Residues from anabolic agents in food-producing animals: a focused evaluation of “semi-natural” occurrence of hormonal active compounds

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ABSTRACT

In recent years, a development of techniques and methods for detecting the presence of residues of substances with strong pharmacological actions in several animal matrices is carrying out. Additionally, an improvement of the analytical performances has allowed to achieve lower decision limits and capability of detection values, resulting in the ability to analyse the presence of analytes in extremely low concentrations (in the order of parts per trillion, ppt).

The increase of the sensitivity permitted to detect the presence of substances that, under certain concentration levels, could be considered endogenous, even if they were defined only synthetically produced.

Therefore, the analysis of substances that could be recognised as having a dual nature, both endogenous and exogenous, represents a challenge for researchers involved in the residue analysis. The presence of these substances in urine of animals (especially bovine, horses and pigs) could be a consequence of an illicit treatment, but also a result of endogenous production due to different factors, as stress, faecal contamination, ingestion of food containing precursors of the substances to be investigated.

Currently, a debate on the origin in European of different analytes including, in particular, boldenone, prednisolone and the thiouracil, is ongoing.
The use of these substances in zootechnical field for illicit purposes of treatment in order to obtain an increase of the animal’s weight, is prohibited by European legislation because the presence of residues represent a risk for the consumers health.

Despite the severe European regulations for the prohibition of the use of substances for fattening purposes in breeding, several cases of positive animals to these substances have been reported. However, considering the high number of non-compliant samples, a hypothesis of abuse would be difficult to formulate. Consequently, the natural or endogenous origin was suggested for these substances.

This thesis has the main aim to investigate the endogenous nature of two well-known semi-natural substances: boldenone, an anabolic steroid in urine of bovine, prednisolone in feedstuff for bovine and in urine and adrenal glands of pigs. These two steroids share a structurally analogy because both of them respectively differ from the most common steroid, testosterone and the corticosteroid cortisol for only one double bond in the first ring of the structure.

The first work is focused on the development and validation of an analytical method for the direct determination of androstadienedione, free forms and conjugated forms of boldenone, whose is followed by a work performed in collaboration with the University of Torino (with the participation of the group of prof. Biolatti) to check the endogenous presence of
β-boldenone in urine of young bulls by the investigation of phase II metabolites (glucuronide and sulfate) under particular environmental conditions of stress. Surprisingly, no positive samples were detected in the most intense level of stress that was at the slaughterhouse. The study confirms the endogenous production of boldenone glucuronide and sulfate in the collection time corresponding to a low or zero level of stress (at the farm).

The hypotheses that try to explain the presence of semi-natural prednisolone in animals, as cattle, referring to an endogenous or a neo-formation ex vivo in urine of animals contaminated with faeces. However, the potential ingestion of feed containing prednisolone should be taken into account. Considering the limited literature, we wanted to analyse different commercially available complementary feedstuff for husbandry, stored in both farm and in controlled conditions in the laboratory to observe if the presence of the corticosteroid of our interest in the feed samples was due to environmental conditions of storage. Data obtained from our study confirm the crucial role of storage prednisolone was detected in almost all (95%) feedstuffs collected at the farm.

The presence of prednisolone in urine, mainly due to stress, has been demonstrated in cattle, in horses and humans, but only one paper is available regarding pig. The fourth study of the present thesis has the purpose to detect the presence of prednisolone in urine of pigs collected at the farm and at the
slaughterhouse, where the adrenal glands were also sampled. Additionally, the presence of prednisolone was considered related to particular condition of stress for the animals. A second objective of the work was to verify a possible illegal treatment due to the presence of corticosteroids. The trial, funded by Regione Lombardia, has provided for the evaluation of the difference in the frequency of detection and concentration of corticosteroids in urine of animals at the farm compared to the urine of the same animals at the slaughterhouse.

The work confirmed the presence of endogenous prednisolone in pigs, since found in most of the adrenal glands. Moreover, there was a relationship between the presence of prednisolone and cortisol in urine directly related to the intensity of the stress. In fact, the highest percentage of positivity to prednisolone in urine was collected at the time of slaughter. In the study, only one sample was considered suspicious for abuse prednisolone, given the high concentration in the urine sample.

A further problem discussed in European laboratories regards the determination of cut-off levels of thyreostatic substances in urine and thyroids of bovine in order to define the natural origin.

The analysis of thyreostats both in the thyroid and urine of bovine and the comparison of the results obtained in the two matrices could be a good way to satisfy this need. Therefore, the last part of the thesis concerns the development of a method for the
determination of five thyreostats: thiouracil, methylthiouracil, propylthiouracil phenylthiouracil and tapazole.

Several methods have been published regarding the extraction procedures for thyreostats, but a derivatisation of the analytes prior to extraction, resulting in a possible loss of analytes, is generally performed. The methods developed and proposed in this thesis, allows the direct extraction of thyreostats without a derivatisation step and, since the extraction methods for the two matrices are very similar, allow a comparison between the results obtained in urine and thyroid glands. Therefore, the work carried out could be a great tool useful not only in the context of monitoring study, but also for research studies concerning the natural production of thyreostats.
RIASSUNTO

Negli ultimi anni si è osservato uno sviluppo delle tecniche e metodologie per la ricerca di residui di sostanze ad azione farmacologica in diverse matrici animali. Il miglioramento delle performances analitiche ha permesso di raggiungere bassi livelli di rivelazione, quindi la possibilità di analizzare la presenza di analiti a concentrazioni estremamente basse (nell’ordine di parti per trilione, ppt). L’abbassamento dei limiti di sensibilità ha permesso di evidenziare la presenza di sostanze che sotto determinati livelli di concentrazione possono essere considerate endogene. Una sfida per coloro che si occupano di analisi di residui è quindi la ricerca di sostanze che possiedono una duplice natura e, che sotto certe condizioni, possono essere ritenute endogene o possono essere considerate semi-naturali perché prodotte dall’animale in seguito a vari fattori come ingestione di alimenti contenenti precursori delle sostanze da indagare.

Attualmente, è in corso un dibattito a livello europeo sull’origine di diversi analiti, tra i quali, in particolare, il boldenone, il prednisolone e il tiouracile. L’uso di tali sostanze in campo zootecnico per scopi illeciti di trattamento, al fine di ottenere un aumento del peso degli animali, è vietato dalla legislazione europea poiché i residui, presenti nei prodotti di origine animali, rappresentano un rischio per la salute del consumatore. Nonostante la ferrea regolamentazione europea per
il divieto in allevamento dell’uso di sostanze a scopi auxinici, diversi casi di animali positivi a tali sostanze sono stati riportati ma, considerato l’elevato numero di positività tale da non poter collegare la presenza della sostanza a un abuso, è stata proposta l’ipotesi di origine endogena o semi-naturale.

La presente tesi vuole andare ad indagare la natura endogena o semi-naturale di due note sostanze: il boldenone, steroide anabolizzante, nell’urina di vitellone da carne; il prednisolone nell’urina e ghiandole surrenali del suini. entrambi steroidi che differiscono dai più noti testosterone e cortisolo per la presenza di un solo doppio legame nel primo anello della loro struttura.

Il primo lavoro verte sullo sviluppo e validazione di un metodo analitico per la diretta determinazione di boldenone coniugato e libero e androstadienedione, a cui segue un lavoro realizzato in collaborazione con l’università di Torino con la partecipazione del gruppo del prof. Biolatti, per verificare la produzione endogena di β-boldenone attraverso la determinazione dei metaboliti di II fase, glucuronato e solfato, in urina di vitelloni sottoposti a particolari condizioni ambientali di stress. Lo studio condotto ha confermato la presenza endogena dei coniugati del boldenone in condizioni di assente o relativamente basso stress, mentre non è stata osservata alcuna positività al boldenone solfato e glucuronato nei campioni prelevati al macello, quando gli
animali sono sottoposti ad elevati livelli di stress dovuto alle diverse procedure che anticipano la macellazione.

Le ipotesi che cercano di spiegare la presenza seminaturale di prednisolone in animali come i bovini, fanno riferimento ad una produzione endogena o una neo-formazione ex vivo nelle urine di animali contaminate dalle feci. Tuttavia, anche la possibile ingestione di mangime contenente prednisolone dovrebbe essere considerata. Considerate le scarse informazioni al riguardo, abbiamo voluto analizzare diversi mangimi complementari disponibili in commercio e conservati sia in azienda agricola che in condizioni controllate in laboratorio per verificare se l’eventuale presenza di steroide nel mangime fosse dovuta alle condizioni ambientali di conservazione I dati ottenuti confermano il ruolo determinante delle modalità di conservazione poiché la quasi totalità di campioni risultata positiva al prednisolone è stata campionata in azienda.

Ad oggi, la presenza di prednisolone nelle urine come esito di produzione endogena o correlata a una condizione di stress, è stata dimostrata nel bovino, nel cavallo e nell’uomo, ma scarse informazioni si hanno relativamente al suino. Il quarto lavoro della presente tesi riguarda la determinazione della presenza di prednisolone in urine di suino prelevate in allevamento ed in urine e ghiandole surrenali campionate in macello e di stabilire se la presenza di prednisolone fosse correlata a condizioni di stress per l’animale o a un trattamento illecito. Un secondo
obiettivo del lavoro svolto è stato quello di verificare un possibile trattamento illecito dovuto a presenza di corticosteroidi. La sperimentazione, finanziata dalla Regione Lombardia, ha previsto la valutazione della differenza nella frequenza di ritrovamento e nella concentrazione del corticosteroide nelle urine di animali in allevamento rispetto alle urine degli stessi animali al macello. Il lavoro ha confermato la presenza endogena di prednisolone nel suino, in quanto riscontrato nella maggioranza delle ghiandole surrenali. Inoltre, è stata osservata una relazione fra presenza di prednisolone e cortisolo in urina direttamente correlata alla intensità della stress. Infatti, la percentuale maggiore di positività al prednisolone nelle urine è stata verificata in sede di macello. Nello studio, il sospetto di un eventuale abuso prednisolone è stato avanzato solo per un animale a causa dell’elevata concentrazione dell’analita nel campione di urina.

Un ulteriore problema dibattuto nei laboratori europei riguarda la determinazione di un livello di cut-off del tiouracile in urina e tiroidi per definirne l’origine naturale. Verificare la presenza di tireostatici nella tiroide dei bovini e confrontare i risultati ottenuti mediante l’analisi delle urine, potrebbe essere un buon metodo per rispondere a questa esigenza. Pertanto, l’ultima parte della tesi riguarda lo sviluppo di una metodica per la determinazione di cinque tireostatici, tiouracile, propiltiouracile, metiltiouracile, feniltiouracile e tapazolo.
Diversi metodi sono stati pubblicati riguardo le modalità di estrazione dei tireostatici, ma è pratica comune eseguire una derivatizzazione degli analiti prima della loro estrazione, comportando una possibile perdita degli analiti stessi. Il metodo che è stato sviluppato e inserito nella presente tesi, permette l’estrazione diretta di tireostatici senza condurre una derivatizzazione e permette un confronto fra i risultati ottenuti con i due metodi. Quindi, il lavoro condotto potrebbe essere un ottimo strumento non solo in un contesto di monitoraggio, ma anche per le ricerche incentrate sull'origine naturale di tireostatici.
CHAPTER 1

Introduction
1.1 Food Safety in livestock

News on the quality of the meat on the market seem to confirm a negative role of the globalisation on several production fields, and a condition of risk for the consumer’s health. The public awareness regarding problems of food, breeding and treatment of the animals is now arising; therefore, when on March 17, 2015 several Italian newspapers have reported the following news, no one was surprised: “from early morning hours, an operation of N.A.S. (Nucleo Anti Sofisticazione) in various intensive farming of dairy cows of Northern Italy is ongoing. People under investigation are considered responsible for the illegal trade and use for animal breeding of veterinary drugs. A total amount of 55 kg of illegal drugs was found. In 2014, a first investigation had already thrown light on a spread illicit trading of bovine growth hormone or, best known as somatotropin, from non-European countries and sold to breeders of dairy cattle along with other veterinary drugs derived from the illegal market and illegally introduced in Italy. The aim to use this kind of substances was to obtain an increase of 20% in milk production. Consequently, it means a prosecution for falsification and sale of adulterated food substances. The Financial Police raided the stables where doping substances were administered, seizing 845 veal calves, mainly of the Belgian Blue breed. The Financial Police found bottles containing 17α-oestradiol benzoate, an anabolic and substance
with carcinogenic activity. Bovine urine samples analysed resulted non-compliant for the presence of the anabolic compound above cited. After slaughter, histological analyses were also performed. The results showed the presence of metaplasia in the sexual glands. Finally, drugs without the obligatory approval were found. The payment of a penalty of 109 000 € for violation of regulations relating to pharmacovigilance has been imposed to the farmers” [1]. Despite the cited news, according to the report published in June 25, 2015 by the Italian Ministry of Health, almost all bovine samples analysed for the routine control were compliant. Out of 40 806 samples tested according the requirement of the NRP (National Residual Plan), only 44 samples showed irregularities. 16 276 out of 40 806 samples were analysed for the detection of residues of unauthorized substances and only a percentage of 0.11% resulted non-compliant. Of these, 15 are resulted non-compliant for the presence of residues of category A of unauthorized compounds (34.1 %) and 29 for the detection of residues of substances in category B of Veterinary drugs and contaminants (65.9%) [2].

However, the Italian husbandry should not be criminalized in total, because the conditions of foreign breeding are often more extreme than the Italian ones. In the United States, for example, the use of growth promoters is allowed and the treatment are commonly made [3, 4]. It is well known the elevated costs for breeding could cause difficulties to the farmers, who do
not consider the animal as only a machine producing milk and meat, and they do not want to give “junk food” to the consumers.

In order to guarantee the farmers, the animal’s heath and, in particular, the consumer’s health, it is necessary to apply a sophisticated system of Food Safety, because the research of chemicals and hormones in meat or in another matrices, as the substances identified in the trafficking reported, are very difficult due to the number and differences of active compounds to investigate. Today, questions of Food Safety are at the centre of the International and Community scientific debate, because substances, such as veterinary drugs, are ones of the most important chemicals hazard in food of animal origin. Veterinary drugs, as other chemicals, are potentially dangerous based on their degree of pharmacological activity and capability of remain in foods as residue. The possible accumulation of these drugs or their metabolites in edible tissues may establish and increase the risk of exposure for consumers \[5\].

De Brabander has given a clearer, but generic explanation of residue in a detailed review, “a residue is a trace of a substance, present in a matrix after some kind of administration” \[6\]. More precisely, Codex Alimentarius Commission defined the residue as following: “Residues of veterinary drugs include the parent compounds and/or their metabolites in any edible portion of the animal product, and include residues of associated impurities of the veterinary drug concerned” \[7\].
Nevertheless, there are some endogenous substances with a threshold level due to their natural origin, as the corticosteroids, and other molecules, whose exogenous origin is now into question and are complicated challenge for the Health Authorities [8].

As established by the famous sociologist Ulrich Beck, the modern society is a risk society that contributes to the development and making of a global risk. The role of every Member States and, particularly of Health Authorities, is to manage and, possibly, decrease the risks existing at the various levels [9, 10].

In this context, the Food Safety is becoming one of the most important questions to be addressed. It can be divided into to the categories of Food Safety and Food Security, on a matter of supplying and food self-sufficiency of the company or of national communities that invoke. Food Safety is an umbrella term that encompasses many facets of handling, preparation and storage of food to prevent illness and injury. Included under the umbrella are chemical, microphysical and microbiological aspects of Food Safety. Food Security has been defined by the Food and Agriculture Organization (FAO, 2003) of the United Nations (UN) as; "Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life. Household food security is the application of this concept to the family level, with individuals within households as the focus of concern" [11].
In its turn, Food Safety can be subdivided into many different levels, whose one of the most important is the Risk Analysis, defined for the purposes of the Codex Alimentarius Commission as "A process consisting of three components: risk management, risk assessment and risk communication" [12-15].

The use of hormonal growth promoters in food-producing animals evokes fears and is dangerous for human health. More information and a better knowledge on human health risks are essential and necessary to establish regulation on the use of drugs and control programs. Risk assessments and management, with their plethora of levels and components, as hazard identifications, hazard characterizations, exposure assessments, and risk characterizations, play a major role in order to guarantee of Food Security and Food Safety and, at the end, to protect and preserve public health [5].

A new challenge for the scientist is to evaluate the so-called pseudoendogenous or semi-natural substances or the “grey-area substances” that have double nature of endogenous but also exogenous substances. The task of the scientist is to detect the abuse of these substances, known to be endogenous in certain conditions. One difficulty arises from the fact that when they are defined as products naturally in a particular type of animal, a simple qualitative evidence of their presence does not necessarily prove the abuse.

Furthermore, a threshold of these compounds should be
established with the aim to distinguish a condition of illicit treatment from a natural production, depending on the sensibility and specificity of the instruments by which the analyses are performed [8].

References


2. Ministero Della Salute; Dipartimento Della Sanità Pubblica Veterinaria, Della Sicurezza Alimentare E Degli Organi Collegiali Per La Tutela Della Salute; Direzione Generale Per L’igiene E La Sicurezza Degli Alimenti E La Nutrizione; Piano Nazionale Per La Ricerca Di Residui, Relazione Finale Anno 2014. Available online on: http://www.salute.gov.it/portale/documentazione/p6_2_2_1.jsp?lingua=italiano&id=2377

3. http://www.fda.gov/AnimalVeterinary/SafetyHealth/ProductSafetyInformation/ucm055436.htm


1.2 Anabolic Androgenic Steroids

The term anabolism originates from Greek ἀνά "upward" and βάλλειν "to throw" and it is a metabolic pathway by which the formation of the molecules is achieved. More specifically, the anabolism is chemically defined as any state in which nitrogen is differentially taken in slender body mass by stimulation of protein synthesis or decreased breakdown of protein in several parts of the body [1,2].

Testosterone (T) is the principal steroid hormone expressing anabolic proprieties and all substances having steroidal structure, similar for its anabolic features and derived from T are known as Anabolic Steroids [2]. The ability to influence the development of secondary sexual characteristics permits to define them also as Anabolic Androgenic Steroids (AAS) [3].

“Play true” is the motto adopted by the World Anti-Doping Agency (WADA) for the awareness campaign against the using of doping of banned athletic performance-enhancing drugs in sports. However, the abuse of these drugs is widely and covertly spread and it is not only a prerogative for the ironman athletes in any disciplines of top-class sports, but it also common in the smallest gym, widespread from young people to seniors. Among the prohibited chemicals substances, included in the WADA Prohibited List 2015, the AAS, collectively classified as “appearance and performance enhancing drugs” (APEDs) are in
the main position by relevance \cite{4,5}.

1.2.1. Chemistry and biosynthesis

The AAS have lipophilic characteristics and low-molecular weight. Steroids share a basic structure, consisting of a skeleton nucleus, cyclopetanoperhydrophenanthrene that derived by the fusion of four rings from the parental compound cholesterol, as reported in Figure 1.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Chemical structure of cyclopetanoperhydrophenanthrene (A) and cholesterol (B).}
\end{figure}

In males, T is the main circulating androgen hormone and it is mostly produced by the Leydig cells of the testis, and less than 10\% is synthesized in the zona reticularis of the adrenal cortex. In female, the course of production of T is principally achieved in the cells of the adrenal cortex and less in ovaries and placenta. T is a C19 steroid with an \textit{–OH} group in the 17 position; its chemical structure is shown in Figure 2 \cite{3}.
Figure I-2. Chemical structure of testosterone.

In blood, T is reversibly bound to two plasma proteins, the sex hormone–binding globulin (SHBG), wherewith has a high-affinity steroid-specific interaction (65%), and with low affinity to the albumin. About 2% of T remains free and available for the interaction with receptor cells.

The biosynthetic pathway of T (Figure 3), as the other steroid hormones, required cholesterol whose side chain is cleaved by 11-α and 21-α hydroxylases of inner membrane, i.e. the mitochondrial cytochrome P450 enzyme (CYP11A), in order to form pregnenolone with the loss of six carbon atoms. Then, pregnenolone is hydroxylated in its 17 position and the cleavage of the side chain gives dehydroepiandrosterone (DHEA). DHEA can be peripherally converted to androstenedione (AED) (oxidation of 3-OH), T, and dihydrotestosterone (DHT), and aromatized to oestrogen. [3].
Figure I-3. Pathway of testicular testosterone synthesis.

1.2.2. Hypothalamus-Pituitary-Gonadal Axis

The control and the regulation of the production and secretion of T is carried out through an active feedback interaction
among the hypothalamus, pituitary, and testis, called Hypothalamus-Pituitary-Gonadal Axis, reported in Figure 4.

Figure I-4. Steroids regulation pathway.

The gonadotropin-releasing hormone (GnRH), synthesized by the hypothalamus, is released in a pulsatile way into the hypothalamic-pituitary portal system and stimulates the release of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropins, henceforth, influence the cells of the testicles to regulate T synthesis and spermatogenesis, respectively. A negative feedback is carried out at both the hypothalamic and the pituitary levels when there is an increase of T levels in serum. In this circumstance,
the T is converted to DHT and aromatized into oestradiol in the hypothalamus. Therefore, both androgens and oestrogens steroids can influence and modulate the release of the GnRH at the two levels, both at the hypothalamus and at the pituitary levels. Particularly in testis, another protein hormone, known as inhibin, is produced. This protein modulates in a negative manner the FSH secretion and it is present in ovarian follicles of female. In female, LH stimulates, together with prolactin, ovulation and the conversion of the ovarian follicle into the corpus luteum. Indeed, during the development of the follicle, LH stimulates T and it is inhibited by the oestradiol and progesterone secretion \[^3\].

### 1.2.3. Mechanism of Action

T exerts its action by the binding to an intracellular receptor. This complex binds to specific site of sequence of DNA in the nucleus, enabling the transcription of androgen-responsive genes; then a 5α-reductase converts T to DHT in target cells. There is also DHT circulating in the blood that is about 10% of the T level. After the conversion to DHT, the hormone binds to a receptor protein in the nucleus and this complex is activated and binds receptor on the nuclear matrix, causing a chain of reactions
as protein synthesis and cellular metabolism \cite{3, 6}.

### 1.2.4. Metabolism, degradation and excretion of androgens

The metabolism of AAS occurs in two phases, I and II in order to convert them into more polar molecules and to simplify their elimination. In the Phase I reactions, steroids are changed into more polar compounds from the body by enzymatically catalysed reactions (e.g., oxidation, reduction, or hydroxylation), while Phase II reactions or conjugation reactions, involve the addiction to the anabolic steroid or its metabolite of glucuronic acid or sulfate moiety \cite{7}. The elimination of plasma T occurs principally in the liver, where it is rapidly metabolised into androstenedione (AED) and dehydroepiandrosterone (DHEA) and conjugated as water-soluble compounds either glucuronides or sulfates (glucuronides, particularly) and then excreted by the kidney as the urinary 17-ketosteroids or into the gut by way of the liver and bile \cite{3}.

### 1.2.5. Anabolic androgen steroid actions

T and other androgen steroids mainly exert two effects on the organism, the anabolic and the virilising effects, but they also
exert an inhibitory feedback effect on pituitary LH secretion. The AAS actions are below summarised (Figure 5):

- **Androgenic effects** - Androgens permits the formation of secondary sex characteristics, including changes in hair distribution, body configuration, and genital size that develop in boys at puberty.

- **Anabolic Effects** - Androgens cause masculinising and increase of muscle mass trough an increase of the protein anabolism, and increased bone density and strength, and stimulation of linear growth and bone maturation. Secondary to their anabolic effects, androgens cause modest electrolyte retention [7].

![Figure I-5. Effects of anabolic androgenic steroids.](image-url)
1.2.6. Anabolic steroid in livestock and legislation aspects

A leitmotiv of the Food Safety is to guarantee the consumer’s health through a monitoring “from the farm to fork”, or the entire food chain. However, socio-economic reasons, correlated to the high costs of the livestock management, could contribute to enlarge the use of additives, as vitamins, antibiotics or steroids, added to animal food in order to improve breeding efficiency, and guarantee a production in concordance with the market requirements. Moreover, it is true that the abuse of these substances in the black market might not be excluded. Residues of chemicals might be present in edible matrices and exert dangerous actions of different levels of toxicity, hence representing a health hazard.

In 1977, breast augmentation in young girls and boys in an Italian school was reported as consequence of contaminated meals with oestrogens. This event put attention on the health hazards correlated to the residues of drugs in products of animal origins and generated interest for the drafting of severe rules concerning the regulatory and, consequently, prohibition of the treatment with anabolic substances [8].

In 1980, an amount of 30 000 jars of baby food was found positive for the presence of diethylstilboestrol due to the treatment of French veal calves. At the beginning of the 80s, the EC Commission proposed strictly stance concerning the hormones,
which should have been completely forbidden, except for these compounds used for therapeutic aims [9].

Therefore, with the purpose to ensure the health of the consumer, in conformity with the Directive 81/602/EEC, the European Union fixed the prohibition of the use of any growth promoter compounds, i.e. anabolic steroids from meat production in 1981 with a “zero tolerance” limit [10]. Among the substances are including 17β-oestradiol, T and esters, progesterone, zeranol, trenbolone, trenbolone acetate and melengestrol acetate. In compliance with the directive, the treatment of farm animals with substances express anti-thyroid activity and oestrogenic, androgenic or gestagenic action, the marketing of meat from treated animals and their slaughter are forbidden.

The Directive 81/851/EEC has laid down common requirements for manufacturing and marketing authorisation, based on the evaluation of the quality, safety and efficacy of the product. Moreover, it required that only official veterinarian could administrate drugs for therapeutic treatments that have to be registered by the veterinarian responsible [11].

The prohibitions on the use of such substances, on the trade of treated animals within the EU, and on the import from third countries was set in 1988 and then spread out to all EU member States, and to meat imported from other States [12, 13]. Directive 96/22/EC of 29 April 1996 (concerning the prohibition on the use in stockfarming of certain substances having a hormonal
or thyreostatic action and of β-agonists) confirmed the ban use of steroidal hormones with promoting growth purpose in animal husbandry. The Council Directive 96/23/EC of 29 April 1996 (on measures to monitor certain substances and residues thereof in live animals and animal products) completely prohibited steroidal hormones as anabolic androgenic substances, including them into the A3 section (Anabolic Androgenic Steroids) as regards the production of animal origin and the livestock. The Category A includes substances with anabolic effect and unauthorized substances for the treatment of the animals in order to induce an increase animal weight and the group involves the “no maximum residue limit (MRL)” substances. In the Group A, substances are divided into four major groups: anabolic steroids, thyreostats, β-agonists or repartitioning agents and Annex IV substances. Group B encloses the veterinary drugs or veterinary medicinal products: antibacterial substances, anthelmintics, coccidiostats, carbamates and pyrethroids, sedatives, non-steroidal anti-inflammatory drugs and other pharmacologically active substances [14, 15].

In 2003, the European Communities amended the Directive 96/22/EC, approving the Directive 2003/74/EC regarding the definitely prohibition of the use of hormones in livestock [16].

Regarding the individual States, already before 1981, the use of hormones had been banned. In Italy, the legislation forbidden their use since 1961, in Denmark since 1963, while
Belgium and Greece had never permitted the use of hormones for fattening aims. Otherwise, countries outside Europe, as the in the United States, Canada, New Zeland, Australia, in some states of South America and Africa, substances having the effects to improve weight gain and feed efficiency in livestock farming are allowed. Particularly, United States, permit the use of steroid hormones, as testosterone, 17ß-oestradiol and progesterone, and synthetic hormone-like trenbolone, zeranol and melengestrol acetate. In United States breeding is approved their use as active component of solid ear implants (17ß-oestradiol in the ester form of benzoate, testosterone - as propionate, progesterone, trenbolone acetate and zeranol) and as feed additives, melengestrol acetate (MGA) for feedlot heifers \(^{[9, 17]}\).

1.2.7. Administration of hormones in cattle

The hormones are characterized by low bioavailability when administered orally; then in the body, they are rapidly conjugated and undergo to metabolic transformation in the liver. Generally, hormones are administered by subcutaneous implantation near the ears (that are discarded in slaughter), which permit to avoid the possibility of the presence of their residues in edible tissues. The subcutaneous implantation is consisting of continuously releasing steroid pellets in the ear (or less frequently, the dewlap) in order to guarantee a gradual and slow release of a fixed dose of
drug. The treatment can be carried out by using either free form or esters forms of steroids; in particular, steroids are usually administered as synthetic ester, mostly as propionic or benzoic acid, but also undecanoate or undecylenate. Esterification normally determines an increase of the half-life of the steroids by 40 to 50% \cite{9, 18}.

A problem in the detection of the synthetic steroid is that they are quickly hydrolysed \textit{in vivo} into natural steroids, and in urine, the detectable metabolites of synthetic steroid are the same of the natural ones \cite{19}. In cattle, the use of hormones is done to veal calves and beef (steers and heifers). The treatment with steroids could be performed with a single steroid or in combination with other hormones in order to improve the efficiency of the steroid \cite{9}.

\textbf{1.2.8. Boldenone: endogenous or exogenous? This is the problem}

In the European Union, the use of steroid hormones as growth promoters for animals is strictly prohibited, as mentioned above. Since the early 80s, several methods have been developed in order to detect the presence of residues of these substances in meat and other matrices with particular attention to the xenobiotic drugs and to anabolic substances having a urinary threshold level due to their natural origin or to drugs permitted only for
therapeutically treatment, as corticosteroids. This plethora of substances poses a challenge for researches and casts doubt on the origin of different substances that Wim Van Thuyne defined in his Ph.D. thesis as “grey-zone substances” [20]. This concept was well defined in a clear review published in 2009 by Scarth et al., where the Authors explained that the task is to identify the illicit abuse of exogenous hormones, synthetically produced, that “are also known to be endogenous under certain conditions, dubbed “pseudo-endogenous” or “grey-zone substances” due to their dual synthetic/endogenous nature”. Based on these considerations, certain elements of discussion on the existence of natural compounds, previously considered as only xenobiotic substances, could be laid. As indicated by the Authors, most of the steroid preparations consist in esters of endogenous steroids that are quickly hydrolysed in urine or plasma to the free endogenous forms and only for few exceptions (hair and injection/implant sites), the direct detection as proof of abuse of these esters compound is possible and can confirm an illicit treatment. However, “Where particular steroids are believed not to be endogenous in an animal at a particular limit of detection (LOD), who is to say that as analytical limits decrease, they will not be discovered as endogenous at a lower concentration?” [21]. The case of boldenone, as well as other 1-dehydro steroids (i.e. 17β-
19-nortestosterone or nandrolone, prednisolone) are perfect examples of this problem.

1.2.9. Chemistry of boldenone

Boldenone (1-dehydrotestosterone or (17β)-17-Hydroxyandrosta-1,4-dien-3-one) or 17β-boldenone (β-bold) is one of the most famous anabolic steroid on the market, known for its anabolic properties, is common used in different preparations as ester forms by athletes to increase muscle mass and improve sports performance, but it is prohibited in sports [22, 23].

β-bold is structurally different from T (androsta-4-ene-17β-ol-3-one) for the dehydrogenation of the carbon in the first position. The chemical structure of the analyte, including the structure of its epimer, 17α-boldenone (or α-bold,) are shown in Figure 6 [23].

![Figure I-6. Chemical structure of α -and β-boldenone.](image-url)
β-bold was synthesised for the first time in 1956 by the dehydrogenation of T using selenium dioxide \[^{[24]}\]. It is commercially available as ready-to-use anabolic preparations either for human, horse or cattle particularly by injection as ester forms, whose the principal is the undecylenate (or undecanoate), but also orally as boldione or Androstadienedione (androsta-1,4-diene-3,17-dione, ADD), the oxidised precursor of boldenone. Examples of boldenone undecylenate used in medical treatments for veterinary use are the following: the Equipoise for horses (Equipoise®), cattle and dogs for Ganabol® Venobol® \[^{[25]}\].

Like the other androgenic steroids, β-bold is classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen, with a carcinogenicity index higher than that of other androgens, such as nandrolone, stanozolol, T \[^{[26]}\]. A recent study has also demonstrated the role of α-bold in the development of human prostate carcinomas implanted in mice \[^{[27]}\].

**1.2.10. Endogenous presence of boldenone**

Despite the European legislation avoids the use of anabolic steroids as boldenone, an illicit administration in order to increase live weight gain, reduce the feed conversion ratio and the trade in the black market of the drugs, results difficult to control, and prevent. Samples positive for the presence of boldenone were
considered as a proof of illicit treatment for a long time because of the only exogenous deemed identity.

Nevertheless, a gradual increase in the findings of samples containing boldenone in different States of the European Union doubted the purely exogenous origin of the drug and gave rise to the question concerning a possible endogenous production or natural presence in urine of boldenone [23].

In 1996, the endogenous nature of \( \alpha \)-bold was reported by Arts et al. The Authors found a percentage of 25% out of a total of 50 urine samples of untreated veal calves, characterised by the natural presence of \( \alpha \)-bold at levels exceeded 1 ng mL\(^{-1} \) (between 0.1 and 2.7 ng mL\(^{-1} \)), while \( \beta \)-bold were always found at a concentration lower than 0.1 ng mL\(^{-1} \) [28].

Since then, a scientific debate about the origin of boldenone is ongoing. Several studies were performed not only to explain the presence of boldenone in urine of untreated cattle, but also to define possible metabolites that could be helpful markers to identify an abuse of \( \beta \)-bold and establish a threshold or a concentration limit by which a condition of unauthorized use of the anabolic hormone could be excluded [23, 21].

Significant information regarding the metabolism of boldenone was acquired in 1983 by the experiments on the horses performed by Dumasia et al. The Authors shown that after the intramuscular administration of radioactive-labelled \( \beta \)-bold to castrated male horses, the metabolites were mainly excreted as
glucu- and sulfo-conjugated compounds. Particularly, the metabolites of boldenone were principally excreted as glucuronic acid conjugated, identified by capillary GC-MS. In the research, the Author expressed the difficulties to hydrolysed the sulfate conjugates with Helix Pomatia, and secondly that the epimer of boldenone tends to be conjugated with the glucuronic acid, while β-bold with sulfate moiety[29, 30].

Studies in vivo were firstly performed by Galletti et al. and, then, by Cartoni at al., who illustrated the elimination of β-bold metabolites in human urine as β-bold metabolite as β-bold free form, consequently the administration of β-bold [23].

Van Puymbroek et al carried out studies both in vitro and in vivo in order to observe the metabolism of boldenone in cattle. The experiments in vitro were performed on microsomal liver preparations and isolated hepatocytes, while the in vivo experiment were done on urine and faeces samples of animal treated with 700 mg of β-bold via intramuscular injection and an animal treated with boldenone undecanoate. The most visible metabolite found in vitro was the oxidised metabolite of β-bold, the ADD in the microsomal preparation, whereas incubations with isolated hepatocytes showed several 6-hydroxylated metabolites of both β-bold (the epimer of bold, α-bold) and ADD. In the experiment in vivo, the Authors observed a similar metabolic profile in the urine and faeces form both of the animals treated. Particularly, they mainly found α-bold and “an unidentified reduction product (5ξ-
“AEVI)” in the samples of the animal treated with boldenone undecanoate and “two reduced metabolites, 5 ξ -AEVI and 5 ξ –AEVII “, in the samples of bovine treated with intramuscular boldenone. A smaller amount of 6-HO-17 β -boldenone was found in urine, but no traces on faeces, as well for 5β-AED [31].

Similar studies were performed on urine and faeces samples of a male calf and a mature cow. The metabolite profile resulted comparable, in fact α-bold e 5 AED were found as principal metabolites [22, 33].

The endogenous production of β-bold in pigs was confirmed by the detection of the steroid in testicles. Unlike 17 β-19-nortestosterone, the production of β bold in pigs is related to the gender, in fact β-bold was not detect in female even if a very low concentration in urine where the steroid could be present as a result of faecal contamination [34]. Regarding the horse, boldenone undecylenate, well known with the commercial name of Equipoise®, is illegally used for treating racehorse. Ho et al. reported the direct evidence of the natural presence of boldenone and its main metabolite, the sulfate conjugate in urine of horse with a concentration lower than 5 ng mL^{-1} [23, 35]. Also in humans endogenous production of boldenone is reported [36, 37].

Recently, Le Bizec and Destrez, achieved studies on β-bold with the purpose of have a clearer overview on its metabolism and to define a criteria to distinguish endogenous production of the steroid from illegal abuse. They suggested sulfo-conjugated form
of β-bold in urine as biomarker useful to define a condition of illicit treatment of the compound, while Blokland et al in 2007 proposed 6β-hydroxy-boldenone. Also in human field, β-bold sulfate was found as tool for fraudulent administration [38-40].

1.2.11. Origin of endogenous boldenone in urine

Several hypothesis on the origin of endogenous boldenone in urine and in the faeces were assumed. Studies were performed both in vitro and in vivo in order to better understand the metabolism involved in the production of boldenone and to recognize metabolites that could be useful marker of illicit treatment. Since 80s, studies concerning the microbial biotransformation were principally focused on the ability of certain microorganisms of expressing selective dehydrogenase enzyme activity, which are able to transform dehydrogenated steroids from certain precursors and substrates. In particular, an interesting alternative of the precursors of steroids used as substrate for the dehydrogenase microorganisms activity are the phytosterols, which are sterols structurally related to cholesterol, but differ from cholesterol in the structure of the side chain [41].

Barthakur et al. described the conversion of β-sitosterol to ADD via dehydrogenase activity of Mycobacterium sp. NRRL B-3683 [42].
Other several microbial enzymatic reduction activities were found using different mycobacterium extracts and fungi, the ability to cleave the sterol side chain in order to obtain AED from sitosterol and in river sediment was also found due, probably, to the presence of phytosterols in plants [23].

In order to give more explanations to the problem related the origin of boldenone, other models were studied. Invertebrate *Neomysis integer* is commonly used for *in vitro* instead of animal tissues. In a study where invertebrate *Neomysis integer* was exposed to β-testosterone and stanazolol, presence of β-bold was found. In 2003, Poelmans showed the ability of *Neomysis integer* to convert β-T to AED and, in its turn to β-T and ADD. When the invertebrate was exposed to ADD, a production of β-bold was revealed [23]. The possibility of an origin from maggot and mould on the feed was not excluded. [43, 44]. The results achieved by Poelmans in 2005 shown the presence of β-bold in the testicles of entire male pig, probably due to aromatisation of oestrogens [34]. Precursors of β-bold can be detected in the faeces of rats fed with phytosterols [45].

The conversion of boldenone from phytosterols, for instance β-sitosterol in vegetable fat, was also considered. The β-sitosterol is commonly used as an additive to animal feed in response to the crises caused by bovine spongiform encephalopathy and is shown through an invertebrate model
capable to converted phytosterols to ADD, and then into bold and T \[^{23}\].

Gastro-intestinal tract hosts a complex and miscellaneous microbial community, capable to convert precursor of steroids or phytosterols into dehydrogenated steroids \[^{44}\]. Several studies focused the attention on the role of microorganism from faeces and examine the bacteria activity of the gastrointestinal tract of the bovine cable to convert steroids in urine when inadequately stored \[^{46}\].

In 2004, Sgoifo Rossi et al. evidenced the possibility that a faecal contamination of urine could provide wrong results because the presence of faeces made urine non-compliant for the presence of α-bold and occasionally for β-bold. Until 2004, in Italy urine sampling was commonly performed by using a zootechnical apron. However, it could be reason of faecal contamination. The Authors suggested to collect urine using a kettle, kept under the body of the animal, after cleaning and trimming of the animal’s coat, thus avoiding any faecal contamination of sheath area. These samples and urine collected by a zootechnical apron were analysed and the results obtained could confirm the necessity of a clean collection of the urine, as shown in Figure 7 \[^{47}\].
In 2006, with the purpose to better understand the role of faecal contamination of urine, Pompa et al. evaluated the eventual presence and variability in concentration of steroids (β-bold, α-bold, ADD, AED, T, epiT) on the basis of the effect of drying the faeces in the urine, skin swabs and faeces of ten Friesian calves. β-bold, α-bold and ADD were never detected in urine samples, whereas AED, T and ET were constantly found. β-bold was detected in all samples of rectal faeces that were directly collected from the rectum (28-89 ng g⁻¹). The epimer of bold was detected in only four animals in faeces rectal (2.6-5.6 ng g⁻¹). Only one calf showed ADD in faeces rectal at 21 ng g⁻¹. A variably increase of the concentrations of the analytes was found in 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. The Authors referred to a de novo synthesis of α-bold and metabolites that could take place naturally in bovine faeces. Due to the possibility to find α-bold and its metabolites in faeces, the Authors confirmed the
necessity to avoid faecal contamination of urine during the sampling and suggested to analyse urine samples rapidly after collection \(^{[48]}\).

In 2008, the same group tried to explain the reason of the presence of the analyte boldenone in faeces. An \textit{in vitro} experiment performing an incubation with several steroids of faecal matter suspended in 0.9\% saline was carried out. The Authors showed that the transformation of steroids could occur in faeces, particularly after faecal expulsion and suggested a possible pathway for the neo-formation of boldenone from the endogenous AED. (Figure 8). They designed AED as key precursor for boldenone synthesis. AED can be converted to the epimer of testosterone (ET) but also T.

The conversion of AED can also result in ADD, which could be an intermediate compound in the production of β-bold, α-bold. However, the mechanism is to be investigated yet \(^{[49]}\).

\textbf{Figure 1-8.} Potential neoformation and interconversions of steroids in bovine faeces (both before and after their emission) and in faeces-contaminated urine.
Van de Kerkhof in 1999 performed a study in order to demonstrate the suggestion of Shänzer (1994) who hypnotized the endogenous nature of boldenone in human urine due to the conversion of ADD. According to the Authors, boldenone could derive from testosterone glucuronide (TG) metabolized in bile and the converted by the intestinal bacteria in the ADD. Then ADD could be transported in liver through the enterohepatic circulation and the secreted in urine where it could be interconverted in boldenone. An experiment in vitro carried out by Van de Kerkhof showed that in fractions of faeces incubated in a medium with a Δ1-dehydrogenase and a precursor as testosterone glucuronide, could be found positive for the presence of ADD, while in urine collected from an athlete, boldenone was found but a very low concentration. In Figure 9 is shown the pathway suggested.[50]

**Figure I-9.** A hypothesis of pathway for the endogenous production of boldenone in urine.
Other different hypothesis on the possible origin of boldenone in bovine urine were assumed. It was reported that some types of wooden crate in which veal calves are housed might contain precursors to boldenone \cite{51}.

However, the debate on the origin of boldenone in urine is still open and the knowledge regarding the mechanism involved in boldenone production is lacking.

At the meeting placed in Brussels in 2003, the main experts in the residue fields representing the Member States made certain recommendations to address the problem of endogenous/exogenous boldenone. The conclusions of the meeting were reported in a review published by De Brabander in 2004 and they are summarized in the following table. Moreover, the passage of more than ten years, the following guidelines are still valid \cite{23, 52}.

<table>
<thead>
<tr>
<th>Normal situation</th>
<th>Strategy for boldenone control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine</strong></td>
<td></td>
</tr>
<tr>
<td>traces of 17α-bold</td>
<td>17α-bold conjugated &gt; 2 ng mL(^{-1}) SUSPICION of illegal use</td>
</tr>
<tr>
<td>no 17β-bold</td>
<td>Presence 17β-bold conjugated at any concentration CONFIRMATION of illegal use</td>
</tr>
<tr>
<td><strong>Faeces</strong></td>
<td></td>
</tr>
<tr>
<td>free unconjugated 17α-bold</td>
<td>?</td>
</tr>
<tr>
<td>free unconjugated 17β-bold (dried faeces)</td>
<td>?</td>
</tr>
</tbody>
</table>

**Table I-1.** Natural occurring situation of boldenone and the strategy for the control of the use of boldenone in cattle.
1.2.12. Legislation aspect regarding boldenone in bovine

In Italy, as in the other Member States of the European Union, the presence of β-bold conjugated at any concentration in urine of calves and bulls is to consider as an evidence of illegal treatment (zero tolerance). Based on the scientific information obtainable, α-bold is considered as endogenous production in bovine urine if it is detected at a concentration lower than 2 ng mL\(^{-1}\) in bovine urine, while there is no evidence today for the endogenous origin of β-bold \[^{52-54}\]. Regarding the sampling procedure, it is required a “clear” collection of the urine sample, without faecal contamination in order to avoid false positive results and then, the samples have to be immediately frozen. The laboratory must to specify the form of boldenone investigated and define it as free or conjugated form, giving information of the animal species, including breed, gender and age of the animal. A Minimum Required Performance Levels (MRPL) for the analytical methods for the detection of β-bold and α-bold in urine of veal calves is now set at 1 ng mL\(^{-1}\). The analytical methods provided by the NRP are both screening methods (ELISA) and
confirmatory methods using liquid chromatography coupled to mass spectrometry (LC-MS/MS)\textsuperscript{[54]}.

### 1.2.13. Analytical methods for the detection of boldenone

The analysis for the detection of β-bold and its metabolites may be carried out using both LC-MS\textsuperscript{n} and GC-MS\textsuperscript{n}, following appropriate extraction and purification methods\textsuperscript{[23]}. Particularly, several studies conducted on urine of calves treated with different preparations of boldenone, esters and/or precursors by the use of GC-MS analysis techniques have shown that β-bold is excreted glucuronide and less as sulphate\textsuperscript{[25,26]}. Biddle et al. indicated two possible markers of treatment as glucuronic metabolites, which are present independently of the route of administration: 6β-hydroxy-17α-boldenone and 5z-androst-1-ene-3z-ol-17-one (a due to the lack of reference standard, have used the "z" to indicate either the α or β configuration). The researchers emphasized the importance of a sensitive method of analysis in order to define the conditions of treatment and were not able to test the limit of 2 ng mL\textsuperscript{−1} defined by European legislation due to lack of reference standards of α-boldenone glucuronide\textsuperscript{[55]}.

Nielen et al. showed the importance of investigating the metabolism of II phase of boldenone, asserting that the presence of β-bold conjugates in urine of calves was the evidence of an illegal treatment\textsuperscript{[56]}.
In further work, metabolism of β-bold in calves was studied after intramuscular and oral administration with boldenone, esters of boldenone and ADD. The Authors have shown by GC-MS analysis that the majority of metabolites were glucuronide (α-boldenone form) and that β-bold sulphate was present only in urine samples obtained from treated animals (this last result was obtained with LC analysis -MS) [38].

β-bold sulfate is proposed as a marker of abuse because it was detected only in treated cattle. However, the study was conducted on a small number of animals (5 calves treated) in a qualitative analysis and analytical performance values for LC-MS analysis were not reported. Destrez in 2009 conducted a study with three male calves treated for oral administration of ADD and by intramuscular injection of boldenone undecylenate. The analytical limits for metabolite sulfate were obtained either by analysis LC-MS / MS (ESI negative acquisition SRM, triple quadrupole) that by LC-HRMS (ESI negative, Resolution 30,000, OrbitrapTM). The analytical limits detected by the two techniques of analysis are here reported: the limits of decision (CCα) 0.2 and 0.1 ng mL⁻¹ and the detection capability (CCβ) 0.4 and 0.2 ng mL⁻¹, respectively. The Authors stated that β-bold sulfate may be a good marker of treatment. The use of high-resolution instrumentation has not improved significantly the performances; the high cost and the low scanning speed of 'OrbitrapTM are reasons to favour the use of an
instrument of detection triple quadrupole. It is therefore to be noted, once again the insignificant number of calves studied [39].

At present, the current official analytical methods to identify β-bold conjugated in calf urine require a preliminary deconjugation step in order to obtain the free form of the steroids, following by LC-MS$^n$ or GC-MS$^n$ analysis.

The hydrolysis of glucuronic acid and sulfate conjugates is usually performed as the first step of the analytical determination of steroids in urine, and the hydrolysis is mainly achieved by enzymatic methods (rather than chemical methods) that involve the cleavage activity by β-glucuronidase and arylsulfatase. These enzymes can be extract from different sources, including bovine liver, bacteria ($Escherichia coli$) or gasteropoda ($Helix pomatia$) and arylsulfatase originates from bacterial sources ($Aerobacter aerogenes$), molluscs or limpets ($Patella vulgata$). The digestive juice of $Helix pomatia$, which contains both β-glucuronidase and arylsulfatase activities, is the most commonly used enzymatic preparation, and it leads to a total deconjugation of urinary steroids. For the determination of steroid hormones that are naturally present in bovine urine in free, glucuronic acid and sulfate forms, the use of a suitable enzyme preparation and optimization of the hydrolysis conditions were necessary. However, the step of deconjugation entails disadvantages, which must be taken into account and may be potential factors, which may lead to misinterpretation. The
enzymatic hydrolysis of the conjugates may be incomplete. The incubation, in particular with *Helix pomatia*, can lead not only to incomplete hydrolysis, but also to the conversion of steroids or degradation [57]. Based on these considerations, analytical strategies performed bypassing hydrolysis step, may be properly developed for the direct detection of phase II metabolites of AAS. The LC techniques allows a broad-spectrum of applications because it allows performing direct analysis of hydrophobic substances without a previous derivatisation and the direct analysis of conjugated metabolites without a prior hydrolysis. The conjugation increases solubility, converting the steroids into more polar compounds and facilitating kidney excretion. Because of polarity, the separation of the conjugated steroids from the other polar substances that are in urine, results difficult and requires many steps of concentration and purification [58, 59]. Recently, an alternative method to the indirect detection of conjugated boldenone in bovine urine was carried out and permitted to distinguish the different conjugated forms. The method, validated according to the European Commission Decision 2002/657/EC, allowed specific and sensitive analysis of conjugated and free forms of α- and β-bold and ADD by a unique step of extraction and purification performing by the use of ImmunoAffinity Columns. The good and satisfactory results in terms of decision limits and detection capabilities may ensure this as an appreciable method to evaluate a condition of illicit treatment from natural
production of boldenone\textsuperscript{[60,61]}. The latest studies cited are reported in Chapter 3.1. and 3.2.

References


17. Available at: http://www.fao.org/DOCREP/004/X6533E/X6533E03.htm#refeccstr


25. Available at: http://www.steroid.com

26. Available at:
http://monographs.iarc.fr/ENG/Classification/ClassificationsAlphaOrder.pdf


International Conference Racing Analysts Veterinarians. Hong Kong


50. van de Kerkhof DH, van der Voort PM, de Boer D, Maes RAA (1999). Recent advances in doping analysis (7)


52. European Commission Outcome on the Meeting on the Control of Boldenone in Veal Calves, Brussels, September 30, 2003

mass spectrometry in urine samples. Analytica Chimica Acta. 552, 116-126


1.3 Glucocorticosteroids

World Anti-Doping Agency (WADA) prohibits the use of corticosteroids when administered by oral, intravenous, intramuscular or rectal routes in sport competition, but the treatments are permitted (only by obtaining the permission of the authorities) out-of-competition or in particular routes before or during competition\(^1,2\). In an attempt to discriminate between permitted and forbidden administrations, WADA established a reporting level of 30 ng mL\(^{-1}\) for glucocorticosteroids and their metabolite\(^3\).

The improper use of glucocorticosteroids in sports is exploited to the relaxing effects that these chemicals cause on the respiratory tract and, at higher doses, to their analgesic effects. The dilation of the airways and the raised pain threshold enable athletes to achieve better performance both in training and in competition.

In veterinary practice, they are frequently used as therapeutic drugs for treatment of bovine ketosis, inflammatory diseases, and induction of parturition, but also illegally used both in the endogenous for in the form of artificial compound as growth promoters\(^4\). Glucocorticosteroids play significant roles in carbohydrate, protein, and lipid metabolism, the immune response, and the response to stress. Natural glucocorticosteroids have a few mineralocorticoid activity and therefore affect fluid and electrolyte balance. While corticosteroids can be highly effective in
suppressing or preventing inflammation, their physiologic and pharmacologic mechanisms of action are mediated by the same receptors. This explains why their pharmacologic and physiologic effects are inherently linked, and why physiologic exposure to corticoids is potentially detrimental to several metabolic, hormonal, and immunologic functions.

1.3.1. Adrenal glands

The adrenal glands are endocrine organs at the superior pole of the kidney and they are the source of the corticosteroids hormones. The adrenal cortex secretes mineralocorticosteroids, glucocorticosteroids, and sex hormones, and it is composed by 3 distinct layers (Figure 10):

- The outer layer is the zona glomerulosa where the production of the mineralocorticoid aldosterone occurs. This hormone is responsible for increasing sodium absorption and stimulating potassium excretion by the kidneys and thereby indirectly regulating extracellular fluid volume.
- The zona fasciculata occupies about 70% of the cortex and it is responsible of the production of
glucocorticosteroids with well-known effects on the metabolism of carbohydrate and protein.

- The zona reticularis, the innermost layer, produces glucocorticosteroids and small amounts of sex androgens, oestrogens and progestins, involving in reproductive function [5].

Figure I-10. Structure of adrenal gland.
1.3.2. Synthesis of glucocorticosteroids

All the metabolic pathways in the biosynthesis of steroid hormones derive from the common precursor cholesterol and they share a cyclopentenoperhydrophenanthrene nucleus. The basic chemical structure of the corticosteroids is shown in the Figure 11.

Based on the number of the atoms of carbon, the corticosteroids are divided in three groups \[6\]:

- **Glucocorticosteroids** or C21 steroids, which have a two-carbon side chain at position 17. i.e. cortisol and corticosterone. The glucocorticosteroids are the main steroids synthesised and they show a hydroxyl group at the 17 position, for this reason they are also defined as 17-hydroxycorticoids or 17-hydroxycorticosteroids. All secreted C21 steroids, classified using Selyes terminology, have both mineralocorticoid and glucocorticoid activity.

- **Androgenic steroid**, C19 steroids, which have a keto or hydroxyl group at position 17
Oestrogens, C18 steroids, which, in addition to a 17-keto or hydroxyl group, have no angular methyl group attached to position 10.

Figure I-11. Synthesis of glucocorticosteroids.

The principally precursor of the corticosteroids, the cholesterol arises from the circulating Low Density Lipoproteins (LDL), which receptors are highly expressed in adrenocortical cells and less is synthesized from acetate. The release of the esterified cholesterol from lipid droplets occurs through the catalytic action of the cholesterol ester hydrolase enzyme. Then, a carrier protein transports the cholesterol to mitochondria where a member of the cytochrome P450 (or CYP11A1 known cholesterol desmolase or side-chain cleavage enzyme) converts it to pregnenolone. Pregnenolone is dehydrogenated in the smooth
endoplasmic reticulum to form progesterone by 3β-hydroxysteroid dehydrogenase, which also catalyses the conversion of 17α-hydroxyprogrenenolone to 17α-hydroxyprogesterone, and dehydroepiandrosterone to androstenedione. A 17,20-lyase region of the same enzyme cuts the 17,20 bond, converting 17α-pregnenolone and 17α-progesterone to the C19 steroids dehydroepiandrosterone and androstenedione. The hydroxylation of progesterone to 11-deoxycorticosterone and of 17α-hydroxyprogesterone to 11-deoxycortisol are catalysed by 21β-hydroxylase, a cytochrome P450 that is also known as P450c21 or CYP21A2. The production of corticosterone and cortisol takes place in the mitochondria of the zona fasciculate from 11-deoxycorticosterone and the 11-deoxycortisol that are 11-hydroxylated by 11β-hydroxylase a cytochrome P450 also known as P450c11 or CYP11B1. Cortisol is produced in greater amounts compared to corticosterone in these species and represents approximately 80% of the glucocorticoid production (Figure 12) [6].
1.3.3. Transport of glucocorticosteroids

Glucocorticosteroids are secreted into the systemic circulation, reversibly bound to a specific α globulin (approximately 80% of human cortisol), called transcortin or corticosteroid-binding globulin (CBG), while 10% is bound to serum albumin and the remaining 10% is the biologically active unbound hormone \[^6\].
1.3.4. The regulation of the synthesis of the glucocorticosteroids: The Hypothalamus-Pituitary-Adrenal Axis

The release of glucocorticosteroids is stimulated by the adrenocorticotropic hormone (ACTH, also called corticotropin), a linear peptide consisting of 39 amino acids, that binds to high-affinity receptors on the plasma membrane of adrenocortical cells. At the physiological level, under conditions of stress (i.e. exercise, fear) or hypoglycemia, the hypothalamus secretes corticotropin-releasing factor that induces the pituitary prior to the production of the hormone ACTH, which in turn, stimulates the release of glucocorticoids in the adrenal glands. This binding is necessary to activate an adenylyl cyclase, resulting to a formation of pregnenolone and its derivatives. The hypothalamus releases the corticotropin releasing factor or CRH that stimulates the anterior pituitary gland to release the hormone ACTH, adrenocorticotropic hormone. The mutual aid of the three glands is defined as Hypothalamus-Pituitary-Adrenal axis (HPA) (Figure 13). Free glucocorticosteroids inhibit ACTH secretion, and the degree of pituitary inhibition is proportional to the circulating glucocorticoid level [6].
1.3.5. Metabolism and excretion of glucocorticosteroids

Corticosteroids are metabolised mainly in the liver, which is the principal site of glucocorticoid catabolism but also in kidney and mammary glands, giving rise to inactive, water-soluble conjugates excreted in urine (75%) and faeces (25%). Most of the cortisol is reduced to dihydrocortisol and then to tetrahydrocortisol, which is conjugated to glucuronic acid [6].

Cortisol (CL) can be interconverted to the non-active hormone, cortisone (CN) through the activity of 11β-hydroxysteroid dehydrogenases (11β-HSD). The enzyme 11β-HSD presents two isoforms: type I catalyses the conversion of CL into CN (Figure 14), while type II controls the catalysis of the conversion of CN into CL. Tetrahydrocortisol (THF), allo-tetrahydrocortisol (ATHF) and tetrahydrocortisone (THE) are the
metabolites of respectively cortisol and cortisone, produced by a two-step reduction of the C4-5 double bond catalysed by 5alpha- or 5beta-reductase (Figure 15), followed by the reduction of 3-keto group catalysed by 3-oxoreductase, which is conjugated to glucuronic acid. THF, allo-THF and THE are made more water-soluble by conjugation with glucuronic acid through a glucuronyl-transferase system responsible for this conversion and the formation of the glucuronides of bilirubin and hormones and drugs. Then, the metabolites are excreted in the urine. Downstream, cleavage of THF and THE to C19 steroids 11-hydroxy or 11-oxo-androsterone or etiocholanolone can occur. Alternatively, reduction of the 20-oxo group by 20α- or 20β-hydroxysteroid dehydrogenase yields α and β-cortols and cortolones, respectively, with subsequent oxidation at the C21 position to form the polar metabolites, cortolic, and cortolonic acids. Hydroxylation at C6 to form 6-β-hydroxycortisol is described, as is reduction of the C20 position, which may occur without A ring reduction giving rise to 20 α- and 20 β-hydroxycortisol. Approximately 50% of secreted cortisol appears in the urine as THF, allo-THF, and THE; 25% appears as cortols/cortolones. Ten percent appears as C19 steroids, and 10% appears as cortolic/cortolonic acids. The remaining metabolites are free, unconjugated steroids (CL, CN, and 6 β- and 20 α/20 β-metabolites of CL and CN).
1.3.6. Glucocorticoid metabolism in farm animals

The published studies on glucocorticoid metabolism in farm animals, particularly in cattle, are mostly focused to evaluate
animal welfare, which possible indicator of the stress that is influenced by several factors as transport, courtship, copulation and hunting and any physiological stressors associated with invasive procedures. Circulating CL is released depending on the intensity of the stressors, metabolized (conjugated) in the liver and excreted in urine and faeces, and in saliva. However, the monitoring of the adrenocortical activity should be made without induce stress to the animals and the non-invasive techniques in order to analyse of faeces and, recently, of saliva are the most suitable [9,11].

Moreover, several Authors have proved that corticosteroid concentrations in saliva are directly related to those in plasma in humans, dogs, pigs, and domestic ruminants [12, 13].

Antignac et al. performed a study on the phase II metabolism of corticosteroids, determining the relative proportions of free, glucuronide, and sulfate metabolites, in bovine urine with the purpose of improving the efficiency and the specificity of their control in cattle in 2002. Two animals were treated with different dexamethasone esters by intra-muscular injection. The results illustrated an evident difference of the total proportion of conjugated forms between endogenous THF (40–65%, mainly as glucuronide form) and endogenous CL (2–8%) or exogenous dexamethasone (4–27%), showing that cortisol is excreted mostly as free form [14].
1.3.7. Therapeutic of corticosteroids in breeding

Prednisolone, methylprednisolone, dexamethasone and betamethasone are synthetic corticosteroids only approved for therapeutic uses. They are widely prescribed in human and veterinary medicine for their anti-inflammatory and anti-allergic properties [15, 16].

Synthetic glucocorticoids, with a 21-carbon steroid skeleton, are chemical derivatives synthesized from cholic acid obtained from cattle or steroidal saponins in plants. Synthetic corticosteroids show a chemical structure of these drugs is very similar to that of natural glucocorticoids with slightly alterations to cortisol skeleton and selectively alter the degree of anti-inflammatory activity. The effects of their resulting anti-inflammatory activity can be even more potent than naturally occurring steroids. The levels of efficacy, potency and pharmacological activity of synthetic hormones are determined by their pharmacokinetic properties. Potency and plasma half-life of natural and synthetic glucocorticoids are shown in Table 2 [16-19].
Table I-2. Relative potencies of corticosteroids used in veterinary practice.

<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Relative Antiinflammatory Activity</th>
<th>Plasma Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone</td>
<td>0.8</td>
<td>30</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.0</td>
<td>90</td>
</tr>
<tr>
<td>Prednisone</td>
<td>4.0</td>
<td>60</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>4.0</td>
<td>200</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>5.0</td>
<td>300</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>5.0</td>
<td>180</td>
</tr>
<tr>
<td>Betamethasone</td>
<td>25.0</td>
<td>100–300</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>25–30</td>
<td>100–300</td>
</tr>
<tr>
<td>Fludrocortisone</td>
<td>10</td>
<td>200</td>
</tr>
</tbody>
</table>

The main changes to the cortisol skeleton in order to increase the corticosteroid activity are the following:

- Introduction of an additional double bond between C1 and C2 (increase of glucocorticoid and anti-inflammatory activity). For example, prednisolone is ~3 to 4-fold more selective than cortisol.
- Additional of hydroxyl group at carbon 11.
- Additional fluorination at the C9 position enhances both glucocorticoid and mineralocorticoid activity.
- Addiction of a group at the position 16.\(^{[19]}\)

Corticosteroids formulations are available for oral, parenteral, and topical use. Prednisone, prednisolone, methylprednisolone, and dexamethasone are well absorbed with oral preparations\(^{[19]}\).
1.3.8. *Glucocorticosteroids and legislation*

European Union banned the use of corticosteroids as growth promoters, allowing their use only for therapeutic aims \(^{[20]}\). The strong pharmacological activity of corticosteroids used in animal breeding makes the residues of these molecules potentially dangerous to meat products consumers \(^{[21]}\). Consequently, the monitoring of the use of corticosteroids is mandatory. The Commission Regulation (EEC) n° 37/2010 of 22 December 2009 (on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin) establishes Maximum Residue Limits (MRLs) for edible matrices (muscle, kidney, liver and milk from different species) with a withdrawal period between treatment and slaughter, as indicated in the Annex and reported in Table 3 \(^{[15,22]}\).

The monitoring control for the presence of corticosteroids is performed by collecting samples at the slaughterhouse on tissues for which MRLs are set, and at farm through the analysis of urine. For this matrix, no MRL has been fixed.
Table I-3. MRLs of Betamethasone, Dexamethasone, Methylprednisolone and Prednisolone, as specified by the Commission Regulation (EU) No 37/2010.

Synthetic corticosteroids might be used particularly at low dosages and via oral administration, as growth promoters either alone or in a cocktail with other active principles, always banned, including β-agonists or steroid [5]. Dexamethasone, which is the most frequently corticosteroid illegally used [23], could ameliorate the overall carcass quality traits through the significant
increase of serum insulin, resulting in a fat deposition and protein catabolism \[5\]. Tarantola et al. reported that the use of dexamethasone at low dosage, both orally and intramuscularly, was found to have no positive effect on performance but it positively influenced meat tenderness and colour \[21\]. Moreover, it was observed that dexamethasone could influence the regulation of β2-receptors, so enhancing the repartitioning effects of β2-agonists.

Among the glucocorticosteroids used in illicit treatment, prednisolone is one of the most debated and differs from CL through Δ1,2, hydroxylation, by which improves its potency and their glucocorticoid effects \[24-26\].

In Italy, from 1988 National Residues Plan (NRP) provides for the surveillance and the monitoring of residues of chemical substances in foods with animal origin. Council Directive 96/23/EC “on measures to monitor certain substances and residues thereof in live animals and animal products” demands to each Member States to implement a National Residues Plan (NRP) \[27\]. This document has to be drafted in order to monitor the process of farming and primary processing of animal products for the detection of residues and of certain substances in live animals, their excrement and liquids biological tissue, animal products, feed and drinking water. The official control system carried out by the competent authorities.
The NRP is designed taking into account the requirements of the legislative decree 16 March 2006, n. 158, implementing Directive 96/22 /EC and 96/23 /EC and its subsequent amendments and decisions 97/747/EC and 98/179/EC, as regards the levels and frequencies of sampling and procedures for the official taking and the management of the samples. It defines the species and categories animals to be sampled, the category of residues or substances to be investigated, the sampling strategies, levels and frequencies sampling, according to the legislation and the directions of the Commission European. The NRP is yearly drawn by the Ministry of Health-Directorate General for Health and Food Safety and Nutrition (the Ministry) that collaborates with the Regions and Autonomous Provinces, National Laboratories reference and Istituti Zooprofilattici Sperimentali [28].

According to Legislative Decree of August 4th 1999 n. 336 with a transposition of EC Directives 96/22/EC and 96/23/EC (concerning the prohibition on the use of certain hormonal substances, thyrostatic and of β-agonists and measures to monitor certain substances and their residues in live animals and their products), the NRP is prepared for the investigation of certain molecules that fall into two categories established by the European Community.

The substances to be investigated and controlled are included into two categories, as established by EU:
- The Category A includes substances with anabolic effects and forbidden substances for the treatment of livestock.

- The Category B includes veterinary drugs, drugs authorized for the treatment of livestock, for which the European Union defines a maximum residue limit that cannot be exceeded in consumer products; environmental contaminants, such as heavy metals and substances that may be absorbed by environment and enter in food chain.

Some Member States include these substances in group A3 (steroids), whereas others allocate them to the group B2f (veterinary drugs and contaminants-other pharmacologically active substances). As regards Italy, corticosteroids belong to B2f, following the suggestion of the European Commission. However, the use of corticosteroids without any kind of control, is considered as illicit treatment [29,30].

1.3.9. Prednisolone: a corticosteroid under magnifying glass

Prednisolone (Δ1,4-pregnadiene-11β,17α,21-triol-3,20-dione) (PL), is one of the most important and debated glucocorticosteroid having gluconeogenetic and anti-
inflammatory activities, which structurally differs from CL only by the presence of the double bound at the position C1-C2 (Figure 16), an adding modification in structure similar to the that of boldenone regarding testosterone. This feature gives an anti-inflammatory activity to the steroid that is 3–4 times higher than that of CL [19].

The use of PL in livestock is allowed only for therapeutically purpose, while it is forbidden as growth promoter. Because the presence of residual traces of PL, as the others corticosteroids, in meat and meat products could be a risk for consumers [8], the use of PL is allowed only for therapeutic purposes in bovine and is regulated by Commission Regulation (EU) N°37/2010 [23], which sets maximum residue limits (MRLs) for milk and the edible tissues (Table 3).

Nevertheless, based on scientific evidence currently available, on 22nd May 2012, the Italian Ministry of Health, recommended a cut-off level 5 of µg L⁻¹ for bovine urine, above which a response of non-compliance could be set and, thus, indicating the potential endogenous origin of PL in urine [31].

![Chemical structure of prednisolone, cortisol and cortisone.](image)

**Figure I-16.** Chemical structure of prednisolone, cortisol and cortisone.
In June 2012, the European Medicines Agency for Veterinary Use (Committee for Medicinal Products for Veterinary Use) recommended to place PL in the Commission Regulation No. 37/2010/EC as substance allowed in horse\textsuperscript{[32]}. 

Until the first ten years of the XXI century, PL was considered only of exogenous origin, however an increased frequency of urine bovine samples positives for PL at slaughtering put in doubt the hypothesis of illegal treatment. A research carried out in Lombardy (Northern Italy) during the years 2008-2009 on 196 bovine urine liver samples taken at the slaughterhouse, reported 72\% non-compliant urine samples for the presence of PL, while all liver samples resulted to be negative for this corticosteroid. Considering the high number of positive samples, the hypothesis of an illicit treatment was excluded and other possible explanations were taken into account, as mainly the role of the stress transport and pre-slaughter on the production and release of corticosteroids. The Authors indicated a possible relationship between stress condition and the detection of PL in urine, collected directly from bladder of three treated Holstein Friesian cows with adrenocorticotropic hormone (ACTH) via intramuscular at two and six hours after treatment. The presence of PL after ACTH treatment seems to suggest the possibility that this molecule could be physiologically produced\textsuperscript{[33]}. It was also noted that the prednisolone could occasionally be found even if animals are apparently not submitted stress\textsuperscript{[24]}. The metabolic
pathway that would lead to the production of endogenous prednisolone has not yet been clarified. Bertocchi et al. performed a comparative study on cow urine samples collected at the farm and urine and adrenal glands (of cattle positive for corticosteroid in breeding) taken at the slaughterhouse from the same animals, assuming a conversion of endogenous CL to PL. The adrenal glands were positive for the presence of PL and could therefore be the seat of endogenous synthesis of PL obtained as an intermediate metabolite in the synthesis of endogenous corticosteroids, although data shown showed that the involvement of the adrenal glands seem to play a minor role in the endogenous production of PL. Moreover, the Authors hypothesized that presence of trace amounts of the corticosteroid in urine could be a result of intestinal dehydrogenation of cortisol operated by bacterial conversion in the gut and then transported in the liver by the hepatic portal vein, and thus secreted via the urine \[15,25\].

Furthermore, the formation of PL may also be the result of a poor preservation of the sample. However, the bacterial dehydrogenation does not explain the positive to the slaughterhouse, where urine samples are taken directly from the bladder and thus, theoretically, free of faecal material \[34\]. The formation of PL from CL could be also a result of microbiological contamination of soil bacteria. Bredehoft et al published \textit{in vitro} experiments by which the Author demonstrated that urine positive for the presence could be obtained by \(\Delta^1\)-dehydrogenase activity
of bacteria as *Rhodococcus erythropolis* that were able to convert cortisol into PL [35].

Additionally, a possible presence of PL in urine due to the involuntary administration of prednisolone with complementary feed is also conceivable. In 2014, the detection of PL in samples of feedstuff under poor storage, thus indicating a determinant role of the environmental conditions in the possibility of PL neo-formation in feedstuff and, consequently, in urine of animals fed with non-compliant feed [36] This study is reported in Chapter 3.3.

The possibility of the endogenous origin of PL has also been described for equine and human urine [37, 38]. Finally, Delahaut et al. (2014) reported that the Belgian Federal Agency for the Safety on the Food Chain (FASFC) found PL at a mean concentration of 0.96 µg L\(^{-1}\) in 73% of 393 samples of porcine urine collected at the slaughterhouse. The same Authors described the results of a preliminary study concerning the presence of PL in sows before and after intramuscular administration of PL or tetracosactide hexaacetate. The urine collection was performed at the farm before and after the treatment and at the slaughterhouse, where the liver was collected as well. The presence of PL in porcine urine was confirmed in all samples prior to the treatment and in most of them after the treatment, but, in liver, PL was only found after administration of prednisolone or tetracosactide hexaacetate. The Authors proposed the PL/CL ratio in liver samples as an indicator for detecting illicit PL administration to
pigs and suggested confirming these observations in a study on a larger number of animals \([39]\). The endogenous production of PL in pigs seemed also confirmed in a recent study on 80 pigs, whose urine both at the farm and at the slaughterhouse, and adrenal glands were object of analysis for the presence of the corticosteroids. PL was detected in 89% of the samples. The possibility that PL is endogenously produced in pigs was directly demonstrated by its presence in the adrenal glands, the organ in which CL is produced \([40]\). The study performed is reported in Chapter 3.4 of the present thesis.

1.3.10 Method of analysis of corticosteroids

Numerous methods have been developed for the determination of endogenous and exogenous corticosteroids in different matrices. As recommended by the National Residues Plan, urine is the matrix for control a misuse of glucocorticosteroids in live animal, while tissues as liver and kidney are used for the analysis after slaughter \([22, 27]\). Corticosteroids are chemically similar to the AAS, therefore the analytical methods used for the determination of corticosteroids are analogous to the ones for AAS. The common pathway followed to analyse corticosteroids adopts a preliminary step of enzymatic hydrolysis prior of the extraction process in order to obtain the free compounds. However, numerous studies reported
the possibility to analyse directly the analyte without deconjugation step \[^{41,42}\]. Subsequently, liquid-liquid extraction or solid liquid extraction are carried out using different organic solvents, i.e. tert-butyl-methyl-ether, diethyl-ether. A step of purification is commonly performed in order to obtain interference-free samples to analyse. The SPE methods described to purify the sample are several, such as the use of simple C18, polymeric-based sorbents, HLB Oasis or the more specific immunoaffinity columns. The urine, which is the required matrix for the monitoring purpose, is traditionally checked, but nowadays new kind of matrix are chosen as alternative available matrices due to the possibility to have more information from there and to find the analyte more concentrated, for examples hair, plasma and serum), faeces, feed, milk, water. Working with solid and more complex matrices, a step pf defatting is necessary, such as using n-hexane.

Different system of detection are used depending of the purpose of the analysis required. A preliminary immune enzymatic method (ELISA) is ordinarily done for a screening analysis. However, the low specificity of an ELISA test and the possibility to obtain false positive results, due to a compounds cross-reactivity, causes problems regarding the interpretation of the results. As confirmatory levels, the techniques used are MS-based system of detection, preceded by specific and selective separation procedure. In earlier times, the optimization of GC–MS methods
was prevalent for the analysis of corticosteroids, but the thermally lability and their low volatility cased difficulties on the determination and need a long derivatisation step, prior the GC-MS analysis. Currently, the application of LC-MS\textsuperscript{n} is principally preferred, particularly with ion source using electrospray ionization (ESI) in negative mode. For the analysis of corticosteroids the use of UPLC is now rising. The challenge remains for the mass spectrometer analysts is the ability to separate isomers as dexamethasone and betamethasone, because with common reverse phase columns, it results difficult. Therefore, the use of different chromatographic column, characterized by different sorbent, are preferred, for example graphite-based columns could be used to achieved the separation of the isomers. Monolithic and polymeric reversed-phase columns are used, even if rarely \cite{43-44}.

**References**


3. WADA Technical Document – TD2014MRPL \cite{World Anti-Doping Agency Web site}. Available at:


from untreated cows. Food Additives & Contaminants: Part A. 29:12 1893-1900


dexamethasone on productive traits and meat quality of veal calves. Animal Science. 79: 93–98


28. D.lgs. 16 marzo 2006, n. 158 Attuazione delle Direttive 2003/74/CE concernente il divieto di utilizzazione di talune sostanze ad azione ormonica, tireostatica e delle sostanze beta agoniste nelle produzioni animali


31. Italian Ministry of Health, Circular Letter. Department of Public and Veterinary Health about the opinion of the Consiglio Superiore di Sanità, Sezione IV, 22 May 2012
32. EMA/CVMP/404452/2012. MRL summary opinion. Prednisolone. Equidae. Committee for Medicinal Products for Veterinary Use, EMA, June 2012, pp. 1


1.4  Thyreostats

1.4.1. Thyroid gland

The bovine thyroid gland is one of the endocrine glands and it is constituted by two lobes connected by an isthmus between the 2° and 4° tracheal rings, as shown in Figure 17. A normal gland weighs between 20-65 grams in a bovine adult while a young cattle or calves could have a gland weighing 12-31 grams [1].

![Figure 1-17. Anatomy structure of the thyroid gland.](image)

Regarding the microscopically structure, the thyroid gland is composed by spherical follicles that are the functional units of the gland. (Figure 18) The follicles are lined by a layer of epithelial cells, secreting active iodine hormones: triiodothyronine (T3) and thyroxine (T4) bound to glycoprotein Thyroglobulin (TG). Close to the thyrocytes, the cells parafollicular or C cells synthesize calcitonin peptide, involving in the calcium, and
phosphate and sodium balance of the organism. The thyroid hormones profoundly increase the metabolic rate of the metabolic activities of almost all the tissues of the body: increase the oxygen consumption and heat production, stimulate the metabolism of cholesterol, carbohydrates, stimulate the normal growth and body development and have effect on the cardiovascular and nervous system \[2\].

![Figure I-18](image.png)

**Figure I-18.** Microscopically structure of thyroid gland.

### 1.4.2. Iodide Pump (Iodide Trapping)

Iodine is a necessary element for the synthesis of the thyroid hormones; therefore, a correct thyroid function essentially depends on a proper iodine intake from the diet. From the blood circulation, iodine is transported into the thyroid glandular cells and follicles, whose basal membrane has the specific pump for
taking iodine into the cells. This process is called iodide trapping [2].

The extraction of iodine from the plasma and its concentration by the thyroid cells is an active process of concentration against an electrochemical gradient, which implies a waste of energy ensured by the system ATPase Na / K dependent. This is a mechanism saturable, energy-dependent, which produces an increase of the intracellular level of iodine from 20 to 40 times higher than in plasma. The ability of the thyroid gland to concentrate iodine is controlled by the activity of a sodium / iodide symporter (NIS), recently identified as a protein located on the membrane of the thyrocytes [3].

1.4.3. Thyroglobulin, and Chemistry of Thyroxine and Triiodothyronine Formation

The iodide ions are converted by the enzyme peroxidase to an oxidized form of iodine, either nascent iodine (I0) or I3-, a form of iodine capable to link the aminoacid tyrosine.

The TG, synthesized from the endoplasmic reticulum and Golgi apparatus and secreted into the follicles, is bounded to the iodine trough the organification. An iodinase enzyme catalyzes the binding of the iodine to a tyrosine of the TG. Tyrosine is first iodized to monoiodotyrosine (MIT) and then to diiodotyrosine (DIT). The coupling of two DIT will form a molecule of
triiodothyronine (T4) while the coupling of a MIT and a DIT form a molecule of thyroxine (T3), the major hormonal product of the coupling reaction that remains part of the thyroglobulin molecule, while triiodothyronine represents about one fifteenth of the final hormones. The normal thyroid gland produces all the circulating T4 and approximately 20% of the entire circulating T3. Many of the biological activity of thyroid hormones is due to the effects of T3 on the target cells, which has a great affinity for the receptor for thyroid hormones and is about from 4 to 10 times more potent than T4. Since 80% of the T3 serum is derived from the deiodination of T4 in tissues and since the thyroid hormone receptor preferentially binds the T3, T4 is considered a prohormone. Then, T3 and T4 are released into blood circulation [2].

1.4.4. Storage of Thyroglobulin

The thyroid gland has ability to store large amount of hormones in order to guarantee a supply of thyroid hormones for 2 or 3 months, depending on the needs of the organism. In fact, after the hormone synthesis, each thyroglobulin molecule contains
up to 30 thyroxine molecules and a few triiodothyronine molecules, a useful form to be stored [2].

1.4.5. *Release of Thyroxine and Triiodothyronine from the Thyroid Gland*

Thyroxine and triiodothyronine has to be cleaved from the TG in order to be released into the circulating. Under physiological conditions, the TBG binds almost completely T4 and T3: the small fraction unbound (free or: F = free) of the activity is responsible for a biological hormone. Proteases digest iodinated thyroglobulin, releasing the hormones T4 and T3, the biologically-active agents central to metabolic regulation [2].

1.4.6. *Transport of Thyroxine and Triiodothyronine to tissues and transcription*

In the bloodstream, carrier proteins, synthetized from liver, form complexes with thyroxine and triiodothyronine. Mainly, there are three specific carrier proteins: thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA), and albumin. The hormones are released slowly depending on the high affinity of the plasma-binding proteins for the thyroid hormones. The catabolism of thyroid hormones occurs in the liver. Most of the actions of thyroid hormones are mediated by the interaction of
T3 with specific nuclear receptors. The receptors for thyroid hormones belong to a family of nuclear transcription factors hormone-response elements, which are structurally and functionally to steroid hormones. After binding of the nuclear thyroid hormone receptor, the complex binds to a regulatory region of the gene (thyroid-hormone response elements - TRE) and starts a series of events that lead an increased transcription of the DNA, translation of the mRNA and protein synthesis. Hence, the thyroid hormones increase the metabolic activities of almost all the tissues of the body \[2\].

**1.4.7. Physiological effects**

The thyroid hormones on the organism are various and are briefly summarized \[2\]:

- Stimulation of Carbohydrate Metabolism
- Stimulation of Fat Metabolism: – Decreases the concentrations of cholesterol, phospholipids, and triglycerides. – Increases numbers of low-density lipoprotein receptors on the liver cells, leading to rapid removal of low-density lipoproteins from the plasma. – Increases significantly the rate of cholesterol secretion in the bile and consequent loss in the faeces
- Increased metabolism in the tissues causes more rapid utilization of oxygen than normal and release of greater than normal quantities of metabolic products from the tissues. These effects cause vasodilation in most body tissues, thus increasing blood flow
- Cardiac output also increases. Thyroid hormone has a direct effect on the excitability of the heart, which in turn increases the heart rate

1.4.8. Regulation of Thyroid Hormone Secretion

Thyroid secretion is primarily controlled by thyroid-stimulating hormone (TSH), secreted by the anterior pituitary gland in response of the hypothalamus releases hormone (TRH) through the Hypothalamic–Pituitary–Thyroid axis. Anterior pituitary gland cells produce the thyroid stimulating hormone (TSH), a glycoprotein that interacts with specific receptors on the cells of the thyroid gland and stimulates the synthesis and secretion of thyroid hormones. The synthesis and release of TSH of the pituitary gland are influenced by hormones from the thyroid and by hypothalamic peptide TRH. The activity of the thyroid gland is regulated by a negative feedback, in which the thyroid hormone binds to specific receptors on pituitary gland cells inhibiting the secretion of TSH and the hypothalamus TRH. The interactions
among the hypothalamic-pituitary-thyroid maintain a stable amount of circulating thyroid hormones. Therefore, aberrations of TSH levels almost always indicate the presence of a pathology thyroid hidden. The effects of TSH on the thyroid are numerous and complex. It stimulates proliferation mobile and more increases the synthesis of thyroid peroxidase, thyroglobulin and uptake Iodine all 'inside of the follicular cells and its incorporation in the thyroid. Then, the synthesis and release circulating thyroid hormone is dependent on factors intrinsic to the gland, such as availability of iodine, and factors extrinsic to it, as the negative feedback. This is made through different mechanisms [2]:

1. The hypothalamus by TRH (Thyrotropin Releasing Hormone)
2. The pituitary by TSH (Thyroid Stimulating Hormone)
3. The same thyroid hormones, or better, their blood level.

1.4.9. Thyreostats

Thyreostats are drugs commonly used in human medicine as treatment for people with Graves’ disease (autoimmune disorder) and in general in the control of hyperthyroidism. The consequence of the treatment with thyreostats is a decrease of the circulating thyroid hormones (T3 and T4) due to an inhibition of the function the thyroid gland [4].
Thyreostats are a complex of synthetic compounds that are used in order to interfere with the function of the thyroid gland with a following decrease of the production and then inhibition of the thyroid hormones T3 and T4, which causes a wide range of actions mainly correlated with the growth and the metabolism development. In the veterinary field, the administration of the thyreostats are made in case of high production of thyroid hormones, as in the hyperthyroidism condition, especially for cats and dogs \[4\]. Regarding milk and meat producing animals, thyreostats are given with the feed during the last 4-8 weeks before the slaughter. The thyreostats are easily and quickly absorbed, are able to alter the energetic metabolism by the inhibition of thyroid hormones. Despite of the severe legislation regarding the use of thyreostats, their illegal administration cannot be excluded. The treatment with antithyroid agents is made in order to obtain an increase of animal weight due mainly to an increase absorption and retention of water in the edible tissue, as well as filling of the gastrointestinal tract. The results is to obtain meat that weighs more, but with poor nutritional proprieties \[5\]. However, thyreostatic drugs cause hypertrophy of the thyroid gland, easily detectable after slaughter of the animal and a thyroid gland weighing more than 60 g was considered as a suspect of fraudulent use of the anti-thyroid agents \[6\]. Furthermore, the presence of thyreostats in edible tissues represents a hazard to human health related to consumption of contaminated meat from thyreostats \[7\].
Teratogenic and carcinogenic effects have been reported after exposition of edible tissues derived from treated animals, and cases of aplasia cutis, characteristic disease of the scalp, were registered in Spain due to a contaminated meat ingestion [7-12].

The main thyreostats used in animal livestock are divided into two groups:

1. **Natural source of sulfur compound**

   Thiocyanates and oxazolidine-2-thiones (OZT’s) are considered not only endogenous but also synthetic because they are substances that are capable to be synthetized. Cruciferous or Brassicaceae vegetables are unique in that they are rich sources of glucosinolates, sulfur-containing compounds thyroid inhibiting synthetic compounds [13].

2. **Xenobiotic thyreostats**

   The most frequently used thyreostatic drugs include the very potent thyroid-inhibiting compounds 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), and 1-methyl-2-mercapto-imidazole (tapazole, TAP) [4-6]. The chemical structures of these substances are shown in Figure 19.
Thyreostats are polar amphoteric thioamide, with a low molecular weight (114-204 Da) a heterocyclic tautomeric structure and are mostly derived from thiouracil and mercapto-imidazole. The sequence consisting in nitrogen–carbon–sulphur known as thioamide is considered as accountable for the thyroid-inhibiting activity (Figure 20). Especially, the anti-thyroidal agents include three types of compounds:

I. Sulfated compounds to position 2, aromatic heterocycles containing two nitrogen atoms to position 1 and position 3 (pyrimidine), including the thiouracil (TU) and its analogues to which is added a group R at the position 6. (ethyl, dimethyl, propyl or phenyl group).
II. Group of the 2-mercaptobenzimidazole (MBI), characterized by an aromatic cycling structure with a benzene and sulphated imidazole group.

III. Group of the 1-methyl-2-mercaptoimidazole, or well known as tapazole (TAP), a methylated an sulfated imidazole compound.

![Chemical structure of thioamide sequence.](image)

**Figure I-20.** Chemical structure of thioamide sequence.

### 1.4.10. Endogenous occurrence of thiouracil

Anti-thyroidal agents could be produced from precursors present in Cruciferous or Brassicaceae plants and relative analogues. Because of their cheapness and high protein content, Brassicaceae are common added in feed. Cabbage, mustard and rapes, the most known Brassicaceae, contain glucosinolates, which are secondary metabolites involved in the tissues defence system. The catabolism of glucosinolates is characterised by the hydrolysis
via plant myrosinase, as β-thioglucosidase or by bacterial hydrolysis of the microflora of the gut. The hydrolysis of the glucosinolates produces oxazolidine-thiones, nitriles, epithionitriles, thiocyanates, isothiocyanates, thiourea, that are different on the bases of the pH condition of the field production. The products of the glucosinolates catabolism express anti-thyroidal proprieties, as performed by the synthetic thyreostats. These metabolites, and particularly the thiocyanate, are able to inhibit the thyroid functions and cause a reduction in the production of the thyroïdal hormones. Other metabolites could interfere with different functions of the organism, including motor capability; they could have nephrotoxic and mutagenic effects. Hence, the presence of glucosinolates in feed should be minimal. Furthermore, the presence of TU was found in untreated animal (bovine, pigs, and horses) not subject to a diet rich in Brassicaceae. A question regarding the non-clear or dual origin of TU raises, as well the difficulties in the TU determination on case of fraudulent use of endogenous origin. In addition, the thiocyanate could be synthetically produced and their characteristic thioamide group, that is the corresponding of that in the synthetic drugs, is responsible for the inhibition of the thyroid gland activity [14].

A relationship between presence of Brassicaceae in feed and thioracil in urine has been however demonstrated by Pinel [12], Vanden Bussche [15] and Kiebooms [14,16]. The Community Reference Laboratories (CRLs) in 2007 proposed a recommended
concentration of 10 μg L$^{-1}$ in urine and 10 μg kg$^{-1}$ in thyroid for the purpose of control.$^{[17]}$

Recently, Wauters et al. have reported concentrations up to 18.2 μg L$^{-1}$ in the 99% percentile from 3894 bovines and they suggested to increase the recommended concentration to 30 μg L$^{-1}$.$^{[18]}$ Actually, the Italian National Residue Plan already provides this level of concentration as the capability of detection for the thyreostats in urine.$^{[19]}$

### 1.4.11. Thyreostats and legislation

Presence of residues of thyreostats in edible tissues are a risk for consumer’s health of food of animal origin, and based on this risk, the European legislation established the prohibition of the illicit use of thyreostats for farm animals.$^{[20, 21]}$ Consequently, the method to determinate the presence of thyreostats are rigorously regulated. A minimum required performance limit (MRPL) of 10 μg L$^{-1}$ in urine is required for the analytical methods. In 1974, Belgium forbidden the use of thyreostats and the EU extended the prohibition of the use of thyreostats in all Member States seven years later, as Council Directive 81/602/EC$^{[21]}$ and, then, revised by Council Directive 96/22/EC$^{[22]}$. Furthermore, it states the ban import of meat from third countries hailing from animals treated with in the EU prohibited substances. In order to guarantee the control of the illicit use of thyreostats, the EU declares specific
measures of control have to be observed and realised by each EU Member States, as described by Council Directive 96/23/EC. The thyreostats are included in Directive 96/23/EC in Annex I as substances belonged to the Group A of the substances having anabolic effects and unauthorized substances. Concerning thyreostats, a minimum required performance limit (MRPL) has been suggested (but not already fixed by the EU authorities) at 100 μg L⁻¹ or μg kg⁻¹ in urine and thyroid gland, respectively. The Community Reference Laboratories (CRLs) in the EURL guidance paper in 2007 proposed a recommended concentration (RC) for TAP, TU, MTU and PTU of 10 μg L⁻¹ in urine and 10 μg kg⁻¹ in thyroid for the purpose of control. RC is considered an up-to-date reference value for setting analytical requirements to be fulfilled by the analytical methods used for the determination of thyreostats. Nevertheless, Wauters et al. have recently reported concentrations up to 18.2 μg L⁻¹ in the 99% percentile from 3894 bovines and they suggested to increase the recommended concentration to 30 μg L⁻¹. Actually, the Italian National Residue Plan already provides this level of concentration as the capability of detection for the thyreostats in urine, while regarding
the thyroid glands, a minimum required performance limit (MRPL) of 100 μg.Kg⁻¹ is required and set [19].

1.4.12. Method of analysis of the thyreostats

Regard to the importance of the effects that thyreostats cause to human health when present as residues in edible matrices, different analytical approaches were developed to determine thyreostats, as required by European legislation. Prior to the development of mass spectrometry techniques, the abuse of thyreostatic drugs, causing a condition of hypothyroidism, was mainly verified by histological analysis of samples of thyroid, as a semi-quantitative parameter and the observation symptoms resulting from the administration of these drugs. At the very beginning, differentiation (compliant/non-compliant) was based on a combination of subjective factors: symptomatology, detection of thyroid hormones, thyroid micro- (histological changes) and macroscopy (weight, goiter). Eventually, the weight shifted towards analytical methodologies for the detection of thyreostats.

Before the development of the spectrometric system of detection, in the early 70s a colorimetric method was firstly applied. However, the unsatisfactory performance parameters of the limit of detections obtainable through this method and the suitability to use the colorimetric method only for the thyroid matrix have compelled the researchers to choose another system
of detection. Afterwards, thin layer chromatography (TLC) improved the level detection and gave the possibility to analyse thyreostats in in the order of 10 µg kg$^{-1}$.

Nowadays, the evaluation of the thyreostatic residues in matrices of animals is mainly performed by gas or liquid chromatography as separation methods combined to mass spectrometry as detection techniques, able to reach a lower LOD in the range of 1-10 µg kg$^{-1}$[24].

Normally, the extraction of thyreostats is carried out by using polar solvents more suitable to their chemical characteristics as methanol, acetonitrile or ethyl acetate. Further steps of purification or clean up with different kind of solid phase extraction (SPE) are reported[24]. Due to the small molecular mass and high polarity of the thyreostats, several authors proposed a derivatisation before or after the clean-up, mostly made with 3-iodobenzylbromide (3-IBBr), in case of HPLC-MS/MS analysis[24]. This procedure induces the stabilisation of the chemical structure of the molecule under a specific and single tautomeric form, the reduction of the molecular polarity in order to increase the separation characteristics on the reversed-phase column in case of HPLC-MS detection and the increase of the molecular mass[25]. Nevertheless, its application before the analysis could cause a loss of analytes, particularly due to the several evaporation steps required. Furthermore, the avoidance of the derivatization simplifies and shortens the whole analysis procedure[26].
An innovative analytical method based on QuEChERS extraction and ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry detection was recently carried out. This technique do not require further SPE clean-up or derivatization reactions. The method resulted simple and easy to be conducted and able to achieve calculated decision limits (CCα) are below the recommended concentration \[^{27}\].

**References**


CHAPTER 2

Aims
2. Aims

Problems relating to Food Safety are acquiring importance and becoming an increasingly frequent phenomenon. Terms as “mad cow disease”, “dioxin-contaminated chickens” are getting more familiar and are arising a public interest. Recently in 2013 the announcement of the horse meat passed off as beef, the well known as the horse meat scandal, produced a speeded stir. This problem of the horsemeat in frozen beef burgers and in beef lasagne has been for the first time reported in United Kingdom and subsequently, the analysis have also revealed a widespread phenomenon that involved more than 20 countries. In addition to commercial fraud, the most troubling aspect of the case concerns the use of meat from racehorses, sometimes treated with Phenylbutazone, a potent anti-inflammatory drug, for whom residues in edible matrices could be a dangerous risk for the consumer health \(^{[1,2]}\). These last news reports, including the trade and selling of bovine and pigs meat treated with illicit drugs or corticosteroids and antibiotics in Italy of the last two years, are further evidences of the need to strengthen the capability of the control system and increase the levels of increasing levels of protection guaranteed to the consumer \(^{[3,4]}\).

The drugs used in the veterinary field are regulated as stated by the European and national legislation. Several veterinary drugs do not represent a risk for the consumer. Nevertheless, their
use and administration have to be severely controlled, and the eventual presence of residues of drugs in edible matrices could be a result of illicit treatment or in the case of authorized treatments, the inappropriate use of the drugs due to a wrong evaluation of the withdrawal times before slaughtering. Therefore, it is necessary to develop sophisticated and innovative analytical methods for different classes of compounds, whose residues could be present in the edible matrices and in the products of animal origin, causing a risk to human health. In addition, the monitoring of the consumer exposition to the drugs, the impact of the chemicals on the environmental and the security in the trade of the animals, result further important focal points to be addressed \[5\].

Nevertheless, the demands of the market are not always in keeping with the available offer and fraudulent use of the drug treatment in order to fattening the animals, increasing their growth rate and, therefore, to obtain a meat sold at the highest price, it cannot be excluded \[6\].

In 1996, the prohibition of the use of beta-agonists and the substances having anabolic and thyreostatic actions in livestock farming were states with the Directive 96/22/EC, which was followed by the Directive 96/23/EC that establishes the measures that EU Member States should take to monitor substances and their residues in both live animals and animal products. In the Annex I of Directive 96/23/EC are listed the substances objects of the regulation and they are organised into

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two groups. Group A or banned substances consist of the growth promoters substances subdivided into four groups: r anabolic steroids (group A1, A3, A4), Thyreostats (group A2), β-agonists or repartitioning agents (group A5) and Annex IV substances. The group B comprises veterinary drugs, environmental contaminates, and corticosteroids belong to group B2f despite their possible abuse to fatten animals [7,8].

The anabolic steroids hormones play a role similar to the endogenous testosterone, causing an increase of the anabolic metabolism, an increase of the thin body mass and a reduction in whole body and regional fat mass.

The monitoring of the use of the growth promoting substances registered into the Group A and B2f has a central priority, due to their implication in the public health. However, it is necessary to improve the scientific knowledge and develop analytical methods in order to better understand the presence of certain substances having a double identity of endogenous and exogenous substances. As well described by Van Thuyne Wim in his thesis, these “pseudoendogenous substances” are “situated in a grey zone” and characterized by “a urinary threshold level, or compounds that are allowed if a proper medical cause justifies their use, such as corticosteroids”. These substances have been considered for a long time with exogenous origin. Nevertheless, thanks to the improvement of the technique and the consequent reducing of the limit of detection, they are also discovered as
endogenous or pseudoendogenous substances in some animal species or in particular conditions, and are “an emerging challenge for the future”.\(^9,10\)

The aim of this thesis is to contribute to the comprehension of the possible endogenous or natural origin of three borderline or semi-natural substances: boldenone in bovine urine, prednisolone in feedstuff for bovine and in porcine urine and adrenal glands and the development a new method for the detection of thiouracil in bovine urine and thyroid glands.

**Boldenone in bovine urine**

17β-boldenone (1-dehydrotestosterone) or β-bold is an anabolic steroid with androgenic anabolic activity, structurally different from 17β-testosterone for the dehydrogenation of the carbon in the first position of the A ring \(^11\). The European Directives 96/22/CE and 96/23/CE ban bold as growth promoter, including it into the A3 section as regards the production of animal origin and the livestock \(^7,8\). Until 1996, β-bold has been considered only of synthetic origin, but several studies confirmed the endogenous production of boldenone in its epimer form (17α-boldenone) in urine as a result of the involvement of bacteria from faeces or due to the role of maggots and moulds on feed, environmental contamination. Recently, natural production of traces in
urine of 17β-bold is suggested due to the conversions of phytosterols precursors \[11\]. In two studies on evaluation of the β-bold metabolism, Le Bizec and Destrez proposed β-bold -sulfo conjugate in urine as a useful marker proof of illegal treatment \[12,13\]. At present, α-bold is considered as endogenous production in bovine urine if it is detected at a concentration lower than 2 ng mL\(^{-1}\) in bovine urine \[11\], where the steroid is present as sulfo or glucuronic-conjugated form \[14\]. Regarding bold, the European and Italian legislation established the zero tolerance limits and state that the only presence of 17β-bold at any form of conjugation in urine is a sufficient evidence of illicit administration \[8,15\].

The determination of conjugated steroids are mainly performed after a preliminary step through hydrolytic enzymes (glucuronidases and sulfatases) from \textit{Escherichia coli} or \textit{Helix Pomatia}. Nevertheless, the deconjugation may be incomplete and steroid conversion, degradation or artefact formation may occur \[16, 17\]. Therefore, one of the aims of the present study is to improve the knowledge concerning the origin of boldenone in bovine urine, trying to understand if the β-bold II metabolites could be marker of abuse or naturally produced. This purpose was followed in two research papers, reported in Chapter 3.1 and 3.2.
The first one focused on the improvement of the methodological approach to investigate the presence of boldenone in urine. Particularly, the development and the validation of an innovative method of extraction by using immunoaffinity columns (IACs) were carried out. Immunoaffinity columns (IACs) are a separation method, which permits to obtain a very selective extraction and guarantee a purified extract of compounds in only one-step. Consequently, IACs perform a more reliable technique than the common liquid-liquid extraction and SPE clean up, optimizing a sensitive, specific, reproducible LC-MS/MS technique of analysis. It allowed the detection in a single chromatographic run of a wide range of steroids, included free forms of $\alpha$- and $\beta$-bold, as well as $\alpha$-bold conjugates and androstadienedione (ADD). The validation has been made according the Decision of Commission 2002/657/EC $^{[18]}$. Concerning the second scientific paper, the validated method was used to in order to verify the possible endogenous origin of $\beta$-bold II phase metabolites in an extended study in vivo on 56 young bulls. Urine samples from 56 animals were collected at different intervals at the farm, where the animals were under veterinary control, and at the slaughterhouse, where histological alterations to the bulbourethral and prostate
glands were also investigated. The main variable evaluated was the influence of the stress in the eventual bold production. Therefore, we evaluated the occurrence of β-bold glucuronide and sulfate in young bull urine, with the aim of understanding whether they could be of endogenous origin, and to check for a possible relationship with particular environmental and stress condition.

**Prednisolone in pigs in complementary feedstuff for bovine and in urine and adrenal glands**

Prednisolone (Δ14–pragnadiene-11β, 17-triol-3, 20 dione) is a glucocorticoid with a gluconeogenetic and anti-inflammatory activity 3-4 times higher than the activity of cortisol, from which structurally differs for the presence of a double bond at the position Δ1-2 of the A ring, a feature in common with the structure of boldenone with respect to the testosterone [19]. In the Commission Regulation (EEC) 37/2010 are established maximum residues limits (MRLs) of Prednisolone for cattle: 4 µg kg⁻¹ in muscle and fat, 10 µg kg⁻¹ in liver and kidney and 6 µg kg⁻¹ in milk [20]. For urine, no MRLs have been set (it is not an edible matrix). Albeit its presence at any concentration should not be allowed, a 5 µg L⁻¹ cut-off level is recommended, by the Italian Ministry of Health.
The only exogenous origin of prednisolone was put in doubt through studies that have shown the possibility of finding prednisolone in urine of untreated cattle. Studies suggested poor collection and storage conditions of the urine samples could be reasons of the prednisolone detections in urine. Besides an endogenous production or of *ex vivo* formation in urine of prednisolone, the hypothesis of an accidental ingestion of contaminated complementary feedstuff with prednisolone has to be taking into account. Considering the complexity of the feed composition, we investigated the presence and origin of prednisolone plant complementary feed samples for cattle. Particularly, we investigated the eventual findings of prednisolone in different commercially available complementary feedstuffs, stored at the farm and/or in the laboratory, with the purpose of understand and verify if its presence was due to neo-formation during storage. The study id reported in Chapter 3.3.

Moreover, the role of stress in cortisol production and, consequently in the conversion in prednisolone is well known.

The possibility of the endogenous origin of prednisolone has also been described for equine and human urine, while there is still a lack of knowledge regarding the origin of prednisolone in pigs. Until
2014, only one work has been performed about the endogenous presence of prednisolone in pigs (sows). The Authors carried out the study on a limited number of animals (10), whose urine samples were analysed before and after the treatment prednisolone or a synthetic analogue of adrenocorticotropic hormone, and at the slaughterhouse. The livers were analysed, as well. All porcine urine samples collected before the treatment were confirmed positive for the presence of prednisolone and in most of them after the treatment. Instead, regarding the liver, prednisolone was only detected after treatment. The prednisolone/cortisol ratio in liver samples was suggested as a useful criterion to distinguish a case of fraudulent treatment with prednisolone. It has been suggested to confirm these observations in a study on a larger number of animals [23].

In order to clarify the possible endogenous origin of prednisolone and the influence of stress on the production of this corticosteroid in pigs, the study proposed in this thesis (Chapter 3.4) has investigated the presence of prednisolone in urine samples collected from the same 80 pigs at the farm and at the slaughterhouse. Furthermore, their adrenal glands were analysed, assuming an endogenous production of prednisolone in this organ.
A simple liquid-liquid extraction (for urine samples) and solid-liquid extraction (for adrenal glands) were performed in order to extract the analytes from urine and from adrenal glands, and then a LC-MS/MS analysis was done.

A simple validated method to analyse thyreostats in bovine urine and thyroid glands

The administration of the thyreostats to cattle was banned since 1981 following to the severe pharmacological activity and the effects given by residues of the drugs in the edible matrices for the health of consumers [24]. The thyreostats induce an inhibition of the synthesis of hormones of the thyroid gland with a consequently decrease of the basal metabolism, gastrointestinal motility and an increase of the extracellular water retention. These effects are fraudulently exploited for fattening purposes. At present, No Minimum Required Performance Level (MRPL) or Maximum Residue Limit (MRL) has been established for thyreostats and, in particular the thiouracil [25]. Over recent years, the thiouracil was frequently found in bovine urine at a lower concentration than 10 μg L⁻¹. Several studies confirmed a relationship between presence of Brassicaceae containing glucosinolates in
feed and thiouracil in urine, so assuming that thiouracil at low concentration could have “semi-natural origin” [26,27]. In the CRL Guidance paper of December 2007 of the Community Reference Laboratories suggested a recommended concentration (RC) of 10 μg L⁻¹ in urine and 10 μg kg⁻¹ in thyroid were suggested for monitoring purposes [28]. Newly, a retrospective study performed by six European Member States (France, Poland, the Netherlands, United Kingdom, Norway and Belgium) in the European Union Reference Laboratory (EURL) on the official thiouracil data (2010-2012) collected from bovines, pigs and small livestock, suggested to increase RC from 10 to 30 μg L⁻¹ [29]. The Italian National Residue Plan already provides this level of concentration as the capability of detection for the thyreostats in urine [15]. Because of their chemical characteristics, the analysis of thyreostats is often carried out through a previous derivatisation step with 3-iodobenzylbromide to the extraction procedure, which might results in a loss in terms of recovery of the analytes. Confirmatory methods are now mostly perform with GC or LC-MS/MS system of detection [25]. In 2011 Vanden Bussche et al. proposed a method for direct detection without derivatisation of thyreostats in urine [30]. However, unique methods for the
direct extraction of the thyreostats from urine and thyroid glands from bovine have not yet been reported.

Therefore, the purpose of the fifth work included in this thesis in Chapter 3.5, was to perform a simple, sensitive, specific and reproducible method for the extraction of five most frequently illegally used thyreostats (2-thiouracil, 6-methyl-2-thiouracil, 6-propyl-2-thiouracil, 6-phenyl-2-thiouracil, 2-mercaptobenzimidazole or tapazole and 5,6-dimethyl-2-thiouracil, used as internal) without derivatisation, and by HPLC-MS/MS analysis in bovine urine and thyroid glands.

The quantitative method was validated following the criteria established in the 2002/657/EC Commission Decision and SANCO/2004/2726-revision 4 [18,31].

The development of a simple and rapid method of analysis not only for the urine, but also for the thyroid glands could be a useful tool for studies regarding the distinguish between illicit treatment and the natural origin of thiouracil in bovine urine and thyroid glands and improve the understanding of the current ambiguous identity of thiouracil.
References

1. Available online from: http://ec.europa.eu/food/food/horsemeat


stockfarming of certain substances having a hormonal or thyrostatic action and of β-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC.


androstenedione in bovine urine using immunoaffinity columns clean-up and liquid chromatography tandem mass spectrometry analysis. Talanta. 131:163-169


21. Italian Ministry of Health, Circular Letter. Department of Public and Veterinary Health about the opinion of the Consiglio Superiore di Sanità, Sezione IV, 22 May 2012


CHAPTER 3

Research papers
3.1. *Determination of α- and β-boldenone sulfate, glucuronide and free forms, and androstadienedione in bovine urine using immunoaffinity columns clean-up and liquid chromatography tandem mass spectrometry analysis*

*Published in:*  
3.1. Determination of α- and β-boldenone sulfate, glucuronide and free forms, and androstanediol in bovine urine using immunoaffinity columns clean-up and liquid chromatography tandem mass spectrometry analysis

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3.1.1. Abstract

The debate about the origins of boldenone in bovine urine is ongoing for two decades in Europe. Despite the fact that its use
as a growth promoter has been banned in the European Union (EU) since 1981, its detection in bovine urine, in the form of α-boldenone conjugate, is considered fully compliant up to 2 ng mL⁻¹. The conjugated form of β-boldenone must be absent. In recent years, the literature about boldenone has focused on the identification of biomarkers that can indicate an illicit treatment. β-boldenone sulfate is a candidate molecule, even if the only studies currently available have taken place in small populations. In this study, a method for the determination of sulfate and glucuronate conjugates of β-boldenone was developed and validated according to the European Commission Decision 2002/657/EC and applied to α-boldenone sulfate and glucuronide, α- and β-boldenone free forms and androstadienedione (ADD), too. The clean-up with immunoaffinity columns enabled the direct determination of the conjugates and free forms and allowed specific and sensitive analyses of urine samples randomly selected to verify this method. The decision limits (CCα) ranged between 0.07 and 0.08 ng mL⁻¹, the detection capabilities (CCβ) between 0.08 and 0.1 ng mL⁻¹. Recovery was higher than 92% for all the analytes. Intra-day repeatability was between 5.8% and 17.2%, and inter-day repeatability was between 6.0% and 21.8% for the studied free and conjugated forms. This method has been developed as a powerful tool with the aim to study the origin of boldenone in a trial on a significant number of animals.
Keywords: boldenone sulfate, boldenone glucuronide, boldenone, androstadienedione, bovine urine, food safety.
3.1.2. Introduction

The use of substances that have hormonal activity for growth promotion in farm animals has been prohibited in the European Union (EU) since 1981 [1]. The bans on the use of such substances, on the trade of treated animals and their meat within the EU, and also on the import from third countries was confirmed in 1988 [2, 3]. A typical substance with hormonal action is 17β-boldenone (1-dehydrotestosterone or androsta-1,4-dien-17β-ol-3-one) (β-bold), an anabolic steroid that differs from testosterone only by the double bond between carbons 1 and 2 of the steroid A ring as shown in Figure 1. In 1996, Arts et al. [4] reported the natural occurrence in calf urine of 17α-boldenone (α-bold) at concentrations ranging from <0.1 to 2.7 ng mL⁻¹. Since then, a number of studies and regulations followed, aiming to explain the presence of boldenone (bold) in bovine urine, to indicate a biomarker metabolite for illicit treatment, and to establish levels of the hormonal substance that could exclude administration to the animal [5, 6]. In particular, in September 2003, the thesis of the natural production of this steroid was proposed by the experts within the EU, who stated that scientific knowledge was sufficient to conclude that the presence of α-bold in urine and faeces of bovine animals has a natural origin. They set the ‘natural threshold’ of 2 ng mL⁻¹ in the urine of veal calves below which α-bold conjugate (boldenone conjugates are water soluble forms of
boldenone bound to e.g. glucuronic acid formed by metabolism in the animals) come from sources other than illegal treatment. The presence of β-bold conjugates at any concentration in the urine of veal calves was indicated as the result of an illegal treatment \[^7\].

The presence of conjugates of α- and β-bold, without specifying the nature of the ionized group (sulphate, glucuronide) is not the only option considered by the scientific community. Biddle \[^8\] in 2005 performed a study on beef cattle treated with three preparations of bold: intra-muscular bolus administration of β-bold, followed by oral administration of the supplement androstadienedione (1,4-androstadiene-3,17-dione) (ADD), and finally intra-muscular administration of β-bold undecylenate. He concluded that highly sensitive methods would be required to detect the abuse of bold using β-bold glucuronic acid conjugate as a marker; they could not confirm the EU recommended level of 2 ng mL\(^{-1}\) for α-bold glucuronic acid conjugate due to the lack of the reference standard. Finally, two markers, present in the glucuronate fraction regardless of route of administration, were specially indicated in this study: 6β-hydroxy-17α-bold and 5z-androst-1-ene-3z-ol-17-one (the letter ‘z’ indicates position α or β, due to the lack of the reference standard). Another study \[^9\] investigated the metabolites of bold in treated cattle after intramuscular and oral treatment with bold, bold esters and ADD. The Authors showed that the majority of metabolites, analysed by GC-MS, were glucuronide conjugates and that β-bold sulfate was
present only in urine from treated animals (this last result obtained by LC-MS/MS). They therefore suggested to use β-bold sulfate as an indicator of bold administration, after larger scale studies. However, the study was conducted in a predominantly qualitative way, the analytical limits in the LC-MS/MS were not reported; therefore, the question: “Who is to say that as analytical limits decrease, (particular steroids) will not be discovered as endogenous at a lower concentration?” [6] has a fundamental importance. In 2009, Destrez et al. [10] performed a study on treated male calves with oral administration of ADD or with intramuscular injection of bold undecylenate. The analytical limits for β-bold sulfate were set by both LC–MS/MS (negative ESI, SRM acquisition, triple quadrupole) and LC-HRMS (negative ESI, R 30,000, Orbitrap™): the decision limits (CCα) were 0.2 and 0.1 ng mL⁻¹ and detection capability (CCβ) 0.4 and 0.2 ng mL⁻¹, respectively. The Authors concluded that once again β-bold sulfate demonstrated to be the candidate marker of a treatment. In an effort to develop a study on an extended population, deemed necessary also by the Authors cited above, we developed an LC-MS/MS method with triple quadrupole technology that had the lowest analytical limits possible for the detection of β-bold sulfate in
bovine urine. The method was also developed for α-bold sulfate, α- and β-bold glucuronide, ADD, α-bold and β-bold (Figure 1).

![Chemical structures of the seven analytes.](image)

**Figure 1.** Chemical structures of the seven analytes.

The validation was made according the Decision of Commission 2002/657/EC [11].

### 3.1.3. Materials and Methods

#### 3.1.3.1. Materials

All solvents were of HPLC or HPLC-MS grade quality and supplied by Fluka (Sigma-Aldrich, St.Louis, MO, USA).
Formic acid (98–100%) was from Riedel-de Haën (Sigma-Aldrich, St.Louis, MO, USA). The chemicals for the preparation of artificial urine were from Sigma-Aldrich (St.Louis, MO, USA). β-bold sulfate (triethylamine salt), β-bold glucuronide, and α-bold were from LGC Standards (Teddington, UK), and ADD and β-bold were from Fluka (Sigma-Aldrich, St.Louis, MO, USA). The sulfate and glucuronide forms of α-bold, provided by research partners, were prepared by a two-step synthesis: the epimerization of β-bold (Steroid SpA, Cologno Monzese, Milan, Italy) using a modified Mitsunobu protocol, according to Dodge and Lugar [12]; and sulphation, according to Sanaullah and Bowers [13] or glucuronation, according to Casati et al. [14], of α-bold. Internal standards were β-bold sulfate-d3 for sulfate forms, β-Bold-d3 for free forms (LGC Standards, Teddington, UK) and epitestosterone (EpiT) glucuronide-d3 for α- and β-bold glucuronide (National Measurement Institute, Pymble, NSW, Australia). Stock solutions of each analyte and of the internal standards were prepared in methanol at a concentration of 1 mg L\(^{-1}\), and stored at −40°C. Working solutions were prepared daily by diluting the stock solutions. Immunoaffinity columns (IAC) were from Randox. Concentrated wash and storage buffers were supplied with the columns and diluted following the manufacturer’s instructions before use (DM 2185, Randox Laboratories, Antrim, UK).
3.1.3.2. Artificial urine preparation

We were unable to find bovine urine samples in which all analytes were totally absent. Artificial urine was prepared in our laboratory for the validation studies, as described by Fabregat et al. [15]. Briefly, 0.1 g of lactic acid, 0.4 g of citric acid, 2.1 g of sodium bicarbonate, 10 g of urea, 0.07 g of uric acid, 0.8 g of creatinine, 0.37 g of calcium chloride·2H₂O, 5.2 g of sodium chloride, 0.0012 g of iron II sulfate·7H₂O, 0.49 g of magnesium sulfate·7H₂O, 3.2 g of sodium sulfate·10H₂O, 0.95 g of potassium dihydrogen phosphate, 1.2 g of dipotassium hydrogen phosphate, and 1.3 g of ammonium chloride were dissolved in 1 L of ultrapure water.

3.1.3.3. Urine sampling and storage

Urine samples were collected from untreated, under veterinary control, Charolaise or Limousine young bulls. Urine (about 100 mL) was collected into long-handled sterile containers. A visual inspection was made to check the urine clarity, in order to exclude the presence of raw materials or faecal contamination [16]. Only clear urine was put in 150-mL containers, cooled to 4°C and taken to the laboratory for storage at −40°C until extraction and analysis.
3.1.3.4. Sample preparation, extraction and purification

Five millilitres of centrifuged urine was added of internal standard to a final concentration of 2 ng mL\(^{-1}\). The IAC was washed with 5 mL ethanol:water (70:30, v/v) and equilibrated with 3 × 5 mL wash buffer (flow rate ≤3 mL min\(^{-1}\), i.e. about one drop per second). After adjusting the pH to 8 with 0.1 N NaOH, the urine was loaded (gravity flow). Wash buffer (2 × 5 mL) and water (1 × 5 mL) were used to wash the column. The elution of the bound analytes was then made by the application of 4 mL ethanol:water (70:30, v/v) (flow rates ≤3 mL min\(^{-1}\)) collected in a 15-mL polypropylene tube. The eluate was evaporated in a rotary evaporator. The dried extract was reconstituted in 500 µL of methanol:water (50:50; v/v) and transferred to an autosampler vial. The injection volume was 10 µL. The IAC could be again used, beginning from the equilibration described above, after a wash step with 2 × 5 mL ethanol:water (70:30, v/v).

3.1.3.5. LC-MS/MS analysis

LC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), consisting of a Surveyor MS quaternary pump with a degasser, a Surveyor AS autosampler with a column oven, and a Rheodyne valve with 20-µl sample loop. Chromatographic separation was achieved by using a Synergi Hydro RP reverse-phase HPLC column (150 x 2.0 mm, i.d. 4µm), with a C18 guard column (4 x 3.0 mm)
(Phenomenex, Torrance, CA, USA), kept at 30°C. The flow rate was 200 μl min⁻¹, and the mobile phase consisted of 0.1% aqueous formic acid (solvent A) and methanol (solvent B). The gradient program is reported in Table 1. The overall run time was 22 minutes.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Eluent A %</th>
<th>Eluent B %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>15</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>22</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 1. Gradient Timetable. A: 0.1% aqueous formic acid; B: methanol.

The mass spectrometer was a triple quadrupole TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray interface (ESI) set both in the positive (ESI+) and in the negative (ESI-) ionization mode. Acquisition parameters were optimized in the ion spray mode by direct continuous pump-syringe infusion of standard solutions of the analytes at the concentration of 1 μg mL⁻¹, a flow rate of 20 μL min⁻¹ and flow rate of the MS pump of 100 μL min⁻¹ in the ion source of the mass spectrometer. Conditions were as follows: capillary voltage 3,5 kV; ion transfer capillary temperature 340°C; nitrogen as sheath and auxiliary gas at 30 and 10 arbitrary units,
respectively; argon as collision gas at 1.5 mTorr; and peak resolution 0.70 Da FWHM. The scan time for each monitored transition was 0.1 s, and the scan width was 0.5 amu. Four diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in the multiple reaction monitoring (MRM). The selected diagnostic ions, one of which chosen for the quantification, and collision energies are reported in Table 2.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion [M-H]⁻ or [M-H]⁺ (m/z)</th>
<th>Product ions CE (m/z)</th>
<th>ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-bold</td>
<td>365</td>
<td>9747, 17739, 34940,</td>
<td>(-)</td>
</tr>
<tr>
<td>β-bold</td>
<td>365</td>
<td>9747, 17739, 34940,</td>
<td>(-)</td>
</tr>
<tr>
<td>β-bold</td>
<td>368</td>
<td>9854, 18041, 35240, 35331</td>
<td>(-)</td>
</tr>
<tr>
<td>α-bold</td>
<td>463</td>
<td>12132, 13521, 26913,</td>
<td>(+)</td>
</tr>
<tr>
<td>β-bold</td>
<td>463</td>
<td>12132, 13521, 26913,</td>
<td>(+)</td>
</tr>
<tr>
<td>EpiT</td>
<td>468</td>
<td>10937, 25623, 27416,</td>
<td>(+)</td>
</tr>
<tr>
<td>ADD</td>
<td>285</td>
<td>12122, 14713, 15114,</td>
<td>(+)</td>
</tr>
<tr>
<td>α-bold</td>
<td>287</td>
<td>12123, 13514, 17315,</td>
<td>(+)</td>
</tr>
<tr>
<td>β-bold</td>
<td>287</td>
<td>12123, 13514, 17315,</td>
<td>(+)</td>
</tr>
<tr>
<td>β-bold-d3</td>
<td>290</td>
<td>9148, 12127, 13814,</td>
<td>(+)</td>
</tr>
</tbody>
</table>

**Table 2.** MS/MS condition for the MRM acquisitions of analytes and internal standards. Ions for quantification are in bold. CE (eV): collision energy.

In Figure 2, the LC-MS/MS reconstructed chromatogram of an artificial urine sample spiked with 0.1 ng mL⁻¹ of each analyte is shown together with the ion spectra; on the right the internal standards (2 ng mL⁻¹) are also reported. Acquisition data
were recorded and elaborated using Xcalibur™ software from Thermo.

**Figure 2.** Reconstructed LC–MS/MS chromatograms of a spiked artificial urine sample with the respective ion spectra. Left side: standard analytes (concentration $= 0.1 \text{ ng mL}^{-1}$). Right side: relative internal standards (concentration $= 2 \text{ ng mL}^{-1}$).
3.1.3.6. Method validation

The developed method was validated according to Commission Decision 2002/657/EC \cite{11}. Parameters taken into account were as follows: instrumental linearity, precision, recovery, decision limit (CCα), and detection capability (CCβ). The instrumental linearity was evaluated by drawing six points calibration curves in solvent, containing analytes concentrations corresponding to 0.25-0.5-1.0-2.0-3.0-4.0 ng mL\(^{-1}\) and a fixed amount of ISTDs corresponding to 20 ng mL\(^{-1}\). The validation study was done using artificial urine as explained in 3.2.3.2., so the evaluation of specificity could not be made. Matrix calibration curves were obtained by spiking urine samples with the seven analytes, resulting in three analytical series, each series with three concentration levels (0.05-0.1-0.2 ng mL\(^{-1}\)) and six samples per concentration level (6 samples × 3 concentration levels × 3 series = 54 analyses). Method recovery and precision were evaluated using these matrix curves results; recovery was expressed in terms of percentage of measured concentration-to-fortified concentration ratio and precision as the coefficient of variability (CV). The same results were used to calculate the decision limit (CCα) and detection capability (CCβ) according to the matrix calibration curve procedure described in the Commission Decision
2002/657/EC as clarified in the document SANCO/2004/2726-revision 4\textsuperscript{[17]}.

### 3.1.3.7. Evaluation of artificial urine suitability

Three curves were prepared to evaluate the suitability of artificial urine. One curve with low doses (0.05; 0.1; 0.2 ng mL\textsuperscript{−1}) of the seven analytes in artificial urine, two curves with high doses (0.5; 1.0; 2.5 ng mL\textsuperscript{−1}) in artificial and bovine urine, respectively. The slopes and the Y-intercepts for each analyte of each curve were then compared with the unpaired t-test.

### 3.1.4. Results and Discussions

The presence in bovine urine of \(\alpha\)-bold and, primarily, \(\beta\)-bold in their free forms or as II phase metabolites, is a matter of debate in Europe. The need to study the conjugated metabolites of boldenone in urine as well as the development of a method that can distinguish the different conjugated forms, appears mandatory to distinguish between illicit treatment and the natural origin of boldenone in bovine urine. The developed method uses a simple extraction step with IAC and allows for the determination of ADD, sulfate, glucuronate, and free forms of \(\alpha\)- and \(\beta\)-boldenone in only one chromatographic run at concentration levels suitable for research purposes.
3.1.4.1 Sample preparation, extraction and purification

The need for an analysis that directly determined the free sulfate and glucuronate forms of bold led us initially to direct our efforts on clean-up methods like liquid/liquid extraction. Unfortunately, some results were achieved only for bold sulfate but not for bold glucuronide, while a different extraction altogether had to be made to detect the free forms. Direct determination methods of bold sulfate with good analytical limits are present in literature, as already stated \cite{10}; a unique method that could together determine sulfate, glucuronate and free α- and β-bold has not been developed even if Buiarelli et al. \cite{18}, in 2005 proposed a method for β-bold free and conjugate forms, free α-bold and 5β-androst-1-en-17β-ol-3-one in bovine and human urine. The use of the IAC was, at least initially, due to a casual occurrence. Briefly, the IACs used in this study are in fact intended for the clean-up of urine and serum in order to detect the corticosteroids dexamethasone, betamethasone and flumethasone. In the course of the development of a method for the determination of these corticosteroids with bovine urine through IAC extraction and LC-MS/MS analysis with the full mass monitoring, we noticed the presence of interfering compounds. We hypothesized, because of the $m/z$ values and due to the similarities in the chemical structures of anabolic steroids to corticosteroids, the compounds were the conjugate forms of boldenone. Subsequent trials performed by IAC clean-up of urine
fortified with the standards confirmed previous suppositions and
gave surprising results in terms of analytical limits. Therefore, we
decided to refine the method as described in 3.1.3.4 and 3.1.3.5
and to validate it. IAC extraction is considered expensive, so we
also considered the possibility that not all ten runs recommended
by the manufacturer could be made, accounting for the different
use from that indicated. We observed a constant response for the
first eight runs, a tolerable decline in accuracy in the next two runs
(that we roughly evaluated lower than 9%) and, subsequently, a
variable and unpredictable fall in the column performance. Using
a column for 10 cycles before discarding it is therefore advisable
and keeps the cost per sample comparable to other techniques.

3.1.4.2. LC–MS/MS

Data acquisitions were performed on the pure standard
compounds in full scan (m/z range 50–500) using the first
quadrupole to choose the precursor ion. In MS/MS experiments
m/z product ion scans were recorded between m/z 50 and 500 and
the four product ions with the higher signal-to-noise ratio (s/n) for
each analyte or internal standard were then chosen for analysis,
performed in the MRM mode, because of its high sensitivity and
specificity. The precursor ions, the four diagnostic product ions
and the collision energies are reported in Table 2. The four ions
fulfilled the requirements provided by the Decision of
defined in Annex I of Directive 96/23/EC\textsuperscript{[19]}. A minimum number of 3 ions (the precursor and 2 product ions) and of 4 identification points (IPs) are in fact required: as each one of the four product ions is equal to 1.5 IPs and the precursor is equal to 1.0 IP, the abundant value of 7 IPs was reached. The relative ion intensities also always complied with the maximum tolerances permitted. The ion for quantification was the most abundant of the four diagnostic ions.

3.1.4.3 Method validation

The instrumental linearity for the seven analytes was evaluated with calibration curves in solvent in a concentration range from 0.25 to 4.0 ng mL\textsuperscript{-1} using standard solutions in methanol:water (50:50; v/v), containing a fixed amount of ISTDs (20 ng mL\textsuperscript{-1} each). Regression coefficients of curves indicated a good fit for all analytes (\(\alpha\)-bold sulfate, \(r^2=0.982\); \(\beta\)-bold sulfate, \(r^2=0.991\); \(\alpha\)-bold glucuronide, \(r^2=0.978\); \(\beta\)-bold glucuronide, \(r^2=0.988\); ADD, \(r^2=0.988\); \(\alpha\)-bold, \(r^2=0.995\); \(\beta\)-bold, \(r^2=0.991\)).

The matrix calibration curves built for each analyte were linear in the range from 0.05-0.2 ng mL\textsuperscript{-1}. The regression lines, obtained using the least square method, had good fit for all analytes (\(\alpha\)-bold sulfate, \(r^2=0.977\); \(\beta\)-bold sulfate, \(r^2=0.995\); \(\alpha\)-bold glucuronide, \(r^2=0.985\); \(\beta\)-bold glucuronide, \(r^2=0.990\); ADD, \(r^2=0.995\); \(\alpha\)-bold, \(r^2=0.982\); \(\beta\)-bold, \(r^2=0.994\)).
Specificity and matrix effect were not evaluated; the presence of at least one of the studied analytes in any bovine urine sample required the use of artificial urine, as already specified in 3.1.3.2. Some bovine urine samples randomly selected were however checked, and the presence of one peak at the analyte retention times was shown not to be due to an interfering substance, i.e. the peak had all the characteristics of the analyte as provided by the Decision of Commission 2002/657/EC [11].

Precision, calculated by applying the one-way analysis of variance (ANOVA), was expressed as CVs, in terms of intra-day and inter-day repeatability. The results reported show that the intra- and inter-day repeatabilities for all the analytes were below 17.2% and 21.8%, respectively. These CVs were lower than 22% as proposed by Thompson [20]. The high values were probably due to the low concentrations used for the method validation: The levels chosen were addressed to a subsequent research aimed to understand if conjugated bold in bovine urine have a natural or endogenous origin of and to eventually set a natural threshold. The mean recoveries ranged between 96% and 103% for $\alpha$-bold sulfate, 97% and 102% for $\beta$-bold sulfate, 96% and 106% for $\alpha$-bold glucuronide, 93% and 109% for $\beta$-bold glucuronide, 95% and 104% for ADD, 92% and 110 % for $\alpha$-bold, and 99% and 100% for $\beta$-bold. The data for all the analytes are reported in Table 3.
Table 3. Method trueness and precision.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration level (ng mL⁻¹)</th>
<th>Recovery % (n=18)</th>
<th>Repeatability intra-day (CVᵢ n=6)</th>
<th>Repeatability inter-day (CVᵢ n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-bold sulfate</td>
<td>0.05</td>
<td>96</td>
<td>16.2</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>103</td>
<td>17.2</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>99</td>
<td>11.0</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>97</td>
<td>10.1</td>
<td>15.4</td>
</tr>
<tr>
<td>β-bold sulfate</td>
<td>0.1</td>
<td>102</td>
<td>9.8</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>100</td>
<td>12.8</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>106</td>
<td>15.2</td>
<td>21.8</td>
</tr>
<tr>
<td>α-bold glucuronide</td>
<td>0.1</td>
<td>96</td>
<td>10.1</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>101</td>
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<td>11.2</td>
</tr>
<tr>
<td></td>
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<tr>
<td>β-bold glucuronide</td>
<td>0.1</td>
<td>93</td>
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<td>12.3</td>
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<tr>
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<td>101</td>
<td>10.2</td>
<td>10.2</td>
</tr>
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<td></td>
<td>0.05</td>
<td>95</td>
<td>7.5</td>
<td>14.4</td>
</tr>
<tr>
<td>ADD</td>
<td>0.1</td>
<td>104</td>
<td>6.6</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>99</td>
<td>7.9</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
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<td>110</td>
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<td>19.4</td>
</tr>
<tr>
<td>α-bold</td>
<td>0.1</td>
<td>92</td>
<td>9.8</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>101</td>
<td>11.2</td>
<td>11.2</td>
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<td></td>
<td>0.05</td>
<td>100</td>
<td>11.1</td>
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<td>β-bold</td>
<td>0.1</td>
<td>100</td>
<td>8.1</td>
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</tr>
<tr>
<td></td>
<td>0.2</td>
<td>100</td>
<td>5.8</td>
<td>6.0</td>
</tr>
</tbody>
</table>

CCα was calculated as described in the document SANCO/2004/2726 revision 4 [17] using parallel extrapolation to
the x-axis at the lowest experimental concentration. $CC\alpha$ and $CC\beta$ values are reported in Table 4, which shows the low analytical limits obtained.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$CC\alpha$ (ng mL$^{-1}$)</th>
<th>$CC\beta$ (ng mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-bold sulfate</td>
<td>0.08</td>
<td>0.10</td>
</tr>
<tr>
<td>$\beta$-bold sulfate</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>$\alpha$-bold glucuronide</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>$\beta$-bold glucuronide</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>ADD</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>$\alpha$-bold</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>$\beta$-bold</td>
<td>0.07</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 4. $CC\alpha$ and $CC\beta$ validation data.

3.1.4.4. Evaluation of artificial urine suitability

After the first preliminary tests, we realized that we could not find bovine urine samples where all the studied analytes were totally absent. At least one peak for each ‘blank’ bovine urine at the retention times of the analytes was found. The estimated concentrations were moreover not much lower than 0.05 ng mL$^{-1}$, the lowest concentration considered in the validation study. In order to avoid errors in the parameter calculation, we could not therefore simply subtract the peak areas
to the corresponding ones that resulted in the spiked sample because of the similar values. The use of artificial urine was thus mandatory, even if an evaluation of its suitability was required. In a first step, we built two curves with high doses (0.5; 1.0; 2.5 ng mL\(^{-1}\)) in artificial and bovine urine, respectively. The choice of this concentration range was made taking into account the estimated concentrations of the peaks found in bovine “blank” urine. We therefore considered it satisfactory to use a range the minimum concentration of analyte which was at least 10 times higher than the estimated concentrations in the “blank” bovine urine. The correlation coefficients of all the curves so prepared were higher than 0.99. The slopes and Y intercepts of each analyte in the two different matrices, compared with an unpaired t-test, did not result in significant differences. However, we could not yet consider the two matrices similar, as we were using doses higher than those used for validation. As a second step, we compared the slopes and Y intercepts of the high dose and low dose curves of each analyte in artificial urine. In this case, too, no difference was found. Therefore, we verified that high and low doses of each analyte, if combined, belonged to the same curve and subsequently that artificial urine was suitable for method validation. Slopes and Y intercepts of this “wide range” curves were compared with the slopes and Y intercepts of the high and low concentration curves. No difference was found for each
analyte. Accordingly, this curves were used as calibration curves for quantification of the samples described in 3.1.3.4.

3.1.4.5. Application of the method

In order to verify the developed method in real conditions, we analysed four urine samples, randomly collected from the young bulls under veterinary control, for the detection of the seven studied molecules. The reconstructed chromatograms and ion spectra relative to one sample are shown in Figure 3.

![Chromatograms and Ion Spectra](image)

**Figure 3.** Reconstructed LC–MS/MS chromatograms and respective ion spectra of the analytes detected in a urine sample.

The concentration values found in the four sample ranged from 0.15 to 0.63 ng mL$^{-1}$ for α-bol sulfate (3 positives); from 0.09 to 0.26 ng mL$^{-1}$ for β-bol sulfate (3 positives); from 0.12 to 0.58 ng mL$^{-1}$ for α-bol glucuronide (4 positives); from
0.08 to 0.48 ng-mL$^{-1}$ for β-bol glucuronide (4 positives); and from 0.47 to 2.1 ng mL$^{-1}$ for ADD (4 positives); α-bol was found in one only sample (0.53 ng mL$^{-1}$) where β- bol was detected, too, at a concentration lower than CCβ.

For further confirmation of our results, we asked the Drug Residue Laboratory of the official organization Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna (IZSLER) to test five other urine samples already analysed by us for the presence of only β-bold conjugates. The method used by IZSLER was a fully validated method according to the Commission Decision 2002/657/EC with SPE extraction [21]. It must be stressed that the method involved deconjugation with β-glucuronidase and indirect and non-specific determination of conjugate forms by LC-MS/MS: the differences in the sample preparation and analysis only permitted a qualitative comparison. The method outlined in this paper always detected β-bold glucuronide and three times it detected β-bold sulfate, while the method used for comparison found β-bold conjugate four times out of five. The possibility that incubation with β-glucuronidase could cause inter-transformations of steroids [22] and that β-boldenone could be neo-formed or metabolized cannot be discarded. It must be highlighted however that, when the samples were ordered by increasing concentrations found by the two different methods, the sequence was significantly the same as shown in Table 5.
A simple method that uses an IAC extraction and LC-MS/MS triple quadrupole analysis in the MRM mode, for the simultaneous determination of α- and β-bold sulfate, α- and β-bold glucuronide, α- and β-bold, and ADD in bovine urine was developed and validated according to EU Decision 2002/657/EC [11]. The method was verified on real samples. A further qualitative confirmation by a different laboratory on five urine samples that we had already analysed was made just for the conjugated forms of β-bold. The results were also satisfying in actual conditions and demonstrated that IAC clean up can be used for the subsequent direct analysis of α- and β-bold and their

Table 5. Results of the comparison between the IAC extraction plus LC-MS/MS analysis and the SPE extraction after hydrolysis plus LC-MS/MS analysis.

<table>
<thead>
<tr>
<th>Urine sample</th>
<th>β-bold sulfate (ng mL⁻¹)</th>
<th>β-bold glucuronide (ng mL⁻¹)</th>
<th>β-bold conjugate (ng mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.14</td>
<td>0.19</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>nd</td>
<td>0.34</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>nd</td>
<td>0.55</td>
<td>0.50</td>
</tr>
<tr>
<td>4</td>
<td>0.14</td>
<td>0.44</td>
<td>0.82</td>
</tr>
<tr>
<td>5</td>
<td>0.21</td>
<td>0.58</td>
<td>1.52</td>
</tr>
</tbody>
</table>

nd: not detected

3.1.5. Conclusions


sulfate and glucuronate conjugates, and for ADD for research purposes. The next step will be the study of the bold II phase metabolites as biomarkers of an illicit treatment ‘to unambiguously distinguish treated from non-treated animals’ \[9\] on a representative number of animals.

3.1.6. Acknowledgments

The Authors wish to thank Nicola Brina and Piero Giorgi whose involvement allowed ‘Coop Italia Società Cooperativa’ to fund the study.

Elisa Pasquale is the recipient of a Cariplo Ph.D. fellowship in Animal Production in the Laboratory of Inspection of Food of Animal Origin at the University of Milan.

3.1.7. References


3.2. Presence of β-boldenone sulfate and glucuronide in untreated young bulls

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3.2. Presence of $\beta$-boldenone sulfate and glucuronide in untreated young bulls

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3.2.1. Abstract

The administration of boldenone to bovines, either for growth promotion or therapeutic purposes, has been banned in the European Union since 1981. It is, however, a pseudoendogenous hormone, thus its detection in bovine urine, in the form of $\alpha$-boldenone conjugates, is considered fully compliant up to 2 ng mL$^{-1}$. Greater attention has been placed on $\beta$-boldenone, the anabolic active epimer, whose conjugated form must be absent in urine. Recently, the identification of a biomarker representing unquestionable evidence of illicit treatment with bold or its precursor androstadienedione has been a major topic in the
literature regarding the detection of residues in bovine urine, and β-boldenone sulfate is a candidate molecule. In this study, we used a method previously validated according to the European Commission Decision 2002/657/EC for the determination of sulfate and glucuronide conjugates of β-boldenone. We assessed the occurrence of these molecules in young bull urine, with the aim of understanding whether they could be of endogenous origin, and to check for a possible relationship with particular environmental and stress conditions. Urine samples from 56 young bulls were collected after transport stress, under non-stressful conditions and after transport and slaughter stress. Histopathological investigation of the hormone target organs, i.e. the bulbourethral and prostate glands, was also performed. The results indicate an inverse relationship between the presence and concentration of β-boldenone sulfo- and gluco-conjugates in urine, and stress conditions, expressed by the absence of detection at the slaughterhouse. No significant macroscopic and histologic lesions were detected. Our study indicates that β-boldenone sulfate could be a biomarker of treatment only at the slaughterhouse, while at the farm, in untreated animals, (i.e. after a five-month period under the control of Official Veterinarians), sulfate and glucuronide metabolites were found with a frequency of 78% and 46%, respectively, showing the endogenous origin of boldenone.

**Keywords:** β-boldenone sulfate, β-boldenone glucuronide, young bull urine, LC-MS/MS analysis.
3.2.2. Introduction

The steroid β-boldenone (β-bold), also called 1-dehydrotestosterone or androsta-1,4-diene-17β-ol-3-one is an anabolic steroid, which differs in structure from testosterone by dehydrogenation at the C1-C2 position of the cycloperhydrophenanthrene ring as indicated in Figure 1, where the conjugated forms of bold are shown. β-bold exhibits strong anabolic activity and for this reason can be used by athletes in doping preparations (principally as ester form, i.e. as undecylenate ester) [1].

![Chemical structures of the analytes](image)

**Figure 1.** Chemical structures of the analytes.

In the veterinary field, β-bold, like other anabolic steroids, is among the most important drugs tested for at horseracing and equestrian events [2], and is known to be illicitly administered as a growth promoter to meat-producing cattle [3,4]. Bold belongs to the A group of Council Directive 96/23/EC, which includes substances having anabolic effect and unauthorised
The presence of β-bold conjugates in veal calf urine is considered proof of illicit treatment, while a threshold of 2 ng mL\(^{-1}\) has been set for α-bold conjugates in the urine of veal calves, under which it could be considered endogenous and not proof of illegal administration. A detection capability (CC\(\beta\)) of screening methods or a decision limit (CC\(\alpha\)) for confirmatory methods for β-bold conjugates of 1 ng mL\(^{-1}\) in urine is required for surveillance purposes \(^{[3, 6, 7]}\).

The presence of bold in veal calf urine was first demonstrated by Arts et al. \(^{[8]}\) who detected α-bold in the urine of untreated calves in concentrations ranging from <0.1 to 2.7 ng mL\(^{-1}\), while β-bold was not observed at levels exceeding 0.1 ng mL\(^{-1}\). Sterk et al. confirmed these results on two bovines of unspecified gender and age \(^{[9]}\).

However, it is necessary to elucidate the mechanisms that lead to the presence of endogenous bold in cattle urine. Faecal contaminations of calf urine could be a source of false positives for free α-bold and β-bold, due to the transfer of boldenone and/or its precursors from faeces to urine \(^{[10-12]}\). It has therefore been recommended to pay particular attention to the procedure of bovine urine sampling to prevent faecal contamination in order to avoid boldenone false positives \(^{[3]}\).

The endogenous occurrence of β-bold, α-bold and related substances with hormonal activity in cattle urine and faeces could be linked to the conversion of phytosterols and other steroid substances \(^{[5]}\).
precursors in feed [13-16]. In 2004, Nielen et al. stated the importance of investigating the phase II metabolites of bold, asserting that the presence of β-bold conjugates (without specifying the kind of conjugated moiety) in the urine of calves can be considered evidence of illicit treatment [17]. Le Bizec et al. [18] and Destrez et al. [19] suggested, in a study carried out on a limited number of calves that the presence in urine of the sulfo-conjugate fraction could be useful to distinguish between natural situations and the illegal use of β-bold in cattle.

The determination of conjugated forms of anabolic androgen steroids like β-bold is mainly based on the analysis of the free form after a preliminary step of deconjugation, with the use of specific hydrolytic enzymes (glucuronidases and sulphatase) from Escherichia coli or Helix pomatia. After extraction, the analysis of steroids can be performed by GC-MS/MS or LC-MS/MS. However, the enzymatic hydrolysis may be incomplete. Moreover, steroid conversion or degradation and artefact formation may occur [20, 21].

As an alternative to the indirect detection (i.e. after enzymatic hydrolysis) of β-bold conjugates, we have developed and validated a method which uses immunoaffinity purification coupled to LC-MS/MS in order to perform direct analysis of the glucuronide and sulfate forms of β-bold [22, 23]. In contrast to the present study, in that study the free forms of α- and β-bold, as well
as α-bold conjugates and androstadienedione (ADD) were also considered.

In order to verify the possible endogenous origin of β-bold II phase metabolites in young bulls, we carried out a study on urine samples from 56 animals collected at different times at the farm, where the animals were under veterinary control, and at the slaughterhouse. As already shown for the relationship between prednisolone and cortisol \cite{24}, we hypothesised that an increase in the release of androstenedione (AED) by the adrenal glands, stimulated by pituitary adrenocorticotropic hormone (ACTH), could lead to the production of bold through the formation of ADD.

Additionally, we performed a histological examination of the accessory sex glands of each animal, a screening test introduced in Italy in 2009 by the Ministry of Health to control the illegal use of sex hormones \cite{25}. This strategy is based on the biological effects of the different steroid classes in target organs. Groot and Biolatti \cite{26} and Cannizzo et al. \cite{27} reported morphological alterations to the accessory sex glands of boldenone-treated animals. The official monitoring of residues in cattle throughout the European Union in 2012 found 0.25% non-compliance for the use of illegal growth promoters, including sex steroids, corticosteroids and β-agonists. In particular, in the group of steroids (A3), there were 0.09% non-compliant samples in all
animal and product categories. These figures may underestimate the real incidence of steroid abuse in meat cattle breeding.

The conditions that could lead to the detectable presence of β-bold glucuronide and sulfate in the urine were hence considered: uncontrolled, stressed animals upon arrival at the farm, animals in non-stressful conditions after an adequate period of adaptation, and animals stressed by transport and slaughtering operations. In this last case, histological alterations to the bulbourethral and prostate glands were also studied.

3.2.3. Materials and methods

3.2.3.1. Chemicals and reagents

Methanol (HPLC-MS grade), ethanol (HPLC grade) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid 98-100% was from Riedel-de-Haën (Sigma-Aldrich, St. Louis, MO, USA). Ultrapure water was prepared with a Milli-Q Plus apparatus (Millipore, Molsheim, France).

β-bold sulfate (triethylamine salt) and β-bold glucuronide were procured from LGC Standards (Teddington, UK). Internal standards were β-bold sulfate-d3 for the sulfate form and epitestosterone (EpiT) glucuronide-d3 for β-bold glucuronide (National Measurement Institute, Pymble, NSW, Australia). Stock solutions of each analyte and of the internal standards were
prepared in methanol at a concentration of 1 mg L\(^{-1}\), and stored at \(-40^\circ\)C. Working solutions were prepared daily by diluting the stock solutions. Immunoaffinity columns (IAC) were from Randox. Concentrated wash and storage buffers were supplied with the columns and diluted following the manufacturer’s instructions before use (DM 2185, Randox Laboratories, Antrim, UK).

3.2.3.2. Animal housing and urine collection

The study was performed on 56 young Charolaise and Limousine bulls, initially weighing 300 to 350 kg; animals were reared in France, transported to the farm and slaughtered in an abattoir, both located in Piedmont. The young bulls were fed with a diet usually employed in zootechnical practice. Ad libitum access to water was allowed to the animals. Throughout the experimental period, the animals were under the control of Official Veterinarians of the National Health Service, who also collected urine at different times. The first collection was performed upon arrival (T1) at the farm. The second urine collection was performed at the farm after a five-month adaptation period (T2) in order to assess untreated, unstressed animals. The first and the second urine samples at the farm were collected into long-handled sterile container and collection was performed in the morning hours under conditions of natural micturition, as recommended by the Italian National Residues Plan \[28\]. The third urine sampling
was performed at the slaughterhouse after a period of seven months (T3) of residence at the farm and when the weight of the animals was about 550-600 kg. This last urine sample was collected directly from the urinary bladder immediately after slaughter. A visual inspection was made to check the turbidity or the presence of raw materials. Only clean urine was sampled, frozen and taken to the laboratory for storage at -40°C until extraction and analysis.

3.2.3.4. Tissue collection

The bulbourethral and prostate glands of each animal were collected after slaughter. Tissue samples were fixed in 10% neutral buffered formalin overnight at room temperature and paraffin embedded according to routine histological procedures. Representative sections of each sample were stained with haematoxylin-eosin (HE).

3.2.3.5. Sample preparation, extraction, LC-MS/MS analysis and method validation

Urine extraction and analysis were performed as previously described by Chiesa et al.[23]. Briefly, a 5 mL centrifuged urine sample spiked with internal standards (2 ng mL⁻¹) and adjusted to pH 8 with 0.1 N NaOH was loaded into a previously washed IAC column (5 mL ethanol: water; 70:30, v/v) and equilibrated (3 x 5 mL wash buffer). The column was then
washed (wash buffer, 2 x 5 mL and water, 1 x 5 mL). The elution was performed with 4 mL ethanol:water (70:30, v/v) (all the flow rates were \( \leq 3 \) mL min\(^{-1}\)). The eluate was evaporated until dry in a rotary evaporator, reconstituted in 500 µL of methanol:water (50:50; v/v) and transferred to an autosampler vial. A volume of 10 µL was analysed by LC-MS/MS. The LC apparatus and chromatographic conditions were: Surveyor AS autosampler and Surveyor MS quaternary pump (ThermoFisher Scientific, San Jose, CA, USA), Synergi Hydro RP reverse-phase HPLC column 150 x 2.0 mm, i.d. 4µm, with a C18 4 x 3.0 mm guard column (Phenomenex, Torrance, CA, USA), kept at 30°C. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and methanol (solvent B) with a flow rate of 200 µL min\(^{-1}\). The gradient program was: from 0 to 1 minutes A was kept at 40%, then decreased to 5% in 11 minutes, and maintained for 1 minute, then A was increased again to 40% from 13 to 15 minutes; the last 7 minutes were in isocratic elution (A=40% and B=60%). The run length was 22 minutes. The MS/MS apparatus and conditions were: triple quadrupole TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray interface (ESI) set both in positive and negative ionisation mode; capillary voltage 3.5 kV; ion transfer capillary temperature 340°C; nitrogen as the sheath and auxiliary gas at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr and peak resolution 0.70 Da FWHM; the scan time for each monitored transition was
0.1 s and the scan width was 0.5 amu. The acquisition was performed in multiple reaction monitoring (MRM) after selecting, for each analyte and internal standard, four diagnostic product ions, one of which used for quantification (Table 1). Data were acquired and elaborated by Xcalibur™ software from Thermo. Figure 2 shows the reconstructed chromatograms with the ion spectra of β-bold glucuronide and sulfate in the solvent. The validation protocol, performed according to the European Commission Decision 2002/657/EC [22], is described by Chiesa et al. [23].

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ions [M-H] or [M-H]^+ (m/z)</th>
<th>Product ions (m/z)</th>
<th>ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-boldenone sulfate</td>
<td>365</td>
<td>974, 17739, 34940, 35039</td>
<td>(-)</td>
</tr>
<tr>
<td>β-boldenone glucuronide</td>
<td>463</td>
<td>12122, 13521, 26013, 28712</td>
<td>(+)</td>
</tr>
<tr>
<td>β-boldenone sulfate-d3 (IS)</td>
<td>368</td>
<td>9814, 18041, 35240, 35331</td>
<td>(-)</td>
</tr>
<tr>
<td>EpiT glucuronide-d3 (IS)</td>
<td>468</td>
<td>10997, 25623, 27416, 29221</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Table 1. MS/MS conditions for the MRM acquisitions of analytes and internal standards. Ions for quantification are in bold. CE (eV): collision energy
Figure 2. Reconstructed LC–MS/MS chromatograms with ion spectra of β-bold sulfate and β-bold glucuronate in solvent at a concentration of 0.5 ng mL$^{-1}$.

3.2.3.6. Statistical analysis

All statistical analyses were performed by taking into account only the samples in which β-bold sulfate and glucuronate were found. The Kolmogorov–Smirnov test was performed to check the normality of the positive results from each dataset and the non-parametric Spearman correlation test was used to verify the effective pairing of the datasets. The differences in the results obtained from the analysis were checked using the Wilcoxon matched-pairs signed-rank test. The null hypothesis was set at P>0.05. GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego, CA, USA) was used to perform these calculations.
3.2.4. Results and Discussion

3.2.4.1. Method validation

The parameters of the validation are reported here in a summarised way: linear matrix calibration curves for each analyte were in the range from 0.05-0.2 ng mL$^{-1}$ ($r^2 = 0.99$ for both analytes) (6 samples×3 concentration levels×3 series = 54 analyses). Intra-day and inter-day repeatability, representing the precision, were calculated by one-way analysis of variance (ANOVA), expressed as CVs, and were below 14.7% and 17.0%, respectively. The mean recoveries ranged between 93% and 109%. The CC$\alpha$ value, calculated as described in the document SANCO/2004/2726 revision 4[29], was 0.07 ng mL$^{-1}$ and the CC$\beta$ value was 0.09 ng mL$^{-1}$ for both analytes. The values of the decision limit (CC$\alpha$) and detection capability (CC$\beta$) were significantly lower than the actual recommended concentration set at 1 ng mL$^{-1}$ for β-bold[7].

3.2.4.2. Urine analyses

The reconstructed chromatograms with ion spectra of β-bold sulfate and β-bold glucuronate in a urine sample at the three different times of collection are shown in Figure 3. The results of this study are reported in Table 2.
Figure 3. Reconstructed LC–MS/MS chromatograms with ion spectra of β-bold sulfate and β-bold glucuronide in a urine sample at different steps of collection T1 (A), T2 (B) and T3 (C). The internal standard is at a concentration of 2 ng mL⁻¹. The concentrations of β-bold sulfate at T1 and T2 were 0.16 and 0.32 ng mL⁻¹, respectively. The concentrations of β-bold glucuronide at T1 and T2 were 0.61 and 0.92 ng mL⁻¹, respectively.
Table 2. Number of young bull urine samples analysed and found positive for β-boldenone sulfate and glucuronide at different times on the farm (T1=arrival; T2=after five months) and after slaughtering (T3=7 months); mean, minimum and maximum concentrations of the two conjugated forms.

<table>
<thead>
<tr>
<th></th>
<th>β-boldenone sulfate</th>
<th>β-boldenone glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Farm T1</td>
<td>Farm T2</td>
</tr>
<tr>
<td>Samples</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>Positive (%)</td>
<td>18 (32%)</td>
<td>44 (78%)</td>
</tr>
<tr>
<td>Mean±SD (ng mL⁻¹)</td>
<td>0.18±0.041⁻²⁵</td>
<td>0.26±0.17⁺</td>
</tr>
<tr>
<td>Minimum (ng mL⁻¹)</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Maximum (ng mL⁻¹)</td>
<td>0.31</td>
<td>0.94</td>
</tr>
</tbody>
</table>

*: Different from the corresponding form in urine at T2 (P<0.001).
⁺: Different from glucuronide at the same time (P<0.01).
⁻⁻⁻: Different from glucuronide at the same time (P<0.0001).

The most evident datum is the lack of detection of both β-bold conjugates in urine samples collected at the slaughterhouse. Moreover, the II phase metabolites were found with a lower frequency at the collection time T1 than T2, i.e. 32% and 78% for β-boldenone sulfate, and 18% and 46% for β-boldenone glucuronide, respectively. The three collection times were chosen due to their correspondence to different stress conditions: the collection at T1 occurred after transport stress, at T2 there were no stressors, at T3 the animal had transport and slaughter stresses. The cause of stress can be psychological (restraint, handling, unfamiliar smell, breakdown of social groupings, crowding) or physical (hunger, thirst, noise, etc.)[30-32], and animals respond to stressful challenges in their environment through interacting mechanisms that include...
neuro-hormonal parameters\textsuperscript{[33]}. It can be supposed that, even if the listed causes of stress are related to both transport and slaughtering, the cattle perceives with greater intensity the stimuli at the slaughterhouse (i.e. the smell of blood). In addition, the young bulls underwent a long period of transport, with grouping in assembly centres and subsequent transfer to the farm where the first urine collection was performed on the day of arrival. An adaptive response to stressors as a function of their duration can be expected \textsuperscript{[34]}. Due to these considerations, a ranking can be made based on the stress suffered at different sample collection times: T3 (higher stress conditions) > T1 (lower stress conditions) > T2 (presumable absence of stress). If the frequencies in detection of \(\beta\)-bold sulfate and glucuronate are considered, an inverse relationship was found. It is therefore conceivable that the starting hypothesis expressed in the introduction, i.e. a positive correlation between stress and ACTH-induced boldenone release, has to be turned upside down. In the urine collected at T2, when the animals were under control for five months, we found more positive samples, demonstrating the production of endogenous bold and, by the comparison with T1 and T3 samples, an inhibitory role of stress on bold production. The detected concentrations of \(\beta\)-bold sulfate were to the frequencies, i.e. they were statistically higher when the frequencies of detection were higher. A similar observation does not seem feasible for the glucuronide metabolite, as there was no statistical difference. The high standard deviations,
however, did not allow for finding a difference between the average values at T2 and T1. The means per se and the maximum values were higher at T2, as for β-bold sulfate. The T2 concentrations of β-bold sulfate were higher than the T1 maximum concentration in 11 out of 44 samples i.e. in about 25% of the samples, while the T2 concentrations of β-bold glucuronide were above the T1 maximum concentration in 9 out of 26 samples, i.e. in about 35% of the samples. This higher percentage seems to indicate an actual difference between the concentrations of β-bold glucuronide at the first two time points. Also for the concentrations, a negative correlation between stress and β-bold endogenous production was suggested; this hypothesis is strengthened by the consideration that the animals at T1 had just been put under veterinary control for bold treatment, thus we cannot exclude a lower frequency of detection and concentration of β-bold conjugates of endogenous origin at the first sampling.

The concentration of the sulfate form found in our study was higher than has been described in literature. In a study in 2004, performed using GC-MS, Sterk et al. stated that almost all boldenone excreted in urine is present as the glucuronic acid conjugate. In 2009, Destrez et al. proposed β-bold sulfate as a candidate marker of treatment\(^{[19]}\). Both studies were performed on treated (bold or ADD) bovines. Particularly the second study, performed using LC-MS/MS, showed a CC\(\alpha\) value of 0.2 ng mL\(^{-1}\) and a CC\(\beta\) of 0.4 ng mL\(^{-1}\). These higher analytical limits could
have caused misleading conclusions, considering that we found β-bold sulfate in 37 urine samples at a concentration lower than the CCα indicated in the previous study, and 21 out of these 37 samples were collected in the absence of stress.

Finally, even if determining the endogenous presence of α-bold sulfate and glucuronate was not the aim of the study, because of their nature as conjugates of the epimer of β-bold, the LC-MS/MS method detected their eventual presence. α-bold sulfate was found just once at T2; α-bold glucuronate was found at the three collection time points, generally together with β-bold glucuronate.

3.2.4.3. Gross pathology and histopathology

No macroscopic alterations were detected in the genital tracts and accessory sex glands of the examined subjects. In 18.1% (n=10) of cases, slight prostate ectasia was detected, but sometimes this lesion was associated with other changes, such as slight hypersecretion, which was observed in 5.5% (n=3) of the examined animals. Slight hypersecretion of the bulbourethral glands was observed in 16.4% (n=9) of cases, and slight ectasia was detected in 3.6% of the examined glands.

3.2.5. Conclusions

The analyses performed on urine samples from 56 young
bulls at three collection times showed that β-boldenone conjugates, both the sulfate and glucuronide forms, can be naturally present with variable concentrations in urine collected at the farm.

The finding that β-boldenone conjugates were not found at the slaughterhouse is of note. A negative influence of stress on bold endogenous production is conceivable. The results obtained from urine collected at the farm confirm this hypothesis. This study indicates that β-bold sulfate presence is not per se a biomarker of treatment when urine sample collection is performed at the farm; it still needs to be ascertained if a cut-off level can be set for both β-bold conjugates. The presence β-bold sulfate and glucuronide in urine at the slaughterhouse could contrariwise represent a useful parameter for the control of illicit treatments. To this aim, a study on urine collected at the slaughterhouse from treated animals should be performed. No relevant morphological alterations to the sexual organs and associated glands were detected in these animals. These results are compatible with the physiological findings typical of untreated animals.

3.2.6. Acknowledgements

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3.2.7. References


samples of blank urine from livestock. Fresen Journal Analytical Chemistry. 360: 454-455


organization and communication. Proc. Euro Food Chem XII, Bruges, Belgium. 74-77


47. Ministero Della Salute; Dipartimento Della Sanità Pubblica Veterinaria, Della Sicurezza Alimentare E Degli Organi Collegiali Per La Tutela Della Salute; Direzione Generale Per L’igiene E La Sicurezza Degli Alimenti E La Nutrizione; Piano Nazionale Per La Ricerca Di Residui, Relazione Finale Anno 2009


50. - Ministero Della Salute; Dipartimento Della Sanità Pubblica Veterinaria, Della Sicurezza Alimentare E Degli Organi Collegiali Per La Tutela Della Salute; Direzione Generale Per L’igiene E La Sicurezza Degli Alimenti E La Nutrizione; Piano Nazionale Per La Ricerca Di Residui, Relazione Finale Anno 2013

51. European Union, European Commission, Health & Consumer Protection, Directorate General Directorate E,
Safety of the Food Chain, Document SANCO/2004/2726-
revision 4, December 2008, Guidelines for the
Implementation of Decision 2002/657/EC

52. Grandin T. 1997. Assessment of stress during handling and

introductory text. CAB International, Oxfordshire, United
Kingdom

54. Adzitey F. 2011. Effect of pre-slaughter animal handling
on carcass and meat quality. International Food Resource
Journal. 18: 484-490

transport. Veterinary Research Communication. 27: 519-
524

and slaughter. Italian Journal of Animal Science. 8: 241-
252
3.3. Presence of prednisolone in complementary feedstuffs for bovine husbandry

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3.3. Presence of prednisolone in complementary feedstuffs for bovine husbandry

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3.3.1. Abstract

**BACKGROUND:** According to European Union legislation, prednisolone, a steroid that belongs to the glucocorticosteroid group, is banned as a growth promoter in cattle husbandry and therefore should not be present in bovine
feedstuffs. As our preliminary investigations detected prednisolone in this matrix, we performed a study on different commercially available complementary feedstuffs, stored at the farm and/or in the laboratory, in order to verify whether its presence was due to neo-formation during storage.

**RESULTS:** Prednisolone was detected in almost all (95%) feedstuffs collected at the farm. When the feedstuffs were stored at the laboratory, the frequency (31%) and the concentration of prednisolone-positives were lower. This difference, likely due to different environmental conditions, implies the possibility of its neo-formation.

**CONCLUSION:** Our data indicate that the neo-formation of prednisolone can occur in feedstuff, and that the frequency and concentration could be related to the storage conditions. The individuation of an objective parameter that is useful for the identification of the compliance of feed is therefore mandatory.

**Keywords:** pigs urine, pigs adrenal glands, endogenous prednisolone, cortisol, LC-MS².
3.3.2. Introduction

The intensive production of food animals has triggered the development of minutely elaborated diets and has induced increased utilisation of veterinary drugs for therapeutic or preventive purposes.

The ban of any growth-promoter in the EU, accomplished on January 1st 2006 with the last four antimicrobial agents - Monensin sodium, Salinomycin sodium, Avilamycin and Flavophospholipol

set moreover very precise limits to the use of drugs or medicated feeds in animal husbandry, with the aim of ensuring “a high level of consumer protection with regard to food and feed safety”, and “animal health and animal welfare” as well as limiting anti-microbial resistance.

The concern of the EU legislator was the control of the use of veterinary drugs in food producing animals, the enactment of regulation on feedstuff hygiene, on the use of additives in animal nutrition, and on the presence of undesirable substances such as inorganic contaminants, nitrogenous compounds, dioxins and polychlorobiphenyls in animal feed, as stated by the Directive 2002/32/EC and its subsequent amendments. The monitoring of residues in feed and food of banned or undesirable substances requires a heavy effort by Official Control Organisations, whose investigations are regulated by the National Animal Feed Plan and the National Residues Plan in each EU Member State. The work
of these Organisations is however made more difficult by the possible presence of active principle of drugs, which may be included in the category of pseudoendogenous substances, i.e. synthetically produced hormones that are also known to be endogenous under certain conditions, due to their dual synthetic/endogenous nature.\textsuperscript{[9]} This is the case for thiouracil, a thyreostatic drug that was banned in the EU in 1981 for use in livestock for fattening purposes; this drug, and other naturally goitrogen substances, may originate from the ingestion of Brassicaceae, glucosinolate-rich plants. Myrosinase, an endogenous enzyme of these plants freed from the cell vacuoles after disruption, or myrosinase-like intestinal bacterial activity during digestion, causing glucosinolate hydrolysis, can induce the presence of thiouracil in the urine of livestock.\textsuperscript{[10]}

Also, the anabolic steroid boldenone has been heavily studied since Arts et al.\textsuperscript{[11]} showed its possible endogenous origin in calves. Some Authors hypothesised an ex vivo neoformation in contaminated urine.\textsuperscript{[12]} A study on human athletes who tested positive for boldenone showed, through GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry), its endogenous presence in urine, and suggested its formation in the gut, defined as an "endocrine active side organ".\textsuperscript{[13]} The role of phytosterols in the diet was studied on veal calves;\textsuperscript{[14]} it was shown that these sterols do not significantly increase the urinary level of 17\(\alpha\)-boldenone, nor induce the formation of 17\(\beta\)-boldenone, both
in their conjugate forms. The EU regulations require the presence of the total conjugate fraction in bovine urine as an unambiguous demonstration of boldenone administration\textsuperscript{[15]} and, to demonstrate the difficulties experienced by Control Laboratories, more recent studies have shown that the detection of only the sulfo-conjugate fraction of 17β-boldenone should unequivocally demonstrate treatment with the anabolic steroid ester.\textsuperscript{[16, 17]}

In these pseudoendogenous substances, prednisolone must be mentioned. This corticosteroid was demonstrated to be produced by cattle under stress conditions, \textsuperscript{[18,19]} was found in 612 out of 780 racehorse urine samples at concentrations around 1 ng mL\textsuperscript{-1},\textsuperscript{[20]} in all urine samples of 34 untreated human volunteers of both genders \textsuperscript{[21]} and, finally a possible ex vivo neoformation in human urine \textsuperscript{[22]} and in bovine faeces \textsuperscript{[23]} was demonstrated. Besides its endogenous origin, it was recently suggested that exogenous prednisolone administrated in bovine, could influence the metabolism of some natural corticosteroids.\textsuperscript{[24]}

Currently, studies of the natural presence of prednisolone in feed are not available in the literature: although the possibility of an endogenous production or of \textit{ex vivo} formation in the urine cannot be excluded, the involuntary administration of prednisolone with complementary feed should be accounted for. The term “complementary feed” is precisely described in Article 3, Paragraph 1 of the Regulation (EC) No 767/2009\textsuperscript{25} as: “compound feed which has a high content of certain substances
but which, by reason of its composition, is sufficient for a daily ration only if used in combination with other feed”. Therefore, specific, “dense” composition of complementary plant feedstuffs can serve as a good basis to start with the examination of the presence of corticosteroids in this milieu.

Bearing this in mind, we undertook an investigation of the presence and origin of prednisolone in complementary plant feedstuff samples.

3.3.3. Experimental

3.3.3.1. Reagents and Chemicals

Cortisol and prednisolone were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Internal Standard prednisolone-d6 was from CDN Isotope (Pointe-Claire, Quebec, Canada). All other chemicals were from Fluka ChemieGmbH (Buchs, Switzerland). Standard stock solutions were prepared in ethanol (1 mg mL-1) and stored at −18°C. Working solutions were prepared daily by diluting the stock solutions with methanol:water (50:50, v/v).

3.3.3.2. Sample selection

The experiment was designed according to available feed samples. Initially, feeds were collected at the farms (FARM group)
and included into two samples sets. The first set included five feed samples that were randomly collected in farms during hot summer months. After collection, the samples were stored in the laboratory, without any consideration of storage temperature. In the late autumn, the samples were analysed. The second set consisted of 15 samples of cattle feed of four different compositions. These samples were stored at the farm in the summer and autumn, collected in the late autumn, taken to the laboratory and, unlike the first set, immediately analysed. A second analysis was carried out after a month of storage at room temperature.

On the bases of preliminary results obtained for the FARM group, a new experimental group was formed, which included feeds stored in the laboratory (LAB group). The LAB group included 18 samples of cattle feed of different compositions, which were collected in the spring. These samples taken to the laboratory before their delivery to the farm. Upon their arrival in the laboratory, these samples were and immediately analysed. A five weeks storage period at room temperature followed, with sampling on every 7th day.

3.3.3.3. Complementary feed composition

We used commercially available, vegetable complementary feeds. All of the information about the feedstuff compositions came from the manufacturer’s certificates. The total
number of feeds considered in the experiment was 38. The types of feed were 16, named with the letters of the English alphabet from A to P, as some samples came from different batches of the same feed type. Feed A was for veal calves weaning, feed B was for veal calves weaning and young beef, feeds C to G were for young beef, feeds H to O for adult beef and feed P for dairy cows. Feeds A, B, C, D and F came from different farms, the remaining were obtained directly from the manufacturer.

All feeds contained calcium carbonate, sodium chloride, sodium bicarbonate, magnesium oxide and calcium salts of fatty acids. Feeds G and M also contained dicalcium phosphate and feed H calcium sulphate.

All feeds, except K and N, contained wheat as flour middling (B, D, E, O, P), bran (A, C, E-G, I, J, L, M, P) or middling (H).

Corn was present in all feeds except J, K and N; in B, C, E and F this was present as gluten feed, in E and G as germ, in I and L as bran, in A and E as corncob. In the remaining feeds, the presence of corn was generically indicated. In O, corn was genetically modified (GM).

All feeds but M contained soy as dehulled soybean flour (A- D, F-L, N and P), soybeans (E, P), soybean oil (G), and soybean hulls (A). In O, soy was GM.

Sunflower meal was present in all feeds except D. Feeds K and O contained barley flour; GM canola flour (O) and rice bran
were also present. Sugarcane or beet molasses were in B, D, E, G, I, J, M-O; sugar beet pulp was in A, E, F, H, L and N.

Streptomyces cerevisae was in I and L-N; wheat distillers in I; sulphur bloom and saponified vegetable oil in L; Yucca schidigera, brewers grain, linseeds and carob in M.

The analytical constituents were: proteins from 14.5% (O) to 35.0% (K); lipids from 1% (K) to 9% (M); cellulose from 5.10% (D) to 12.0% (L); ashes from 6.20% (A) to 35.0% (K); calcium from 0.9% (A, D, E) to 3.5% (M); phosphorus from 0.40% (E) to 0.80% (G); sodium from 0.30% (E) to 4.8% (K); magnesium from 0.30% (A) to 0.90% (M); methionine from 0.20% (A) to 0.60% (P). Feed H was supplemented with selenomethionine (22.75 mg kg\(^{-1}\)).

Vitamins A, D3 and E were present as additives in all complementary feeds (from 6500 to 125000 UI kg\(^{-1}\), from 750 to 25000 UI kg\(^{-1}\) and from 25 to 1400 mg kg\(^{-1}\), respectively). B vitamins were present at different concentrations in feeds H, I, K-N and P. Choline was present in feeds H, L and M. In L, vitamin K was also reported. Feeds B, H, J and K contained urea (from 18000 to 40000 mg kg\(^{-1}\)). Selenium, zinc, manganese, iron, copper, and iodine were present. Feed L contained sorbent and binding materials, while flavourings were present in feed N.
3.3.3.4. **Sample extraction**

A 2 g portion of cattle feed (pellets or flour), transferred to a 50 mL polypropylene tube, was spiked with 40 μL of a 100 ng mL⁻¹ internal standard solution. After the addition of 20 mL water, the sample was shaken for 1 minute until complete dispersion was achieved. A solution (4 mL) of 80/20 tert-buthylmethylether:ethyl acetate (v/v) was added, and the resulting mixture was shaken in a vertical rotary shaker for 20 min and centrifuged for 15 min at 3000 g. The tube was kept at -18°C for about 1 h, until freezing of the aqueous phase and lipid solidification. The organic liquid supernatant was then transferred into a glass 10 mL tube. The sample was dried under vacuum in a centrifugal evaporator. The residue was dissolved in 200 μL of a mixture of methanol:aqueous formic acid 0.1% (50:50 v/v), 800 μL of petroleum ether was added, and then the solution was vortexed for 30 s and centrifuged for 2 min at 3000 g. The lower aqueous phase was collected with a disposable 1 mL syringe and transferred to the autosampler vial.

3.3.3.5. **LC–MS³ analysis**

Analysis conditions have been previously described elsewhere. [18] Briefly, the HPLC system comprised a quaternary pump equipped with a degasser and a Surveyor AS autosampler (Thermo Electron, San Jose, CA, USA). The chromatographic separation was performed using a HPLC column (100mm×2.1mm i.d., 3 μm particle size Allure Biphenyl) (Restek Corporation,
Bellefonte, PA, USA) in an oven set at 30°C with an isocratic elution [40% aqueous formic acid (0.1%) and 60% methanol at a flow rate of 0.2 mL min⁻¹]. An LCQDecaXpMax ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA) was operated in negative electrospray ionisation (ESI-) mode with the following conditions: sheath and auxiliary gas (nitrogen) flow rates of 40 and 18 arbitrary units, respectively; a spray voltage of 5.50 kV; an ion transfer capillary temperature of 245°C; a capillary voltage of −23 V; and a tube lens offset of −77 V. Helium was used for collision-induced dissociation. All of the investigated compounds showed, in full scan MS, very abundant formiate adducts ([M+HCOO]⁻). Consequently, these ions were used as precursor ions for the MS² fragmentation: for each analyte, the most abundant ion detected after collision was then used as a precursor for the MS³ fragmentation. The analysis was performed in consecutive reaction monitoring (CRM). The precursor ions were the formiate adducts of the studied compounds ([M+HCOO]⁻), and are shown in Table 1 together with the product ions and collision energies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MS² precursor ion (m/z)</th>
<th>Collision energy (%)</th>
<th>MS³ Precursor ion (m/z)</th>
<th>Collision energy (%)</th>
<th>Products ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone</td>
<td>405</td>
<td>25</td>
<td>329</td>
<td>26</td>
<td>313, 295, 280, 187</td>
</tr>
<tr>
<td>Cortisol</td>
<td>407</td>
<td>35</td>
<td>331</td>
<td>25</td>
<td>315, 297, 189</td>
</tr>
<tr>
<td>Prednisolone-d6</td>
<td>411</td>
<td>25</td>
<td>333</td>
<td>26</td>
<td>317, 299, 284, 191</td>
</tr>
</tbody>
</table>

Results in bold type are the ions for quantification.

**Table 1.** Ions for prednisolone, cortisol and the internal standard prednisolone-d6 detected by LC-MS3 in consecutive reaction monitoring mode.
The quantifications were made on one ion. Representative chromatograms and mass spectra of a spiked feed sample are reported in Figure 1.

![Figure 1](image)

**Figure 1.** Reconstructed chromatogram and consecutive reaction monitoring (CRM) mass spectra of a blank feed sample spiked with 2 ng g\(^{-1}\) prednisolone and cortisol. The concentration of the internal standard prednisolone-d6 is 2 ng g\(^{-1}\).

### 3.3.3.6. LC/HRMS analysis

The presence of prednisolone was qualitatively confirmed by High Resolution Mass Spectrometry (HRMS) in four samples in full MS scan mode; all data were processed with a mass tolerance of 5 ppm. The exact mass of the prednisolone formiate adduct is 405.19187 Da. The chromatographic separation was performed on a reversed-phase SunfireW column (150 2.1 mm, 3.5 mm; Waters, Milford, MA, USA), with a mobile phase consisting of a mixture of 75% water with 0.1% formic acid and 25% acetonitrile at a flow rate of 0.3 mL min\(^{-1}\). The HRMS instrumentation was an Exactive™ Benchtop high-resolution mass...
spectrometer equipped with an HESI-II source (Thermo Fisher, San José, CA, USA) operating in negative mode. The method is exhaustively described elsewhere.\textsuperscript{20}

3.3.3.7. \textit{LC–MS\textsuperscript{3} method validation}

The presence of the studied corticosteroids in feed samples was checked by the analytical method described above. A calibration curve was thus prepared with blank samples, which were spiked to give known concentrations of prednisolone and cortisol (0.10, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 5.0 and 10 ng g\textsuperscript{-1} feed). Three replicates were measured on three different days after liquid–liquid extraction. The following parameters were calculated: precision, expressed as intra- and interday coefficient of variations (CV\%), on four blank feed samples, spiked with 0.6 ng g\textsuperscript{-1} feed, roughly corresponding to twice the detection capability (CCβ); recovery (%), on the same four samples, expressed as the percentage of measured concentration to a fortified concentration ratio; decision limit (CCα) and detection capability (CCβ); between-run accuracy, on three different days using four different samples spiked with 0.6ng g\textsuperscript{-1} feed (twice the CCβ).

The method used was validated following the Commission Decision 2002/657/EC,\textsuperscript{26} with modifications proposed by Galarini \textit{et al.},\textsuperscript{27} who calculated CCα and CCβ, starting with the determination of the “minimum required
performance level” (mrpl), which indicates the concentration above which the curves must be built.

3.3.3.8. Statistical analysis

Means, medians and standard deviations were calculated for every set/group of feeds. In order to determine if a difference existed in prednisolone concentrations, we compared the different sets/groups of feeds. The Kolmogorov and Smirnov method was used to verify the normality of the value distribution. When a comparison was made between two sets/groups, we always used the Mann-Whitney test as at least one of the populations did not pass the normality test. To compare three sets of values, we performed the ordinary Analysis of Variance (ANOVA) if the normality test was passed by all sets, or the Kruskal-Wallis test (non-parametric ANOVA) in all other cases. The software used was GraphPad InStat™ version 3.00 (GraphPad Software, San Diego, California USA; www.graphpad.com).

3.3.4. Results And Discussion

Although there is a need for sensitive, accurate and quick analytical methods to monitor the abuse of corticosteroids, only a limited number of analytical methods have been published for feedstuff. Animal feed is a very complex matrix; not only does the composition differ for each type but starting materials also differ
for each production batch, leading to each sample of feed having its own characteristics. This means that the interfering compounds differ from sample to sample, which makes method development challenging. Therefore, we paid special attention to the sample handling and extraction procedure. The parameters calculated for method validation are reported in Table 2. All validation data for prednisolone and cortisol determination in feedstuff were adequate and indicated good performance of the developed analytical procedure. The level of cortisol was below the decision limit in all of the analysed samples.

![Table 2. Validation performance characteristics of prednisolone and cortisol](image)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Prednisolone</th>
<th>Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity R2</td>
<td>0.98</td>
<td>0.97</td>
</tr>
<tr>
<td>Intra-day CV (%)</td>
<td>7.4</td>
<td>9.5</td>
</tr>
<tr>
<td>Inter-day CV (%)</td>
<td>12.7</td>
<td>14.2</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>91</td>
<td>85</td>
</tr>
<tr>
<td>CCα (ng g⁻¹)</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>CCβ (ng g⁻¹)</td>
<td>0.29</td>
<td>0.29</td>
</tr>
</tbody>
</table>

CCα, decision limit; CCβ, detection capability; CV, coefficient of variation.

Prednisolone was detected in all samples from the preliminary study (first set, FARM group) and could be quantified in four. The mean ± SD value was 1.6±1.5 ng g⁻¹ (Table 3). The unexpected presence of prednisolone in these samples strongly suggested the possibility of its neo-formation, similarly to the faecal matter as we already observed. The samples were
randomly collected from farms, and then transferred in the laboratory. The time and temperature of their storage at the farm were neither uniform nor exactly monitored; the period was from 1 to 2 months. The storage period in the laboratory was 2 months, also without any caution to the storage temperature. Therefore, neo-formation could occur during both of the indicated intervals.

In order to gain a clearer picture of where and when prednisolone was formed, a new approach was designed; the results are given as a second set of the FARM group. As the values obtained for this set were not normally distributed, the Mann-Whitney test was used to compare them to the first set. The 15 samples showed a prednisolone concentration value of 1.6±1.3 ng g⁻¹ (mean±SD), which did not differ significantly from the first set (table 3). The second set of the FARM group seemed to confirm the initial hypothesis. Prednisolone was in fact detected in 14 out of 15 samples independent of the variable environmental conditions (temperature, humidity, etc.). It has to be noted that the samples of this set had been stored only at the farm when the first analysis was undertaken. The second analysis on the presence of prednisolone was performed after a 1-month storage period in the laboratory at room temperature: all samples were negative except No. 15 (Table 3). The extremely high concentration found in this feed specimen could not be interpreted by the simple addition of the corticosteroid to the feed, as its concentration in the first analysis was about 74-fold lower. A possible explanation for this
could be a high level of precursors or more presumably high microbiological activity due to the particular conditions in the jar. More profound studies should be conducted to clarify why other samples of the same composition did not behave in the same manner (Table 3).

Table 3. Concentrations of prednisolone detected in the feed samples of the FARM group: first set (1 to 5) and of the second set (6 to 20), after a storage period at the farm (first analysis) and at the laboratory (second analysis).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Feed</th>
<th>Arrival at the farm</th>
<th>First analysis</th>
<th>Prednisolone ng g⁻¹</th>
<th>Second analysis</th>
<th>Prednisolone ng g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B</td>
<td></td>
<td></td>
<td>0.97</td>
<td>np</td>
<td>np</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td></td>
<td></td>
<td>1.0</td>
<td>np</td>
<td>np</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>September</td>
<td>November</td>
<td>1.7</td>
<td>np</td>
<td>np</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td></td>
<td></td>
<td>&gt;CCα</td>
<td>np</td>
<td>np</td>
</tr>
<tr>
<td>5</td>
<td>D</td>
<td></td>
<td></td>
<td>4.0</td>
<td>np</td>
<td>np</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td></td>
<td></td>
<td>3.9</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>7</td>
<td>D</td>
<td></td>
<td></td>
<td>0.86</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>8</td>
<td>D</td>
<td>August</td>
<td>December</td>
<td>0.35</td>
<td>January</td>
<td>nd</td>
</tr>
<tr>
<td>9</td>
<td>D</td>
<td></td>
<td></td>
<td>0.73</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>10</td>
<td>E</td>
<td></td>
<td></td>
<td>2.4</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>11</td>
<td>B</td>
<td></td>
<td></td>
<td>0.98</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>12</td>
<td>B</td>
<td></td>
<td></td>
<td>2.1</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>13</td>
<td>C</td>
<td></td>
<td></td>
<td>0.51</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>14</td>
<td>D</td>
<td>October</td>
<td>December</td>
<td>0.82</td>
<td>January</td>
<td>nd</td>
</tr>
<tr>
<td>15</td>
<td>D</td>
<td></td>
<td></td>
<td>3.9</td>
<td>290*</td>
<td>nd</td>
</tr>
<tr>
<td>16</td>
<td>E</td>
<td></td>
<td></td>
<td>0.88</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>17</td>
<td>E</td>
<td></td>
<td></td>
<td>3.6</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>18</td>
<td>B</td>
<td></td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>19</td>
<td>B</td>
<td>November</td>
<td>December</td>
<td>1.7</td>
<td>January</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>B</td>
<td></td>
<td></td>
<td>1.0</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

np: not performed; nd: not detected; *estimated value, out of the calibration range

In order to compare feed samples according to their stay in farm, the samples were merged (samples 1 to 5, 6 to 10, 11 to 17 and 18 to 20, respectively) and ANOVA test was performed; no significant difference was observed (P = 0.81). On the other hand, the Kruskal-Wallis test was performed to evaluate the
prednisolone concentration in the feed samples merged according to their composition. When the mean prednisolone concentrations of feedstuffs B to E were compared, no significant difference was shown. Feed A could not be considered due to the presence of one only sample.

Because of the lack of a significant difference between the prednisolone concentrations in feedstuffs studied in the FARM group, a second experiment was undertaken. Commercially available vegetable feedstuffs (n=18) were randomly chosen, regardless of their composition. The results obtained for this group are shown in Table 4.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Feed</th>
<th>Arrival</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
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<td>nd</td>
<td>nd</td>
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</tr>
<tr>
<td>22</td>
<td>P</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
</tr>
<tr>
<td>23</td>
<td>D</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
</tr>
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<td>B</td>
<td>0.73</td>
<td>nd</td>
<td>nd</td>
<td>0.57</td>
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<td>nd</td>
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<tr>
<td>25</td>
<td>B</td>
<td>nd</td>
<td>1.6</td>
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<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>26</td>
<td>B</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td>1.2</td>
</tr>
<tr>
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<td>G</td>
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<tr>
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<td>G</td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.63</td>
<td>1.2</td>
</tr>
<tr>
<td>30</td>
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<td>nd</td>
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<tr>
<td>31</td>
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<td>0.69</td>
<td>0.54</td>
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<td>nd</td>
</tr>
<tr>
<td>32</td>
<td>J</td>
<td>nd</td>
<td>0.65</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>33</td>
<td>K</td>
<td>nd</td>
<td>nd</td>
<td>3.0</td>
<td>1.8</td>
<td>0.34</td>
<td>nd</td>
</tr>
<tr>
<td>34</td>
<td>L</td>
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<td>0.43</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>35</td>
<td>M</td>
<td>nd</td>
<td>0.43</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
</tr>
<tr>
<td>36</td>
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<td>nd</td>
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<td>nd</td>
<td>0.86</td>
<td>0.75</td>
<td>0.36</td>
</tr>
<tr>
<td>37</td>
<td>O</td>
<td>nd</td>
<td>nd</td>
<td>0.31</td>
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<td>nd</td>
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</tr>
<tr>
<td>38</td>
<td>O</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>&gt;CCα</td>
</tr>
</tbody>
</table>

*nd: not detected*

**Table 4.** Concentrations (ng g⁻¹) of prednisolone detected at different collection times in the feed samples of LAB group during the storage period at the laboratory.
Only one sample showed the presence of prednisolone upon arrival at the laboratory. A total of 108 analyses were performed and prednisolone was found on 34 occasions. Only one sample (No. 22) was always negative. In the other samples, no relationship was found between the collection time and the presence of prednisolone: the corticosteroid was in fact detected between one and four times in each sample. The concentration was either roughly constant, raising, decreasing or with a bell-shaped profile. The mean±SD prednisolone concentrations ranged from 0.74±0.26 ng g⁻¹ (day 28) to 1.13±1.07 ng g⁻¹ (day 14), with no difference shown between the collection days. The positives were 1 upon arrival, 2 on the 7th day and, even if the distribution was random, 7 at any further collection time (Figure 2).

![Figure 2](image)

**Figure 2.** Mean ± SD concentrations ( ) and number of positives ( ) to prednisolone in samples of laboratory (LAB) group, related to the collection day.

The Kruskal-Wallis test was performed to compare prednisolone-positive samples, merged by collection day, but no significant statistical relationship was found again.
Beyond this, the integrated data from positive samples of the FARM group were compared to the corresponding data from the LAB group. The mean±SD values were 1.66±1.28 and 0.95±0.76 ng g⁻¹, respectively, and the Mann-Whitney test (P=0.024) demonstrated a difference in prednisolone concentration between the samples stored at the farm and in the laboratory. Nevertheless, apart from this statistical significance, one fact remains: prednisolone is formed either at the farm or in the laboratory. In the LAB group, in contrast to the FARM group, the sample storage after production was performed only in the laboratory: the neo-formation of prednisolone occurred in this environment as well. However, the frequency was lower, as only 31% of analyses were positive for prednisolone, versus 95% of samples stored at the farm, at least for the short-term. These data suggest that different storage conditions differently evoke prednisolone neo-formation. Also, the variability observed did not exclude the possibility of its degradation. In the second set of the FARM group, 14 samples out of 15 were found to be negative after 1-month of storage in the laboratory. In the LAB group, the higher frequency of prednisolone detection was seen in 7 out of 18 samples, observed from day 14 to day 35. Hence, most of the samples (about 60%) were negative for these collection days and when prednisolone was observed early, it generally disappeared.

The poor stability of the corticosteroids has recently been shown by De Clercq et al.,[28] who, to preserve glucocorticoids in bovine
urine for a long period (20 weeks), recommended filter-sterilising and storage under acidic conditions, preferentially at pH 3 and at a temperature of −80°C (or at least −20°C). This last observation, made on a different matrix, shows the real possibility of the microbiological transformation of corticosteroids. Currently, the only explanation for the higher frequency of prednisone–positive samples in the FARM group in respect to the LAB group could be found in the different sanitary-hygienic storage conditions. Conservation in closed jars, which is performed in the laboratory, preserves the possibility of contamination; while, on the farm, the hygienic conditions are objectively different and obviously more favourable for prednisolone neo-formation. The appearance of prednisolone in a very high concentration in sample No. 15, collected after one month storage in the laboratory, could represent indirect, although controversial, evidence of this observation: in fact, it took place in a closed container where the conditions could have been different compared to all other samples that were stored in closed jars.

Finally, the identification of prednisolone with a low mass resolution spectrometer was fully confirmed in four randomly selected samples, through the accuracy of the measured mass of the formiate precursor ([M+HCOO]$^{-}$) in HRMS analysis, as shown in Fig. 4.
Figure 3. Total ion spectra of the prednisolone peak acquired by HRMS. (A) Standard solution (1 ng mL$^{-1}$), (B) a positive feed sample. The exact mass of prednisolone formiate ([M +HCOO]$^-$) is 405.19187 Da.

3.3.5. Conclusions

Based on the results obtained, we hypothesise that feedstuffs without the addition of drugs may be noncompliant for prednisolone presence upon inspection by the Health Authorities. Due to the low possibility of affecting the storage conditions at the farms, the studies that would indicate objective parameters, e.g. a cut-off level or metabolite markers, are essential. To this aim, special attention must be paid to the definition of the prednisolone
metabolic precursors in the feedstuffs and the nature of their origin. All of this would allow the official control organisations to make the most possible accurate decisions about the cause and importance of the presence of prednisolone in complementary feedstuff.

3.3.6. References


3.4. **Presence of prednisolone in urine and adrenal glands of pigs**

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3.4. Presence of prednisolone in urine and adrenal glands of pigs

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3.4.1. Abstract

The debate about the origin of prednisolone in animal organisms has lasted for five years. The bovine species has been the most studied, but studies on humans and horses are also present in the literature. Even if prednisolone in pigs does not yet represent a problem for control agencies, recently interest has risen with regard to this species. To date there has been just a single study in the literature about this topic, performed on ten sows treated with prednisolone or a synthetic analogue of adrenocorticotropic hormone. We therefore initiated a study on 80 pigs, a number considered representative in relation to the expected frequency (prevalence) of prednisolone detection in urine collected at
slaughter. Prednisolone was detected in urine both at the farm and at the slaughterhouse, with a concentration and frequency higher at slaughter. The presence of prednisolone was also studied in adrenal glands, where the corticosteroids are produced in response to stress, and it was detected in 89% of the samples. These results, together with the similar behaviours of prednisolone and cortisol, i.e. a mutual rise in the two corticosteroids in urine collected at the slaughterhouse and the correlation between the concentrations of the two corticosteroids in the adrenal glands, seem to indicate an endogenous origin of prednisolone in pigs.

**Keywords**: pigs urine, pigs adrenal glands, endogenous prednisolone, cortisol, LC-MS².
3.4.2. Introduction

Cortisol is a steroid hormone produced and released by the adrenal cortex. Cortisol is involved in physiological processes such as immune reactions, the regulation of inflammatory states, and carbohydrate metabolism\textsuperscript{[1, 2]}.

Prednisolone is a glucocorticosteroid whose anti-inflammatory activity is 3-4 times higher than cortisol. The therapeutic use of prednisolone in bovine is regulated by Commission Regulation (EU) N°37/2010\textsuperscript{[3]} which sets maximum residue limits (MRLs), even if its illicit use as growth promoter agent cannot be discarded\textsuperscript{[4]}. For urine, no MRLs have been set but a 5 ng mL\textsuperscript{-1} cut-off level has been recommended, e.g. by the Italian Ministry of Health\textsuperscript{[5]}, following the indications of the European Union Reference Laboratory (RIKILT) of Wageningen and the National Institute for Public Health and the Environment (RIVM) of Bilthoven\textsuperscript{[6]}.

Corticosteroids are allocated to group B2f (other pharmacologically active substances) by EU Council Directive 96/23/EC\textsuperscript{[7]} and monitoring of their administration to livestock is carried out both on urine collected at the farm and urine or liver at the slaughterhouse\textsuperscript{[3, 8]}.

In recent years, an increase in cases positive for prednisolone, reported by some European Union Member States,
has been observed in bovine urine, particularly when sampled at the slaughterhouse [9].

The possibility of in vitro formation of prednisolone from cortisol has been reported, possibly due to poor collection and storage conditions of the urine samples [10-12]. Pompa et al. [13] investigated the relation of stress to the formation of prednisolone from cortisol in dairy cows. The role of stress in cortisol production is well-known. The cortex of the adrenal glands is stimulated by the adrenocorticotropic hormone (ACTH) secreted from the anterior pituitary in response to corticotropin-releasing hormone (CRH) from the hypothalamus. Under unstressed conditions, prednisolone was found sporadically in urine. When the cows were stressed with intramuscularly administered tetracosactide hexaacetate, a synthetic analogue of ACTH, or physically by transport and slaughter, cortisol concentration increased and prednisolone was consistently found in urine, demonstrating the possibility of its endogenous formation.

The possibility of the endogenous origin of prednisolone has also been described for equine and human urine [14, 15].

Finally, Delahaut et al. [16] reported that the Belgian Federal Agency for the Safety on the Food Chain (FASFC) found prednisolone at a mean concentration of 0.96 ng mL⁻¹ in 73% of 393 samples of porcine urine collected at the slaughterhouse. The same Authors described the results of a preliminary study concerning the presence of prednisolone in sows before and after
i.m. administration of prednisolone or tetracosactide hexaacetate. The urine collection was performed at the farm before and after the treatment and at the slaughterhouse, where the liver was collected as well. The presence of prednisolone in porcine urine was confirmed in all samples prior to the treatment and in most of them after the treatment, but, in liver, prednisolone was only found after administration of prednisolone or tetracosactide hexaacetate. The Authors proposed the prednisolone/cortisol ratio in liver samples as an indicator for detecting illicit prednisolone administration to pigs and suggested confirming these observations in a study on a larger number of animals [16].

In order to clarify the possible endogenous origin of prednisolone and the influence of stress on the production of this corticosteroid in pigs, the present study investigated the presence of prednisolone in urine samples collected from the same eighty pigs at the farm and at the slaughterhouse; we also analyzed the adrenergic glands of the same animals, supposing an endogenous production of prednisolone in this organ.

3.4.3. Materials and methods

3.4.3.1. Chemicals and reagents

Cortisol and prednisolone were purchased from Sigma–Aldrich (St.Louis, MO, USA). The internal standard prednisolone-
d6 was from CDN Isotopes (Pointe-Claire, Quebec, Canada). All other chemicals were from Fluka Chemie GmbH (Buchs, Switzerland). Ultrapure water was obtained through a Milli-Q system (Millipore, Molsheim, France). Standard stock solutions were prepared in methanol (1 mg mL\(^{-1}\)) and stored at \(-40^\circ\text{C}\). Working solutions were prepared daily by diluting the stock solutions with methanol/water (50:50, v/v).

### 3.4.3.2. Animals and sampling procedure

The study was carried out on 80 pigs of both genders weighing 100 to 150 kg, coming from northern Italy farms and slaughtered in different abattoirs of Lombardy. Official Veterinarians of Lombard Veterinary Services collected urine and adrenal gland samples, not used for routine analyses. They also verified the lack of treatments in the 90 days before slaughter, by checking the records of purchase, possession and administration of veterinary medicinal products of the animal treatments, maintained by the owners of food producing animals as required by the Directive 2001/82/EC\(^{[17]}\). Urine samples were collected for the first time at the farm into long-handled sterile containers, approximately one week before transport of the animals to the slaughterhouse.

Only clean urine, i.e. clear ad without raw materials, was sampled, frozen and taken to the laboratory for storage at \(-40^\circ\text{C}\) until extraction and analysis.
A second collection was made at the slaughterhouse: urine samples were collected directly from the urinary bladder immediately after slaughter, as well as the adrenal glands. All samples were immediately frozen and taken to the laboratory for storage at −40 °C. Each pig, randomly selected and followed from farm to slaughterhouse, provided three different samples (urine at the farm, urine at the slaughterhouse and adrenal glands) in order to have matched data.

3.4.3.3 Sample size

The urine and adrenal glands investigated in this work came from pigs that were under the veterinarian control for 90 days before slaughter. The sampling therefore had to be made on an appropriate number of animals that would assure detection of the predicted prevalence, i.e. the expected frequency of endogenous prednisolone detection in urine and adrenal glands. The sample size calculation was made according to Bottarelli et al. [18] using the following equation: \( n = Z^2 \times \left[ P(1-P) \right]/D^2 \), where \( n \) is the sample size, \( Z \) is the Student’s t value (1.96, when the level of significance is 5%), \( P \) the expected prevalence and \( D \) the required precision. At the time the experimental protocol was designed, we had no data on the frequency of prednisolone detection in pig urine, but only from bovines (cows at slaughter = 71% positive) [19] and race horses (78.5%) [14]. Based on these frequencies, on their difference between cows and horses, and because the pigs,
i.e. a different species, are studied in this work, we supposed a prevalence for prednisolone detection in pig urine collected at the slaughterhouse of 70% (P=0.7); and a precision of ± 10% (D=0.1). The necessary sample size predicted was 80 animals.

3.4.3.4. Pig urine sample extraction

Sample preparation was conducted as previously reported in Arioli et al. with slight modifications [20]. An aliquot of 2 mL of each urine sample was spiked with prednisolone-d6 as internal standard to a concentration of 2 ng mL$^{-1}$. A 4 mL mixture of tert-butyl methylether: ethyl acetate (4:1, v/v) was then added. After shaking in a vertical rotary shaker for 20 min, the sample was centrifuged at 1300 g for 15 min. The upper organic layer was collected and dried under vacuum in a centrifugal evaporator at a temperature of 30°C. The residue was dissolved in 200 µL of the mixture of methanol/aqueous formic acid 0.1%, 50:50 v/v and transferred to an autosampler vial for the LC–MS$^2$ analysis. The injection volume was 10 µL.

3.4.3.5. Pig adrenal gland sample extraction

Sample preparation was conducted as previously reported in Bertocchi et al. (2013) [19]. A 5 g portion of the adrenal gland was transferred to a 50 mL tube and spiked to a concentration of 10 ng mL$^{-1}$ with the internal standard prednisolone-d6. After the addition of 10 ml water, the sample was homogenized in a
dispersing machine operating at a speed of 13500 rpm for 1 min. A 4 mL mixture of tert-butyl methyl ether: ethyl acetate (4:1, v/v) was then added. After shaking in a vertical rotary shaker for 20 min, the sample was centrifuged at 3000 g for 15 min. The tube was then put in a freezer for about 1 h until lipid solidification. The organic liquid supernatant was transferred to a glass 10 mL tube; the solid lipid layer was placed in a polypropylene 15 mL tube and centrifuged again to recover residual liquid, which was transferred to the glass tube. The aqueous phase was then re-extracted (as described above) and the supernatant liquid was added to the two portions already placed in the glass tube. The sample was then dried under vacuum in a centrifugal evaporator. The residue was dissolved in 250 μL of methanol/aqueous formic acid 0.1%, 50:50 v/v, 1.5 mL of petroleum ether were added, and then the sample was vortexed for 30 s and centrifuged for 5 min at 3000 g. The lower aqueous phase was then quantitatively (200 μL) transferred to an autosampler vial. The injection volume was 20 μL.

3.4.3.6. LC-MS² analysis

Chromatographic separation was performed with a Thermo Finnigan LC system consisting of a Surveyor MS quaternary pump (Thermo Fisher Scientific, San Jose, CA, USA) operating at flow rate of 250 μL min⁻¹ and a Synergi Hydro RP column 150 x 2.0 mm, internal diameter 4 μm (Phenomenex, Torrance, CA, USA), kept at 30°C. The mobile phase was aqueous
formic acid 0.1% (eluent A) and methanol (eluent B). The gradient program, lasting 31 minutes, was as follows: A was at 75% at minute 0, decreased to 20% over 20 minutes, then to 5% for one minute, maintained for 3 minutes, and increased again to 75% from the 24th to 26th minute; the last 5 minutes were in an isocratic elution (A=75%). The mass spectrometer was a TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray interface (ESI) set in the negative ionisation mode. The capillary voltage was 3.2 kV, ion transfer capillary temperature was 340°C, while the sheath and auxiliary gas (nitrogen) had arbitrary units of 30 and 10, respectively. The collision gas was argon at 1.5 mTorr. Three diagnostic transitions were monitored, in multiple reaction monitoring (MRM), for the analytes and internal standard. The quantification was performed on transition with the higher signal-to-noise ratio. Table 1 shows the precursor ions, i.e. the formiate adducts ([M+HCOO]−), the product ions and the collision energies. Data were acquired using Xcalibur™ software from Thermo.
Table 1. MS\(^2\) conditions for the MRM acquisitions of analytes and the internal standard. Ions for quantification are in bold. CE: collision energy expressed in electron volts (eV).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion ([\text{M+HCOO}^-]) (m/z)</th>
<th>Product ions CE (m/z)</th>
<th>ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>407</td>
<td>282(<em>{37}, 297(</em>{33}, 331(_{30}\right</td>
<td>)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>405</td>
<td>187(<em>{30}, 280(</em>{36}, 329(_{18}\right</td>
<td>)</td>
</tr>
<tr>
<td>Prednisolone-d(_6) (IS) &amp; 411</td>
<td>284(<em>{37}, 299(</em>{32}, 333(_{19}\right</td>
<td>) (−)</td>
<td></td>
</tr>
</tbody>
</table>

3.4.3.7. Method Validation

The method was validated for prednisolone and cortisol, according to Commission Decision 2002/657/EC requirements \cite{21}. The instrumental linearity was evaluated by preparing eight point calibration curves in the mobile phase containing a fixed amount of internal standard prednisolone-d\(_6\) (2 ng mL\(^{-1}\)) and analytes at concentrations corresponding to 0.01-0.05-0.1-0.2-0.5-1-2-5 ng mL\(^{-1}\).

Matrix calibration curves were obtained by spiking urine samples and adrenal glands with the analytes, resulting in three analytical series, each with three concentration levels (0.05-0.1-0.2 ng mL\(^{-1}\) for urine and 0.1-0.2-0.3 ng g\(^{-1}\) for adrenal glands) and six samples per concentration level (6 samples \(\times\) 3 concentration levels \(\times\) 3 series = 54 analyses for each matrix). Method recovery and precision were evaluated using these matrix curve results;
recovery was expressed in terms of percentage of measured concentration to fortified concentration and precision as the coefficient of variation (CV) calculated by applying one-way analysis of variance (ANOVA) for the intra-day and inter-day repeatability. The decision limit (CC\(\alpha\)) and detection capability (CC\(\beta\)) were calculated according to the procedure described in the Commission Decision 2002/657/EC as clarified in the document SANCO/2004/2726-revision 4 [22].

Specificity identification was achieved by detecting the peaks in the blank matrix chromatograms matching the relative retention time observed for the spiked analytes, compared to standard analytes in methanol, with a tolerance of ±2.5%.

3.4.3.8. Statistical analysis

The Kolmogorov–Smirnov test was performed to check the normality of datasets: depending on whether this test was positive or negative, the correlation of the datasets was verified through the Pearson or the non-parametric Spearman test. The results obtained from farm and slaughterhouse urine were compared using one of the following tests depending on correlation, standard deviation (equal or different), and normality of the datasets: the unpaired t-test; the Wilcoxon matched-pairs signed-ranks test; and the Mann-Whitney test. When three datasets were compared, the non-parametric analysis of variance (Kruskal-Wallis test for unpaired data and non-normal distributions) with
Dunn’s Multiple Comparisons post-test was used. The null hypothesis was set at P>0.05. GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego, CA, USA) was used.

3.4.4. Results and Discussion

3.4.4.1. Method validation

The instrumental linearity for prednisolone ($r^2 = 0.991$) and cortisol ($r^2 = 0.994$) were both satisfactory. The validation parameters, shown in Table 2, demonstrated the good performance of the analytical methods in urine and adrenal glands. As regards specificity, blank and spiked samples did not show any interference (signals, peaks, ion traces) in the region of interest where peaks for cortisol and prednisolone were expected.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>Recovery (%)</th>
<th>CV%</th>
<th>CCα</th>
<th>CCβ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Intra-day</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>Cortisol</td>
<td>98</td>
<td>7.3</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Prednisolone</td>
<td>100</td>
<td>9.3</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>Adrenal Glands</td>
<td>Cortisol</td>
<td>96</td>
<td>13.5</td>
<td>0.20</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Prednisolone</td>
<td>93</td>
<td>7.8</td>
<td>0.12</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Table 2. Validation parameters of the analytical method. CCα and CCβ are expressed in ng mL$^{-1}$ for urine and ng g$^{-1}$ for adrenal glands.
3.4.4.2. Sample analysis

The hypothesis made by Delahaut et al. [16] that prednisolone can be endogenously produced was checked on 80 pigs, a number calculated as already described in the “Sample size” section. In Figure 1, a representative chromatogram and the relative ion spectra of cortisol and prednisolone in a urine sample are shown.

![Figure 1](image.png)

**Figure 1.** Reconstructed LC-MS2 chromatograms and respective ion spectra of the analytes detected in a urine sample. The calculated concentration of cortisol and prednisolone are 9.2 and 0.11 ng mL⁻¹, respectively.

The overall results obtained in this study are reported in Table 3 and 4.
Table 3. Overall analytical results.

<table>
<thead>
<tr>
<th></th>
<th>Prednisolone</th>
<th>Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Farm Urine</td>
<td>Slaughter Urine</td>
</tr>
<tr>
<td>Samples</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Positive (%)</td>
<td>44% (55%)</td>
<td>68% (55%)</td>
</tr>
<tr>
<td>Mean±SD (ng mL⁻¹)</td>
<td>0.23±0.48 ab</td>
<td>0.42±0.29 cd</td>
</tr>
</tbody>
</table>

* Different from the corresponding corticosteroid in urine at the slaughterhouse (Mann-Whitney Test, P<0.0001);

* No correlation with cortisol in farm urine (Spearman r=-0.02, P>0.05);

* Correlation with cortisol in slaughter urine (Spearman r=-0.38, P>0.01).

Table 4. Relationship between prednisolone detection and the place of urine collection for the same pig.

<table>
<thead>
<tr>
<th></th>
<th>Farm</th>
<th>Slaughterhouse</th>
<th>N° of pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Not detected</td>
<td>Not detected</td>
<td>10</td>
</tr>
<tr>
<td>Group 2</td>
<td>Not detected</td>
<td>Detected</td>
<td>26</td>
</tr>
<tr>
<td>Group 3</td>
<td>Detected</td>
<td>Detected</td>
<td>42</td>
</tr>
<tr>
<td>Group 4</td>
<td>Detected</td>
<td>Not detected</td>
<td>2</td>
</tr>
</tbody>
</table>

Cortisol was always detected in urine and its concentration was significantly different (higher) when the sample was collected at the slaughterhouse (P<0.0001). Despite the wide variability (relative standard deviations greater than 80%), the difference was extremely significant, making the data even more meaningful for the influence of stress due to transport and
slaughter on cortisol release. The observed variability could be explained by the circadian rhythm of cortisol secretion in pigs\textsuperscript{[23]} and by inter-individual variability in its urinary excretion. The selection of pigs and the time at which urine samples were collected at the farm and slaughterhouse followed the Official Collection Schedule, so we could not control these factors. As regards prednisolone, the concentration of this corticosteroid in urine from the farm was different with respect to urine from the slaughterhouse (P<0.0001), as well as the frequency of its detection. For both parameters, the value at the farm was lower than that at the slaughterhouse, so demonstrating the influence of stress as for cortisol. Based on the positivity of urine for prednisolone at the farm or slaughterhouse, the data were divided into four groups as shown in Table 4. Group 1 consisted of 10 animals negative both at the farm and at the slaughterhouse; Group 2 consisted of 26 pigs negative at the farm but positive at the slaughterhouse; Group 3 consisted of 42 pigs positive in both cases; Group 4 consisted of just two animals positive at the farm and negative at the slaughterhouse. Table 5 shows the mean ± SD of urine and adrenal concentrations of cortisol and prednisolone in these groups. Group 4 was not considered due to the very small number of values.
Table 5. Cortisol and prednisolone urinary (ng mL\(^{-1}\)) and adrenal (ng g\(^{-1}\)) levels expressed as mean±SD values considering 3 Groups, partitioned accounting for prednisolone detection in urine and place of urine collection for the same animal.

In Table 6 the normality, correlation and comparison tests are reported. Either the Pearson or Spearman test was performed to check the correlation between different datasets, in the first case between datasets that included the results from the same urinary corticosteroid collected at the farm or at the slaughterhouse, respectively (table 6A); in the second case, between datasets that included the results from the two different urinary corticosteroids collected at the same place (Table 6B), in the third case, between datasets that included the results from the two different corticosteroids in the adrenal glands (Table 6C). A comparison of means or medians (depending on the result of the Kolmogorov Smirnov normality test) was made only between datasets for the
same urinary corticosteroid collected at the farm and at the slaughterhouse (Table 6A).

<table>
<thead>
<tr>
<th>Group</th>
<th>Matrix</th>
<th>Analyte</th>
<th>Normality</th>
<th>Correlation</th>
<th>Mean or median comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Farm urine</td>
<td>Cortisol</td>
<td>yes</td>
<td><em>Pearson</em> r=-0.43 P&gt;0.05, NS</td>
<td>Unpaired t test P=0.0091, S</td>
</tr>
<tr>
<td></td>
<td>Slaughter urine</td>
<td>Cortisol</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>no</td>
<td><em>Spearman</em> r=-0.04 P&gt;0.05, NS</td>
<td>Mann-Whitney test P=0.0001, S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortisol</td>
<td>no</td>
<td><em>Spearman</em> r=0.50 P&lt;0.05, NS</td>
<td>Wilcoxon test P=0.0001, S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prednisolone</td>
<td>no</td>
<td><em>Spearman</em> r=0.11 P&gt;0.05, NS</td>
<td>Mann-Whitney test P=0.0001, S</td>
</tr>
<tr>
<td>B</td>
<td>Slaughter urine</td>
<td>Cortisol</td>
<td>yes</td>
<td><em>Spearman</em> r=0.47 P&lt;0.01, S</td>
<td>Distinct corticosteroids</td>
</tr>
<tr>
<td></td>
<td>Farm urine</td>
<td>Cortisol</td>
<td>no</td>
<td><em>Spearman</em> r=0.04 P&gt;0.05, NS</td>
<td>Distinct corticosteroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prednisolone</td>
<td>no</td>
<td><em>Spearman</em> r=0.32 P&lt;0.01, S</td>
<td>Distinct corticosteroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Adrenal glands</td>
<td>Cortisol</td>
<td>yes</td>
<td><em>Pearson</em> r=0.41 P&lt;0.01, S</td>
<td>Distinct corticosteroids</td>
</tr>
<tr>
<td></td>
<td>Adrenal glands</td>
<td>Prednisolone</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>no</td>
<td><em>Spearman</em> r=0.37 P&lt;0.001, S</td>
<td>Distinct corticosteroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Statistical analyses, performed on Groups. A): normality, correlation and comparison of the urinary concentration of the same corticosteroid at the farm and at the slaughterhouse, B): normality and correlation of the urinary concentrations of the two distinct corticosteroids at the farm and or the slaughterhouse C): normality and correlation of the concentration of the same corticosteroid in adrenal glands. The Groups considered are related to prednisolone detection in urine collected at the farm and at the slaughterhouse as shown in Table 4 in the manuscript.

A difference between urinary cortisol collected at the farm or at the slaughterhouse was always found, independent of the group. Urinary cortisol concentrations in the three groups at the farm ranged between 7.7±4.9 and 9.4±7.9 ng mL⁻¹ and were
not significantly different by the Kruskal-Wallis test. However, when the same test was made for urinary cortisol at the slaughterhouse a difference was found (P<0.05) between groups: Group 1 differed from Groups 2 and 3 (P<0.05) (Table 5). It must be noted that in Group 1 urine, prednisolone was never detected, while in Group 2 it was found at the slaughterhouse (0.37±0.24 ng mL\(^{-1}\)) and in Group 3 a significant rise (P<0.0001) in prednisolone concentration was observed between farm (0.24±0.49 ng mL\(^{-1}\)) and slaughterhouse (0.46±0.31 ng mL\(^{-1}\)) urine. Also in Group 2, a rise in prednisolone concentration was actually observed, from “not detected” to 0.37±0.24 ng mL\(^{-1}\). The similar behaviour of prednisolone in Groups 2 and 3 could be related to their similar urinary cortisol concentrations at the slaughterhouse: this could be interpreted as further evidence for the relationship between prednisolone and cortisol. A difference between the urinary levels of each corticosteroid collected at the different places is also observable within the same group (table 6A).

As regards the adrenal glands, no difference was observed between Groups 1 to 3 for both cortisol and prednisolone levels, as the P value of the Kruskal-Wallis test was higher than 0.05. It is worthy of note that in the three groups the Spearman and Pearson tests evidenced significant correlations between cortisol and prednisolone levels in the adrenal glands, so demonstrating an endogenous origin of prednisolone. (Table 6C). Our data on urine collected at the farm do not completely agree with Delahaut et al.
[16], who found a very good correlation coefficient value of 0.81 between prednisolone and cortisol levels in untreated pigs. We could not find this correlation at the farm, but only at the slaughterhouse (Tables 3 and 6B). The positive correlation between prednisolone and cortisol at the slaughterhouse seems to demonstrate a mutual rise in their concentrations, a condition that should exclude treatment with prednisolone, as checked by Official Veterinarians. A doubt about one sample out of 80 could arise: the concentration of prednisolone in urine collected at the farm was 3.3 ng mL\(^{-1}\) and that of cortisol at the slaughter was 0.69 ng mL\(^{-1}\). However, the levels of cortisol and prednisolone in the adrenal glands were 3691 and 2.6 ng g\(^{-1}\), respectively, quite a bit higher than the mean values found in this study, showing no inhibition due to treatment with corticosteroids. Confirmation of the presence of prednisolone in the adrenal glands was made by LC-MS\(^3\) on 8 samples already analysed by LC-MS\(^2\). The analysis, already used for bovine adrenal glands by Bertocchi et al. [19] was performed with an ion trap in the negative ESI mode. The results, performed only through qualitative determination, fully confirmed the ones reported in this work. A reconstructed chromatogram with the relative ion spectra is shown in Figure 2.
Figure 2. Reconstructed LC-MS\textsuperscript{3} chromatograms and respective ion spectra of the analytes detected in an adrenal gland sample. The calculated concentration of cortisol and prednisolone are 4.4 µg g\textsuperscript{-1} and 1.4 ng g\textsuperscript{-1}, respectively.

Finally, the possibility of setting a cut-off level, calculated in an analogous way to the one proposed by de Rijke et al. for cattle should not be discarded \cite{6}. The threshold level for a finding of prednisolone in pig urine would be equal to the mean value of 80 urine samples + 3 x SD. Accounting for both urine at the farm and at the slaughterhouse, the average concentration is 0.35 ng mL\textsuperscript{-1} and the standard deviation is 0.38 ng mL\textsuperscript{-1}. The cut-off value would be 1.51 ng mL\textsuperscript{-1} (0.35 + 3 x 0.38).
3.4.5. Conclusions

The possibility that prednisolone is endogenously produced in pigs was directly demonstrated by its presence in adrenal glands, the organ in which cortisol is produced. Indirect evidence was also provided about the origin of prednisolone that considered its relationship to cortisol levels under different conditions. First, both prednisolone and cortisol urinary concentrations were higher at the slaughterhouse than at the farm because of the stress the animals underwent. Second, Groups 2 and 3, in which a rise in prednisolone urinary concentration was observed at the slaughterhouse, showed a higher concentration of cortisol with respect to Group 1, in which prednisolone was never found. Third, in the adrenal glands, cortisol and prednisolone levels were positively correlated in the three groups. The similar trends in their concentrations and the positive correlation demonstrate the endogenous nature of prednisolone.

In order to understand the mechanism leading to the formation of this corticosteroid in pigs further studies on the metabolites of prednisolone, like 6β-hydroxyprednisolone, 20α-hydroxyprednisolone, and 20β-hydroxyprednisolone, must be carried out.
3.4.6. Acknowledgments

This work was carried out with a grant from the Regione Lombardia for the Research Project: “Verifica della natura endogena del corticosteroide prednisolone in matrici di suino ed indagine sull’utilizzo di corticosteroidi nell’allevamento suino”. Elisa Pasquale is the recipient of a Cariplo Ph.D. fellowship in Animal Production in the Laboratory of Inspection of Food of Animal Origin at the University of Milan.

3.4.7. References


33. Italian Ministry of Health, Circular Letter. Department of Public and Veterinary Health about the opinion of the Consiglio Superiore di Sanità, Sezione IV, 22 May 2012


36. Ministero Della Salute; Dipartimento Della Sanità Pubblica Veterinaria, Della Sicurezza Alimentare E Degli Organi Collegiali Per La Tutela Della Salute; Direzione Generale Per L’igiene E La Sicurezza Degli Alimenti E La Nutrizione; Piano Nazionale Per La Ricerca Di Residui, Relazione Finale Anno 2014


3.5. *Determination of thyreostats in bovine urine and thyroid glands by HPLC-MS/MS*

*Under review in:*

*Chromatographia*
3.5. Determination of thyreostats in bovine urine and thyroid glands by HPLC-MS/MS

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3.5.1. Abstract

The use of thyreostats in livestock is strictly forbidden by European legislation since 1981. The investigation of thyreostats is commonly performed by their detection as derivatives with 3-iodobenzylbromide. However, the derivatisation procedure could lead to a decrease in analyte concentrations. With the aim of simplifying the analysis of five thyreostats in both bovine urine and in thyroid glands, two methods were developed without the derivatisation step. Salting-out assisted liquid–liquid extraction was carried out for both matrices, followed by high-performance liquid chromatography coupled with triple-quadrupole mass
spectrometry analysis. The methods were validated in agreement with the guidelines of Commission Decision 2002/657/EC. For all the thyreostats evaluated, satisfactory results were achieved; the recovery was within 96% to 104% for both the matrices, while precision (coefficient of variation) was less than 20% for urine and 21% for thyroid glands. The limits of decision and capacities of detection for all the compounds were lower than the recommended values of 10 μg L$^{-1}$ and 10 μg kg$^{-1}$, respectively. In urine, the limits of decision ranged from 6.9 to 7.3 μg L$^{-1}$, and the capacities of detection ranged from 8.5 to 9.7 μg L$^{-1}$, while in thyroid glands these values varied from 6.6 μg kg$^{-1}$ to 7.4 μg kg$^{-1}$ and from 8.0 μg kg$^{-1}$ to 9.7 μg kg$^{-1}$, respectively. The results obtained show that the methods described are suitable for the direct detection of thyreostats in bovine urine and thyroid glands.

**Keywords:** thyreostats, bovine urine, bovine thyroid gland, method validation, HPLC-MS/MS
3.5.2. Introduction

Thyreostats are drugs that interfere with the mechanism involved in the synthesis of thyroid hormones and cause a condition of deficiency of circulating thyroxine (T4) and triiodothyronine (T3) \[^{1,2}\], whose production and release are controlled by the hypothalamus–anterior pituitary axis. The hypothalamus secretes thyrotropin-releasing hormone (TRH), which in turn stimulates the anterior pituitary gland to release thyroid-stimulating hormone (TSH) that induces the production of T3 and T4 by the thyroid, which releases them into the bloodstream. These hormones activate the nuclear transcription of a large number of genes, thus causing the synthesis of enzymes, as well as structural and transport proteins. This leads to an increase in metabolism and maintains the physical and psychological development of the organism \[^3\]. The illicit administration of thyreostats causes an improvement in bodyweight gain mainly due to increased absorption and extracellular retention of water in the edible tissues and in the gastrointestinal tract. The most frequently used thyreostatic drugs include the very potent thyroid-inhibiting compounds 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU) and 1-methyl-2-mercapto-imidazole (tapazole, TAP) \[^{4-6}\]. The chemical structures of these substances are shown in Figure 1.
The fraudulent use of thyreostats produces low quality meat. Moreover, the edible tissues derived from treated animals might represent a potential risk to the consumer’s health due to the presence of residues and their teratogenic and carcinogenic effects [7-11].

In 1981, the European Union banned their use in animal production both as growth promoters and therapeutic agents [12] and classified them as “substances having anabolic effects and unauthorized substances” belonging to the group A2 as described by the Council Directive 96/23/CE [13]. However, a relationship between the presence of Brassicaceae in feed and thiouracil in urine has been demonstrated by Pinel et al. [9], Vanden Bussche et al. [14] and Kiebooms et al. [15,16]. The Community Reference Laboratories (CRLs) in 2007 proposed a recommended concentration of 10 μg L\(^{-1}\) in urine and 10 μg kg\(^{-1}\) in thyroid tissue for the purpose of control, as “low concentrations of thiouracil have been detected in bovine animals fed with cruciferous plants, however there is scientific evidence showing that levels above 10 ppb in urine cannot be linked to natural origin due to this contamination” [17]. Recently, Wauters et al. reported concentrations of up to 18.2 μg L\(^{-1}\) in the 99% percentile from 3894 bovines and they suggested that the recommended concentration should be increased to 30 μg L\(^{-1}\) [18]. In fact, the 2015 Italian National Residue Plan already provides this concentration as the limit of detection for thyreostats in urine [19]. Thyreostats are polar
amphoteric thionamides with a heterocyclic tautomeric structure, and are mostly derived from thiouracil and mercapto-imidazole. The sequence consisting of nitrogen–carbon–sulphur, known as thioamide, is considered responsible for the thyroid-inhibiting activity (Fig. 1).

Thyreostats analyses typically consist of separation methods based on gas or liquid chromatography associated with a mass spectrometry system of detection. Normally, the extraction of the substances is carried out by using polar solvents more suitable to the chemical characteristics of the thyreostats, such as methanol, acetonitrile or ethyl acetate. Further steps of purification or clean-up with different kinds of solid-phase extraction (SPE) have been reported. Due to the low molecular mass and high polarity of the thyreostats, several authors have proposed a derivatisation step before or after the clean-up, mainly by using 3-
iodobenzylbromide (3-IBBr) in the case of HPLC-MS/MS analysis \[^6\]. This procedure induces the stabilisation of the chemical structure of the molecule in a specific and single tautomeric form, the reduction of the molecular polarity in order to increase the separation characteristics on the reversed-phase column in the case of HPLC-MS detection, and an increase in the molecular mass \[^{20}\]. However, its application before the analysis could cause a loss of analytes, particularly due to the multiple evaporation steps required. Furthermore, removing derivatisation step simplifies and shortens the whole analysis procedure \[^{21,22}\]. In this paper, we describe the extraction without derivatisation of the five above-mentioned thyreostats in bovine urine and thyroid glands followed by a sensitive, specific and reproducible HPLC-MS/MS analysis. For the full identification and quantification of the analytes, the criteria established in the 2002/657/EC Commission Decision were followed \[^{23}\] and the decision limit (CC\textsubscript{a}) and the detection capability (CC\textsubscript{b}) were calculated according to the matrix calibration curve procedure as clarified in the document SANCO/2004/2726 rev. 4 \[^{24}\].
3.5.3. Materials and methods

3.5.3.1. Reagents and chemicals

All solvents were of HPLC-MS grade quality and purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100%) was from Riedel-de Haën (Sigma-Aldrich). Ultrapure water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). KH2PO4 and NaCl were from Sigma-Aldrich. The analytes 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), 2-mercaptobenzimidazole or tapazole (TAP) were acquired from Sigma-Aldrich, as well as 5,6-dimethyl-2-thiouracil (DMTU), used as internal standard (I.S.). A stock solution of 1 mg/mL was prepared by dissolving the compounds in methanol. Serial dilutions were prepared by diluting the stock solution in the mobile phase, which were then stored at −40°C.

Phosphate buffer, prepared by dissolving 0.25 M KH2PO4 in ultrapure water, was adjusted to pH 7 and then saturated with 0.1% DL-dithiothreitol (DTT; Sigma-Aldrich) as in Vanden Bussche et al. [11].
3.5.3.2. Sample collection

Urine and thyroid gland samples from Friesian Cows aged 32 to 63 months were collected in a Lombard abattoir after slaughtering, immediately frozen and taken to the laboratory for storage at −40°C until analysis.

3.5.3.3. Sample extraction

Urine

One millilitre of bovine urine was transferred to a 15-mL glass tube and spiked with 10 ng of internal standard (DMTU) in order to give a final concentration of 10 μg L⁻¹, then vortexed and left for 5 minutes to equilibrate. The samples then underwent denaturation conditions at 65°C for 30 min, after the addition of 1 mL of PBS buffer with 0.1% DTT at pH 7. NaCl (2 g) was added to the solution to mixture as a salting-out reagent.

The extraction was performed by twice repeating these steps: addition of 5 mL tert-butyl methyl ether, centrifugation at 2000 x g for 5 min at 4°C, and collection and transfer of the upper organic layer to a 10-mL polypropylene tube. The extract was dried under vacuum in a rotary evaporator apparatus (Heidolph Instruments GmbH & Co., Schwabach, Germany) at a temperature of 40°C. The residue was dissolved in 200 μL of the mobile phase (methanol: 0.1% aqueous formic acid, v/v 50:50) and transferred to vials for HPLC. The injection volume was 10 μL.
**Thyroid gland**

The thyroid gland samples were minced with surgical scissors and homogenised. The sample (1 g) was weighed in a polypropylene tube and 10 ng of internal standard (DMTU) were added, and then the sample was vortexed and left for 5 minutes to equilibrate, then 5 mL of methanol was added. The samples were vortexed, placed in an ultrasonic bath for 10 min and then centrifuged at 2000 x g at 4°C for 10 min. The organic liquid supernatant was then filtrated and transferred to a 15-mL glass tube and 5 mL of PBS buffer with 0.1% DTT at pH 7 were added. The samples underwent denaturation conditions at 65°C for 30 min. NaCl (4 g) was added to the solution as a salting-out reagent. Tert-butyl methyl ether (2 x 10 mL) was added. The sample was centrifuged at 2000 x g for 5 min at 4°C. The upper organic layer was collected and transferred to a 50-mL glass evaporating flask. Lastly, the extracts were combined and dried under vacuum in a rotary evaporator apparatus at 40°C. The residue was dissolved in 200 µL of the mobile phase and transferred to vials for the autosampler. The injection volume was 10 µL.

### 3.5.3.4. HPLC-MS/MS analysis

A Synergi Hydro RP reverse-phase HPLC column C18 (150 x 2.0 mm, i.d. 4 µm) with a C18 4 x 3.0 mm guard column (Phenomenex, Torrance, CA, USA) at a column oven temperature of 30°C was used for the separation, which was performed by an
HPLC system that included a Surveyor MS quaternary pump with a degasser, a Surveyor AS autosampler with a column oven, and a Rheodyne valve with a 20-μl sample loop (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and methanol (solvent B), and the flow rate was set at 200 μL/min. The gradient program is shown in Table 1. The overall run time was 30 minutes.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Eluent A (%)</th>
<th>Eluent B (%)</th>
<th>Flow rate (μL min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>20</td>
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<td>70</td>
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<td>90</td>
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<td>90</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>30</td>
<td>90</td>
<td>10</td>
<td>200</td>
</tr>
</tbody>
</table>

A: 0.1% aqueous formic acid; B: methanol

Table 1. Gradient table for HPLC method

The HPLC system was connected to a TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) triple-quadrupole mass spectrometer with an electrospray interface (ESI) set in the positive (ESI+) ionization mode. The acquisition was made in the multiple reaction-monitoring (MRM) mode. The specific acquisition parameters of all the analytes were optimised by means of direct infusion of standard solutions of the analytes at a concentration of 1 μg/mL, a flow rate of 50 μg/mL and a flow rate of the MS pump of 100 μg/mL. The capillary voltage was 3.2 kV;
the capillary temperature was 340°C; nitrogen was used as the sheath and auxiliary gas at 30 and 10 arbitrary units, respectively, and argon as the collision gas at 1.5 mTorr; peak resolution was 0.70 Da FWHM. The parent ions, product ions, and collision energy values for each analyte are shown in Table 2. The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. The mass spectrometer data acquisition and processing were carried out using Xcalibur™ 2.0.7 SP1 software from Thermo Fisher Scientific Inc.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion ([M-H]^+) (m/z)</th>
<th>Product ions CE ((m/z))</th>
<th>ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAP</td>
<td>115</td>
<td>56, 72, 74, 83, 88, 15</td>
<td>(−)</td>
</tr>
<tr>
<td>TU</td>
<td>128</td>
<td>57, 60, 70, 82, 87, 17</td>
<td>(−)</td>
</tr>
<tr>
<td>MTU</td>
<td>143</td>
<td>60, 72, 84, 86, 82, 13</td>
<td>(−)</td>
</tr>
<tr>
<td>PTU</td>
<td>171</td>
<td>60, 67, 86, 112, 19</td>
<td>(−)</td>
</tr>
<tr>
<td>PtTU</td>
<td>205</td>
<td>77, 86, 103, 105, 125</td>
<td>(−)</td>
</tr>
<tr>
<td>DMTU (I.S.)</td>
<td>157</td>
<td>60, 72, 86, 98, 118</td>
<td>(−)</td>
</tr>
</tbody>
</table>

L.S.: internal standard

CE (eV): collision energy

**Table 2.** MS/MS conditions for the MRM acquisitions of analytes and the internal standard. Ions for quantification are in bold.

### 3.5.3.5. Method validation

The HPLC-MS/MS method was validated according to the guidelines of Commission Decision 2002/657/EC[^23].
Specificity, instrumental linearity, precision (intra-day and inter-day repeatability), recovery (accuracy), measurement of uncertainty, decision limit (CCα) and detection capability (CCβ) were assessed.

MS identification criteria were verified throughout the validation study by monitoring relative retention times, signal-to-noise ratios (S/N) and ion ratios. The instrumental linearity was evaluated through calibration curves in solvent at six levels, (1.0, 5.0, 10, 20, 50, 80, 100 µg L\(^{-1}\)) and 10 µg L\(^{-1}\) of DMTU as I.S.

The method validation parameters were determined with fortified blank urine and thyroid gland samples at three concentration levels (5.0, 10, 15 µg L\(^{-1}\) and µg kg\(^{-1}\)) in six replicates on three different days (6 samples × 3 concentration levels × 3 series = 54 analyses). Method recovery and precision were evaluated using the matrix curves; recovery is calculated as ratio between the measured concentration to fortified concentration and expressed in percentage, and precision in terms of intra- and inter-day repeatability expressed as the coefficient of variability (CV). The same data from the matrix calibration curves were used to calculate the decision limit (CCα) and the detection capability (CCβ) according to the matrix validation curve procedure described in the Commission Decision 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4\(^{[23,24]}\).
3.5.4. Results and Discussion

Thiouracil may be considered a semi-endogenous compound, and its ‘natural’ presence at concentrations lower than 10 μg L\(^{-1}\) in urine of untreated animals is associated with a cruciferous-based diet \(^{[6,14]}\). The development of a simple, rapid and sensitive method for the detection of thyreostats in matrices of bovine origin is required and mandatory, particularly to discriminate between illicit treatment and the natural origin of thiouracil in bovine urine and thyroid glands. The performed method uses a simple liquid–liquid extraction, preceded by a deconjugation step, and allows for the determination of TAP, TU, MTU, PTU, PhTU and DMTU in only one chromatographic run at concentration levels suitable for monitoring purposes.

3.5.4.1. Sample preparation

Despite the diversity of the matrices analysed, we carried out two similar methods to prepare urine and thyroid glands in order to have the same steps for each matrix.

Regarding the denaturation step, as reported by Vanden Bussche et al. \(^{[11]}\), the cleavage of the disulfide bonds of the proteins in urine can be performed by the addition of a reducing agent, such as DTT. The protocol developed in the present work follows this suggestion for both urine and thyroid glands.
The polarity of the thyreostats requires the use of an organic polar solvent to extract them from the matrices: we evaluated the applicability of different solvents by several tests using ethyl acetate, chloroform and tert-butyl methyl ether. Comparing the signal intensity of the analytes extracted with the three different solvents, tert-butyl methyl ether was chosen as the best solvent for the extraction. The poorest results were obtained by the extraction performed with ethyl acetate by which we could not extract most of the thyreostats.

In order to facilitate the phase separation and to reduce the miscibility of the analytes in the aqueous phase, this protocol adopted the approach of salting-out-assisted liquid–liquid extraction (SALLE), adding salt (NaCl) prior to the liquid–liquid extraction to favour the transfer of the analytes into the organic solvent [25-27].

3.5.4.2. Method validation

The analytical procedures developed were subjected to the validation process according to the Commission Decision 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4 [23,24].

The HPLC–MS/MS-reconstructed chromatograms together with the ion spectra of the thyreostats in solvent (10 μg L⁻¹) and of the thyreostats in urine and thyroid glands are shown in Figures 2 and 3, respectively. DMTU as the internal standard (10 μg L⁻¹) is also reported. The chromatograms show the absence of
interference peaks at the expected retention times of the thyreostats, hence illustrating a good specificity and selectivity of the method. The analytes were detected and confirmed based on their proper relative retention times and their ion ratios. The relative retention times were within a tolerance limit of 2.5% and the relative ion intensities were within the maximum permitted tolerances \cite{28}.

LC-MS/MS chromatograms and ion spectra of thyreostats in solvents at a final concentration of 10 μg L\(^{-1}\).
LC-MS/MS chromatograms and ion spectra of a blank urine (A) and thyroid gland (B) sample spiked with thyreostats at a final concentration of 5 μg L⁻¹ and μg kg⁻¹, respectively.

For the HPLC-MS/MS confirmation of substances listed in Group A of Annex I of Directive 96/23/EC [13], a minimum of four identification points (IPs) is required. In the present work, we monitored five products ions with the highest intensity [23]. Each one of the five product ions is equal to 1.5 IPs, making a total of 7.5 IPs. The ion giving the highest signal-to-noise ratio was selected for the quantification. The MRM transition intensities were compliant with the maximum tolerances permitted. The parameters obtained for the method validations are given in Tables 3, 4 and 5. Linearity was verified by using squared correlation
coefficients ($r^2$): The regression coefficients of the curves that were built to check the instrumental linearity were higher than 0.982, which indicates a satisfactory linearity for all the analytes. Good linearities were also achieved in urine and in thyroid glands and showed values higher than 0.978 and 0.973, respectively, thus demonstrating a suitable and adequate correlation between the concentration and the acquired response in the sample for both matrices. The precision of the method, which was calculated by applying one-way analysis of variance (ANOVA), was evaluated in terms of intra- and inter-day repeatability, and is expressed as the coefficients of variation (CV) from the replicate samples. Their values were lower than 22%, as proposed by Thompson [28], demonstrating an acceptable precision for the method. The recoveries showed good values ranging from 96% to 104% in urine and from 96% to 104% in thyroid glands. The results regarding the precision and recovery of TAP are noteworthy both in urine and the thyroid glands. The CV values are better than those reported in the literature, as are the recovery values. In particular, the highest values of the recovery could be due to the decrease in the number of the evaporation steps that may cause of considerable losses of TAP, as suggested by Abuìn et al. [21].
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration level (µg L⁻¹)</th>
<th>Recovery % (n = 18)</th>
<th>Repeatability intra-day (CV: n = 6)</th>
<th>Repeatability inter-day (CV: n = 18)</th>
</tr>
</thead>
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<tr>
<td>TAP</td>
<td>5</td>
<td>99</td>
<td>8</td>
<td>20</td>
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<td>8</td>
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<tr>
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<td>101</td>
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<tr>
<td>MTU</td>
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<td>104</td>
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<tr>
<td>PTU</td>
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<td>PrTU</td>
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<td></td>
<td>15</td>
<td>100</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

CV: coefficient of variation

**Table 3.** Analytical performance (method trueness and precision) data for thyreostat determination in urine.
Table 4. Analytical performance (method trueness and precision) data for thyreostat determination in thyroid glands.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration level (μg L⁻¹)</th>
<th>Recovery % (n = 10)</th>
<th>Repeatability intra-day (CV: n = 8)</th>
<th>Repeatability inter-day (CV: n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAP</td>
<td>5</td>
<td>99</td>
<td>8</td>
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<td>10</td>
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<tr>
<td>PTU</td>
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<td>104</td>
<td>12</td>
<td>19</td>
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<td>PrTU</td>
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<td></td>
<td>15</td>
<td>100</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

CV: coefficient of variation

The decision limit (CCα) and detection capability (CCβ) are very important, debated and decisive points to evaluate. For the estimation of these values, the document of the Commission Decision 2002/657/EC (2002) explains both the definition and procedure. However, the approach proposed in the document to evaluate these limits – based on the extrapolation of the calibration curve procedure according to ISO 11843 – may lead to an underestimation of the parameters, as already explained by Galarini et al. [29] and other Authors [31-32].
Therefore, CCα (and, consequently, CCβ) was determined using a parallel extrapolation to the x-axis at the lowest experimental concentration as clarified in the document SANCO/2004/2726-rev. 4 [24]. Decision limits achieved with this approach were thus experimentally determined, and therefore not underestimated. A comparison with previously published data concerning the detection of non-derivatised thyreostats should consider the differences in the method of CCα determination. Table 5 shows the obtained CCα and CCβ values, which are lower than the minimum required performance limits (MRPLs) proposed in the CRL guidance document of 2007 in urine and in thyroid glands [17]. Moreover, the TAP analytical limits are lower than those reported in literature for the two matrices, such as MTU in the thyroid gland [11,21,22,31]. Finally, it is worth noting that the validation parameters obtained with our method are comparable between the two different matrices.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CCα  (µg L⁻¹ and µg kg⁻¹)</th>
<th>CCβ  (µg L⁻¹ and µg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Thyroid gland</td>
</tr>
<tr>
<td>TAP</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>TU</td>
<td>7.3</td>
<td>7.4</td>
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<tr>
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<td>7.0</td>
</tr>
<tr>
<td>PTU</td>
<td>7.2</td>
<td>7.4</td>
</tr>
<tr>
<td>PhTU</td>
<td>6.9</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Table 5. Decision limits (CCα) and detection capabilities (CCβ) calculated for thyreostats in urine and in thyroid glands.
3.5.5. Conclusion

The methods for the simultaneous direct identification and quantification of five thyreostats without derivatisation in both urine and thyroid gland samples were specific and sensitive. Moreover, the validated methods guarantee a better performance for TAP in both matrices than those reported in the literature. The choice to develop a method without derivatisation and clean-up steps was made due to the advantages in terms of costs and the time of analysis. The simultaneous determination of five thyreostats in two matrices using similar methods could be useful to make comparative analyses more reliable, because the process variables are the same for urine and thyroid glands.

Furthermore, the measurement of the endogenous TU in urine and thyroid is possible as the analytical limits are all below 10 μg L⁻¹ and 10 μg kg⁻¹, and particularly considering that the CCα – which was determined as clarified by the document SANCO/2004/2726-rev. 4 [24] – is not an estimate, but an experimentally verified concentration with all the characteristics required by the Commission Decision 2002/657/EC [23] for a substance to be quantified.
3.5.6. Acknowledgments

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Elisa Pasquale is the recipient of a Cariplo Ph.D. fellowship in Animal Production in the Laboratory of Inspection of Food of Animal Origin at the University of Milan.

3.5.7. References


CHAPTER 4

General conclusion and research

prospective
4. General conclusions and research perspective

Boldenone, prednisolone and thiouracil (as main representative of the thyreostats) are substances well known to be fraudulently used in livestock for fattening purposes. Despite their different chemical classes, hormones (boldenone and prednisolone) and thyreostats, these substances share a common feature: a double identity, both exogenous and endogenous, as to be considered pseudoendogenous or with semi-natural origin \cite{1-3}.

Nowadays, the approaches to investigate the illicit use of a substance are qualitative, by the identification of the substance or its metabolites of interest in the analysed matrix, and quantitative, based on a cut-off level of concentration for the detection of an eventual abuse. However, the detection of the illicit treatment of the so-called pseudoendogenous and semi-natural substances is challenging and represents a problem for official control, because the approach of the “zero tolerance” appears to not be valid for these substances. Additionally, a continuous improvement of the techniques to detect the presence of drug residues that leads to a decreasing of the detection limits has to deal with the difficulty to understand when a sample should be considered compliant or non-compliant under a certain concentration.

During the three years of doctoral fellowship, my whole research has been dedicated to a better comprehension of these
substances, trying to give a contribution to the development of new methods to analyse them, and to understand their nature.

In particular, we have dealt with the problem of the origins of these substances considering the following questions:

I. “Where particular steroids are believed not to be endogenous in an animal at a particular limit of detection (LOD), who is to say that as analytical limits decrease, they will not be discovered as endogenous at a lower concentration?\textsuperscript{[4]}”

II. Is it still valid the “zero tolerance” for the pseudoendogenous and semi-natural substances?

III. Based on literature, a proper sampling procedure allows a decrease of the probability of false-positive results to boldenone and prednisolone. The sampling procedure as well as the all the events associated to the slaughtering (transport, blood smell, fear) are now thought as essential factors to take into
account in case of monitoring and official control. In the context of steroids analysis, one of the main element to study seems to be the stress. Therefore, a question arises: has the stress always a positive influence in the eventual presence of these substances in urine?

1) The improvement of the analytical methods requires a constant research of the best technique and methodologies to perform in each step of the analysis. Very clear extracts, without any interferences due to the matrix, combined with a sophisticated and specific chromatographic separation, and sensitive system of detection, as a triple quadrupole, are all elements necessary to achieve optimal analytical performances. The application of new methods allows to obtain higher sensitivity and specificity values and, consequently, a reducing of the decision limits (CCα). Considered the chemical proprieties of our analytes of interest and the techniques described in scientific literature, we have developed and optimized new analytical methods that gave advantages in terms of time of execution, cost, and very satisfactory analytical performances. The detection capabilities
(CCα) calculated for the research of β-bold sulfate and β-bold glucuronide were for both metabolites 0.07 ng mL⁻¹. In the chapter 3.1. our purpose was to improve the analytical techniques in order to carry out a further study on the investigation of boldenone II phase metabolites in urine of young bulls. The ability to detect low concentrations, as a result of the methodological advancement, allowed to observe significant presence of β-bold sulfate and glucuronide in urine samples of bovine in different conditions and degree of stress.

A technical improvement was also realised for the detection of prednisolone (CCα calculated of 0.07 ng mL⁻¹) in the study included in the chapter 3.4., regarding its possible endogenous origin in urine of pigs. While concerning the CCα of prednisolone for adrenal glands of pigs was of 0.12 ng g⁻¹.

The development of new simple method for the detection of thyreostats has been carried out considering the chemical properties, chromatographic behaviour of the analytes, and the characteristics of the two matrices studied (urine and thyroid glands) in order to detect them without the most common derivatisation procedure that is generally performed (Chapter 3.5.). Therefore, all thyreostats showed CCα,
both in urine and in thyroid glands, below the recommended concentration of 10 ng mL\(^{-1}\) in urine and 10 ng g\(^{-1}\) in thyroid glands. The decision to avoid the step of derivatisation led to CC\(\alpha\) not extremely low, if compared to the values already reported in literature. However, it is worth of note that the method to calculate the decision limit is not homogenous in all laboratory. We chose to determine the CC\(\alpha\) as explained by the document SANCO/2004/2726-rev. 4 \[5\]. Therefore, our limits were experimentally verified, already useful for an eventual quantification of the substances.

2) During the early years of this decade, several EU Member States reported an increase in the frequency of detection of boldenone (mainly as \(\alpha\)-bold) in urine of cattle \[6\]. In particular, the so-called “boldenone problem” was deeply felt in Italy. In the course of the biennium, 2001 and 2002, within the framework of the Italian National Residues Plan, a considerable number of urine samples of calves was confirmed positive to \(\alpha\)-bold and, less, to \(\beta\)-bold \[7\]. Then, data provided by the Italian National Residue Plan between the years 2008-2008, stated an alarming rise in the occurrence of prednisolone residues in
cattle \[^8\]. Afterward, the probably endogenous nature of prednisolone has been also proposed for horse and human. In the Belgian Residue Control Plan in 2011 and 2012 urines of pigs were found positive for prednisolone, and a study performed by the Belgian Federal Agency for the Safety of the Food Chain (FASFC) revealed that out of 393 porcine urine samples taken at the slaughterhouse, a percentage of 73\% was found positive for prednisolone\[^9\].

Recently, low levels of thiouracil in urine of cattle were frequently registered in the ordinary frameworks of different EU Member States \[^10,11\]. Based on data reported, threshold levels for \(\alpha\)-bold and prednisolone in urine of cattle, and thiouracil in urine and thyroid gland of bovine were proposed, apparently violating the politics of zero tolerance limits regarding banned substances as boldenone and thiouracil.

As described in chapter 3.1 and 3.2., a technical development and a resultant reduction of the analytical limits, allowed to found a significant naturally presence of \(\beta\)-bold sulfate and glucuronide in urine samples of bovine, suggesting that \(\beta\)-bold sulfate is not an indicator of treatment at the farm. Further study could establish cut-off levels for \(\beta\)-bold
II phase metabolites. The zero tolerance for a pseudoendogenous substance as boldenone is probably to re-evaluate because of its natural origin. By the other hand, there are no data in literature regarding eventual promoting effects of these substances at the “natural “levels. Our study, reported in the chapter 3.2. on 56 calves, revealed that no significant morphological alterations to the sexual organs and associated glands were detected in the examined animals. Therefore, it could be concluded that the content in steroid may be so low as not to be sufficient to determine a fattening effect.

A neo-formation of prednisolone can occur in feedstuff for cattle, as discussed in chapter 3.3. Therefore, eventual ingestion of feedstuff rich of the prednisolone may be a reason of non-compliant response for the investigation of prednisolone. Due to the low possibility of modifying the storage conditions at the farms, the studies that would indicate objective parameters, e.g. a cut-off level or metabolite markers, highlighting the need for the definition of the prednisolone metabolic precursors in the feedstuffs and the nature of their origin.

In chapter 3.4, the high frequency of detection of prednisolone in urine samples and, mainly, in the

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adrenal glands permitted to confirm the endogenous presence of prednisolone in pigs and its positive relationship with cortisol, explained with several statistically considerations. Additionally, based on the significant sample size of the animals and following the calculations proposed for cattle, a cut-off value equal to 1.51 ng mL^{-1} for prednisolone in porcine urine was also suggested.

3) The most interesting element of our researches was the opposite influence of the stress in the behaviour of boldenone in bovine and prednisolone in pigs. In Chapter 3.2., the endogenous presence of β-bold sulfate and glucuronide was demonstrated in urine from young calves collected in two different times of sampling corresponding to different degree of stress: medium and low levels of stress. No sample positive for boldenone has been found in the most intense moment of stress, which is at the slaughterhouse. Surprisingly, our preliminary hypothesis, that proposed the stress as a positive factor for the occurrence of boldenone in urine, has been completely overturned and a negative influence of stress on boldenone endogenous production may be now more plausible.
Based on the results obtained in our study, the presence β-bold sulfate and glucuronide in urine collected at the slaughterhouse may be used as marker of fraudulently use of boldenone with the purpose to distinguish treated and untreated animals. This study could suggest a different parameter to evaluate a case of abuse of boldenone contrary to what Le Bizec and Destrez reported, who indicated β-bold sulfate as a candidate marker of unequivocally evidence of abuse of boldenone[12, 13].

Hence, it is necessary to formulate a hypothesis explaining the absence of the detection of boldenone in urine collected at the slaughterhouse.

More studies should be performed to investigate the relationship between cortisol and boldenone in order to understand if an eventual ratio between the two steroids might be useful to define a condition of illicit use of boldenone.

While, regarding prednisolone occurrence in pigs, the results obtained in the study reported in Chapter 3.4. confirmed the positive role of stress in the excretion of prednisolone, as already see in cattle[2].

Further studies could be also carried out on non-conventional matrices as hair, which is now considered worth of note for the
advantages that it offers: easily sampling, possibility to detect the administrated compound, large window of time of detection of substances to be monitored after administration. The segmental analysis of the hair could permit to define the timing of the administration \(^{[14]}\). Other matrices are now very interesting to evaluate, as bile that was recently studied for the investigation of steroids in bovine because of the ease of sampling and for the accumulation of residues of drugs occurring \(^{[15,16]}\). At present, the “omics” technologies, comprising the analysis of the metabolic pathways in order to obtain the entire metabolic profile of the steroid and the analysis carried out with gas chromatography coupled to combustion/isotope ratio mass spectrometry (GC-C/IRMS) would seem as a promising approach for tracing the abuse of boldenone and evaluate its endogenous nature. Besides, a great contribute to the understanding of the metabolism, biochemical reactions and nature of the molecules, is given by the study of the metabolomics untargeted fingerprinting of biological matrices depending on several mass spectrometric techniques (MS–MS, HRMS, and hybrid HRMS systems). This approach as well may be significantly advisable in order to evaluate a useful marker to discriminate between the endogenous occurrence and exogenous administration of a compound \(^{[17]}\).

**References**


Naturally Occurring Thiouracil in Livestock. Journal of Agricultural and Food Chemistry, 63, 1339-46


immunoaffinity column cleanup and two validated liquid chromatography–tandem mass spectrometry analyses. Analytica Chimica Acta, 852, 137–145


CHAPTER 5

Acknowledgements
5. Acknowledgements

The Research is an incredible universe, complicated to understand but engaging and attractive. Then, when the ability of passionate people comes into play, it is possible to see the important applications of several experiments. Three years ago, I had the possibility to know a new and interesting world, where I met professors, researchers and students avid of knowledge. For this reason, I wish to thank my supervisor, Prof. Luca M. Chiesa whose words have accompanied me in every moment of my Ph.D. and have helped me to understand not only the problems correlated to the analysis of residues, but also to see the life in a different way. My thanks are also for all the people of our laboratory, for the always enthusiastic, helpful and smiling Prof. PierAntonio Biondi, for the Dr. Francesco Arioli who helped and corrected me many times, for Dr. Radmila Pavlovic who I consider not only a good researcher but also a friend and a fantastic future mom, for Dr. Sara Panseri whose intelligence and enthusiasm are fundamental in our team and very appreciate by me. And I have to say thank you to Maria who I think will become a very good chemist through her smiling passion for the science, thank to Alessandra who started with me when I didn’t know how much important she was. Thank to Giuseppe who inspired me the desire to visit the Basilicata and thank to all other people I met in these years.
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Moreover, at the end, I have to thank myself because only now I can understand what my grandfather used to say: *omnia voluntas vincit*. However, the road to do is still long.

Elisa