already given birth	1.3-1.9 [75] < 2 [76]	[73]	[73]
Breeding sows	Female breeding pig that has already given birth	Sometimes high (data Dutch national control plan and Dutch food safety authority)		

Urinary metabolites of 17β-NT in the horse were investigated in detail by Teale and Houghton [84], who reported that 17β-NT was mainly excreted as 5α-estran-3β,17α-diol sulphate and glucuronide in horse urine. Therefore, 5α-estran-3β,17α-diol was selected as the target metabolite to determine 17β-NT abuse. Detecting application forms of NT, such as esters of NT in hair can also be used to prove exogenous administration. Steroid profiling of urine and serum samples proved to be a good tool for predicting nandrolone abuse in race horses [15]. Statistical processing of the collected data permitted to establish statistical models capable of discriminating control samples from those collected during several months following administration.

In a study in The Netherlands, over 100 samples of urine from sows were analysed for Nortestosterone and its related metabolites in order to build a reference population. Simultaneously, samples of hair were analysed for NT-esters. None of the hair samples showed the presence of any steroid ester, whereas several samples contained 17β-NT at levels up to 2.9 μg L−1). The fact that these concentrations could be of cryptorchide animals could not be excluded. The low levels of 17β-NT are also seen [75] [Poelmans,2005]. This fact demonstrates that also in female pigs 17β-NT can be endogenous. Besides, in an animal study treating sows with 17β-NT phenylpropionate, urines samples were collected and analysed for a large number of NT-metabolites. Based on these results, a profiling model was developed in which treated sows can be separated from untreated sows (Fig. 4.3). This is done by calculating the ratio of the concentrations of norepiandrosterone and norandrostenedione (green line). If the ratio falls above the 95% confidence interval of the model (red line), the sample can be considered suspect. In the figure, the red dots are the animal test samples. All others are untreated male and female pigs. With this model, it is easy to determine from laboratory analysis whether the origin of nortestosterone is natural or by the administration of NT. This model was developed for pigs; it has not tested whether it applies to other animal species.

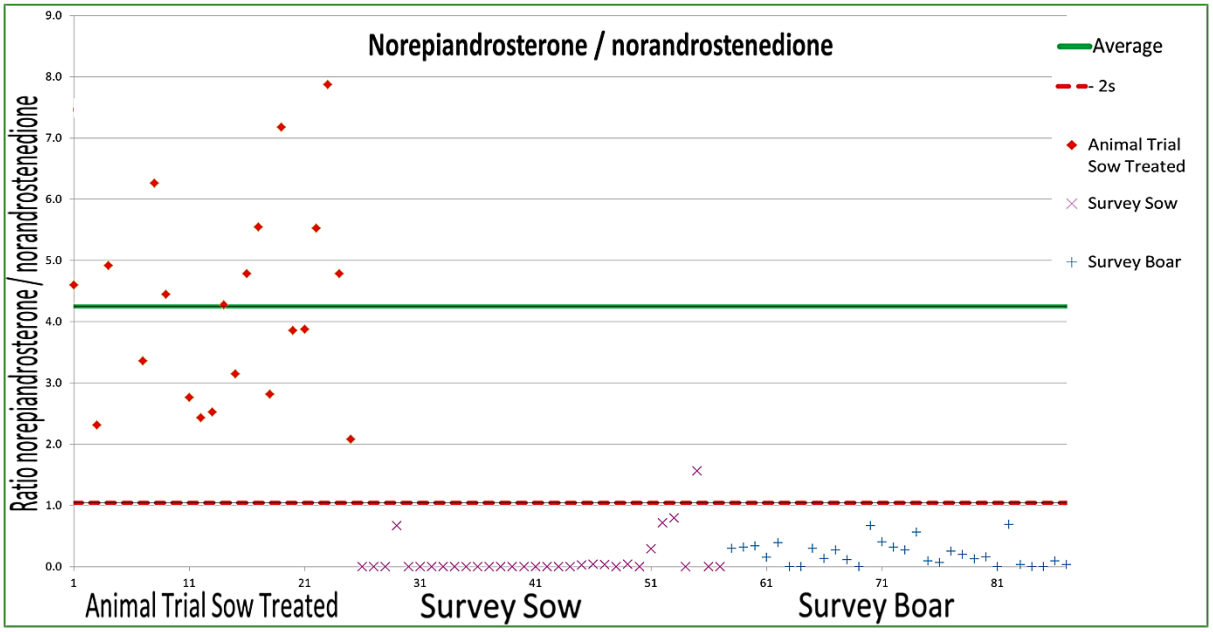


Figure 4.3. Profiling model to determine if 17β-NT is endogenous or exogenous based on the ratio of norepiandrosterone and norandrostenedione

Additionally, the steroid profile of pig urines could indicate whether the overall profile is considered "normal" or with adverse effects (exogenous/administration). For this reason, in a study carried out in The Netherlands, a large number of free steroids and their phase II metabolite were measured in pig urine. By means of statistical models, it can then be determined to which group the animal from which the urine originates belongs. In this study, a model is built with urines whose origin is known. As can be seen in Fig. 4.4A, in the OPLS-DA plot three groups of pigs are shown: males (yellow), females (blue) and steroid-treated pigs (purple). Afterward, this model was applied to unknown pig urines with high nortestosterone (Fig. 4.4B). As can be seen, based on this projection, it can be determined to which group they belong. Hence, it could be concluded that the unknown pigs belong to the male group since, based on their steroid profile, they are located in the plot at the same place as the blank male samples (yellow data in Fig. 4.4A). Therefore, the presence of nortestosterone in the urine is normal for male pigs. In fact, the concentration of nortestosterone can vary in height without this having to be abnormal. Therefore, in this case, it could be concluded that the profiles of the urines examined are within the normal values.

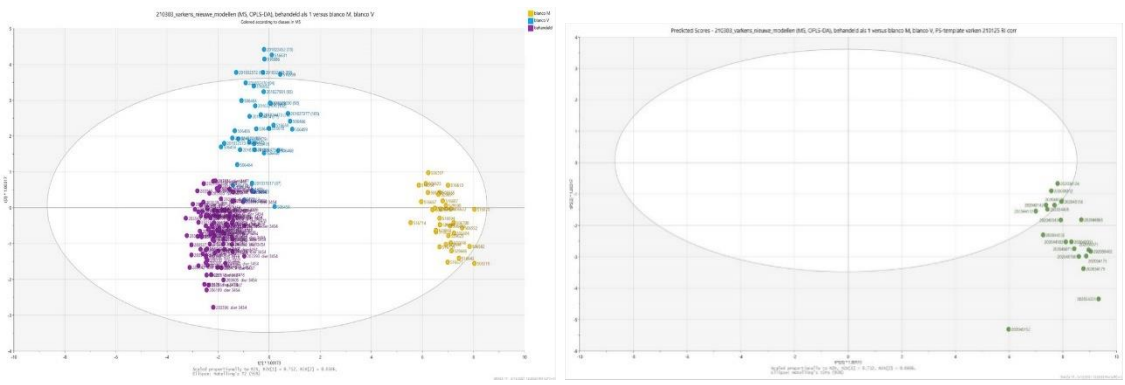


Figure 4.4. OPLS-DA plot left: of three groups of pigs: males (yellow), females (blue) and steroid-treated pigs (purple). OPLS-DA plot right, projection of unknown porcine samples in left OPLS-DA model

Besides, to date, only GC-combustion Isotope Ratio Mass Spectrometry (GC-c-IRMS) is able to detect a distinction between endogenous and administration within cattle. GC-c-IRMS technique uses the carbon isotope ratio, and since synthetic steroids are often formed from plant hormones (phytohormones), the carbon isotope ratio often deviates. This phenomena is owing to the process named isotope fractionation and as plants can be roughly divided into 2 categories, C3 and C4, there is a varying degree of isotope fractionation in these plants. Because after administration of nandrolone, the body starts to metabolize this form, the carbon isotope ratio of the detected NT in the urine changes. When the carbon isotope ratio is compared with an endogenous marker substance, it can be determined whether it deviates from the expected natural carbon isotope ratio. If this is the case, it has been confirmed that it is an administration of nandrolone. However, the distinctiveness decreases over time because the mixing of both the synthetically administered form and the naturally formed form takes place in the body. The above confirmatory analysis technique has been validated according to EU guidelines for bovine urine. Validated IRMS methods are not yet available for the other farm animal species.

Annex 1 summarises the published information on 17α-NT and 17β-NT.

4.4. Proposed Strategy for monitoring

Several options for confirming the abuse of nortestosterone have been suggested in the literature.

- Detection of intact esters on 17β-NT in samples of tissue (application sites), samples of hair or serum [15] currently are the most reliable approach for proving abuse of 17β-NT.
- When for a specific species, inclusive gender, no prior information has been published indicating the 17β-nortestosterone or its marker metabolite occurring endogenously, the confirmed presence can be considered proof for illegal use. The EURL has an overview in which the species and sex of the animals are described (Annex to part 1 of the Reflection paper 2.0).
- Threshold concentrations for specific marker metabolites could be set to discriminate exogenous from endogenous origin. Without confirmation, exceeding threshold values must be considered a suspicious screening result.
- Profiling analysis for a range of metabolites and precursors is an option that has shown its relevance in horses [15] and calves [34] and the models presented by the EURL in this chapter. However, all models need further evaluation by adding data from different member states.

The reliability of threshold concentrations or profiling analysis can only be evaluated in terms of false compliant or false non-compliant result rates. As discussed previously, such evaluation in the classical sense can only be made when a confirmatory technique, or at least a technique with a defined low error probability, becomes available. Alternatively, validation could be performed on the basis of an extensive evaluation of reference samples obtained from animal experiments.

The use of Isotope ratio MS currently is considered a potentially useful approach. However, currently, detection limits hamper the use when low concentrations are found.

The diagram below (Fig. 4.5) shows schematically the approach that can be followed after finding nortestosterone in the urine of cattle and pigs. For sheep, goats, and horses, there is no interpretation of the results possibly different or not occurring naturally. If this deviates, a follow-up will have to take place. When a nortestosterone ester is found, this is always evidence of abuse.

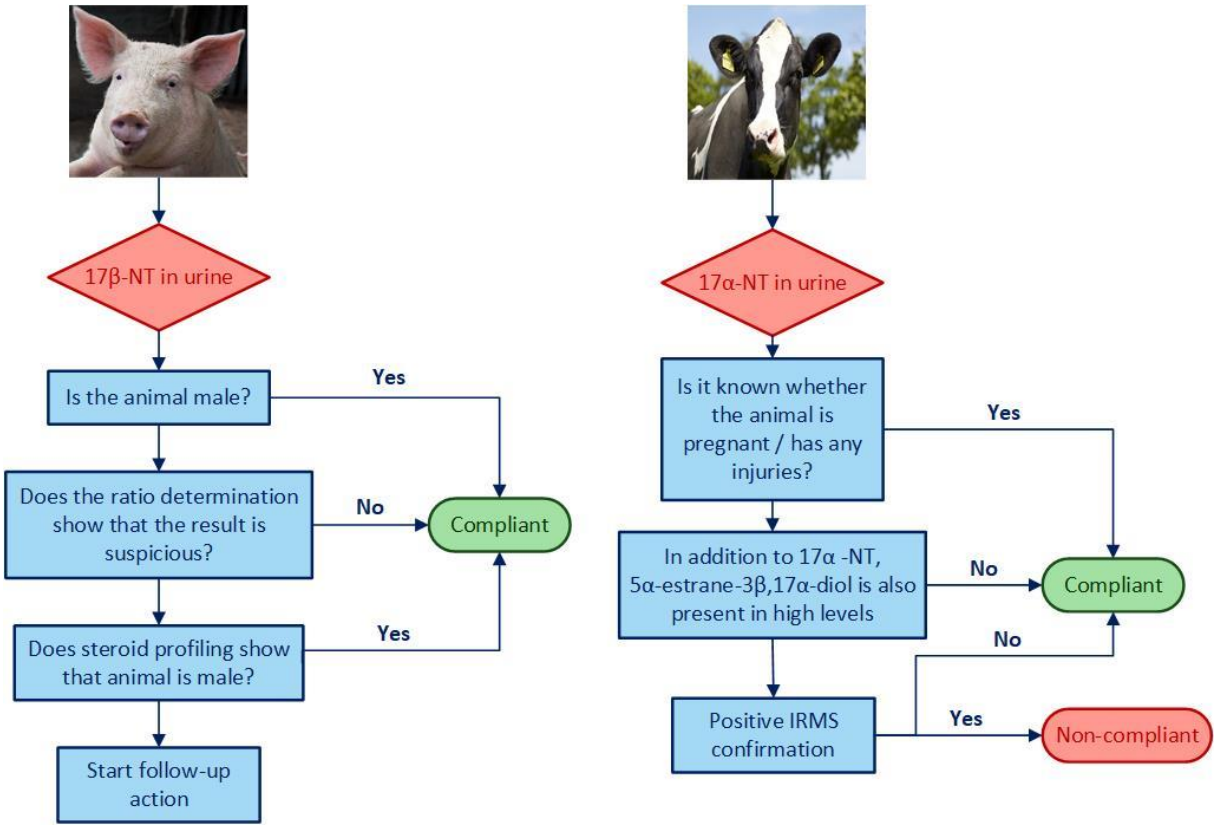


Figure 4.5. Flowchart Decision on nortestosterone finding in porcine and bovine animals.

Research questions for full implementation

To implement a strategy when finding nortestosterone residues and discriminating illegally administered from endogenously produced, research is needed in the following subjects:

Analytical methods

1. Validation of GC-c-IR-MS methods for 17β- and 17α-nortestosterone in urine.
2. Research into the analysis of steroid esters in hair and /or serum

EURL Recommendations

- Continue research on threshold values for specific biomarkers (species and sex dependant).
    - Further data collection from national programmes
- Continue the use of profiling techniques and models for data evaluation.
- Continue research on the applicability of analysing for steroid esters (EURL/NRL/OL network), e.g. in hair.

- Validate the use of GC-c-IRMS (EURL priority, in cooperation with specific project group).
- Continue research on the use of steroid esters in hair and serum.

Evaluation of screening methods effectively is only possible in combination with the corresponding confirmatory method.

5. Boldenone

5.1. Introduction

17β-Boldenone (17β-Bol), also denoted as 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 (Figure 5.1). Important steroids closely related to 17β-Bol and 17β-T are the 17β-boldenone epimer, i.e. 17α-boldenone, androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED)(Figure 5.1). These two di-keto substances, ADD and AED, are precursors of 17β-Bol and 17β-T, respectively, in humans and different animal species. 17β-Bol, esters of 17β-Bol (e.g. undecylenate ester) and ADD are for sale as anabolic preparations. 17β-Bol improves cattle growth and feed conversion and, therefore, might be abused to achieve more efficient meat production. Boldione (ADD) is sold on the Internet for use by bodybuilders as a product with an even greater anabolic potency than 17β-Bol itself.

Around 2002, EU Member states started reporting non-compliant results for boldenone. During that period, an expert group was formed, and they investigated the possible origin of these boldenone findings. For this reason, De Brabander *et al.* published a review paper on the possible origins of boldenone [85].

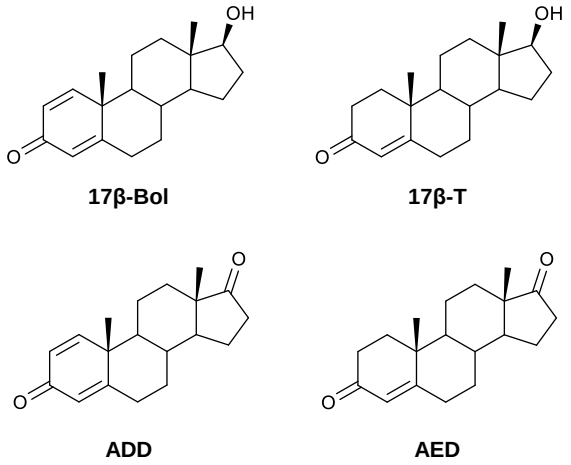


Figure 5.1. Structures of 17β boldenone (17β-Bol) and 17β-testosterone (17β-T) and of androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED).

Boldenone is not registered as a veterinary drug for food-producing animals. Boldenone belongs to the A1c group of CDR (EU) 2022/1644. No Maximum Residue Limit or Reference Point for Action is established. In the MMPR Guidance paper of 2021 a recommended concentration of 1 µg L^{-1}or kg in urine and muscle and 2 µg kg^{-1}in liver was proposed. With the remark that this is for the marker metabolite 17β-boldenone-glucuronide in bovines. In 2003 the expert working group wrote a boldenone review [85].

The outcome given by the authors is "The Commission presented the outcome of the meeting of 30 September 2003 on the control of boldenone in calves. The group of experts concluded that only 17β-boldenone conjugates in the urine of young calves could be used as proof of illegal treatment, provided that some specific sampling procedures were applied to avoid faecal contamination. The Committee endorsed these conclusions to be used as guidance for control of boldenone by the MS". For 17β-boldenone-glucuronide a recommended concentration for control in urine of 1 µg $L^{-1}$was set. Findings of 17α-boldenone higher than 2 µg L^{-1} have to be investigated further.

5.2. Analytical methods

For the analysis of boldenone and its metabolites animal biological matrices, several methods based on LC−MS/MS are available in which most methods also include a number of steroids such as nortestosterone, and testosterone. Most of these methods focus on the determination of 17-boldenone and its conjugates in urine [86-88,61] although some studies are also focused on meat [89], bile [90,91], liver [92], serum [93] and hair [94-96] matrices. In LC-MS analyses there are also methods published on analysis of intact phase II metabolites [97,98]. In the human sports doping field a number of published methods use IRMS to distinguish endogenous boldenone and metabolites from synthetic boldenone [99].

5.3. Research Summary

Boldenone is metabolised into a number of metabolites depending on the animal species. Le Bizec *et al.* and Destrez *et al.* found nine metabolites in urine after administration through several routes of boldenone, boldenone-esters and boldione with 17-α-boldenone being the metabolite marker in bovine urine [100,97]. Unfortunately, these metabolites were also found in animals that were known not to have been treated. Looking at phase II metabolites, Le Bizec showed that 17β-boldenone was the only metabolite present as a sulphate conjugate in urine of treated animals. In 2009, Le Bizec published a paper on direct measurement of this sulphate conjugate. Blokland *et al.* proposed the 6-hydroxy-boldenone-sulphate as marker metabolite [101].

In castrated male horses, the formation of metabolites was investigated by excretion of radioactive metabolites in urine [102]. Studies on human volunteers showed that after the administration of 17β-Bolenone, it was excreted as a conjugate in urine [103]. Galletti *et al.* and Cartoni *et al.* described the in-vivo formation and excretion of metabolites of 17β-Bol and 17β-Bol itself in urine after an oral administration of 17β-Bol to human volunteers [104].

In vitro and *in vivo* metabolism of 17β-Bol were investigated by Van Puymbroeck *et al.* [105]. The main metabolite produced by microsomes was ADD, while in the isolated hepatocytes, 6-OH-17β-Bol and 6-OH-ADD were identified. This research group also examined the excretion of 17β-Bol in a calf and a cow. The main metabolite found in urine was 17α-Bol. Several reduced, oxidized (such as ADD) and hydroxylated metabolites were also found. Faeces samples were investigated for the presence of 17α-Bol and 5β-AED, which do not naturally occur in bovines,and other reduced metabolites. No 17β-Bol, was detected in bovine faeces. Faeces samples showed a different metabolite profile in comparison with urine samples. No hydroxylated or oxidized products were found [106]. In the urine of a male calf treated intramuscularly with 200 mg 17β-Bol-undecylenate, 17α- and 17β-Bol, and also ADD and 5β-AED were identified. In a second experiment, a mature cow was treated intramuscularly with 700 mg free, unesterified 17β-Bol. The metabolic profile in urine was comparable. Besides 17α- and 17β-Bol, 5β-AED was found at levels comparable with 17β-Bol, while ADD was found at lower concentrations. The differences in the excretion profile in faeces were considerable. 17α-Bol and 5β-AED were the most predominant metabolites, whereas 17β-Bol and ADD were not detected (Van Puymbroeck 2000).

The presence of metabolites is most frequently investigated in the urine matrix. An overview of metabolites found in treated and untreated animals, is shown in Table 5.1.

Table 5.1. Metabolites of boldenone are present in urine in different untreated and treated animal species. Reproduced from [85].

	Untreated		Treated	
	Male	Female	Male	Female
Pig	17β-Bol	–	n.k.	n.k.
Cattle	17α-Bol	–	17α-Bol, 17β-Bol and metabolites	17α-Bol, 17β-Bol and metabolites
Horse	17β-Bol	n.k.	n.k.	n.k.

–, No metabolites present; n.k., Not known.

17β-Bol was confirmed in both intact male pig [107] and horse. In calves, confirmed 17α-Bol, and traces of 17β-Bol [28]. Boldenone is also found in environmental samples taken close to pig farms and in waste water sampled close to paper mills [108,109]. Besides, in humans endogenous production of boldenone is reported [110,103].

Possible origin of boldenone

Different hypotheses were postulated and tested on the possible origin of boldenone. The involvement of micro-organism from faeces was studied *in vitro* and *in vivo*. Also, models using shrimp, Neomycis Integer were used. In nearly all cases using this model 17β-Bol could be measured after exposure of 17β-T [111]. In the Netherlands, France, Italy and Belgium studies were performed into metabolism and excretion. Boldenone was found in high concentrations by Nielen *et al.* [112]. Also, conversions of Phytosterols in 17β-Bol were postulated and checked by Poelmans *et al.* [113] and Verheyden *et al.* [114]. Verheyden also postulated formation of Boldenone by maggots and moulds on feed [114]. In the intact male horse and in pigs boldenone is probably formed through aromatisation of estrogens in the testis. Poelmans *et al.* demonstrated the presence of boldenone in pig testis [113].

Draisci *et al.* analysed urine samples for boldenone, epiboldenone and androsta-1,4-diene-3,17-dione by LC-MS from 25 untreated animals [115]. Boldenone (LOQ 0.2 μg L^{-1}), epiboldenone (LOQ 0.5 μg L^{-1}) and androsta-1,4-diene-3,17-dione (LOQ 0.2 μg L^{-1}) were not detected above the LOQ in any of the urine samples from the untreated animals. Pompa *et al*. studied the concentrations of boldenone, epiboldenone, androsta-1,4-diene-3,17-dione, testosterone and epitestosterone in the urine, skin swabs and faeces of Friesian calves and also assessed the effect of drying the faeces on the resulting faecal steroid concentrations [116]. In urine, LODs for all steroids were 0.1 μg $mL^{-1}$and in faeces LODs for all steroids were 0.5 μg kg^{-1} (based on S/N 43). Boldenone, epiboldenone and androsta-1,4-diene-3,17-dione in urine were not detected in any of the samples from ten calves. Boldenone was detected in faeces sampled directly from the rectum (rectal faeces) in all the calves at concentrations ranging from 28 to 89 μg kg^{-1}. Epiboldenone in rectal faeces was not detectable in six calves and between 2.6 and 5.9 ng g^{-1}in the other four animals. Androsta-1,4-diene-3,17-one was not detected in the rectal faeces from nine calves while one calf had 21 ng g^{-1}. Results from faeces scraped from the skin, faeces taken from the stall floor and faeces stored for up to 13 days at room temperature in a cowshed showed that the concentrations of all steroids increased significantly (but variably) over time. This is especially true of epiboldenone and androsta-1,4-diene-3,17-dione, which by day 13 of

storage are present in high concentrations, while boldenone was reduced to not detectable by day 13. This study exemplifies the need to avoid fecal contamination of urine during sampling and ensure swift storage and analysis of any samples taken. The conclusions for cattle of this study are summarized in Table 5.2.

Table 5.2. Natural occurring situation of boldenone and the strategy for the control of the use of boldenone in cattle. Reproduced from [85].

	Normal situation	Strategy for boldenone control
Urine	Traces of 17α-Bol	17α-Boldenone conjugate > 2 ng mL^{-1} SUSPICION of illegal use
	No 17β-Bol	Presence of 17β-Bol conjugate at any level CONFIRMATION of illegal use
Faeces	Free unconjugated 17α-Bol	
	Free unconjugated 17β-Bol	?
	(in dried faeces)	

Le Bizec *et al.* and Destrez *et al.* performed studies of the 17β-Boldenone metabolism [100,97]. A marker for distinguishing naturally formed and exogenously administered boldenone was proposed, namely, 17β-Boldenone-sulfo conjugate. In the same period, the EURL performed studies [101] and proposed 6β-hydroxy-boldenone as a marker. In human studies of Gomez *et al*. show boldenone-sulphate as a marker for exogenous administration [110].

Besides, the discrimination of the exogenous origin of boldenone from consumption of inappropriately stored feed samples or through putative microbial action is also of great importance in adverse analytical findings. In human doping, exogenous origin is confirmed by carbon IRMS [99], but it is not possible in horses since the plant material that they eat are derived from similar carbon sources as it has been demonstrated for bovines [117]. Therefore, in a recent study by Viljanto *et al.* a differentiation of boldenone administration from ex vivo transformation in the urine of castrated male horses has been developed [118]. Authors aimed to assess whether Δ1-progesterone and 20(S)-hydroxy-Δ1-progesterone could have potential to distinguish *ex vivo* transformation of boldenone from its administration in gelding urine samples. In this way, the storage of samples at room temperature resulted in an increase of boldenone, Δ1-progesterone and 20(S)-hydroxy-Δ1-progesterone but this did not occur when they were stored in refrigerators. These results were used to propose Δ1-progesterone and 20(S)-hydroxy-Δ1-progesterone as biomarkers to distinguish steroid administration in gelding urine when boldenone concentration exceed the action limit. Hence, authors suggested that if Δ1-progesterone and 20(S)-hydroxy-Δ1-progesterone exceed 50 pg mL^{-1} and 100 pg mL^{-1} ex vivo transformation or the consumption of altered feed was the main source of boldenone rather than steroid administration [118].

Annex 1 summarises the published information on 17β-Boldenone and 17α-Boldenone.

5.4. Proposed Strategy for monitoring

Several options for confirming abuse of boldenone have been suggested in the literature.

- 17β-boldenone conjugates in urine of young calves can be used as a proof of illegal treatment.
- For the detection of intact esters on boldenone in tissue samples (application sites), hair samples are the most reliable approach for proving abuse of boldenone.
- When for a specific species, inclusive gender considerations, no prior information has been published indicating that boldenone or its marker metabolite occur endogenously, the confirmed presence hereof can be considered proof for illegal use, based on current knowledge. The EURL has an overview in which the natural occurrence dependent of the species and sex of the animals are described (Annex to part 1 of the reflection paper).

Screening

- Threshold concentrations for specific marker metabolites can be set discriminating exogenous from endogenous sources. Without confirmation, exceeding threshold values must be considered a screening suspicious result.
- Profiling analytical results for a range of metabolites and precursors is an option to be further studied. As for the use of threshold values, such a result can only be used for screening.

Determining the reliability of the use of threshold concentrations or profiling analysis can only be made in terms of false compliant of false non-compliant result rates. As discussed previously, such evaluation can only be made once a confirmatory technique becomes available. The final status of an analytical method will have to be based on the outcome of such a study.

The use of IRMS currently is considered as a potentially useful approach. However, current detection limits hamper the use when low concentrations are found.

Research questions for full implementation

To implement a strategy when finding boldenone residues and discriminating illegally administered and endogenously produced boldenone, research is needed in the following subjects:

Screening methods

- Research into unambiguously biomarker such as conjugated metabolites such as sulphate conjugates or 6-hydroxy-boldenone(sulphate)
- Calculation of threshold concentrations for biomarkers.
- Investigation of natural occurrence in ovine and caprine

Confirmatory methods

- Validation and development of Isotope Ratio Mass Spectrometry methods for 17β- and 17α-boldenone in urine.
- Continue research on the use of steroid esters in hair and serum.

EURL Recommendations

- Continue research on threshold values for specific biomarkers (species dependant).
    - Further data collection from national programmes

- Continue the use of profiling techniques and data evaluation.
- Continue research on the applicability of analysing for steroid esters (EURL/NRL/OL network), e.g. in hair.
- Validate the use of GC-c-IRMS (EURL priority, in cooperation with specific project group).
- Continue research on the use of steroid esters in hair and serum.

6. 1-Testosterone

6.1. Introduction

The use of 1-testosterone (17β-hydroxy-5α-androst-1-en-3-one) as a growth promoter for humans is well described [119] and probably occurs on a large scale. Its usage as growth promoter in cattle was uncertain until a few years ago, until its presence was confirmed in an injection preparation seized at a farm. Besides being a growth promotor of itself, 1-testosterone is part of the metabolic pathway of boldione in humans. In Figure 6.1 an overview is given of the metabolic pathway in humans of boldione, it includes also the metabolic pathway of boldenone and 1-testosterone [110].

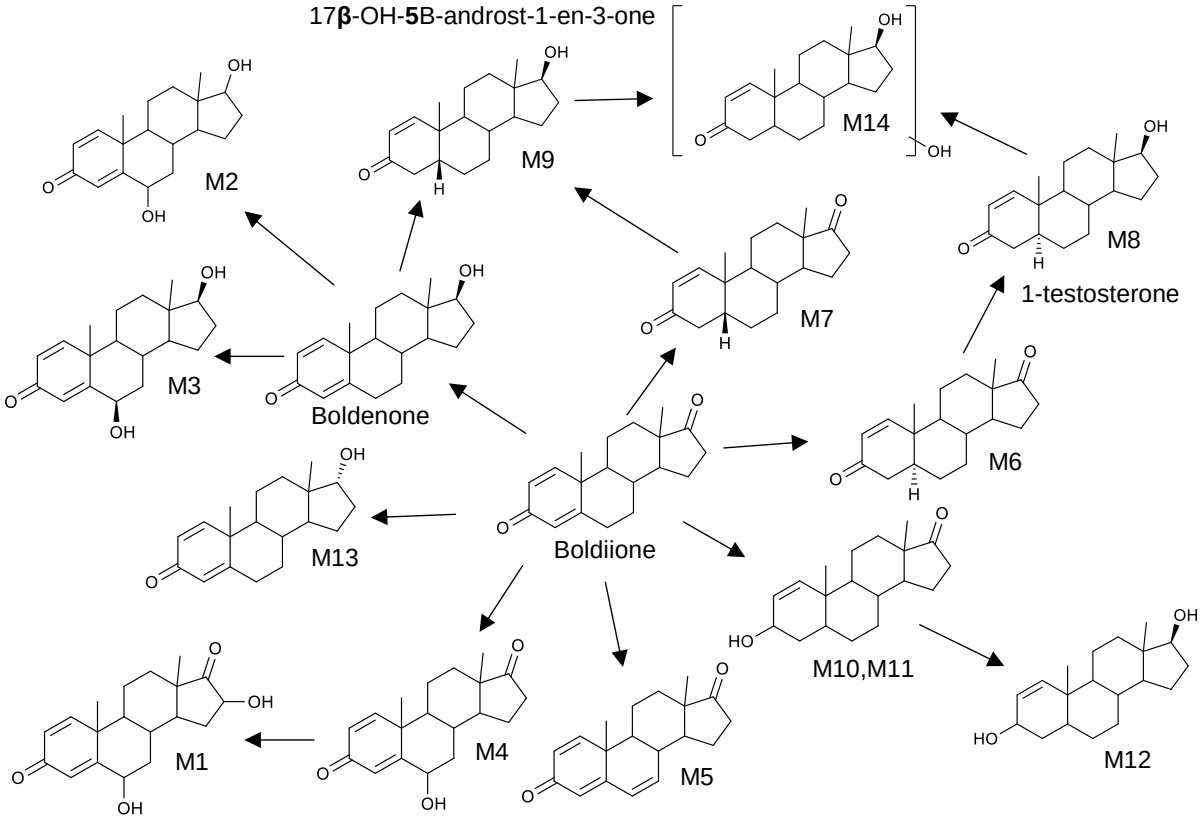


Figure 6.1. Overview of the metabolic pathway in humans of boldione [110].

Results obtained until now indicate that 17α-1-testosterone is probably present endogenously in urine samples. In a study by [114]. It was determined that faeces contains high amounts of α-boldenone and traces of 1-testosterone (17β-hydroxy-5α-androst-1-en-3-one).

1-Testosterone is not registered as a veterinary drug for food producing animals and belongs to the A3c group of CDR (EU) 2022/1644. No Minimum Required Performance Limit (MRPL), Maximum Residue Limit (MRL) or Reference Point for Action (RPA) is established and the compound was not included in the MMPR Guidance paper of 2021 [6].

6.2. Analytical methods

There are few methods published to detect 1-testosterone in samples of urine [120,121,30]. These methods are based on GC-MS or LC-MS/MS. Sample matrix is limited

to urine and as far as we know, there are no methods published to detect 17α-1-testosterone in other matrices. In a ring test conducted by the EURL for residues in 2012 it was noted that there was substantial variation in the quantification of 17α/β-1-testosterone. This is probably due to the lack of an isotopically labelled analogue for use as an internal standard.

6.3. Research summary

In the study of Le Bizec *et. al.*, after treatment of cattle with boldenone or boldione three isomers of 17-hydroxy-5-androst-1-en-3-one were identified e.g. 17α-hydroxy-5β-androst-1-en-3-one, 17β-hydroxy-5 β-androst-1-en-3-one and 17β-hydroxy-5α-androst-1-en-3-one, from which the latter has close resemblances with 1-testosterone on basis of retention time and LC behavior [97]. Since, 1-Testosterone can be formed from boldione treatment in humans [110] and maybe from boldenone in cattle [97] treatment implies that other forms of treatment of an animal, than with 1-testosterone itself, can also be the cause of an adverse 1-testosterone finding.

Note: Although 17β-hydroxy-5 β-androst-1-en-3-one is considered as a "safe" marker for boldenone abuse [30] it's presence in urine can also be formed after administration of boldione.

The main metabolite of 1-testosterone (17ß-1-testosterone, 17ß-hydroxy-5α-androst-1-en-3-one) is 17α-1-testosterone (17α-hydroxy-5α-androst-1-en-3-one). Both compounds are not registered as veterinary drugs for food-producing animals. 17α/ß-1-testosterone belongs to the A1c group (Steroids) of CDR (EU) 2022/1644. No Maximum Residue Limit (MRL) or Reference Point for Action (RPA) is established for these compounds. In the MMPR Guidance paper of 2021 [6] there is no recommended concentration set for this compound.

To determine the metabolism of 1-testosterone, an animal experiment was performed by the EURL. The main metabolite identified by GC-MS and confirmed by NMR analysis was 17α-1-testosterone (17α-hydroxy-5α-androst-1-en-3-one) (Figure 6.2).

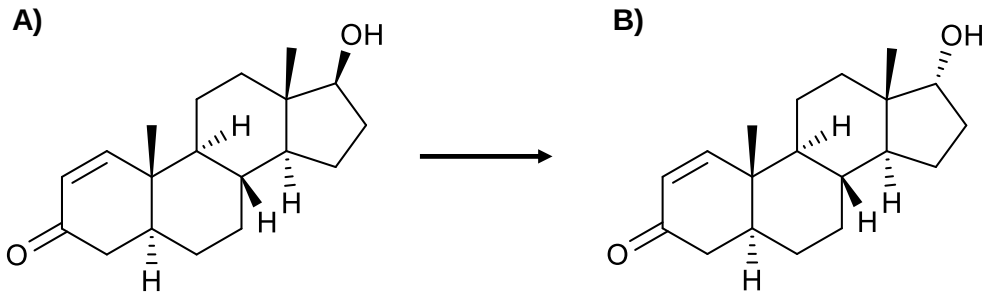


Figure 6.2. Metabolism of 1-testosterone (17β-hydroxy-5α-androst-1-en-3-one) (A), main metabolite 17α-1-testosterone (17α-hydroxy-5α-androst-1-en-3-one) (B).

After "unravelling" the metabolism in a heifer, reference materials were prepared for a ring test which needed the preparation of blank samples of urine. During analysis of such urine samples, 17α-1-testosterone was detected in a few of them. To be sure that the finding of 17α-1-testosterone was correct more samples were analyzed (origin Dutch population of cattle) and also several confirmatory techniques were used. The presence of 17α-1-testosterone was confirmed in approximately 7% of the samples (n=120). The analysis of a well-defined set of samples obtained from a population of animals of which it can be guaranteed that they are not treated, remains to be performed. The typical concentration

range of these compounds was between 0.1-3 ng mL^{-1}in urine. For confirmation GC-MS/MS, LC-MS/MS, GC-HRMS (magnetic sector instrument) and GCxGC-ToF-MS were used. Also the clean-up was checked to be sure that 17α-1-testosterone was not formed due to enzymes or other conditions applied during clean-up. It was determined that the clean-up did not influence the outcome and that indeed 17α-1-testosterone was detected. Furthermore, compounds with close structural relation to 17α-1-testosterone were added at high concentrations to samples of urine. After analysis of these samples, it was confirmed that 17α-1-testosterone was not formed during clean-up through the conversion of one of these compounds. At this stage, it was concluded that 17α-1-testosterone was probably present endogenous in samples of urine. As far as we know, this is the first time that the presence of 17α-1-testosterone has been confirmed in urine of cattle. In a study by Verheyden *et al.* it was found that feces containing high amounts of α-boldenone contains some 1-testosterone (17β-hydroxy-5α-androst-1-en-3-one) [114].

Although the presence of 17α-1-testosterone was confirmed in samples of urine, more research has to be conducted before it can be stated that the origin of 17α-1-testosterone is truly endogenous. Metabolism studies show that 1-testosterone is formed after administration of boldenone and probably boldenone and is also found in feces together with boldenone.

Annex 1 summarises the published information on 1-Testosterone.

6.4. Proposed Strategy for monitoring

Although the presence of 17α-1-testosterone was confirmed in urine samples of bovine animals, more research has to be conducted before it can be stated that the origin of 17α-1-testosterone is truly endogenous. A large number of urine samples from animals known as untreated animals remains to be analysed. Where 17α-1-testosterone is present, a control strategy has to be developed. The active parent compound, 17ß-1-testosterone has not yet been detected in presumed blank samples of urine. However, metabolism studies [110] have shown that 17ß-1-testosterone is formed after administration of boldione and probably boldenone and is also found in feces together with boldenone, making 17ß-1-testosterone a complicated marker for abuse. However, as long as all potential precursors are considered exogenous steroids, it still may be a valuable marker.

Decreasing detecting limits could show that this compound is present endogenously in low concentrations. Therefore - for the moment - it is recommended to use a threshold value of 1 µg $L^{-1}$for this compound for screening. An evaluation of the use of threshold values can only be made in terms of false compliant or false non-compliant result rates. Such evaluation can only be made when a confirmatory technique becomes available. The use of Isotope Ratio MS currently is considered the most effective approach. Detection limits hamper its application in case of trace concentrations.

Research questions for full implementation

To implement a strategy when finding 17α-1-testosterone residues and discriminating illegally administered from endogenously produced, research is needed on the following subjects:

Screening methods

- Determining whether 17α-1-testosterone (and 17ß-1-testosterone) is truly endogenous by measuring the concentration in urine of a large population of guaranteed non-treated animals.
- Improve quantification by synthesising an isotopically labelled analogue.
- Research into the use of other discriminating metabolites or conjugates.
- If applicable calculation of threshold concentrations.

Confirmation methods

- Development of Isotope Ratio Mass Spectrometry methods for 17α/ß-1-testosterone in urine.

EURL Recommendations

- Continue research on threshold values for specific biomarkers (species and sex dependant).
    - Further data collection from national programmes
- Continue the use of profiling techniques and data evaluation.
- Continue research on the applicability of analysing for steroid esters (EURL/NRL/OL network), e.g. in hair.
- Validate the use of GC-c-IRMS (EURL priority, in cooperation with specific project group).
- Continue research on the use of steroid esters in hair and serum.

7. Zeranol

7.1. Introduction

Zeranol (α-zearalanol) and its primary metabolite in bovine animals, Taleranol (β-Zearalanol), are members of the group of Resorcylic Acid Lactones (RALs), also comprising α- and β-zearalenol, zearalanone and zearalenone. Zearalenone is also known as the *Fusarium spp.* Toxin (F2-toxin) and is commonly found in animal feed. Zeranol is approved to use as growth promoter for livestock in USA and Canada. Implantation of Zeranol in calves causes an increase in mean live weight gain.

Zeranol is prepared commercially from zearalenone, one of a number of structurally similar toxins produced by *Fusarium spp.* Fungi Zeranol and zearalenone are known to give identical metabolites explaining the fact that these metabolites, including Zeranol itself, can also occur naturally in deer, goats, sheep, ovine, bovine, and horses urine and bovine bile following metabolism of *Fusarium spp.* Toxin [122,123]. In Figure 7.1 the metabolism of RALs is given based on a study performed by Kennedy *et al.* (1998)[123].

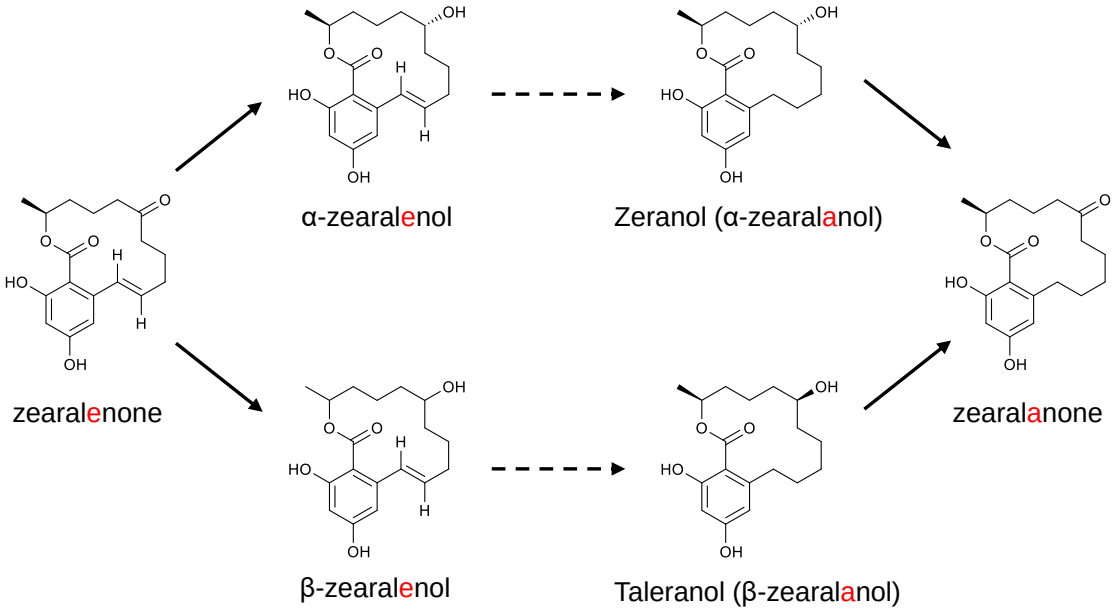


Figure 7.1. Overview of the metabolism of RALs, dashed arrows represent the conversion of the toxins towards zeranol.

In Europe Zeranol is not registered for veterinary use in food-producing animals and belongs to the A4 group of CDR (EU) 2022/1644. No Maximum Residue Limit (MRL) or Reference Point for Action (RPA) is established for this compound. In the MMPR Guidance paper of 2021 a recommended concentration was proposed of 1 µg L^{-1}or kg in urine and muscle and, 2 µg kg^{-1}in liver.

7.2. Analytical methods

Detection of RALs is based on GC-MS [124] or LC–MS/MS [125]. However, a recent study by Echarte *et al.* evaluate the performance of both GC-MS and LC-MS/MS techniques for the determination of zeranol and related mycotoxins and demonstrated that LC-MSMS showed better performance so had a stronger capability to roll out unauthorized use of target compounds. So far, zeranol has been analyzed in matrices such as urine [125-128],

muscle, liver, kidney, hair and milk[129-135]. Sample clean-up consists of liquid-liquid extraction (LLE) [136,64], solid phase extraction (SPE) [137,138,64], immunoaffinity column (IAC) extraction [125] and QuEChERS [135]. Several fully validated methods are published capable of analysing all six compounds with decision limits (CCα) below 1 µg L^{-1}in urine [125]. Besides detecting all six targeted RALs, it is also possible to include them in a targeted multi-residue method, including other compounds. In a study performed by Geis-Asteggiantea *et. al.* over 100 veterinary drug residues in bovine muscle were detected, including zeranol, using UHPLC-MS/MS [129]. There are also multi-methods based on GC-MS/MS [136] capable of detecting several compounds, although they are limited to a maximum of 20 compounds. The drawback of these methods is that they only include α/β-zearalanol, neglecting the other RALs. From a theoretical point of view, it should be fairly simple to include all RALs in these multi-methods. Besides targeted multi-residue methods, performing an untargeted analysis and filtering out interesting masses or using a hybrid-mass spectrometer to collect specific MS/MS spectra using an inclusion list is possible. The latter case is demonstrated by Praveen Kumar *et al.* [127].

7.3. Research Summary

In an EFSA opinion (2011) about zearalenone a lot of information was presented on its natural occurrence. Zearalenone was reported at quantifiable concentrations in 15% of 20.000 samples of grain. The concentrations of zearalenone in the group 'Unprocessed grains' were considerably higher than in the group 'Grains for human consumption'. The chronic total dietary exposures to zearalenone ranged from 2.4 to 29 ng kg^{-1} body weight (b.w.) per day. Exposure of vegetarians to zearalenone could be up to 2-fold higher than for the general population. Zearalenone is rapidly and extensively absorbed from the gastrointestinal tract in mammals. Foetuses and neonates could be more susceptible than adults to the estrogenic effects of zearalenone. Metabolism produces α-zearalenol, a metabolite with greater affinity for estrogen receptors than the parent compound, and β-zearalenol, a metabolite with lower affinity. Toxicity studies of zearalenone and its main reductive metabolites demonstrate that estrogenic activity is the critical mode of action. Of the laboratory and domestic animals studied, pigs are the most sensitive species for estrogenic effects, with females being more sensitive than males. Adverse effects on testosterone synthesis, sexual behavior, sex organ weights, testicular histology, spermatogenesis, reproductive-tract, fertility and embryo survival have been observed. Estimates of chronic dietary exposure to zearalenone based on the available occurrence data are below or in the order of the tolerable daily intake (TDI) for all age groups and do not pose a health concern.

In 2004, a FAIR EU project was finalised with the goal to develop methods to discriminate between abuse of zeranol and ingestion of mycotoxine contaminated feed. The outcome of this project was a statistical model based on the metabolite pattern [131,139] using concentrations of all RALs present in urine. Discrimination between natural contamination and abuse is based on differences in the metabolite pattern of α/β-zearalanol (zeranol and taleranol) + zearalanone versus α/β-zearalenol + zearalenone. Authors highlighted that when the combined concentrations of α/β-zearalanol + zearalanone are higher than those of α/β-zearalenol + zearalenone it is an indication of illegal use. This model was extensively validated for urine from cattle and is used in routine residue control programs in case of a non-compliant finding. Although well validatation, the model has no legal basis. The model is only applicable for bovine urine samples. It is a screening tool and the result can help in deciding if a non-compliant finding requires a follow-up action or that the non-compliant finding was due to ingestion of mycotoxine contaminated feed.

The metabolism of zearalenone in pigs is described in a study of Zöllner *et al.* [140]. In this study, it was found that the metabolism is identical to the one depicted in Figure 7.1. After feeding zearalenone contaminated feed to the pigs some trace amounts of zeranol and taleranol were found in urine. Also samples of the liver and meat were investigated but neither zeranol nor taleranol and zearalanone were present in any of the liver samples. In the meat samples, trace amounts of all RALs were detected. It was noted that the occurrence of RALs was dependent on the type of muscle analyzed. Samples from the femoral region contained only zeranol at low concentrations (0.5-2.1 μg kg^{-1}), samples from the back contained a higher concentration of zeranol (0.5-13.3 μg kg^{-1}) which were also accompanied by relatively high amounts of α-zearalenol (0.5-14.5 μg kg^{-1}) and occasionally traces of zearalenone and taleranol (0.5-1 μg kg^{-1}). A possible explanation given by the authors for this observation might be the fact that muscle tissue from the back is better supplied with blood, which can be regarded as the most important carrier system for the analytes in the animal body. Thus, higher amounts of analytes were directly transported to this tissue and, consequently, were more likely to be incorporated in this compartment.

Recently, in order to improve the knowledge about metabolic patterns of RALs in porcine, two animal studies using different scenarios of exposure have been carried out in the EURL-Wageningen Food Safety Research. For this purpose, on one hand, pigs received feed contaminated with ZEN, the mycotoxin that is metabolized to α-ZAL and on the other, α-ZAL was administrated to a porcine. The study lasts four weeks consisting of one week acclimatization and three weeks treatment. Next to each treated pig a companion pig was be placed, so there were two animals per pen. The companion animals will not be treated, but will be sampled to get information of the contamination depending on administration route. Urine samples from both treated and nontreated animals are collected daily during the four weeks from Monday till Friday. In the first study with zearalenone administration, during the acclimatization period the presence of zearalenone was predominant and after administration, in addition to zearalenone the natural occurring toxins, zearalanone and α-zearalanol forbidden substances were also present at high signal. In the case of zeranol administration, the presence of zearalenone and α-zearalenol toxins could be seen as well as the rest of the prohibited compounds such as zearalanone and β-zearalanol. In this case, the signal of forbidden substances drop down significantly from the administration day to the 5th day of administration and therefore the detection of the forbidden substances after illegal administration could be limited.

Statistical model to discriminate Zeranol abuse from natural contamination.

To distinguish illegal use of Zeranol from the consumption of *Fusarium spp.* toxin contaminated food, an EU project, FAIR5-CT-1997-3443, was undertaken. A statistical model was developed after screening and confirmation of samples of urine collected from different areas in Europe [124,139]. Most samples were of bovine origin. However, some samples were also of ovine, porcine and caprine origin. In total 8008 samples were analyzed, originating from different countries within Europe.

Samples were classified as: 'False-positive'; Fusarium spp. toxins present, Zeranol (or Taleranol) absent. 'Equivocal'; Fusarium spp. toxins present, Zeranol (or Taleranol) present, and 'True-positive'; Fusarium spp. toxins absent, Zeranol (or Taleranol) present. In table 7.1 a summary of the results of this survey are shown.

Table 7.1. Summary of the confirmatory results of the 461 samples that tested positive during screening [139].

	Country 1	Country 2	Country 3	Country 4	Total
Samples tested	65	146	94	156	461
False-positive	23 (35.4%)	84 (57.5%)	82 (87.2%)	94* (60.3%)	283* (61.4%)
Equivocal	42 (64.6%)	61 (41.8%)	11 (11.7%)	60 (38.5%)	174 (37.7%)
True-positive	0	1 (0.7%)	1 (1.1%)	2 (1.2%)	4 (0.9%)

*Including one porcine sample.

On the equivocal samples, sub-set statistical analysis was performed. The statistical model built is based on comparing the sum of Zeranol and Taleranol mass concentrations with the sum of the Zearalenone and its two major metabolites, α- and β-Zearalenol (Figure 7.2).

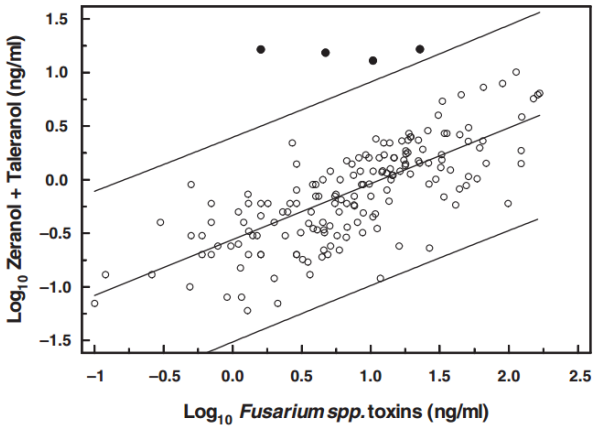


Figure 7.2. Graphical representation of the statistical model. The samples represented by a solid circle fell outside the 99% confidence interval and can be considered as suspect [139].

The statistical model built could help laboratories and also competent authorities to decide if the origin of a positive Zeranol finding was due to illegal use of Zeranol or natural contamination in Zeranol. This tool can be obtained from the EURL for residues. This model is fully validated for bovines. During the animal study carried out in 2022 for porcine, the obtained concentrations of RALs were tested following the aforementioned model and the obtained ratio values can be seen in Fig. 7.3.

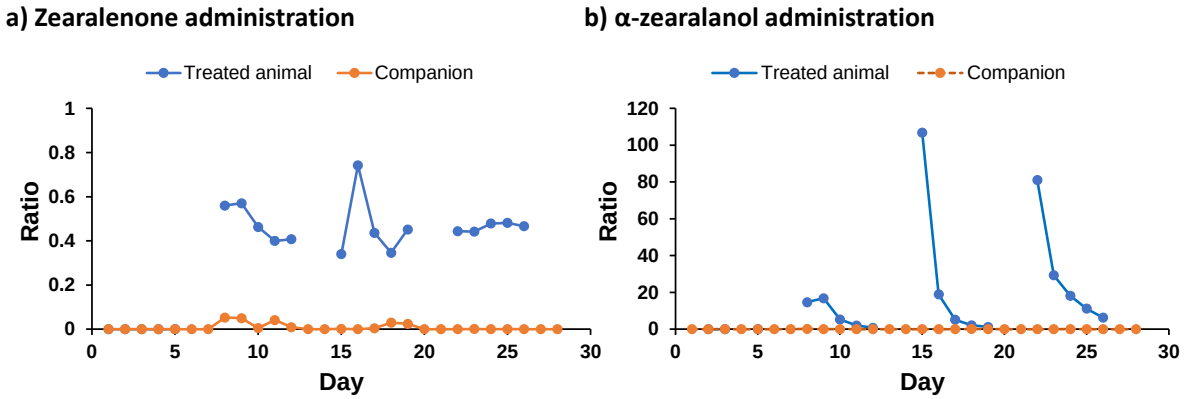


Figure 7.3: Ratio of α/β-zearalanol and α/β-zearalenol + zearalenone in a) zearalenone administration study and b) α-zearalenol administration study for treated (blue) and companion (orange) animal.

As can be seen in Fig. 7.3a and Fig. 7.3b (orange), the obtained ratio for companion animals in both studies resulted to be 0. This result demonstrates how, if an animal has not been directly exposed to the prohibited RALs, it will not show signs of it. Therefore, it does not mean that none of the animals from the same farm have been exposed either. In the case of the animal treated with zearalenone toxin (Fig. 7.3a, blue), the ratio during the first week corresponds to 0 but from the administration day ongoing it is changed and increased up as a sign of some exposure. Nevertheless, in this case, the value of the ratio is constant and below 1 in all cases. This results demonstrated the aforementioned theory about possible contamination with the value lower than 1. In the last case, with the animal treated with α-zearalenol (Fig. 7.3b, blue), during the control week the ratio was zero but a large rise in the ratio value can be observed on the days of administration (up to 115) followed by a significant decrease during the 5 days post administration. It should be noted that during these 5 days, the value of the ratio has always been above 1, so that at any subsequent time the model would come out as a positive sample due to administration or in need of follow-up.

Annex 1 summarises the published information on Zeranol and Taleranol.

7.4. Proposed Strategy for monitoring

So far the only strategy to detect abuse is based on using a statistical model based on the quantitative metabolite profiles of RALs in urine for cattle [124,139]. Samples were classified as: 'False-positive'; *Fusarium spp.* toxins present, zeranol (or taleranol) absent. 'Equivocal'; *Fusarium spp.* toxins present, zeranol (or taleranol) present, and 'True-positive'; *Fusarium spp.* toxins absent, zeranol (or taleranol) present. The statistical model is based on comparing the sum of zeranol and taleranol mass concentrations with the zearalenone and its two major metabolites, α- and β-zearalenol. In practice, some simpler versions are sometimes being used for screening, e.g. based on the ratio between the sum of zeranol and taleranol (indicating treatment) to the sum of α- and β-zearalenol (indicating feed contamination). The statistical model used could help laboratories and competent authorities decide if the origin of a positive zeranol finding was due to the illegal use of zeranol or natural contamination in zeranol. This tool can be obtained from the EURL for residues.

This approach is simple and fast and can be used in combination with GC-MS/MS or LC-MS/MS measurements. The method, however, is not court-proof. Follow-up actions have to be taken in case of a suspicious finding. This could be to go back to the farm and analyse samples of feed and determine if these contain the *Fusarium spp.* toxins. If the feed contains the *Fusarium spp.* toxins it is likely that this is the source of the non-compliant finding, but if no *Fusarium spp.* toxins are present, the source is probably due to administration. There is no absolute confirmation method available yet.

A promising technique, which may discriminate abuse from contamination, is by use of GC-c-IRMS. There could be a difference in isotopic pattern between naturally occurring and administrated substance, but this will require further work to confirm.

Research questions for full implementation

To implement a strategy when finding RALs residues and discriminate illegal administration from ingestion of contaminated feed, research is needed in the following subjects:

Screenings methods

- Screening on use of the metabolite profile in urine is a good and proven concept. However, the statistical model should be updated with data from different countries and species.
- Development of Isotope Ratio Mass Spectrometry methods for zeranol and taleranol in urine, provided this techniques gives sufficiently significant different isotope ratios.

EURL recommendations

For all Member States

Implement RAL profiling in addition to analysing for Zeranol and Taleranol and provide data to the EURL

General

- Update and extend the reference datasets for the RAL profiling (different regions, different species).
    - Data collection from national programmes
- Evaluate the approaches for screening (setting limits for ratios).
- Evaluate the potential of IRMS as a confirmatory method.

8. Prednisolone

8.1. Introduction

Prednisolone, a corticosteroid with glucocorticosteroid activity, is used for the treatment of a wide range of inflammatory and auto-immune conditions. The use of corticosteroids in livestock is regulated in the European Union for therapeutic purposes. Prednisolone is part of registered veterinary drugs containing amoxycilin and clavulinic acid for intra-mammary administration. The maximum residue limit for the therapeutic use of prednisolone in bovine animals is 4 µg kg^{-1}in muscle and fat, 10 µg kg^{-1} in liver and kidney and 6 µg kg^{-1} in milk [141]. In several EU Member States, corticosteroids were/ are classified as group A3 substances (anabolic/unauthorized substances) because of their steroidal structure, whereas other EU member states classify them as B2f (other pharmacologically active substances) [3]. No recommended concentration for control was established in the Community Reference Laboratories Guidance Paper (2021) [6].

The illegal use of corticosteroids as growth promoters cannot, however, be excluded. Corticosteroids can prolong the effect of growth-promoting substances, such as anabolic steroids and β-agonists, in the last weeks before slaughter. In addition, low doses of glucocorticoids can result in improved feed intake, increased live weight gain, reduced feed conversion ratio, reduced nitrogen retention and increased water retention and fat content [142-144].

8.2. Analytical methods

To date, several methods are available to determine prednisolone and its metabolites in biological matrices by LC-MS. The most used technique is LC-MS/MS and it has been employed to analyze the target compounds in urine [145-149], liver [145,144,150], bile [90,91], faeces [151], teeth [152] and milk [153] of different species such as bovine, calves, cows and bulls. Additionally, few studies used LC-HRMS for the determination of prednisolone and its metabolites in bovine urine [154-157], plasma [157] and complementary feedstuffs for bovine husbandry [158].

8.3. Research Summary

Over the last years, low concentrations of prednisolone have been reported in bovine and porcine urine by a number of laboratories in EU member states. Concentrations vary but are reported to be below approximately 3 µg L^{-1}. In 40% of bovine urine samples from the Dutch national control plan, concentrations of prednisolone between 0.11 – 2.04 µg L^{-1} were found. In literature, a number of studies investigate the mechanism of formation of prednisolone in bovine, porcine and equine urine, including *in-vitro* conversion of cortisol by bacteria from faeces and soil, and *in-vivo* formation of prednisolone due to stress) [159]. Findings of prednisolone in concentrations up to 5 µg L^{-1} indicate that it can originate from other sources than illegal treatment with growth promoters [146]. This chapter presents an overview of the current scientific knowledge on the occurrence of prednisolone in cattle.

In recent years, low concentrations of prednisolone in bovine and equine [146,160,161,150] urine have been reported by several European Union Member states. Also in porcine urine low concentrations of prednisolone have been found [113] (Annual report of the Belgian Hormone Commission 2011). Because prednisolone differs in

structure from cortisol by only one double bond, the formation of prednisolone could resemble the process described for the formation of boldenone from testosterone [162,124] in the presence of faeces in the urine sample. The possibility of *in-vivo* formation of prednisolone from endogenously present cortisol (see Figure 8.1) has indeed been reported [163-165,145,154,160,161,159,166].

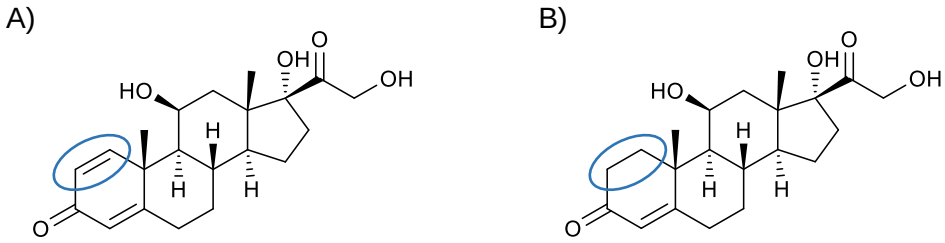


Figure 8.1. Molecular structures of (A) prednisolone and (B) cortisol.

The hypothesis that faecal contamination causes the formation of prednisolone from cortisol in urine is supported by the *in-vitro* experiment of Arioli *et al.* in which aqueous faecal solution was incubated with cortisol and cortisol-glucuronide, leading to deconjugation of the glucuronide and 1-delta dehydrogenation resulting in formation of unquantifiable traces of prednisolone [163]. In 2010, Arioli published an article in which the influence of faecal contamination on real samples shows that prednisolone can be formed. This is also the case in the article of de Rijke *et al.* [146]. Also, the influence of storage conditions on the formation of prednisolone is described [163,151,146]. This is also consistent with the animal experiment with dexamethasone of Ferranti *et al.*, in which prednisolone was found both in urine samples from control animals taken at a slaughterhouse (56% positive at concentrations of 0.4-1.4 µg L^{-1}) and among animals treated with dexamethasone (90% positive at concentrations of 0.4-1.5 µg L^{-1}) [160].

Cannizzo *et al.* described an experiment in which 15 or 30 mg of prednisolone per day was administered orally to beef calves for a period of 30 to 35 days. No prednisolone (>1 µg L^{-1}) was found for both dosages in urine and liver and no prednisolone was found in urine sampled at the slaughterhouse [165]. Histological investigations showed that for prednisolone, no effect on thymus tissue was observed. It was concluded that prednisolone was difficult to detect both with the histological and the analytical methods used.

Stress is related to the formation of prednisolone from cortisol. This was first investigated by Pompa *et al.* in an animal experiment with dairy cows (n=3, 44-106 months) [159]. Prednisolone was detected occasionally in unstressed situations. When stress was simulated by injection with tetracosactide hexaacetate, a synthetic analogue of adrenocorticotropic hormone, prednisolone was constantly found in concentrations between 1.01-4.08 µg L^{-1}. Besides, Capra *et al.* recently evaluated the effects of truck transportation and slaughtering into 15 untreated cows on the occurrence of prednisolone and its metabolites in cow urine, liver and adrenal glands by LC-MS/MS [145]. Authors find that the stressful conditions of transportation and slaughtering promote the increase of the concentration of cortisol and cortisone. Besides, prednisolone was only determined in two urine samples at a concentration (<0.6 µg L^{-1}) below the established to avoid false non-compliances by EURL. This can be considered as proof that endogenous formation of prednisolone can occur. Additionally, De Clercq *et al.* studied the effect of stress in bovine urine samples by comparing the presence of cortisol and prednisolone levels in urines at

the farms and urines of bovines subjected to natural stress in slaughters or pharmacologically induced stress by administration of a synthetic analog of adrenocorticotropic hormone [154]. These studies showed that no presence of prednisolone was found in urines collected at farms, but in 82 samples collected upon slaughter or following administration, prednisolone could be determined. However, from 82 samples, only in 1 sample showed a prednisolone level (6.45 µg L^{-1}) above the suggested threshold by the EURL. The authors also evaluated the urinary metabolite pattern of the three cattle groups and found out that 169 metabolites (in which 3 were defined as steroids) play a key role in the urinary patterns in response to stress. Therefore, the authors suggested metabolomic fingerprinting as a powerful tool to classify unknown bovine urine samples. Another study into the formation of prednisolone from cortisol describes *in-vitro* experiments with different bacterial strains that resulted in formation of prednisolone, e.g. with the soil bacteria *Rhodococcus erythropolis* [164].

The first findings of prednisolone in the horse were described by Fidani *et al.* [161]. In 78% of the horse urine samples investigated prednisolone was detected in low concentrations, with an average of 1 µg L^{-1}. Its origin may be endogenous but no proof for this was provided by the authors. Further, in the 2011 annual report of the Belgian Hormone Commission (Hormonencel) it was stated that prednisolone was found in low concentrations in porcine urine, but no further scientific information was provided [113]. In an Italian field survey from Vincenti *et al.* when the urine of 131 guaranteed untreated cows aged between 2.5 and 8 years old from beef and dairy farms were investigated for prednisolone, no prednisolone (>0.7 µg L^{-1}) was found in 124 samples, and the remaining 7 samples showed only traces of prednisolone between 0.1 and 0.3 µg L^{-1} [166]. Interestingly, in these 7 samples the cortisol concentrations were also higher.

Delahaut *et al.* describe an animal experiment in which prednisolone was found in pigs, before and after administration [167]. In liver prednisolone was only found after administration of prednisolone or Synacthen® (tetracosactide hexaacetate). The cortisol concentration in liver remained constant. The ratio prednisolone/cortisol in liver seems to be a potential marker for treatment. Until four days after treatment, this can be used.

From the studies discussed above, it can be concluded that prednisolone is found in bovine urine of animals from different ages, in both beef and dairy cattle, and also in porcine and equine urine samples, although to date only a few reports have appeared in pigs and horses. A relationship between prednisolone with stress and cortisol was established, but this was not observed in all studies. A relation between prednisolone and faecal contamination has also been provided, with *in-vitro* proof, possibly indicating that the formation of prednisolone was caused by bacterial transformation due to bacteria from faeces or soil. Animal experiments with dexamethasone and prednisolone showed no histological changes and very low, <1.0 µg L^{-1}, prednisolone residues in urine. Ludwig *et al.* demonstrated that a 4-plex biomarker Flow Cytrometric Immune Assay showed an elevation in IGF-1 concentrations of 16% after 43 days of treatment [168]. Leporanti *et al*. proposed 20-beta-dihydroprednisolone as a biomarker to detect illegal use [169]. After administration of prednisolone-acetate IM this metabolite was detected for 6 days at concentrations up to 27 ng mL^{-1} in urine. Also, 20α-dihydroprednsiolone, 6-beta-hydroxy-prednisolone and 20-beta-prednisone were found but concentrations were lower. After the administration of prednisolone to bovines prednisolone and a small amount of prednisone was detected. Recent studies from a Belgian group showed that in calves prednisone and in adult cows 20-β-dihydro-prednisolone were found [167].

Annex 1 summarises the published information on Prednisolone.

8.4. Proposed Strategy for monitoring

Several options for detecting abuse of prednisolone have been suggested in literature.

- Delahaut *et al.* showed that the ratio of prednisolone to cortisol can detect the misuse of prednisolone [167].

- Based on literature, the EURL proposed a threshold concentration of 5 µg L−1 for prednisolone in urine of porcines and bovines.

These approaches have in common that the result has to be interpreted as a result of screening analysis.

Research questions for full implementation

To implement a strategy when finding prednisolone residues in cases its use was not prescribed, and discriminating illegally administered and endogenously produced research is needed in the following subjects:

- Research into the use of other metabolites such as 20-α- or 20-β-dihydroprednisolone.
- Research into use of prednisolone-cortisol ratio or other ratios.
- Quantitative validation of the method of analysis.
- Determination of threshold limits for the selected marker.
- Development of LC-IRMS methods for prednisolone in urine.

EURL recommendations

- Continue research on ratios of marker metabolites as diagnostic tool is continued.
  - Collect data from national programmes
- Evaluate the potential of IRMS as a confirmatory method.

9. Recombinant Bovine Somatotropin

9.1. Introduction

Somatotropin (ST), also known as growth hormone (GH), is a single-chain polypeptide hormone of 191 aminoacids long and approximately 22 kDa. It controls the differentiation, growth and metabolism of many cell types of vertebrate species [170]. Therefore, it plays an important role in the control of growth and reproduction. It is a hormone produced by the anterior pituitary gland and belongs to the same hormonal family as prolactin and placental lactogen [171]. One of its characteristics is stimulation of mammary gland growth and regulation of milk production [172]. As ST stimulates mammary gland growth and milk production, exogenous administered ST will also stimulate this production. In the 1980's a joint cooperation between Monsanto Co. and Genetech Inc. researched the possibility to produce recombinant bovine ST (rbST) with biotechnological techniques [173]. This biotechnology then offered the opportunity to produce rbST in large amounts, which led to commercialization of the rbST protein. Various forms have been produced over the years, four forms have been submitted to Joint Expert Committee on (JECFA) for evaluation namely, somagrebove, sometribove, somavubove, and somidobove. Nowadays two forms are commercially available Posilac® and BoostinS®. Posilac®, developed by Monsanto, is chemically almost identical to the endogenous form, with only a distinct N-terminal end as shown in Figure 9.1. BoostinS® (Hilac), is produced by LG Life sciences and is identical to the endogenous form [174]. Until now, research was mainly focussed on Posilac®. After administration of this formulae, found rbST concentrations in the blood were lower than 10 ng mL^{-1} [175], and for milk even lower concentrations are expected.

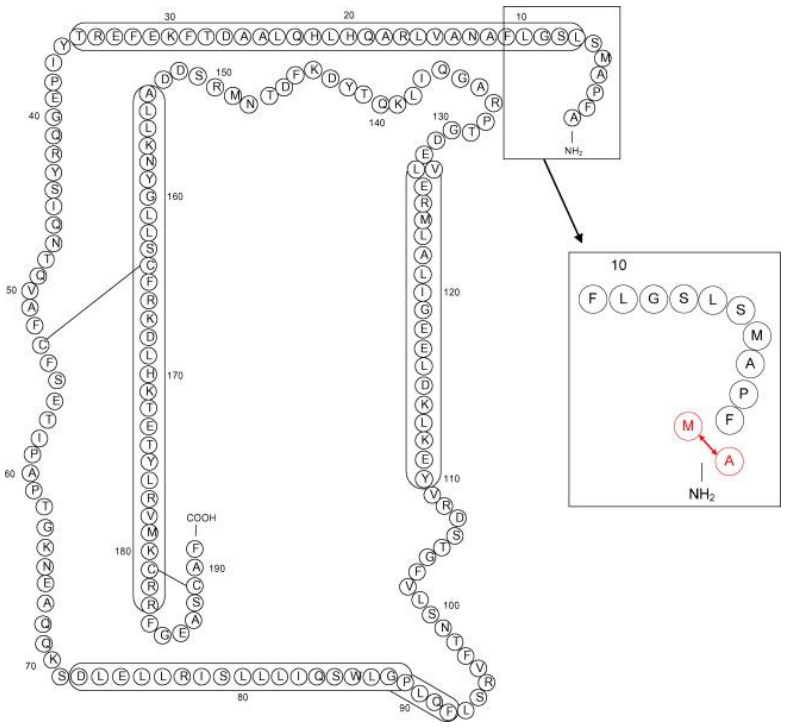


Figure 9.1. Schematic representation of the bovine growth hormone molecule with the N-terminal end in detail with the alanine (A) replaced by methionine (M) as in Monsanto recombinant rbST (Reproduced from [175].

9.2. Analytical methods

Screening methods

Several methods, compliant with the CDR (EU) 2021/808 [3], are available for indirect biomarker based detection as follows: a fourplex flow cytometric immuno-assay (FCIA) using IGF-1, IGFBP2, osteocalcin, and rbST-induced antibodies as biomarkers [168] and an automated clinical chemistry blood parameter measurement (IGF-I, urea, NEFA, insulin and cholesterol) [176]. Furthermore, also several ELISA methods for serum [177,178] and an FCIA method for milk [179] for rbST induced antibodies are available. Nowadays, research for rbST abuse detection is also focussed on gene-expression [180], this screenings technique however, is not yet compliant with the CDR (EU) 2021/808 [3].

For direct rbST detection, a competitive ELISA is described with a LOD of 66 µg L^{-1} in buffer. This ELISA is, however, not yet able to reach the required sensitivity of below 5 µg L^{-1}, needed to detect rbST in milk [181].

Confirmatory methods

Three confirmatory methods were described for serum analysis of rbST, with differentiation on the sample preparation, followed by trypsin digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements. Sample preparation used by Le Breton *et al.* [175] was a precipitation technique, Smits *et al.* [182,183] and Robert *et al.* [184] both used affinity purification techniques. Moreover, new suggestions are made to improve milk sample preparation by eliminating the micellar networks from casein in milk before digestion and LC-MS/MS measurement [185]. All methods enabled the measurement of 2 transitions of the rbST specific peptide, with this difference that Smits et al. (2015) and Robert et al. (2017) enabled to measure and confirm rbST in the serum for more than a week, whereas Breton *et al.* enabled confirmation to 4 days [175].

9.3. Research Summary

Recombinant bovine somatotropin (rbST) can be used to enhance growth and lactating performance in cattle. Within the EU, rbST is banned since 2000, Decision 1999/879/EC [186]. The ban on rbST use does require methods to control this ban; therefore, methods for determining rbST in food (products) are urgently needed. Direct rbST detection is complex due to the similarity with the endogenous hormone (bST) and the strong fluctuations of bST in serum; screening methods are focused on detecting rbST-dependent biomarkers, instead of rbST itself. As rbST-dependent biomarkers have a longer half-life, they offer a promising alternative, as reported in literature for steroid abuse and in sports doping [187,188,34,189]. Ludwig *et al.* showed that for indication of rbST abuse, a combination of only two biomarkers in serum, i.e. osteocalcin and anti-rbST antibodies, was powerful and sufficient enough to identify over 95% of the rbST-treated cows as truly positive [168]. The most specific biomarker of those two, the rbST induced antibodies, is also used for single biomarker detection in serum and milk. As the anti-rbST antibodies were also found in raw milk from rbST-treated cows. These single biomarker assays aren't sufficient enough to reach the 95 % true positive rate in serum and milk of single cows [179,178]. But when analysing tank milk samples from rbST-treated cows, which is a mixture of milk of multiple cows, over 95% true positive rate can be reached [179].

The results obtained with these screening methods need to be confirmed, to provide absolute proof of the identity of the compound of abuse. Until now two confirmatory methods for detection of rbST in blood samples, using a liquid chromatography-mass

spectrometry (LC-MS/MS) method, were described [175]. However, this method couldn't detect rbST for the full period between 2 rbST administrations. Therefore, a novel approach is needed to enable the detection of low levels of rbST in bovine serum/plasma. In human doping control, promising results are obtained for the detection of traces of peptide hormones by immunoaffinity purification prior to liquid chromatography-mass spectrometry [190]. This approach of combining immunologically based affinity purification and chemical confirmation to obtain a new confirmatory method for the detection of rbST in bovine serum was used by Smits *et al.* [183] and Robert *et al.* [184].

The ability to measure in milk samples is most desirable for both screening and confirmatory methods, as milk samples are non-invasive and easy to obtain. Moreover, the whole stable is tested at once when sampling tank milk. Developing confirmatory methods for milk is, however difficult. Smits *et al.* shows that CCα of Met-rbST in milk, 2.3 ng mL^{-1}, is much higher than in serum, 0.8 ng mL^{-1}, while lower concentrations are expected [182]. When endogenous bST is measured, a significant increase was seen in BoostinS® treated animals, with concentrations up to 15 ng mL-1. Due to its similarity with the endogenous bST, it will be challenging to prove BoostinS® use. Unfortunately, in Posilac® treated animals, rbST concentrations in milk were below CCα and undetectable.

9.4. Proposed Strategy for monitoring

Several options for detecting abuse of rbST have been suggested in the literature as described in the former paragraphs. These detection methods can be separated into two types of assays, screening assays and confirmatory assays. For monitoring purposes recommendation is to start with a screening assay, and if non-compliant results are found to, use a confirmatory method to enable pinpointing rbST abuse as also suggested by van Ginkel *et al.* [191].

EURL recommendations

- All the current research question should also take into account the more recent issue of LG-life sciences rbST (similar to natural one).
- Metabolomics research for alternative biomarkers to be used in the screening step.
- Explore the influence of E.coli infections on farms on the false positive rate in screening assays.
- Gaining more knowledge about inter-individual variations in rbST concentrations in samples from rbST treated cows.
- Obtain more knowledge about excretion curves of rbST after administration; from the slow release formulae to the bloodstream and subsequently to the milk.
- Research and validation of rbST confirmation in raw milk samples from farms/tanks, as these milk samples are easy to obtain.
- rbST detection in other matrices, for instance in milk products after processing, like milk powder and cheese.
- Getting reference standards of all commercial available used rbST's.
- Develop a test for milk using threshold values of rbST in milk using sensitive peptides obtained after tryptic digestion of rbST. In case of exceeding this threshold, follow-up action should be conducted at the farm

10. IGF-1

10.1. Introduction

Proteins and peptides with growth-promoting properties are a class of compounds that can be illegally used by athletes and farmers looking for prohibited enhancement muscle growth. In this chapter, we discuss the case of insulin-like growth factors (IGFs), as example of peptides with growth-promoting proprieties. IGFs are part of a specific class (S2), ''peptide hormones, growth factors, and related substances'', which are prohibited compounds by the World Anti-Doping Agency (WADA). These substances have a direct or indirect effect on muscle growth.

IGFs are bioactive hormones that are endogenously produced as single-chain peptides with high sequence similarity to insulin. They are part of a complex system that cells use to communicate with their physiologic environment. This system consists of two cell-surface receptors (IGF1R and IGF2R), two ligands (Insulin-like growth factor 1 (IGF-I) and Insulin-like growth factor 2 (IGF-2), a family of six high-affinity IGF-binding proteins (IGFBP-1 to IGFBP-6), as well as proteases - associated IGFBP degrading enzymes [192]. More than 99% of circulating plasma IGF-1 and -2 is associated with IGFBPs [193]. Although IGF-1 and IGF-2 are structurally related and bind to the same receptor and IGFBPs, they have different functions. For example, IGF-1 plays an important role in childhood and adult growth, and IGF-2 has a primarily effect during gestation.

Both IGF-1 and IGF-2 are biomarkers proposed for the detection of growth hormone (GH) administration and for a diseased state (e.g. breast cancer, GH-related disease) [194-196], consequently, specific methods of analysis of these compounds are in high demand. IGF-1 is mainly secreted by the liver as a result of stimulation by growth hormone. Both IGF-1 and GH levels in plasma are affected by factors such as gender and age in comparison with the circulating levels of IGF-2 which are more stable [197-199,196]. IGF-2 is less GH dependent than IGF-1, therefore, IGF-1/IGF-2 ratio can be used to detect GH misuses [196].

In particular, IGF-1 and its synthetic variants are important biomarkers for doping control due to their effects on growth hormones; therefore, their analysis is important for the detection of growth hormone and IGF-1 misuses [196]. The synthetic IGF-1 analogues (des1-3-IGF-1, R^3-IGF-1 and longR^3-IGF-1) were developed in order to hinder their association with IGFBP after administration. However, they are not approved for use and are distributed illegally on the black market or via internet [200,201,193].

In Table 10.1, the amino acid sequences of the species of interest and of the synthetic variants of IGF-1 are listed. The human, bovine, porcine and equine IGF-1 (mass = 7648 Da) are identical (see Table 10.1A). Cysteins that form disulphide bridges are highlighted in orange. Differences between these species are highlighted in green. Amino acid sequence of IGF-2 (mass=7469 Da) differs slightly for these species, between one and three amino acids (see Table 10.1B). Cysteins that form disulphide bridges are highlighted in orange. Differences between these species are marked in yellow.

Although IGF-1 and -2 are not identical, due to their high similarity, comparable detection strategies can be applied. Nevertheless, the large variation of the endogenous proteins, their high homology with the exogenous administrated variants, alteration in psychological range and fast degradation after administration makes the development of the detection strategies of these compounds challenging.

Table 10.1. A) Amino acid sequence of endogenous IGF-1 and its synthetic variants. . B) IGF-2 amino acid sequence of human, porcine, equine and bovine.

A)

Compound	Aminoacid sequence
IGF-1 human/pig horse/bovine	GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFR SCDLRRLEMYCAPLKPAKSA
Des1-3-IGF-1	ETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSC DLRRLEMYCAPLKPAKSA
R3-IGF-1	GPRTLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFR SCDLRRLEMYCAPLKPAKSA
Long-R3-IGF-1	MFPAMPLSSLFVNGPRTLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRR APQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA
Des1-10-Long-R3-IGF-1	FVNGPRTLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDEC CFRSCDLRRLEMYCAPLKPAKSA

B)

Compound	Aminoacid sequence
IGF-2 human	AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRVSRRSRGIVEECCFRSC DLALLETYCATPAKSE
IGF-2 pig	AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRNSRRSRGIVEECCFRSC DLALLETYCATPAKSE
IGF-2 horse	AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRINRRSRGIVEECCFRSC DLALLETYCATPAKSE
IGF-2 bovine	AYRPSETLCGGELVDTLQFVCGDRGFYFSRPSSRINRRSRGIVEECCFRSC DLALLETYCATPAKSE

10.2. Analytical methods

For screening and confirmation of IGFs, ligand binding assays (LBA) are preferred due to their simplicity of use and low limit of detection achieved [202]. Limitation of LBA are susceptibility to interference from IGFB proteins, the non-discriminating power between different isomers, and exogenous and endogenous proteins with high percentage of homology [203,204,194,205] possibly leading to false positive results. Mass spectrometry-based analysis gives accurate results and can also discriminates between different protein isoforms and homologues [206-208].

The combination of several biomarkers is usually necessary to provide correct clinical and doping research information. In this context, LC-MS/MS with multiple reaction monitoring (MRM) is the method of choice since this enables the quantification of a tens to hundreds

proteins in a single analysis. Mass spectrometry-based methods are specific, fast and more sensitive for screening and confirmation than the traditionally LBA based methods. Both bottom up (enzymatically digested) and to-down (intact protein) methods were developed for identification and quantification of IGFs in serum (or plasma) and urine with fully isotope labelled proteins or synthetic stable isotope labelled peptide homologues used as internal standards [209,210,197,211,200,212,213,207,208,214-219,195,196,193,220,221].

Urine vs. plasma (or serum)

IGFs were detected by LC-MS/MS techniques from both urine and plasma (or serum) samples [196]. The concentration of IGF-1 and -2 in human urine were estimated to be between 20 to 400 pg mL^{-1}, being about 1000-fold less concentrated than in plasma, 20 - 1000 ng mL^{-1} [193,190]. This low concentration represents a challenge for sample preparation, and thus most of the methods are using plasma or serum as matrix. In veterinary control, urine was considered the matrix of choice because it is easy to obtain and there are no legal restrictions for its collection; however since the concentration of many peptides and proteins is higher in serum or plasma, this analysis is becoming more accepted. In urine samples, proteins need to be concentrated due to their low amounts and because of the high amount of salts that needs to be pre-filtrated before MS analysis [193]. In plasma samples, the wide dynamic range of blood proteins requires application of extraction methods to allow analysis of the low concentrated proteins such as IGFs. Acetonitrile –ACN- precipitation, liquid-liquid extraction or solid-phase extraction (SPE) method are mostly used prior to digestion and LC-MS/MS analysis in order to extract IGFs from serum/plasma samples [222,195]. Immuno affinity methods are also used in combination with MS, in order to specifically isolate IGFs from both urine and plasma or serum samples and to improve the limit of detection [215,193,190]. As already mentioned, circulating plasma IGFs bind strongly to IGFBP therefore, different conditions were evaluated for disruption of the protein binding in plasma/serum [222,197,213,207,223].

10.3. Research summary

The LC-MS/MS methods discussed below can be used to detect abuse of IGFs or the abuse of growth hormones with IGF-1 being mostly used as one of the biomarkers for identification and/or quantification. Most of the methodologies for MS-based IGF assays presented below are targeting human applications.

Bottom-up analysis

Bottom-up approaches use specific enzymes to cleave the proteins into smaller peptides before MS analysis and it is a well-established, sensitive method for the characterization of proteins. Here we present an overview of the bottom-up methods developed for detection and quantification of IGFs. Kirsch *et al.* developed a simple LC-MS/MS method to quantify IGF-1 from plasma samples and achieved a LOQ of approximately 2000 ng/ml. In this work no sample purification was performed before tryptic digestion of the proteins [214]. The LOQ level was improved to 100 ng $mL^{-1}$when ACN precipitation was applied before tryptic digestion of the samples [208]. Both methods use heavy tryptic peptides from IGF-1 for quantification. Cox *et al.* developed a LC-MS/MS method (ACN precipitation was performed) for the quantification of serum IGF-1 in multiple laboratories and on different instrument configurations to determine the interlaboratory precision, which was found to be acceptable [212,217]. It was found that the method of calibration used in different laboratories (CV between 11% and 16 %) causes higher variability than sample preparation. Variation from the sample preparation was minimized by using ^{15}N-IGF-1 for

quantification. Rat serum was used for the calibration curve of IGF-1, since the amino acid sequences of rat and human IGF-1 are not similar and therefore, no standard addition calculations are necessary. Clinical analysis of IGF-1 and -2 by SRM LC-MS/MS showed that liquid-liquid extraction of only 7µl plasma samples is feasible, which allowed the authors to develop a throughput of 80 samples per day and good sensitivity [195]. Solid phase extraction (SPE) methods give good recoveries for IGFs, especially for IGF-2 [222,207], suggesting SPE is a better extraction technique than ACN precipitation. Immunoassay purification of the samples can further lower the detection and quantification limit of IGFs. A LOQ of 10 ng $mL^{-1}$for IGF-1 was achieved when a monolythic column activated with anti-IGF-1 antibodies fixed in pipet tips was used for sample purification [215]. Niederkofler *et al.* describes a robust method with an intra- and inter- assay precision of achieving with CVs < 10% [218]. This SRM LC-MS/MS method uses MSIA (mass spectrometric immunoassay) tips for full characterization and validation of IGF-1 from human serum samples. A LOQ of 5 ng $mL^{-1}$was achieved and the lowest limit of detection was 1 ng mL^{-1} [218]. LongR^3-IGF-1 was utilized as internal standard.

Top-down analysis

Top-down approaches provide information about the intact protein molecule and it is less-time consuming since less sample preparation is needed (i.e. no enzymatic digestion of the protein). The first initiatives for intact MS analysis of IGF-1 were reported in 2001 [224-226] .

The only method for detection of both endogenous IGFs and Long-R^3-IGF-1 (20-50 pg mL^{-1} and a precision lower than 20 %) in urine is presented by Thomas *et al.* [193], using nano-UHPLC-MS. Urinary degradation products, Des1-10- Long-R^3 –IGF-1 and Des1-3 –IGF-1, were also detected in this study. SPE combined with magnetic beads-based immunoaffinity purification was needed to acquire low detection limits. Thomas et al. also developed a qualitative method for IGF-1 and its synthetic analogues in plasma samples [220], reaching a limit of detection of 0.5 ng mL^{-1} and a precision lower than 25 %. ACN precipitation combined with immunopurification with magnetic beads was used for IGF extraction from plasma in combination with a similar LC-MS/MS approach as the one developed for the determination of IGFs in urine. Different in-vitro metabolic studies were performed for all species and in-vivo experiments for LongR^3-IGF-1 only. From the truncated metabolites detected and confirmed by in-vivo experiments with rodents, (Des1-11)-LongR^3-IGF-1, a metabolite of LongR^3-IGF-1 is the longest detectable in-vivo. A method for detection and quantification of endogenous and synthetic IGF-1 in plasma was presented by Bredehoft *et al.* [209]. An LOD=20 ng mL^{-1}, LOQ=50 ng $mL^{-1,}$ and a precision lower than 15 % was achieved using paramagnetic beads-based for immunoaffinity purification before LC-MS/MS analysis. The methods presented above used immunoprecipitation in order to reach low limits of detection. Two nice examples that combines protein purification with SPE are presented by Bystrom and Tanna. Bystrom *et al.* achieved low quantification limits of IGF-1 and -2 in human plasma (15 ng mL^{-1} and 30 mg mL^{-1}, respectively) and LOD of ng $mL^{-1}$and 8 ng mL^{-1}, respectively by coupling SPE to TOF-MS, after acid ethanol extraction [197,211]. This approach was also used by Haweks *et al.* in order to measure both IGF-1 and -2 concentrations in cord blood samples of infants and correlated this information with their growth [227]. Tanna *et al.* developed a MS method of a triple quadrupole, without immunoaffinity isolation, and reached a quantification limit of 5 ng/ml and detection limit of 1 ng $mL^{-1}$for IGF-1 with a CV lower than 15 %, in the limits of bioanalytical requirements [223]. He achieved these results by working on the optimization of both, sample preparation and chromatography. A simple preparation approach (ACN precipitation, 0.6

% SDS denaturation and mix-mode SPE) provided high recovery, >90 %. This value is much higher than the recoveries obtained by more popular acidic disruptors (approx. 40 % with 5% ac. acetic). The use of a sub-2-µm column provided a very good chromatographic separation and sharper peaks improving the sensitivity of the analysis. The expected plasma level of IGF-1 analogues after administration is estimated to be between 0.5 and 20 ng mL^{-1} [220], therefore this method is interesting for both endogens and analogous IGFs analysis due to its robustness and simplicity in combination with a limit of quantification in the ng/ml range. A nano-UHPLC MS/MS method for quantification of intact IGF-1 from small volumes of serum samples (<25 µl) by ACN sample precipitation is also established [216]. A LOQ of 50 ng mL^{-1} was reached. Pratt *et al.* also reached low LOQ for both IGF-1, 5.9 ng mL^{-1}, and for IGF-2, 8.4 ng mL^{-1}, by antibody-free LC-MS/MS and using a two-step selective protein precipitation on the plasma samples [219].

Important subjects to mention are sample collection and shipping. A less invasive approach, that quantifies IGF-1 (LOQ of 50 ng mL^{-1}) from dry blood spot (DBS) by LC-MS/MS is presented by Cox *et al.* [200]. ACN extraction was optimized in order to improve the sensitivity of the assay. This method is designed to be used for detection of GH abuse in sport. Current developments for DBS analysis of hormones are important to simplify sample collection and shipping methods.

Animals

In the last decade, the development of methods for detection of the abuse of proteins with growth-promoting proprieties was achieved for the doping control of athletes [196]. This knowledge can now also be applied for veterinary control were testing of these methods is limited or absent. In the veterinary world, the abuse of growth promoting proteins and peptides allows the enhancement of milk production [173,179]. Their use for muscle growth in cattle is speculated because of the lack of analytical methods there are no positive cases reported till now to the best of our knowledge. In horse racing the abuse of growth hormones is considered already for some time [228,229]. The IGF-1 levels in horse plasma were determined and these values are similar with the levels detected in humans. The upper limits of IGF-1 in horse were determined around 700-800 ng mL^{-1} [224,230,231] developed a method for determination and quantification of IGF-1 in horse by using LC-ESI-MS. Sample preparation consisted of acid extraction followed by immuno affinity purification. This method was tested for samples with a IGF-1 concentration between 300-900 ng mL^{-1}. A protein-transposing software was developed for quantitative determination by LC-ESI-MS. For confirmation of the results, protein sequencing with LC-ESI-MS/MS was developed.

10.4. Proposed strategy for monitoring

Several strategies for detection, identification and quantitation of IGFs were suggested in literature. Since the levels of IGFs in urine are very low, methods for plasma/serum samples are preferred.

Historically, immunoassays have been used for the quantification of IGFs. In the last years, LC-MS/MS based approaches, especially MRM, are the methods of choice for the identification and quantification of IGFs.

For identification and quantification of the endogenous hormones IGF-1 and IGF-2 in bovine serum by MRM LC-MS/MS at a level of 50 – 1000 ng/ml, a combination of disruption of IGFs from the binding protein (e.g. acetic acid), removing the high abundant proteins by protein precipitation (e.g. acetonitrile) and the reduction, alkylation and trypsin

digestion of the supernatant provides good results. Unique tryptic peptides are widely used for the quantification of several proteins in a single MRM analysis. Synthetic stable isotope labelled peptide homologues are used to assist identification and ion signal correction. Validation and quantification are performed using recombinant IGF-1 and IGF-2, via the construction of a calibration curve.

This approach [212,195] is sensitive enough to obtain quantitative information for both IGF-1 and IGF-2 in a single analysis and can provide important information for the detection of growth hormone abuse. It also has the advantage that it can be further combined with the detection of other biomarkers in plasma.

Bottom-up methods using isotope-dilution in combination with MRM of tryptic peptides are preferred, however this approach is more laborious than top-down approach. Consequently, for small proteins such as IGF, intact LC-MS/MS was also developed. The recent top-down method for identification and quantitation of IGF-1 [223] performed on a Xevo TQ-XS MS (Waters, UK) and using a sub 2 µm C_{18} column has a simple sample preparation approach: ACN precipitation, SDS denaturation followed by mix-mode SPE. With this method a limit of quantification of 5 ng mL^{-1}, a limit of detection of 1 ng $mL^{-1}$for IGF-1 and CVs lower than 15% is reached, in agreement with recommended criteria for bioanalytical quantification. This low LOQ value is promising for doping research since the analogues forms of IGF are circulating at low levels (0.5-20 ng mL^{-1}) in plasma.

Research questions for full implementation

In order to implement a strategy when finding higher IGFs values than expected from illegally administrated endogenous or analogues IGFs, research is needed in the following subjects:

- Calculation of threshold concentration for biomarkers (species, age and sex dependent).
- Validation of the LC-MS/MS method for tryptic digested IGF-1 and -2.
- Further development for establishing the appropriate type of sample preparation and LC-MS/MS method for detecting, identifying and quantifying all forms of IGFs in plasma/serum.
- Establishing a strategy to discriminate between endogenous IGF-1 and IGF-2 and illegally administered IGF-1 and IGF-2. Either by LC-IRMS or by finding a biomarker.

11. Final Remarks

This Reflection paper 2.0 describes the state of the art in 2022 for the selected compounds. Of course this is only a limited selection. There are more compounds which have an endogenous origin or are related to being formed in the animals.
See below for a table with compounds that can be present in animals not only due to administration. These compounds will be added to the Reflection paper in new updates.

Table 11.1. Compounds that can be present in animal not only due to administration.

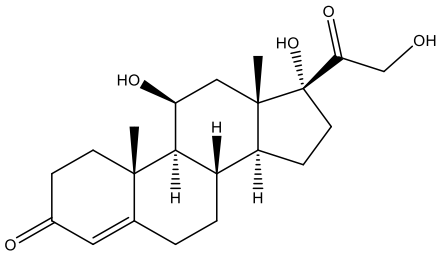
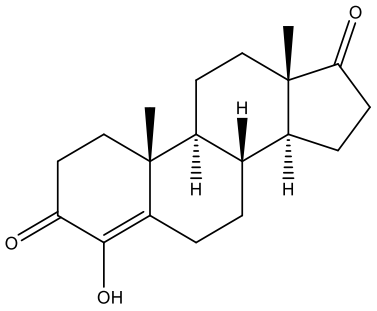
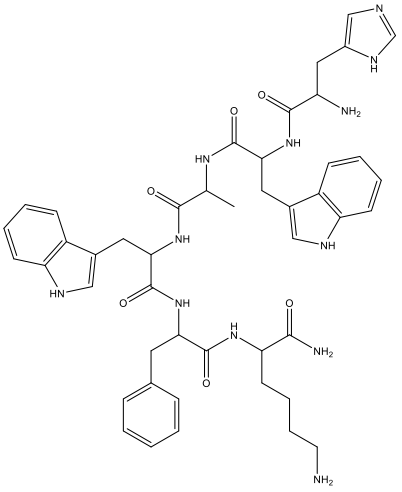
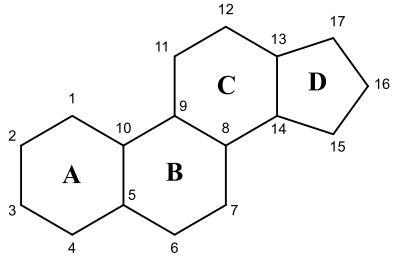
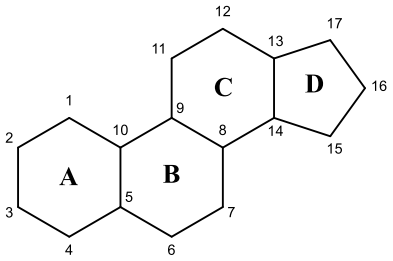
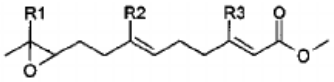
Compound	Chemical formula	Chemical structure
Cortisol	$C_{21}H_{30}O_5$	
Formestane	$C_{19}H_{26}O_3$	
Growth hormone releasing peptide	$C_{46}H_{56}N_{12}O_6$	
Pro-hormones	Steroid skeleton	

Table 11.1. (cont.) Compounds that can be present in animal not only due to administration.

Compound	Chemical formula	Chemical structure
Ecdysteroids	"similar" to the steroid skeleton	
Juvenile hormone mimics	Can be used as growth promoter during production of insects	 JH O : R1 = Et , R2 = Et , R3 = Et JH I : R1 = Et , R2 = Et , R3 = Me JH II : R1 = Et , R2 = Me , R3 = Me JH III : R1 = Me , R2 = Me , R3 = Me

Annex 1. Overview qualitative information on natural occurrence

Compound	Matrix	Natural occurrence information
17β-Testosterone	Aquaculture	Endogenous, no current criteria for discrimination
	Bovine	Endogenous, limited quantitative criteria available for serum and muscle. Currently no criteria for discrimination based on urine analyses. Some laboratories have implemented GC-cIR-MS for confirmation.
	Horses	Endogenous, no current criteria for discrimination
	Porcine	Endogenous, no current criteria for discrimination
	Poultry	Endogenous, no current criteria for discrimination
	Sheep / Goats	Endogenous, no current criteria for discrimination
17α-Testosterone	Aquaculture	No specific data
	Bovine	Endogenous, no current criteria for discrimination. Some laboratories have implemented GC-cIR-MS for confirmation.
	Horses	Endogenous, no current criteria for discrimination
	Porcine	Not likely to be found in this species
	Poultry	Endogenous, no current criteria for discrimination
	Sheep / Goats	Endogenous, no current criteria for discrimination
17β-Estradiol	Aquaculture	Endogenous, no current criteria for discrimination
	Bovine	Endogenous, limited quantitative criteria available for serum and muscle. Currently no criteria for discrimination based on urine analyses. Some laboratories have implemented GC-cIR-MS for confirmation.
	Horses	Endogenous, no current criteria for discrimination
	Porcine	Endogenous, no current criteria for discrimination
	Poultry	Endogenous, no current criteria for discrimination
	Sheep / Goats	Endogenous, no current criteria for discrimination
17α-Estradiol	Aquaculture	No specific data
	Bovine	Endogenous, no criteria for discrimination. Some laboratories have implemented GC-cIR-MS for confirmation.
	Horses	No specific data
	Porcine	Not likely to be found in this species
	Poultry	Endogenous, no current criteria for discrimination
	Sheep / Goats	Endogenous, no current criteria for discrimination

Annex 1. Overview qualitative information on natural occurrence

Compound	Matrix	Natural occurrence information
Progesterone	Aquaculture	Endogenous, no current criteria for discrimination
	Bovine	Endogenous, limited criteria for discrimination
	Horses	Endogenous, limited criteria for discrimination
	Porcine	Endogenous, limited criteria for discrimination
	Poultry	Endogenous, limited criteria for discrimination
	Sheep / Goats	Endogenous, limited criteria for discrimination
Thiouracil	Aquaculture	No specific data
	Bovine	Can be present in animal urine. When 2-thiouracil is present over the threshold of 10 µg L^{-1} check for the presence of 6-Methylthiouracil as a biomarker for treatment. Feed can be the source of 2-thiouracil due to the presence of certain food components.
	Horses	No specific data, but formation from constituents in the feed is not regarded as unlikely.
	Porcine	Can be present in animal urine. When 2-thiouracil is present over the threshold of 30 µg L^{-1} check for the presence of 6-Methylthiouracil as a biomarker for treatment. Feed can be the source of 2-thiouracil due to the presence of certain food components.
	Poultry	No specific data
	Sheep / Goats	Can be present in animal urine and feed due to the presence of certain food components.
Mercaptobenzimidazol	Aquaculture	No specific data
	Bovine	When findings of mercaptobenzimidazol in bovine urine are seen a follow up onsite visit must be conducted due to possible contamination of soil, water and feed with mercaptobenzimidaol
	Horses	When findings of mercaptobenzimidazol in bovine urine are seen a follow up onsite visits must be conducted due to possible contamination of soil, water and feed with mercaptobenzimidazol
	Porcine	When findings of mercaptobenzimidazol in bovine urine are seen a follow up onsite visits must be conducted due to possible contamination of soil, water and feed with mercaptobenzimidazol
	Poultry	No specific data
	Sheep / Goats	When findings of mercaptobenzimidazol in bovine urine are seen a follow up onsite visits must be conducted due to possible contamination of soil, water and feed with mercaptobenzimidazol

Annex 1. Overview qualitative information on natural occurrence

Compound	Matrix	Natural occurrence information
17β-Nortestosterone	Aquaculture	No specific data
	Bovine	No indications for natural occurrence except in a special situation: presence after acute injury of male bovine animals [bulls and steers].
	Horses	Endogenous in intact male animals
	Porcine	Endogenous in non-castrated male pigs. At low concentrations also in barrows and sows
	Poultry	No specific data
	Sheep / Goats	Currently under investigation
17α-Nortestosterone	Aquaculture	No specific data
	Bovine	Endogenous in very young calves and pregnant cows. after acute injury of male bovine animals [bulls and steers].
	Horses	Endogenous at low levels in female animals.
	Porcine	Not likely to be found in this species
	Poultry	No specific data
	Sheep / Goats	Endogenous at low levels in female animals.
17β-Boldenone	Aquaculture	No specific data
	Bovine	Can be of natural origin, in which case unconjugated.
	Horses	Endogenous (intact on male animals)
	Porcine	Endogenous in non-castrated male pigs. Insufficient information on barrows and sows
	Poultry	No specific data
	Sheep / Goats	Currently under investigation, but no proof of natural occurrence yet
17α-Boldenone	Aquaculture	No specific data
	Bovine	Endogenous
	Horses	No specific data
	Porcine	Not likely to be found in this species
	Poultry	No specific data
	Sheep / Goats	Endogenous, no current criteria for discrimination

Annex 1. Overview qualitative information on natural occurrence

Compound	Matrix	Natural occurrence information
Zeranol	Aquaculture	No specific data
	Bovine	Can be present due to formation via Fusarium toxin compounds. Use excel sheet to calculate origin
	Horses	No specific data
	Porcine	Can be present due to formation via Fusarium toxin compounds. Use excel sheet to calculate origin
	Poultry	No specific data
	Sheep / Goats	Can be present due to formation via Fusarium toxin compounds.
Taleranol	Aquaculture	No specific data
	Bovine	Can be present due to formation via Fusarium toxin compounds. Use excel sheet to calculate origin
	Horses	No specific data
	Porcine	Can be present due to formation via Fusarium toxin compounds. Use excel sheet to calculate origin
	Poultry	No specific data
	Sheep / Goats	Can be present due to formation via Fusarium toxin compounds.
Prednisolone	Aquaculture	No specific data
	Bovine	Can be present
	Horses	Can be present
	Porcine	Can be present
	Poultry	No specific data
	Sheep / Goats	No specific data
Recombinant somatropine	Aquaculture	No specific data
	Bovine	Limited data present
	Horses	No specific data
	Porcine	No specific data
	Poultry	No specific data
	Sheep / Goats	No-specific data
IGF-1	Aquaculture	No specific data
	Bovine	Can be present
	Horses	Can be present
	Porcine	Can be present
	Poultry	No specific data
	Sheep / Goats	No specific data

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