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Development and validation of methods for the detection of residues in unconventional and innovative matrices through LC-MS/MS analyses for safety of food of animal origin.

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"Foodstuffs become blood;

blood becomes heart and brain,

the stuff of thought and attitudes.

Human fare is the basis of human culture and thought.

If you want to improve the people give it,

instead of declamations against sin, better food.

Man is what he eats."

Ludwig A. Feuerbach

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ABSTRACT

Successful animal growth depends on a combination of many factors related to health, management and nutrition. The use of veterinary drugs in food-producing animals for therapeutic purposes is regulated (corticosteroids, antibiotics) or banned (anabolic steroids) in the European Union; however, their use as growth promoters cannot be excluded. Moreover, the eventual presence of residues in food constitutes a fraud and a health issue for the consumers. For these reasons the need to find new accumulation matrices and new sensitive, specific and robust methods that are able to reveal the presence of drug residues is essential, based on the fact that there is a low percentage of non-conformity in the final reports of the National Residues Plan in recent years, although the threat of a disproportionate use of these substances is increasingly on the rise. In the light of these facts, there is the need to implement the framework of controls aimed to food safety, due to the inefficiency of tools for the study of these substances.

Often, the use of conventional matrices, such as urine, liver or muscle, recommended for the official controls of illegal treatment are not completely satisfactory due to the fast elimination rate of the compounds or to the difficulties arising from the compounds characterised also by a pseudoendogenous nature. The debate about the presence of β-boldenone II phase metabolites and prednisolone in urine samples, owing to endogenous or illicit treatment, is currently ongoing within the European Union. These compounds have been appropriately defined “grey-zone substances”, for their double origin. The simple detection of some steroids in urine is currently considered to provide insufficient evidence of illicit treatment. Parameters such as cut-off levels, the presence of metabolites, or both, must be accounted for.

As regards antibiotics, the overuse, over the last decades, as growth promoters in food producing animal have caused favorable condition about the threat of bacterial resistance. The antibiotics can directly affect the consumer in the form of residues from the food chain, or by accumulation in the environment via the application of manure to land as organic fertiliser, via sludge storage or by direct contamination of illicitly additivated water and feed. The main challenge is to monitor contemporally different antibiotic classes, in different steps of the food chain, trying to control this phenomenon.

On the other hand, food contamination by new environmental contaminants should not be neglected. In particular, perfluoroalkyl substances (PFASs) have recently aroused great scientific interest and concern for public health, due to the fact they have been found in appreciable concentrations in human serum. On the basis of EFSA requestes and of analytical problems associated with their determination many studies are recommended to monitor their presence, building a database on PFASs in food, evaluate the contamination levels of the individual compound and finally draw up a reliable risk assesstment of European population.

This work was born with the aim to detect residues of the most commonly used drugs in broad sense, and then extended over time, also following requests from public and private entities, based on realistic situations of risk.

Therefore, based on the mentionated issues, the development, optimisation and validation of multiresidual methods and the direct application on real unconventional matrices allowed us to have a greater amount of information in terms of number, frequency, and concentration of different classes of veterinary drugs than in conventional matrices. We confirmed the presence of pseudoendogenous compounds and their precursors in the unconventional matrix bile, for example. The study of the unconventional matrices, e.g. bovine teeth, has also allowed us to detect esterified forms of some drugs, discriminating them from the active free forms that could have a double, exogenous and endogenous, origin. Finally, this work demonstrates the utility of an eventual introduction, through the food of animal origin chain, of several monitoring points of different types of residues, consisting of non-edible matrice analyses that are not destructive of the product intended for the consumer. On the other hand, the sensitivity and good performance of the developed LC-HRMS methods for the emerging PFASs, could help further studies and also EFSA to increase the number of quantifiable data useful to extend a risk assessment in its final reports.

CHAPTER 1

Introduction

1. INTRODUCTION

1.1. Food safety and analysis of residues

In recent years, food safety has become a frequently recurring phenomenon, familiar to the general public also as a result of media attention. In the European Union (EU), consumer protection is a matter of extreme importance. This is expressed in the precautionary principle [1] based on the Treaty of Amsterdam [2].

In the modern agricultural practises and intensive breeding systems several agrochemicals and veterinary drugs are being used or administered on a large scale, while industrialization has led to an increased potential exposure of food to chemicals from both industrial and different environmental sources. If we think to the European Council Regulation n°315/93 [3] definition of food contaminant as "*any substance not intentionally added to food which is present in such food as a result of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food or as a result of environmental contamination*" we can understand how this issue is so wide and complicated to deal with.

All these aspects also create a synergic increase of health risks to humans and animals and in concern regarding food safety. There are well known cases of incidence of food toxicity, which caused either acute or chronic effect for consumers. Some examples of such effects relate to the observed precocious sexual development in children of Puerto Rico and Italy due to the presence of estrogenic compounds in food [4, 5] or the toxic effects of French and Spanish people for the high content of β-agonists in the liver [6, 7].

As a response to these matters, have been implemented the regulations on use of chemicals, their residue levels and their monitoring in food.

In this context the residue analysis play an important role to reach the required level of security, to collect reliable data and to allow adequate risk assesstment and subsequent protection action.

A clear but generic definition of residue was given by De Brabander in his review: "*a residue is a trace of a substance, present in a matrix after some kind of administration*" [8]. Codex Alimentarius Commission precised the term 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" [9]. Whether applied to study the accumulation in matrices of animal origin after several treatments, or to study the metabolite profile, or the effect on trace level in food, the analysis of residues has its last aim the ability to estabilish if food is safe or not for human consumption, to safeguard animal welfare, and to ensure eventual illicit frauds as well.

In the light of that mentioned above, there is the need to develop sensitive, selective and robust analytical methods for a wide variety of residues, as well as anabolic steroids and antibiotics in innovative matrices of animal origins. The results of such surveillance is to ensure that residues, if they are present if matrices of animal origins, respect the estabilished maximum residue limit (MRLs) where indicated, or in case of prohibited substances to monitor the compliance with the regulations.

1.2. The use of veterinary drugs in breeding

Veterinary drugs are usually employed for therapeutic, metaphylactic, prophylactic purposes as well as for improved breeding efficiency. Although most of them are regulated in the European Union and can only be administered under strict control in specific circumstances under prescription of responsible veterinarian, sometimes they are illegally used also as growth promoters [10]. In this last case, feed conversion efficiency is improved as well as the gain in protein deposition increasing the lean to fat ratio. The result is usually a meat of poorer quality due to the increase in connective tissue production and collagen cross-linking for the reduction in protein degradation that allows more time for collagen molecules to cross-link and thus, increase the toughness of the meat [11]. The fat amount is also substantially reduced with the subsequent loss in juiciness and poorer flavour development. Moreover these substances produce a noticeable retention of water that is released during cooking. It is evident that there are important benefits for the farmers when using these illegal substances, but it is also evident that there are important prejudices for the processing industry, like lower quality of products and very important prejudices to the consumers, not only for the worse quality or the higher water content but for the presence of residues and the consequent

harmful health effects [12]. For all these reasons, there is an evident interest of both official organisms and food industry to control the presence of these substances in farms and foods of animal origin.

The EU has strictly regulated controls on the use of veterinary drugs, particularly in food animal species, by issuing several Regulations and Directives.

Council Regulation 2377/90/EEC [13], established maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin.

Council Directive 96/23/EC [14] contains guidelines for controlling veterinary drug residues in animals and their products with detailed procedures for EU Member States to set up national monitoring plans, including details on sampling procedures. For any type of animal or food, there are two main groups of substances that must be monitored (Table 1):

- unauthorized substances having anabolic effect that belong to Group A (are defined by Council Directive 96/22/EC [15] and Annex IV of Council Regulation 2377/90/EC [13]); and,
- veterinary drugs with established MRLs that belong to Group B.

Criteria to define the performance of analytical methods and the interpretation of results have been established in Commission Decision 2002/657/CE [16].

Table 1. List of monitored substances in animal productions.

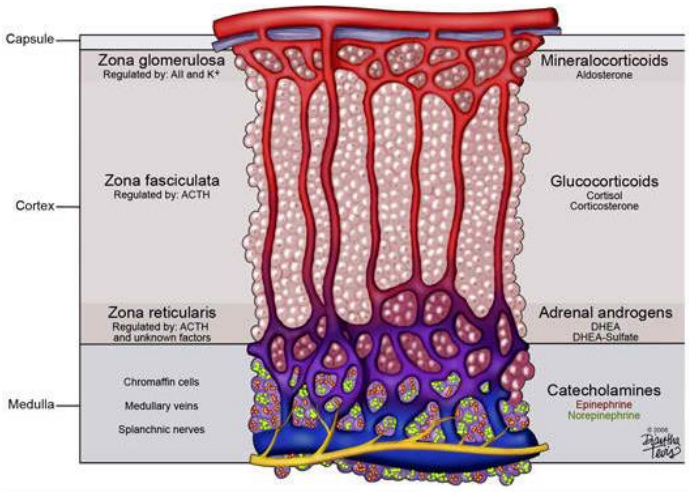
Group A: substances having anabolic effects and unauthorized substances
• Stilbenes, stilbene derivatives, and their salts and esters
• Antithyroid agents
• Steroids
• Resorcylic acid lactones including zeranol
•Agonists
• Compounds included in Annex IV to Council Regulation 2377/90/EC [13]
Group B: veterinary drugs and contaminants
• Antibacterial substances, including sulphonamides and quinolones
• Other veterinary drugs
Anthelmintics
Anticoccidiostats, including nitroimidazoles
Carbamates and pyrethroids
Sedatives
Non-steroidal anti-inflammatory drugs (NSAIDs)
Other pharmacologically active substances
• Other substances and environmental contaminants
Organochlorine compounds including PCBs
Organophosphorus compounds
Chemical elements
Mycotoxins
Dyes
Others

1.3. Corticosteroids: biosynthesis, regulation and metabolism

Corticosteroids are group of natural and synthetic analogues of the hormones secreted by the hypothalamic-anterior pituitary-adrenocortical (HPA) axis, more commonly referred to as the pituitary gland. These include glucocorticoids, which are anti-inflammatory agents and play significant roles in carbohydrate, protein, and lipid metabolism, the immune response, and the response to stress; mineralocorticoids, which control salt and water balance primarily through action on the kidneys; and corticotropins, which control secretion of hormones by the pituitary gland. Natural glucocorticosteroids have also a mild mineralocorticoid activity and therefore affect fluid and electrolyte balance.

The adrenal cortex secretes mineralocorticosteroids, glucocorticosteroids, and sex hormones, and it is composed by 3 distinct layers (Figure 1).

Figure 1. Structure of the adrenal cortex (Source: http://fontanillacjnotes.blogspot.it/2013/04/structure-of-adrenal-cortex-and.html)



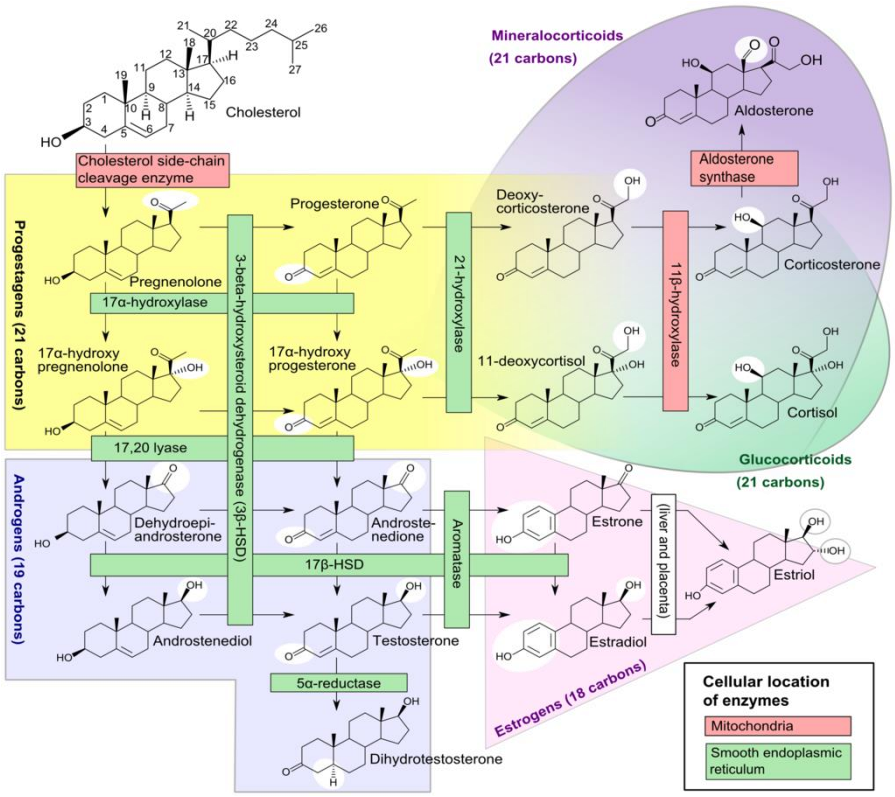
- The zona glomerulosa is in the outer layer were the mineralocorticoid aldosterone is produced to regulate the body's concentration of electrolytes, primarily sodium and potassium, by acting on the distal convoluted tubule of kidney nephrons to increase sodium reabsorption, increase potassium excretion and water reabsorption through osmosis [17].

- 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 [18].

  The synthesis of glucocorticosteroids starts from the common precursor cholesterol (Figure 2). Most steroidogenic reactions are catalysed by enzymes of the cytochrome P450 family. They are located within the mitochondria and require adrenodoxin as a cofactor (except 21-hydroxylase and 17α-hydroxylase).

  Aldosterone and corticosterone share the first part of their biosynthetic pathway. The last part is mediated either by the aldosterone synthase (for aldosterone) or by the 11β-hydroxylase (for corticosterone). These enzymes are nearly identical (they share 11β-hydroxylation and 18-hydroxylation functions), but aldosterone synthase is also able to perform an 18-oxidation. Moreover, aldosterone synthase is found within the zona glomerulosa at the outer edge of the adrenal cortex; 11β-hydroxylase is found in the zona fasciculata and zona glomerulosa [17].

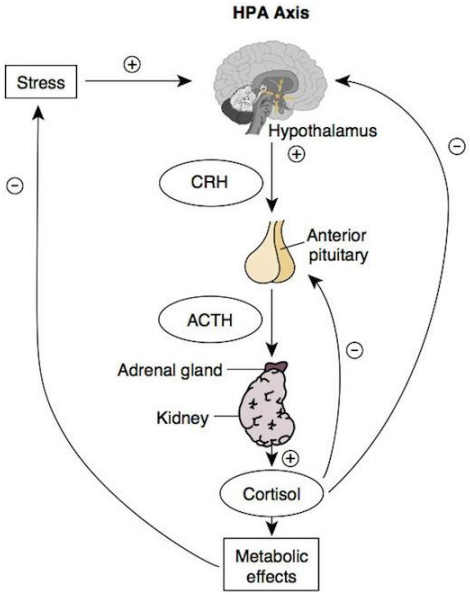
Figure 2. Steroidogenesis, including corticosteroid biosynthesis (Source: https://en.wikipedia.org/wiki/File:Steroidogenesis.svg. Accessed Figure 3: Source: https://embryology.med.unsw.edu.au/embryology/index.php/BGD_Lecture_Endocrine_Histology).



Glucocorticosteroids are secreted into the systemic circulation, reversibly bound (80%) to a specific α globulin, called transcortin or corticosteroid-binding globulin (CBG), while 10% is bound to serum albumin and the remaining 10% is the biologically active unbound hormone [19]. The release of glucocorticosteroids is stimulated by the adrenocorticotropic hormone (ACTH, also called corticotropin), At the physiological level, under stress conditions 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. The mutual aid of the three glands is defined as Hypothalamus-Pituitary-Adrenal axis (HPA). Free glucocorticosteroids inhibit ACTH secretion, and the degree of pituitary inhibition is proportional to the circulating glucocorticoid level (Figure 3). The production and release of these hormones follows a cyclical trend (circadian rhythm), where the highest circulating concentrations generally occur in the early hours of the morning. Circulating cortisol is released depending on the intensity of the stress. In fact, the

literature [20] on glucocorticoid metabolism in animals, especially in cattle, is mainly focused to evaluate animal welfare, as possible indicator of the stress influenced by several factors as transport, copulation, courtship, hunting and any physiological stressors associated with invasive procedures.

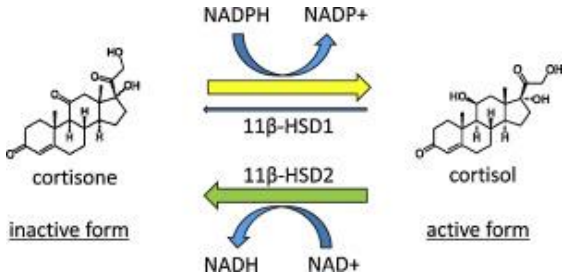
Figure 3. Mechanism of the Hypothalamus-Pituitary-Adrenal Axis (Source: synergyhw.blogspot.com).



Corticosteroids are metabolised principally in the liver, but also in kidney and mammary glands, giving water-soluble inactive conjugates excreted in urine (75%) and faeces (25%). Most of the cortisol is reduced to dihydrocortisol and then to tetrahydrocortisol, subject to glucuronation [19].

Cortisol (CL) can be interconverted to the non-active hormone, cortisone (CN) through the activity of 11β-hydroxysteroid dehydrogenases (11β-HSD), which has two isoforms: type I promotes the conversion of CL into CN, while type II catalyses the conversion of CN into CL [21] (Figure 4).

Figure 4. Interconversion of cortisol and cortisone by the action of 11β-HSD (Source: http://www.jdsjournal.com/article/S0923-1811(16)30131-1/fulltext).



1.3.1 Therapeutic use of corticosteroids in breeding

Adapted modifications to natural corticosteroids have allowed producing a large number of synthetic molecules, with a 21-carbon steroid skeleton, synthesised from cholic acid obtained from cattle or steroidal saponins in plants. These compounds can be used pharmacologically and therapeutically in the human and veterinary field. The latter are produced in order to have more powerful and selective compounds than natural ones. From a therapeutic point of view, glucocorticoids are used predominantly for their anti-inflammatory and antiallergic activity. Cortisone treatment can take place by oral administration, intramuscular, intravenous, subcutaneous, or local administration. These drugs have a rapid absorption when administered orally, reaching the maximum blood peak after 2 hours; via i.m. the maximum blood levels are reached within one hour. The percentage of binding of the synthesis compounds to the plasma proteins is lower than that of the corresponding endogenous hormones (which is about 90%) [22].

This explains the fact that synthetic glycocorticoids have a greater ability to move to the tissues than natural ones, exerting a faster and more intense action.

Therapeutic applications [23] cover the treatment of: collagen diseases (rheumatoid arthritis, lupus erythematosus), allergic diseases (bronchial asthma, hay fever), dermatological diseases (urticaria, dermatitis and psoriasis), haematological diseases (leukemia, autoimmune haemolytic anemia), various diseases (multiple sclerosis, gout, emphysema). However, the use of corticosteroids at the therapeutic doses causes a series of serious effects: excessive sodium and potassium retention, edema (mineralocorticoid effect), increased gastric acidity, hyperglycemia, hypertension, susceptibility to infections, redistribution of adipose tissue, behavioral disorders

(irritability, insomnia, etc.). All these effects are the expression of an over-emphasis on the physiological effects of these hormones. In bovine, in particular, despite the use of a large dose of synthetic glucocorticoids reduces the growth rate of animals, the macroscopic picture shows morphological alterations as well as the reduction in volume and weight of thyme (50-100 g up to the complete atrophy, against 400-800 g of a normal thymus) and adrenal glands. The histological framework, on the other hand, denotes thymus lymphocytic atrophy and depletion, fatty invasion and fibrosis, as well as hypothyroidism of the adrenal cortical [24]. Finally, their teratogenic effect pregnancy is well known [25].

1.3.2. The prednisolone case: pseudoendogenous or illicit treatment?

Prednisolone (PL) is one of the most debated glucocorticosteroid, structurally different from CL only by the presence of the double bound at the position C1-C2 (Figure 5). This variation results in an anti-inflammatory activity 3–4 times higher than that of CL [26].

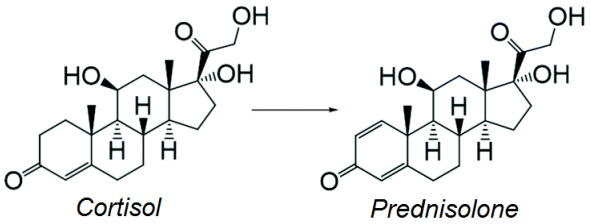
The use of PL is allowed in cattle only for therapeutic purposes and is regulated by Commission Regulation (EU) N°37/2010 [27], that estabilished MRLs for edible matrices.

In 2012 the Italian Ministry of Health, recommended a cut-off level 5 of μg L^{-1} for bovine urine, above which a sample could be considered non-compliant, indicating the potential endogenous origin of PL in urine on the basis of scientific evidences [28].

Until the beginning of the XXI century, PL was considered an exogenous compound, when an increased frequency of positives for PL was detected in urine samples at slaughtering, puting in doubt the hypothesis of illicit treatment. A study carried out in 2008/2009 in the North of Italy (Lombardy) on 196 bovine liver and urine samples taken at the slaughterhouse, showed the absence of PL in all liver sample and 72% of non-compliant for urines. Considering the high number of positive samples one of the possible explanations was the production of PL as a consequence of the stress transport and pre-slaughter stress. The Authors demonstrated this possible relationship by tetracosactide exacetate treatments, a synthetic analogue to ACTH hormone, to simulate stress. From the urine analysis, positivity to PL was accompanied by high levels of cortisol and cortisone [29].

The metabolic pathway of the endogenous production of prednisolone has not yet been clarified. Another study [30] dealt with a comparative study on cow urine samples collected at the farm and urine and adrenal glands (positive for corticosteroid in breeding) taken at the slaughterhouse from the same animals, assuming a conversion of endogenous CL to PL (Figure 5). The adrenal glands were positive for the presence of PL and could therefore be the seat of endogenous synthesis of PL, even if there are contrasting opinions.

Figure 5. Conversion from cortisol to prednisolone (Source: http://www.sciencedirect.com/science/article/pii/S0039128X12003108 with some modifications).



Moreover, the presence of trace amounts of the corticosteroid in urine could be a result of intestinal dehydrogenation of cortisol operated by bacteria [31].

The formation of PL from CL could be also a result of microbiological contamination of soil bacteria, especially when urine samples are taken at the slaughterhouse directly from the bladder, so practically free from fecal contamination [32].

Another possible explanation indicates the possibility of PL neo-formation from natural feed ingredients (phytosterols) under poor storage [33].

The endogenous origin of PL has also been described for equine, pig and human urine [34-37].

Concerning the prednisolone issue resulting from Illegal treatments, a decrease in cortisol and endogenous cortisone levels has been demonstrated due to the increase in the activity of 11β-hydroxy steroid dehydrogenase [38].

1.3.3. Corticosteroids and legislations

European Union banned the use of corticosteroids as growth promoters (either alone or in a cocktail with other active principles, as well as β-agonists or steroid) allowing their use only for therapeutic purpouses [14]. The Commission Regulation (EEC) n° 37/2010

established MRLs for edible matrices (muscle, kidney, liver and milk from different species) setting also a withdrawal period between treatment and slaughter, as indicated in the Annex and reported in Table 3 [27]. The monitoring control for the presence of corticosteroids is performed by collecting the edible tissues, for which MRLs are set, at the slaughterhouse, and at farm through the analysis of urine, even if for this matrix no MRL were set. In Italy, the National Residual Plan (NRP), estabilished in 1988 by the Ministry of Health-Directorate General for Health and Food Safety and Nutrition, is an important surveillance plan designed to detect or verify the use of prohibited substances, the abusive administration of authorized substances, the compliance of residues of veterinary drugs with MRL and maximum levels of environmental contaminants established by national and Community legislation. The official control system is carried out by the competent authorities (Regions and Autonomous Provinces, National Reference Laboratories and Istituti Zooprofilattici Sperimentali).

The NRP defines the species and animal categories to be sampled, the category of residues or substances to be investigated, the sampling strategies, levels and sampling frequencies, according to the legislation and the directions of the Commission European [39]. Italy includes corticosteroids in the Group B2f of monitored substances (Section 1, Table 1), following the suggestion of the European Commission. However, corticosteroids without any indication or limit of control are considered illicit [15, 16].

1.3.4. Analytical approaches for corticosteroids

As mentioned above, the NRP suggests urine as control matrix in the farm and liver and kidney at the slaughterhouse [27, 39]. The analytical determination can be performed with different techniques:

• immunochemical tests (ELISA), used for screening analyses, when a large number of samples are to be processed and get a quick response. However, the low specificity and the possibility to obtain false positives due to the compounds cross-reactivity makes this test less reliable than the others.

• Instrumental techniques ss confirmatory analyses such as:

- GC-MS (Gas Chromatography-Mass Spectrometry), specific and sensitive chromatographic technique constrained by the need to derivatize the corticosteroids in the pre-treatment phase of the sample;

- HPLC (High Performance Liquid Chromatography), chromatographic technique which, when combined with a DAD (Diode Array Detection) detector, is affected by low sensitivity and specificity, but which becomes highly sensitive and specific if coupled to a mass spectrometer. In this last case, there is the possibility of working with two different ionization techniques and interfaces: electrospray ionization source (ESI) and atmospheric pressure chemical ionization (APCI).

In case of instrumental analysis the sample needs some clean-up and purification phases that usually for urine include: a preliminary step of enzymatic hydrolysis to obtain the free compounds, before the exctraction step. However, some studies avoid this preliminary phase of deconjugation [40, 41]. Subsequently, liquid-liquid extraction or solid liquid extraction (SPE) is carried out using different organic solvents, as well as diethyl-ether, tert-butyl-methyl-ether or mixture of these solvents. A purification step, by C18 polymeric-based sorbents HLB Oasis, is usually done to eliminate interferences before the instrumental analysis [42]. The purification technique by immunoaffinity columns is the most specific and effective [43].

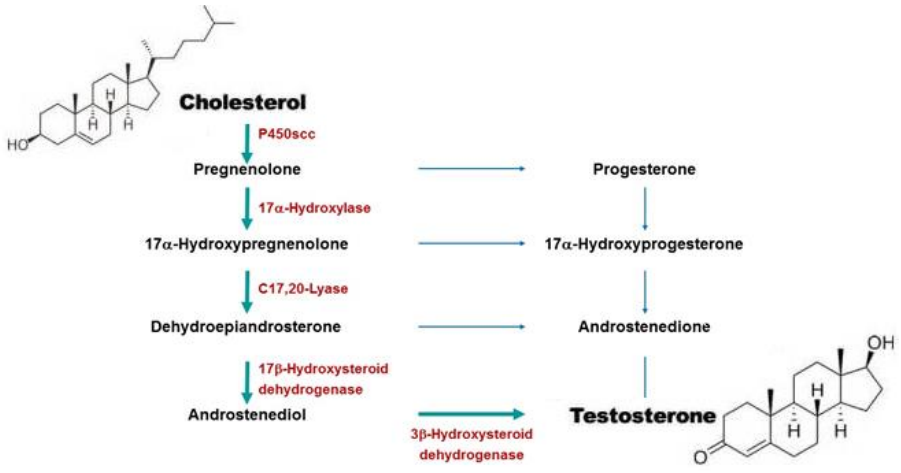
Regards solid and heterogeneous matrices, a defatting step using n-hexan is necessary prior the purification step or the quick, easy, cheap, effective, rugged, and safe (QuEChERS)-based extraction [44] is directly performed.

1.4. Anabolic steroids: biosynthesis, regulation and metabolism

Anabolic steroids, also known more properly as anabolic androgenic steroids (AAS), are molecules that include natural androgens like testosterone as well as synthetic substances that are structurally related and have similar effects to testosterone. Although there was an attempt to dissociate the androgenic and anabolic effects, complete separation, as yet, it has been impossible. However, there are now products available with more androgenic and substances with more anabolic properties. The androgenic effect primarily includes virilizing aspects, including induction of the development and maintenance of masculine secondary sexual characteristics such as the growth of the vocal cords and body hair. The anabolic action affects protein metabolism by stimulation of protein synthesis from amino acids, inhibition of protein breakdown, bone remodeling and growth, and stimulation of bone marrow, which increases the production of red blood cells [45, 46].

Testosterone (T) is the main male sex hormone. Like other steroid hormones T is derived from cholesterol. The synthesis pathway of T is represented in Figure 6. The largest amount of T is produced by testes, but it is also synthesized in smaller quantities by the theca cells of the ovaries, the zona reticulosa of the adrenal cortex, and the placenta. Substantial amounts of the testosterone in women are also produced from estradiol by reverse aromatization in the liver, adipose cells, and other peripheral tissues.

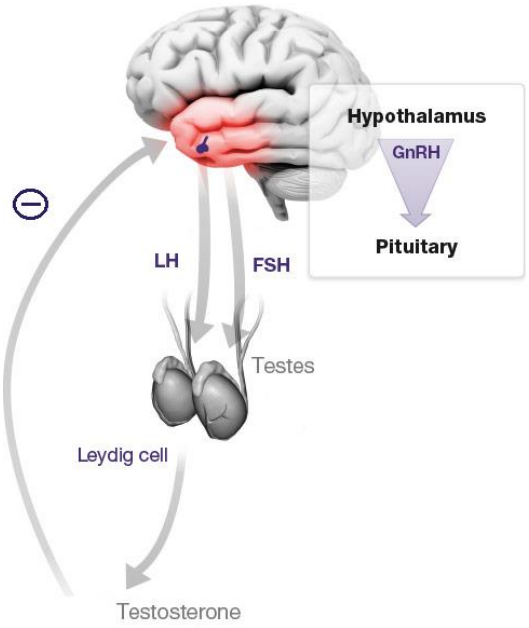
Figure 6. Biosynthetic pathway of testosterone from cholesterol (Source: http://www.endotext.org/chapter/page/9/).



In males, testosterone is synthesized primarily in Leydig cells. The number of Leydig cells in turn is regulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In addition, the amount of testosterone produced by existing Leydig cells is under the control of LH, which regulates the expression of 17β-hydroxysteroid dehydrogenase [47].

The amount of testosterone is regulated by the hypothalamic–pituitary–testicular axis (Figure 7). When testosterone levels are low, gonadotropin-releasing hormone (GnRH) is released by the hypothalamus, which in turn stimulates the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These latter two hormones stimulate the testis to synthesize testosterone. Finally, increasing levels of testosterone, through a negative feedback loop, act on the hypothalamus and pituitary to inhibit the release of GnRH and FSH/LH, respectively [48].

Figure 7. Hypothalamic–pituitary–testicular axis and synthesis of testosterone (Source: https://supplementsinreview.com/testosterone/d-aspartic-acid-testosterone/).



In plasma, 98% of testosterone is bound to protein, with 65% bound to sex hormone-binding globulin (SHBG) and 33% bound weakly to albumin [49]. About 2% of T is free and available for the interaction with receptor cells.

The mechanism of action of T begins with the transportation of free testosterone (T) into the cytoplasm of target tissue cells, where it can bind to the androgen receptor, or can

be reduced to 5α-dihydrotestosterone (DHT) by the cytoplasmic enzyme 5α-reductase. DHT binds to the same androgen receptor even more strongly than testosterone, so that its androgenic potency is about 5 times that of T. [50] The T-receptor or DHT-receptor complex undergoes a structural change that allows it to move into the cell nucleus and bind directly to specific nucleotide sequences of the chromosomal DNA. The areas of binding are called hormone response elements (HREs), and influence transcriptional activity of certain genes, producing the androgen effects.

The mechanism of action of AAS may differ between compounds because of variations in the steroid molecules. These variations are responsible for differences in the specificity of binding to receptor proteins or to interaction with various steroid-metabolising enzymes [51, 52]. With respect to interactions with intracellular steroid receptor proteins, several pathways can be distinguished.First, binding with high affinity to androgen receptors – these steroids are therefore recognised as strong androgens (e.g. 19-nortestosterone). Secondly, several compounds are characterised by binding with low affinity to androgens and therefore are weak androgenic substances. Thirdly, some AAS do not bind to the androgen receptor at all [53]. These steroids are supposed to act after biotransformation to more active compounds or via alternative mechanisms of action. Furthermore, it has been established for AAS that other mechanisms may also be involved.

The metabolism of AAS generally occurs in two phases, I and II in order to convert them into more polar compounds to facilitate their elimination. Both testosterone and 5α-DHT are metabolized mainly in the liver. Approximately 50% of testosterone is metabolized through conjugation into testosterone glucuronide and to a lesser extent testosterone sulfate by glucuronosyltransferases and sulfotransferases, respectively. The conjugates of testosterone and its hepatic metabolites are released from the liver into circulation and excreted in the urine and bile. Only a small fraction (2%) of testosterone is excreted unchanged in the urine [54].

1.4.1. Therapeutic use of anabolic steroids in breeding

Steroid sex hormones are part of the endocrine system and are found in physiological ranges in animal biologic matrices. Therefore, their mere presence in animal need not

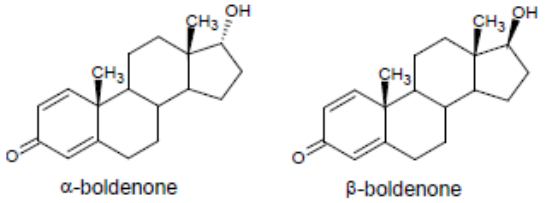
always be taken as a proof of illegal anabolic use. The physiological presence and variation of these hormones according to age, sex and many other factors make identification of abuse of these drugs for anabolic purpose [55]. Therapeutic administration of hormones and their effects on productivity have been investigated for years in numerous studies [56-58]. The administration of anabolics, as growth promoters in breeding, results in meat with a higher content of muscle tissue and lower amount of adipose tissue, i.e. meat of better organoleptic properties [59]. The anabolic effect is obtained through direct and indirect mechanisms of action resulting in enhanced nitrogen retention and increased protein synthesis. The efficiency of animal growth promotion (gain up to 20%) also depends on the animal breeding, age, reproductive status, and route of anabolic steroid administration [60]. When administered orally, the hormones are characterized by low bioavailability for their rapid metabolic transformation. Steroids are usually administered by subcutaneous implantation near the ears 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% [61, 62]. The treatment with anabolic steroids is often explicated either individual substances or as cocktails tin order to have a synergistic effect and try to sidestep controls.

1.4.2. Boldenone: another noteworthy case

Boldenone or 17β-boldenone (β-bold) is one of the most famous anabolic steroid commonly used by injection in different preparations as ester forms (undecylenate or undecanoate), either for human, horse or cattle particularly but also orally as boldione or Androstadienedione (ADD), the oxidised precursor of boldenone [63].

β-bold differs from testosterone 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 8. Like the other androgenic steroids, β-bold is classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen [64].

Figure 8. Structure of β-and α-boldenone (Source: http://www.sciencedirect.com/science/article/pii/S0960076015001478 with some modifications).



The debate about the presence of β-boldenone II phase metabolites in urine samples, owing to endogenous or natural origin, or illicit treatment, is currently ongoing within the European Union since 1996 [65]. In fact, gradual increase of positive samples containing boldenone in different States of the European Union doubted the purely exogenous origin of the drug. This compound togheter with prednisolone (discussed in Section 1.3.2.) have been appropriately defined “grey-zone substances”, for their double origin. This concept was well defined in 2009 by Scarth et al. [66], to identify the illicit abuse of exogenous hormones, synthetically produced, that “are also known to be endogenous under certain conditions, dubbed “pseudo-endogenous” due to their dual synthetic/endogenous nature”.

Several studies were also performed to define possible metabolites to use as markers in this discrimination [63, 66]. Already in 1983, Dumasia et al. [67] showed that metabolites were mainly excreted as glucu- and sulfo-conjugated compounds, after the intramuscular administration of radioactive-labelled β-bold to castrated male horses. Subsequently, they demonstrated that the epimer of boldenone tends to be conjugated with the glucuronic acid, while β-bold with sulphate [68]. Studies in vivo were firstly performed in human [63] and then in cuttle [69] to study the boldenone metabolism.

Also in pigs, the endogenous production of β-bold was confirmed and resulted gender dependent, in fact β-bold was not detect in female even if a very low concentration in urine result as a result of faecal contamination [70].

The simple detection of these substances in bovine urine is currently considered to provide insufficient evidence of illicit treatment. Parameters such as cut-off levels, the presence of metabolites, or both, must be accounted for. The α-epimer of boldenone was proposed, in 2003, as a naturally occurring steroid in bovine animals by experts within the EU, who set the “natural threshold” for the α-boldenone conjugates in urine at

2 ng mL^{-1}: a concentration above this could come from illicit treatment [71]. The authorities responsible for the control of residues in food must, therefore, consider either the possible endogenous production of these molecules or the existence of natural feed ingredients, such as phytosterols, present in vegetable fat, as possible precursors to boldenone [72, 73].

The fecal contamination of urine can also generate false positives because de novo synthesis of α-boldenone and metabolites occurs naturally in bovine faeces [74-76] Le Bizec [77] and Destrez [78] carried out several studies on β-bold with the purpose to clarify its metabolism and to discriminate the endogenous production from illegal abuse. They suggested sulfo-conjugated form of β-bold in urine as biomarker to indicate an illicit administration, while Blokland et al. in 2007 [79] proposed 6β-hydroxy-boldenone. From all considerations, it emerges that the ultimate answer concerning the topics of boldenone in bovine urine has not been accomplished yet; therefore, the topic of their endogenous production in bovine animals needs to be further explored.

1.4.3. Anabolic steroids and legislations

In the European Union, the presence of β-bold conjugated at any concentration in bovine urine is considered as an evidence of illegal treatment, while the detection of α-bold conjugated higher than 2 ng mL^{-1} is considered a suspicion of illegal use [71].

A Minimum Required Performance Levels (MRPL) for the analysis of β-bold and α-bold in bovine urine is 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) [80].

The use of anabolic steroids, in general, is banned by EU for growth promoter purpouses [14, 15].

1.4.4. Analytical approaches for anabolic steroids

To monitor illegal use, urine and manure which are available before the animals are slaughtered and which contain the highest hormone concentrations, are mostly selected. After slaughtering, liver, kidney, hair, fat or meat can be used for monitoring these compunds according to the EU criteria [14, 16]. Chromatographic techniques

combined with mass spectrometry, should be used to confirm the identity of hormone residues detected in the samples. Methods used for control programmes preferably should have a multiresidual character so that new steroids can easily be included [81].

Most literature methods for analysis of urine and liver are based on the analysis of the free steroids, requiring their release from glucuronide and/or sulphate [82, 83]. Helix pomatia juice, whichs contains -glucuronidase and arylsulphatase, is widely used to this end.

Several authors use liquid/liquid extraction (LLE) followed by a solid phase extraction (SPE) purification step [84, 85]. With solid matrices a deproteinization and a defatting step are usually carried out before SPE.

Immuno-affinity extraction has been shown as another feasible procedure for trace analysis of anabolic steroids. As more antibodies become available and as procedures to develop them become more sophisticated it is likely that more methods should be developed [86].

Anabolic compounds have been developed and analysed using GC–MS, for the good sensitivity and sufficient selectivity as a confirmatory technique. However, GC–MS requires derivatization of the steroids by means of silylation, acylation or oxime/silylation, depending on the properties of the individual steroids. The lack of a universal derivatization agent, the failure of some steroids to give a single reaction product, and problems with chemical rearrangement of others, strongly stimulated the development of LC–MS-based methods [81].

1.5. Antibiotics: general aspects, synthesis and mechanisms of action

The term “antibiotic” was invented by Selman Waksman, who discovered the antibiotic streptomycin. According to his definition an antibiotic is a chemical substance that is produced by microorganisms and that have the capacity to selectively inhibit the growth of and even to destroy other microorganisms. Soon scientists developed synthetic compounds that had antibiotic properties but were better than the natural ones, so antibiotics can be defined as molecules that either kill or inhibit growth of microorganisms and causes minimum damage to the host cells. This definition is still very restrictive because of the term “microorganism.” Most scientists would not consider viruses as microorganisms, but there are antiviral drugs available which have the same action mechanism of antibiotics [87]. The terms “antimicrobial,” “antibacterial,” and “antibiotic” are so confusing and interchangeably by US Food and Drug Administration (FDA) while at the same time reports this sclarification: *“The term ‘antimicrobial’ refers broadly to drugs with activity against a variety of microorganisms including bacteria, viruses, fungi, and parasites*” [88]. After Louis Pasteur observed, "if we could intervene in the antagonism observed between some bacteria, it would offer perhaps the greatest hopes for therapeutics" [89], in the late nineteenth century, several researchers have been searching for strategies to kill disease-causing germs. One powerful approach, developed by Pasteur was to use harmless bacteria to destroy harmful bacteria. Another approach was to use dyes as antibacterial agents because they were known to bind bacteria [90]. Arsenical compounds constituted another class of drugs used as chemotherapeuticagents [91]. In 1928, Fleming casually discovered, penicillin, the first scientifically studied antibiotic [92]. He initially characterized some of its antibacterial properties, but its further development was obtained by trained chemists.

An antibiotic should have the following other properties besides being able to kill microorganisms:

- water solubility: the antibiotic must be soluble in water to a sufficient extent to be transported through body fluids to the infected sites;
- selectivity: the antibiotic must kill or inhibit the infecting microorganism but cause minimum harm to the host cells;

- few side reactions: side reactions of the antibiotic should be minimised. These include possible allergic reactions and negative interaction with food or other drugs that the patient may be taking;
- stability: the antibiotic should have a long shelf life to be economically useful;
- low cost: An antibiotic should be low enough for patients to be able to afford it;
- slow resistance development: an ideal antibiotic will be the one to which resistance develops at a slow rate, due to the fact that microorganisms have developed resistance to most antibiotics [87];

While some antibiotics are chemically synthesised, almost all antibiotics, used everyday, are produced by microorganisms. Almost all antibiotics known today, have been isolated from microorganisms present in the soil. Natural antibiotics are products of their secondary metabolic pathways, which are not necessary for their survival.

The pathways for biosynthesis of antibiotics are turned off during exponential growth phase for the abundance of nutrients. However, in this stationary phase of growth, they compete with other microorganisms for the limiting amount of nutrients and so they turn on the pathways for biosynthesis of antibiotics and win the competition by killing the neighboring bacteria. Moreover, the surviving bacteria use the nutrients that are released when the dead bacterial cells lyse.

Microorganisms which product antibiotics need to protect themselves from those antibiotics. Some antibiotics are exported into the environment immediately after their synthesis to keep low their intracellular concentrations. Some antibiotic producing microorganisms also make a resistance protein that inactivates their own antibiotic. Antibiotics in the active form are released outside but if any antibiotic comes back into the cell is inactivated by the resistance protein. Other antibiotics, as well as macrolides, are exported after the sythesis to the outside in an inactive form and then converted to the active one outside. Finally, others microrganisms modify the target of the antibiotic within themselves; e.g they alter their own cell wall using different enzymes that are not targeted by the producted antibiotics [93].

One way to classify antibiotics is based on their effect on growth and survival of the bacteria. An antibiotic is bacteriostatic if it inhibits growth of bacteria but does not kill bacterial cells at a safe and practically achievable concentration. On the other side we

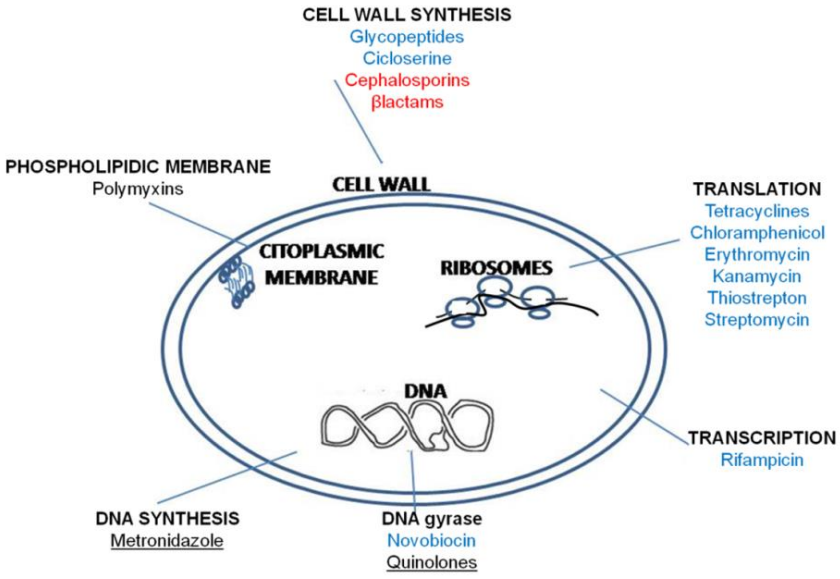
define bactericidal the antibiiotics that irreversible damage bacterial cells thereby killing them at a safe and practically achievable concentration [94, 95].

Another way to classify antibiotics is based on the microbial cell targets that they interact with to cause their inhibition action (Figure 9). Six major categories of antibiotics can be listed:

1) those that inhibit synthesis of bacterial cell wall,
2) those that disrupt the cell membrane,
3) those that inhibit the synthesis of important metabolites,
4) those that inhibit replication through DNA synthesis,
5) those that inhibit transcription acting on RNA synthesis,
6) those that inhibit translation interfering with protein synthesis [87].

Further classification is based on their target specificity: "narrow-spectrum" antibiotics target specific types of bacteria, such as gram-positive or gram-negative, while “broad-spectrum” antibiotics affect a wide range of bacteria.

Figure 9. Targets for antibiotics (Source: https://www.intechopen.com/books/actinobacteria-basics-and-biotechnological-applications/production-of-antibacterial-compounds-from-actinomycetes).



1.5.1. Antibiotic classes

Antibiotics can be divided in these main following classes:

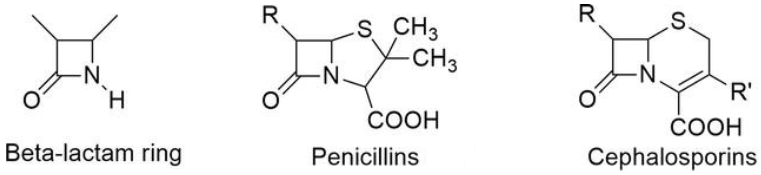
- β-lactams: consist of two classes of thermally labile compounds, penicillins and cephalosporins. Both classes contain a bulky side-chain attached to 6-aminopenicillanic acid and 7-aminocephalosporanic acid nuclei, respectively (Figure 10). The five-membered ring in penicillin contains a sulfur atom and is called the thiazolidine ring. The two rings together is called the penam ring and along with the methyl and carboxyl substituents is called penicillanic acid and is biosynthetically formed from the amino acids cysteine and valine. Penicillins are acyl derivatives of 6-amino penicillanic acid. Other semisynthetic derivatives of penicillin are made from 6-amino penicillanic acid which is obtained by deacylation of penicillin. In cephalosporins the β-lactam ring it is fused to a six-membered ring instead of a fi ve-membered ring. Also cephalosporins have more variable substituents (R1 and R2) in the rings [87].

  The presence of an unstable four-member ring in the β-lactam structure makes these compounds prone to degradation by heat and in the presence of alcohols. Penicillins are also readily isomerized in an acidic environment [96]. About 55 % of all antibiotics used globally belong to this class [87]. Cephalosporins and penicillins are produced by different microorganisms but the pathways for their synthesis are similar. Their mechanisms of action are also similar. β-Lactams inhibit the formation of peptidoglycancross-links in the bacterial cell wall; this is achieved through binding of the four-membered β-lactam ring to the enzyme DD-transpeptidase. As a consequence, this enzyme cannot catalyse the formation of these cross-links, and an imbalance between cell wall production and degradation develops, causing the cell to rapidly die [97, 98]. Penicillins are derived semisynthetically and are active against many gram-positive and gram-negative bacteria. However, they are readily destroyed by the β-lactamases. Many members of the group are acid stable and are administered either orally or parenterally as suspensions in water or oil or as water-soluble salts. The trihydrate forms of the semisynthetic penicillins have greater aqueous solubility than the parent compounds and are usually preferred for both

parenteral and oral use. The combination of β-lactamase inhibitors and broad-spectrum penicillins markedly enhances the spectrum and action efficacy. Penicillins are generally excreted unchanged, but fractions of a given dose (<20%) may undergo metabolic transformations.

Cephalosporins have a broader spectrum of activity than penicillins and are effective against both gram-negative and gram-positive bacteria. Cephalosporins are classified by generations (1–4). Later generations are more resistant to β-lactam destruction and are often characterized by extended but variable spectra. The few acid stable cephalosporins are used either as the free base form for oral administration or as sodium salts in aqueous solution for parenteral delivery. Cephalosporins are distributed into most body fluids and tissues, including kidneys, lungs, joints, bone, soft tissues, and the biliary tract. Plasma half-lives of cephalosporins are quite variable but generally longer than penicillins. Most cephalosporins are renally excreted. Biliary elimination may be also significant [99].

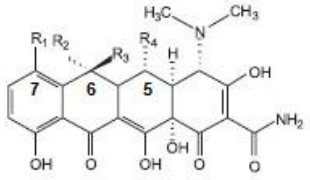
Figure 10. General structure of β-lactams, penicillins and cephalosporins (Source: https://www.intechopen.com/books/antibacterial-agents/classification-of-anti-bacterial-agents-and-their-functions).



- Tetracycline: they are broad-spectrum antibiotics against gram-positive as well as gram-negative bacterias. The basic structure of tetracyclines is a hydronaphthacene skeleton fused with four rings [97]. The different tetracyclines differ amongst them in their substitution patterns at the C5, C6 and C7 positions (Figure 11). Chlortetracycline, doxytetracycline, oxytetracycline, tetracyline are the most commercially available compounds, commonly applied to food-producing animals. Due to the presence of two ketone groups in positions 1 and 11, tetracyclines can readily chelate to metal ions such as calcium, magnesium, aluminum, and iron which prevents its absorption from the digestive system and so should not be administered with food [87]. All of the tetracycline derivatives are crystalline, yellowish, amphoteric

substances that, in aqueous solution, form salts with both acids and bases. The most common salt form is the hydrochloride, except for doxycycline, which is available as doxycycline hyclate or monohydrate. The tetracyclines are stable as dry powders but not in aqueous solution, particularly at higher pH ranges (7–8.5). Preparations for parenteral administration must be carefully formulated, often in propylene glycol or polyvinyl pyrrolidone with additional dispersing agents, to provide stable solutions [99]. There are several tetracyclines all of which have the same mechanism of action. Their mechanism of action is based on the inhibition of protein synthesis through the inhibition of the binding of amino-acyl tRNA to the A-site of the ribosome [87]. Tetracyclines are more effective against multiplying microorganisms and tend to be more active at a pH of 6–6.5. Tetracyclines generally are the drug of choice to treat rickettsiae and mycoplasma. After usual oral dosage, tetracyclines are absorbed primarily in the upper small intestine, and effective blood concentrations are reached in 2–4 hr. Tetracyclines at therapeutic concentrations should not be orally administered to ruminants because they are poorly absorbed and can substantially depress ruminal microfloral activity. Specially buffered tetracycline solutions can be administered by intramuscular and intravenous administration, to produces a long-acting effect. Tetracyclines distribute rapidly and extensively in the body, particularly after parenteral administration. They enter almost all tissues and body fluids; high concentrations are found in the kidneys, liver, bile, lungs, spleen, and bone. Tetracyclines are excreted via the kidneys (glomerular filtration) and the gastrointestinal tract (biliary elimination). Generally 80% of a given dose is unchanged recoverable from the urine. Tetracyclines are also eliminated in bile, feces, milk and saliva [99].

Figure 11. General structure of tetracyclines and main compound substituents (. Source: http://www.sciencedirect.com/science/article/pii/S0039914006001627).



Compound	R_1	R_2	R_3	R_4
Tetracycline	H	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
Chlortetracycline	Cl	CH_3	OH	H
Doxycycline	H	CH_3	H	OH

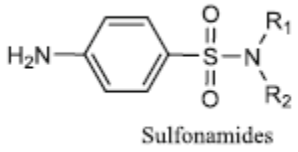
- Sulfonamides: All sulphonamide antibiotics have a free amino group at the para position from the sulfonyl group on the benzene ring (Figure 12). Usually they present a 5- or 6-membered nitrogen containing ring attached to the sulfonylamino group [87].

  Sulphonamides include a large number of synthetic bacteriostatic compounds, which act by competing with p-aminobenzoic acid in the enzymatic synthesis of dihydropholic acid. This leads to a decreased availability of the reduced folates that are essential in the synthesis of nucleic acids [97]. There is usually a time lag before the effect of the sulfa drug can be seen, because the bacterial cell will already have a certain concentration of folic acid before the administration of the drug. Also, other metabolites that require folic acid for their synthesis, such as purines, pyrimidines and amino acids, will also be already present in sufficient amount in the cell when the drug is administered. Another drawback of sulfa drugs is that about 3 % (which is a high percentage) of the general population is allergic to sulfonamides. Some patients also experience nonallergic response to the drugs such as nausea, diarrhea, and headaches [100].

  Sulfonamides are the oldest and remain among the most widely used antibacterial agents in veterinary medicine, chiefly because of low cost and their relative efficacy in some common bacterial diseases. Sulfonamides may be given topically or by oral, intravenous, intramuscular or intrauterine administration, depending on the specific preparation. They are frequently added to drinking water or feed either for therapeutic purposes or to improve feed efficiency. Sulphonamides are often

administered together with trimethoprim, considered as a potentiator. The synergistic action of sulfonamides renders these drugs much more effective than sulfonamides alone. In most species, some of these molecules are administered 1–4 times/day to control systemic infections caused by susceptible bacteria. In other cases, administration of the sulfonamide can be less frequent if the drug is eliminated slowly. Sulfonamides and trimethoprim are rapdly adsorbed and distributed throughout all body tissues. They are rapidly excreted via the urinary tract (>90% in 24 hr) mostly in an unchanged form; because of this, they are primarily used to treat urinary infections [99].

Figure 12. General structure of sulphonamides (Source: https://www.researchgate.net/figure/314119791_fig1_Figure-1-General-structure-of-sulphonamides-RR1H-for-sulphanilamide).



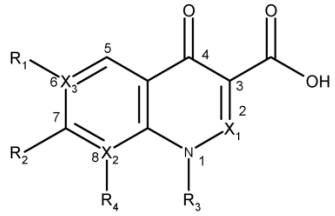
Sulfonamides

- Quinolones: they have position 1 nitrogen in the bicyclical aromatic ring structure, with an alkyl group (ethyl or perhaps cyclopropyl) often attached there (Figure 13). Carboxylic acid at position 3 is required for antimicrobial activity, similarly like a keto group at position 4. They are the highly effective broad spectrum antibiotics that target DNA gyrase as their site of action, a key enzyme in DNA replication [101]. Since there are many quinolones available, a new four-generation classification system has been described for quinolones. The first generation includes nalidixic acid, the first quinolone antibiotic discovered, that achieves only minimal serum concentration and so it's not much used. Second generation quinolones, including ciprofloxacin, can reach high serum levels, have good tissue penetration antibiotics and a broader spectrum of action against gram-negative bacteria. Third generation are effective against both gram-negative and gram-positive bacteria and also against anaerobes, while fourth generation have the broadest spectrum of activity [102].

Quinolones containing a fluorine substituent were developed as better gyrase-targeting antibiotics than the non-fluorinated ones. In fact, fluoroquinolones especially ciprofloxacin, has a much lower minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), so it's effective at much lower concentration [87]. Quinolones are well absorbed following oral administration, with moderate to excellent bioavailability. Elimination half-lives for the quinolones vary from 1.5 to 16 hours. Therefore, most of these drugs are administered every 12 to 24 hours. The quinolones are eliminated by renal and nonrenal routes [103].

Common side effects include gastrointestinal effects such as nausea, vomiting, and diarrhea, as well as headache and insomnia [104]. They are associated with a small risk of tendonitis and tendon rupture [105]; nervous system effects include insomnia, restlessness, and rarely, seizure, convulsions, and psychosis [106].

Figure 13. General structure of quinolones (Source: http://www.mdpi.com/1420-3049/18/9/11153).



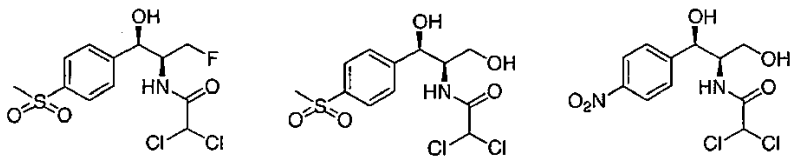
- Amphenicols: are characterised by a phenylpropanoid structure (Figure 14). They exert their action by blocking the enzyme peptidyl transferase on the 50S ribosome subunit of affecting microbial protein synthesis. Examples of amphenicols include chloramphenicol, thiamphenicol, and florfenicolare as broad-spectrum antibacterials, bacteriostatics with closely related chemical structures, used to control enteric and respiratory diseases in cattle, poultry and swine. The first-in-class compound was chloramphenicol, introduced in 1949, initially discovered as a natural product. It has a broad range of activity against most bacteria including anaerobes. One big advantage of the drug is that it can easily penetrate all tissues including the cerebrospinal fluid and so can be used to treat meningitis. It is also one of the very few antibiotics that can enter human cells and so can be used against intracellular bacteria [87]. However, it has been shown to cause a number of toxic side effects in humans such as aplastic anemia and other amphenicols, chemically synthesised,

have been suggested as potential substitutes [107]. Veterinary medicinal products and medicated feeds containing Chloramphenicol were banned in treatment of food-producing animals by the FDA in 1984. A ban has been also implemented in the EU since 1994 [108].

Florfenicol is a semisynthetic derivative of chloramphenicol in which the hydroxyl group at C-3 is replaced by a fluorine atom and the nitro group is also replaced with a sulfomethyl group. Replacement of the OH group prevents acetylation of the antibiotic and so florfenicol is resistant to inactivation by acetyl transferase enzyme. Currently florfenicol is approved for use in veterinary medicine, indicated for the treatment of bovine respiratory disease.. Bioavailability is higher after oral administration than intramuscularly. Florfenicol is excreted as parent drug in the urine and the major urinary metabolite is florfenicol amine. Florfenicol amine is the longest-lived major metabolite in the liver, and, therefore, it was used as the marker residue for withdrawal calculations [109].

Thiamphenicolis is the methyl-sulfonyl analogue of chloramphenicol and has a similar spectrum of activity. Like chloramphenicol, it is insoluble in water, but highly soluble in lipids. It is used in many countries as a veterinary antibiotic in the treatment and control of a wide range of respiratory and alimentary tract infections of bacterial origin in calves, pigs and poultry. These drugs are usually orally administered, through feed or water. The oral product of thiamphenicol is not suitable for the treatment of cattle with functional rumen. Unlike chloramphenicol, thiamphenicol is not readily metabolized in cattle, poultry, sheep, or humans, but is predominantly excreted unchanged. In pigs and rats the drug is excreted both as parent drug and as thiamphenicol glucuronate [110].

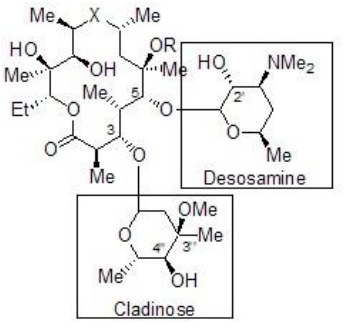
Figure 14. Structure of the main amphenicols (Source: https://www.google.com.na/patents/EP1696934B1?cl=en).



Florfenicol Thiamphenicol Chloramphenicol

- Macrolides: basically are macrocyclic lactones isolated first from Streptomyces spp. The basic chemical structures of macrolides consist of a 12-, 14- or 16-membered macrocyclic lactone to which sugar moieties, including amino and deoxy sugars, are attached (Figure 15). Macrolides are an important class of antibiotics widely used in veterinary practice to treat respiratory diseases and enteric infections in cattle, sheep, swine and poultry [97]. Although the ribosomes of both grampositive and gram-negative organisms are susceptible to macrolide action, these antibiotics are mainly used against gram-positive bacteria since they are unable to enter the porins of gram-negative bacteria. Erythromycin is the most commonly used and the first to be discovered in 1952. The other macrolides are semisynthetic derivatives of erythromycin. Erythromycin contains an amino group, which occurs in the protonated cationic form at neutral pH that is less permeable to cells than the neutral form, explaining why the drugs are more active at alkaline pH. Most macrolides are destroyed by stomach acid and so are administered intravenously or with enteric coating. Macrolides as well as lincosamides inhibit bacterial growth by inhibition of protein synthesis by binding to the 50S ribosome [87]. They are easily absorbed after oral administration and distribute extensively to tissues, especially the lungs, liver and kidneys [96].

Figure 15. General structure of macrolides (Source: http://people.clarkson.edu/~amelman/macrolides.html).

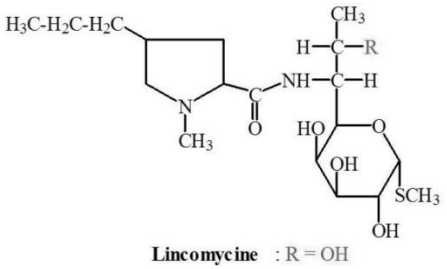


1 R=H, X = CO Erythromycin

- Lincosamides: Their sctructure contains a mycarose sugar (Figure 16). They are bacteriostatic and antagonists of macrolides because bind at the same sites on the ribosomes. As macrolides, in fact, they bind to the 50S ribosomal subunit, inhibiting

early chain elongation by interfering with the transpeptidase reaction [98]. Briefly, they prevent bacteria replicating by interfering with the synthesis of proteins. The first lincosamide to be discovered was lincomycin, isolated from *Streptomyces lincolnensis* in a soil sample from Lincoln, Nebraska, from which derived the name. Lincosamides are normally used to treat *Staphylococcus* and *Streptococcus*, in the treatment of toxic shock syndrome and thought to directly block the M protein production that leads to the severe inflammatory response. Lincosamide antibiotics are one of the classes of antibiotics most associated with *pseudomembranous colitis* caused by *Clostridium difficile.* The lincosamides, as the hydrochloride salts, are bitter to taste, so for oral formulation they are given as the palmitate esters, or formulated in capsules. Activity is enhanced at an alkaline pH. Efficacy is considered time dependent. Lincomycin is incompletely absorbed from the GI tract, especially if administered soon after feeding. They diffuse across the placenta in many species. 50% of a dose of lincomycin is metabolically altered in the liver. Metabolites often retain activity. Liver disease impairs the biotransformation of lincosamides. Unchanged antibiotic and several metabolites may be excreted in bile and urine. Concentrations remain high in the feces for some days, and growth of sensitive microorganisms in the large intestine may be suppressed for up to 2 weeks. Milk is also an important excretory route. No serious organ toxicity has been reported, but GI disturbances do occur; disruption of GI flora is a serious adverse reaction in a number of species and can be lethal. Skeletal muscle paralysis may be seen at high concentrations. Hypersensitivity reactions occasionally are seen. Lincosamides have additive neuromuscular effects with anesthetic agents and skeletal muscle relaxants. Kaolin-pectin prevents their absorption from the GI tract. They should not be combined with bactericidal agents or with the macrolides [99].

Figure 16. General structure of lincosamides (Source: http://unt-ori2.crihan.fr/unspf/2014_Rennes_Tomasi_Macrolides/co/Lincosamides.html).



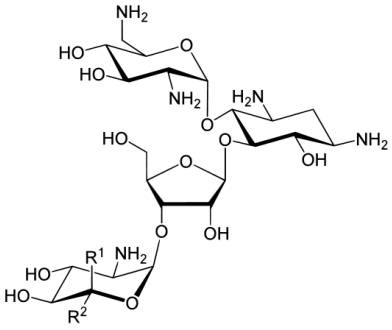
Lincomycine : R = OH

- <u>Aminoglicosides:</u> Chemically, the aminoglycoside antibiotics are characterised by an aminocyclitol group, with aminosugars attached to the aminocyclitol ring in glycosidic linkage (Figure 17). Because of minor differences in the position of substitutions on the molecules, there may be several forms of a single aminoglycoside with relatively minor differences in antimicrobial spectra, patterns of resistance, and toxicities. The amino groups contribute to the basic nature of this class of antibiotics, and the hydroxyl groups on the sugar moieties contribute to high aqueous solubility and poor lipid solubility. If these hydroxyl groups are removed, antibiotic activity is markedly increased. Aminoglycosides are typically quite stable. When the water solubility of an aminoglycoside is marginal, it is usually the sulfate salt that is used for oral or parenteral administration. The pKas of these drugs are generally between 8 and 10, and as a result, they tend to be ionised at physiologic pH, which may limit drug movement, particularly in acidic environments. They need only a short contact with bacteria to kill them and, as such, are concentration dependent in their actions. Their main site of action is the membrane-associated bacterial ribosome through which they interfere with protein synthesis. To reach the ribosome, they must first cross the lipopolysaccharide covering (gram-negative organisms), the bacterial cell wall, and finally the cell membrane. Because of the polarity of these compounds, a specialized active transport process is required. The efficacy of the aminoglycosides is markedly reduced in an anaerobic environment. Aminoglycosides are poorly absorbed (usually <10%) from the healthy GI tract. Aminoglycosides are polar at physiologic pH, limiting distribution to extracellular fluids, with minimal penetration into most tissues. Exceptions include the renal cortex of the kidneys. The aminoglycosides are excreted unchanged in the urine by glomerular filtration, with 80%–90% of

administered drug recoverable from the urine within 24 hours of intramuscular administration.

Elimination varies with glomerular filtration changes associated with cardiovascular and renal function, age, fever, and several other factors. Despite their potential to cause nephrotoxicity, the aminoglycosides are commonly used to control local and systemic infections caused by susceptible aerobic bacteria. Several aminoglycosides are used topically in the ears and eyes and via intrauterine infusion to treat endometritis. Aminoglycosides occasionally may be infused into the udder to treat mastitis. In general, because of their concentration dependency and potential for nephrotoxicity, aminoglycosides are administered once daily to reduce risks [99].

Figure 17. General structure of aminoglicosides (Source: https://commons.wikimedia.org/wiki/File:Neomycin_B_C.svg).



- Nitrofurans: Nitrofurans are synthetic chemotherapeutic agents with a broad antimicrobial spectrum. However, when compared with other antibiotics, their potency is not so effective. The nitrofurans appear to inhibit a number of microbial enzyme systems, including those involved in carbohydrate metabolism, and they also block the initiation of translation. However, their basic mechanism of action has not yet been clarified. Their primary action is bacteriostatic, but at high doses they are also bactericidal. They are much more active in acidic conditions (pH 5.5). Nitrofurans are mainly used orally or topically, due to their very slight water solubility. No nitrofuran is effective systemically. They are either not absorbed at all from the gastointestinal tract or are so rapidly eliminated that they reach inhibitory concentrations only in the urine. Toxic signs seen with excessive doses of nitrofuran are excitement, tremors, convulsions, peripheral neuritis, GI disturbances, poor

weight gain, and depression of spermatogenesis. Various hypersensitivity reactions have been showed. Some nitrofurans are also carcinogenic, and so their use is not convenient [99].

1.5.2. Therapeutic use of antibiotics in breeding

Antibiotic use in animal farms is a major cause of resistance development. According to FDA reports, only about 20 % of the approximately 18,000 tons of antibiotics sold in the United States of America are used by humans while the rest 80 % are used in animals [111]. There are two main types of antibiotic use in breeding: therapeutic use, that provides for the care of several animal infections accompanied by veterinary prescription. Similar to animals, plants may also be subject to infections, which can be cured with antibiotics. These antibiotics are also sprayed on the plants, a process by which most of the antibiotics end up in the soil thereby increasing the antibiotic resistance.

Contribution of therapeutic use of antibiotics to the antibiotic resistance problem actually appears to be insignificant when compared to the subtherapeutic use of antibiotics in food producing animals, not related to any infection. Of course our farm animals are not so sick that they need the excessive amount of antibiotics mentioned above for therapeutic purpouses. A subtherapeutic use consists of a prolonged administration of small antibiotic amounts. Most of this antibiotic is illicitly added to drinking water or animal feed as growth promoter to increase animal body weight with a slower growth rate, which means more profit for the farmers. It is believed by some that subtherapeutic use has a prophylactic effect and is needed for proper health of the animals. This phenomenon of growth promotion was an accidental discovery in 1948 when scientists were testing random food additives to discover new vitamins. Stokstad and Jukes added cellular debris of *Streptomyces auerofaciens* to chicken feed and after the antibiotic chlorotetracycline was extracted from the bacterial culture they observed faster growth of the chicken. Initially they thought that it was due to vitamin B12 present in the additive but later it was understood that the growth promotion was due to the much less amount than therapeutic dose of that antibiotic [87]. Soon it was proved the same effect by subtherapeutic doses of many other antibiotics. On the other side, the mechanism of

growth promotion is not clearly understood. Maybe antibiotics kill bacteria that compete with beneficial bacteria in the intestines of the animals or another hypothesis is that antibiotics do not have to enter the bloodstream to exert the growth promoting effect because its site of action is in the intestines [87].

In any case, it's getting real the negative and worrying effect of of this overuse of antibiotics, which far exceeds the economic advantage of growth promotion.

1.5.3. Antibiotic resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. Resistance is a property of the microbe, not of the person or other organism infected by microbes. As a result, the medicines become ineffective and infections persist in the body, increasing the risk of spread to others. Without effective antimicrobials for prevention and treatment of infections, medical procedures such as organ transplantation, cancer chemotherapy, diabetes management and major surgery become very high risk. Drug resistant infections are already on the rise with numbers suggesting that up to 50000 lives are lost each year to antibiotic-resistant infections in Europe and the US alone. Globally, at least 700,000 die each year of drug resistance in illnesses such as bacterial infections, malaria, HIV/AIDS or tuberculosis [112-114].

The development of antibiotic resistance is not a recent concept. Already in 1945, Fleming in his Nobel Lecture had informed [115]: *"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not suffi cient to kill them*". It wasn't only a prediction because he proved that by creating the resistant mutant bacteria. The magnitude of the problem is now accepted.

Now, we can say that antibiotic resistance evolves naturally via natural selection through random mutation, but it could also be engineered by applying an evolutionary stress on a population. Other factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, and the use of antibiotics as livestock food additives for growth promotion. Antibiotic resistant bacteria may be transferred from animals to humans both of which can be infected by the same pool of resistant bacteria. The spread of resistant bacteria from animals to humans can take place by any of the following ways (Figure 18): by every-day direct

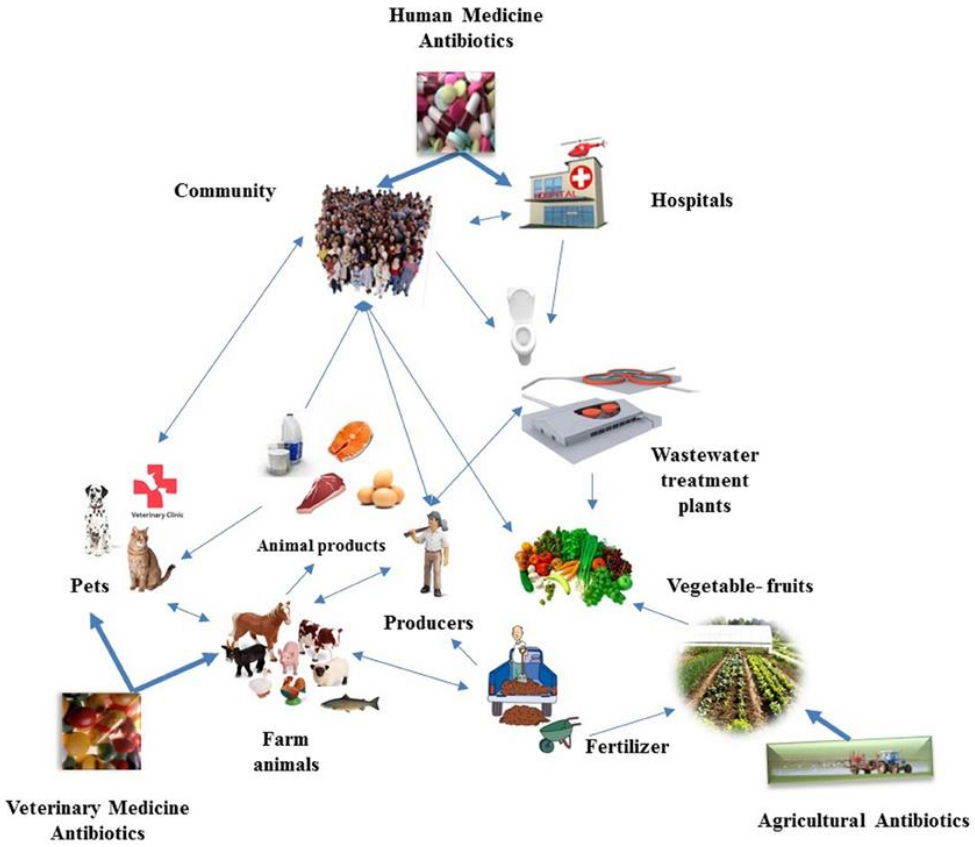
contact of farm workers with the animals; by transfer of resistant bacteria from animal manure to soil and water then to plants, finally to humans through the food chain; by transfer of bacteria from dead non-farm animals or farm animals who died because of disease, to the soil, water and then to plants and finally to humans; by eating contaminated meat that is not cooked properly. In another scenari, bacteria that cause diseases in animals and plants may not infect humans. However, these bacteria may belong to the same family as those that infect humans [87]. It was demonstrates that the presence of subtherapeutic level of an antibiotic induces generation of point mutations in the bacterial genome. Some of these mutations can confer resistance to other antibiotics that may not be related to the antibiotic that the cells have been subjected to; it's result in emergence of resistance to various other antibiotics including multidrug resistance. Whatever is the mechanism of development of multidrug resistant phenomenon in animals, there is a real threat of transfer to humans even if they are not in direct contact with the animals [116]. The most vulnerable are those people who are already taking antibiotics for several infections, because they the lowers immune defences so the infecting multidrug resistant bacteria can more easily cause risks related to the fact that they are not killed by the antibiotic and they don't find competition from any resident bacteria in the body.

"*To contain antibiotic resistance we need to fight on three fronts at the same time: human, animal and the environment*." In these terms, the European Food Safety Authority, the European Medicines Agency and the European Centre for Disease Prevention and Control are concerned about the impact of use of antibiotics on the increase in antibiotic-resistant bacteria and they are trying to improve surveillance across Europe [117].

In the meantime fundamental changes are required in the way that antibiotics are consumed and prescribed, to preserve the usefulness of existing drugs for longer and to reduce the urgency of discovering new ones. Firstly, the specific steps to reduce demand are: a massive global public awareness campaign, improve hygiene and prevent the spread of infection, reduce unnecessary use of antimicrobials in agriculture and their dissemination into the environment, improve global surveillance of drug resistance and antimicrobial consumption in humans and animals, promote new, rapid

diagnostic ways to cut unnecessary use of antibiotics, promote development and use of vaccines and alternatives, improve the numbers, pay and recognition of people working in infectious disease. Secondly, we must increase the number of effective antimicrobial drugs to defeat infections that have become resistant to existing ones through better incentives to promote investment. Finally, none of this will succeed without building a global coalition [112].

Figure 18. How antibiotic resistance spreads (Source: https://www.frontiersin.org/articles/10.3389/fmicb.2013.00096/full).



1.5.4. Antibiotics and legislations

In the European Union, the use of antibiotics in farms is subject to strict rules. Not only preventive treatment is forbidden, but medicines can only be used in the presence of illnesses and after a veterinary prescription, followed by an annotation in the appropriate registers. Authorised drugs are those indicated by Authorities and their use must be limited in time. To minimize the risk for consumers, it is compulsory to comply with the "suspension period", i.e. the period after the suspension of treatment before slaughter [118]. The use of antibiotics at sub-therapeutic doses, as growth promoters, is forbidden [14]. To minimize exposure of humans to antibiotics, Maximum Residue Limitits (MRLs) of antibiotics in different matrices have been established by the European Union [27]. In Table 2 are reported the MRLs for the most used antibiotics in breedings, belonging to the different classes.

Table 2. Estabilished MRLs in the different matrices by European Union for the most used antibiotics.

	Maximum residue limits (MRLs) (µg/kg)						
	Milk	**Eggs**	**Muscle**	**Liver**	**Kidney**	**Fat**	**Fish**
β-Lactams							
Amoxicillin (all food producing species)	4		50	50	50	50	
Ampicillin (all food producing species)	4		50	50	50	50	
Benzylpenicillin (All food producing species)	4		50	50	50	50	
Cloxacillin (all food producing species)	30		300	300	300	300	
Dicloxacillin (all food producing species)	30		300	300	300	300	
Oxacillin (all food producing species)	30		300	300	300	300	
Cefalexin (bovine)	100		200	200	1000	200	
Cefquinome (bovine∞, porcine, equidae)	20∞		50	100	200	50	
Tetracyclines							
Tetracycline (All food-producingspecies)	100	200	100	300	600		
Oxytetracycline (All food-producing species)	100	200	100	300	600		
Chlortetracycline (All food-producing species)	100	200	100	300	600		
Doxycycline (All food-producing			100	300	600	300	

species)						
Sulphonamides						
Total sulfonamide residues	100		100	100	100	100
Aminoglycosides						
Streptomycin (All ruminants*,porcine, rabbit)	200*		500	500	1000	500
Macrolides						
Erythromycin (All food-producing species)	40	150	200	200	200	200
Tylosin (All food producing species)	50	200	100	100	100	100
Quinolones						
Enrofloxacin (bovine and caprine[§],porcin, rabbit, poultry)	100		100	200/300[§]	200[§]/300	100
Marbofloxacin (cattle[#],swine)	75[#]		150	150	150	50
Amphenicols						
Florfenicol (bovine and caprine[§],porcin[¥], poultry[~])			100[~]/200[§]/300[¥]	2000[¥]/2500[~]/3000[§]	300[§]/500[¥]/750[~]	1000
Thiamphenicol (All food producing species)			50	50	50	50

Chloramphenicol				Prohibited substance		
Lincosamides						
Lincomycin	150	50	10	500	1500	50

NOTES: for enrofloxacin is considered the sum with its metabolite ciprofloxacin; for florfenicol is considered the sum with its metabolite florfenicol amine.

1.5.5. Analytical approaches for antibiotics

Before samples are declared to contain concentrations of antibiotics exceeding the MRLs, identification of the individual compounds and their confirmation need to be guaranteed by sufficiently selective and sensitive instrumental methods such as LC–MS/MS or GC–MS. In particular, regards prohibited substances, Commission Decision 2002/657/EC [16] states that "*methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods*". GC–MS has been routinely used in the last 35 years for the analysis of compounds in different matrices. However, almost all of the antibiotics are non volatile or very polar and some are thermally unstable, precluding their analysis by gas chromatography [97]. Consequently, based also on the literature results, LC-MS/MS is instrumentation of choise. Especially the use of the triple quadrupole, equipped by an ESI interface set both in positive and negative mode, allows for sensitivity far below the limits set in the different matrices. In recent years, the use of of liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) has ensured a distinctly enhanced selectivity by its accurate mass measurement and fast scan speed, compared with the other types of tandem mass instrument.

Techniques for residue analysis have changed as different technologies have become available. Owing to the unique and different chemical properties of the antibiotic classes and between the various components of each class, different strategies must be applied to extract target compounds from the different matrices to avoid interferences from matrices and ensure good analytical performance to the method. Regardless of the different matrices suggested by European Authorities, a typical analytical protocol involves the clean-up of the sample by deproteinization and defatting steps, followed by

purification on solid-phase extraction (SPE C18). Different solvents or mixtures of them are used in different proportions in the different literature studies. Most of the work focuses on the analysis of individual classes of antibiotics due to the difficulties that can be encountered during the analysis of different classes, from a chemical-physical point of view [96-97]. Using LC-ESI-MS, the effects of various mobile phase additives, such as formic, acetic acid, trifluoroacetic acid, ammonium acetate, etc., on sensitivity were assessed. Generally, the most intense signals were achieved by adding formic acid to water or methanol, which are the common mobile phases used.

An analytical strategy to take in consideration, reported in literature regarding tetracycline, is the use of chelating agents, e.g. EDTA, in the sample preparation to avoid analyte loss [97]. Especially when we work with milk, the EDTA is well known as a Ca^{2+} chelating agent, which is useful in releasing and extracting tetracyclines. An interesting review of the chromatographic analyses of tetracycline in food has been published by Oka, Ito, and Matsumoto [119] and Anderson et al. [120].

Also for β-lactams several precautions have been adopted in any step of the sample preparation procedure to avoid analyte degradation by heat, the presence of alcohols, especially for penicillins which also readily isomerized in an acidic ambient [97]. Confirmatory ions of β-lactams were obtained by thermal decomposition on the ESI source. Moreover, operating by using classical ESI, in the positive ion mode and at a relatively large potential difference between the sample cone and the skimmer lens, some Authors showed a larger or lesser extent of the characteristic cleavage product of the β-lactam ring of penicillins [96].

1.6. Environmental contamination and emergent pollutant in food chain

Intensive industrialisation of the world and uncontrolled development of multiple human activities have resulted in a lot of contamination sources of both organic and inorganic compounds, which accumulate in the environment, deteriorating the quality of agricultural lands, water and consequently of food [121]. The sources of food pollution are numerous and hard to identify or exclude from our daily environment. Environmental contamination of food takes two forms: long-term, low-level contamination resulting from gradual diffusion of persistent chemicals through the environment, and relatively shorter term, higher level contamination caused by industrial accidents and waste disposal. Chemicals contaminate foods through different routes depending on the chemical and its physical properties, its use, and the source or mechanism of contamination. Measurable health effects depend on the toxicity of the substance, the level at which it is present in food, the quantity of food consumed, and the vulnerability of the individual or population [122].

In last years, an open debate has been raised about the bioaccumulation of persistent organic pollutants (POPs), which are extremely toxic substances for environment and human health at a world scale [123]. They remain intact in the environment for long periods, become widely distributed geographically, and accumulate in the fatty tissue of living organisms. Their physical and chemical properties, particularly their high stability, give them ubiquity and capacity of accumulation and deposition far from their place of release. POPs include a wide range of man-made chemicals which are used worldwide and are indispensable for modern society as well as pesticides, perfluoroalkylated substances (PFASs), polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polycyclic aromatic hydrocarbons (PAHs). Due to their intrinsic features, POPs can be considered of growing interest for both safety and market aspects. The Stockholm Convention, whose goal is to reduce and possibly eliminate the environmental presence of POPs, was implemented in 2004 [124].

There is little toxicological information for most of the chemicals mentioned above, principally with regard to long-term at low-level exposure. Long-term threats or intermittent exposure can destroy ecosystems and often lead to a decrease in

biodiversity and a loss of important functions [125]. In this context, the identification of future hazardous or potentially hazardous chemicals in terms of persistence, toxicity, endocrine disruption potential of both individual compounds and complex mixtures is necessary. Measurements in environmental species may also help assess the potential for human exposure to environmental pollutants, and for predicting the human health risks [126]. In the presence of safety guidelines, two different analytical strategies could be carried out: the first is based on the monitoring of each food commodity to be placed on the market, to verify its compliance with regulatory limits implying the implementation of national monitoring plans. The second strategy deal with the assessment of population exposure, to be compared with tolerable daily intake or pertinent guidelines, reflecting dietary, cooking, and food dressing habits of the population or sensitive groups [127].

1.7. Perfluoroalkyl substances: general aspects

Perfluoroalkyl substances (PFASs) have been recognised as new emergent environmental contaminants after they have been detected in blood samples of both humans and wildlife [128]. In this thesis, we focused our attention only on this kind of environmental contaminants. Since 2010, Commission Recommendation 2010/161/EU have required the Member States to monitor PFASs in food and recommended to transmit these data to EFSA that wishes to outsource an extensive literature search in order to help the Panel on Contaminants in the Food Chain (CONTAM Panel) to update its previous risk assessment, carried out in 2008 [129, 130].

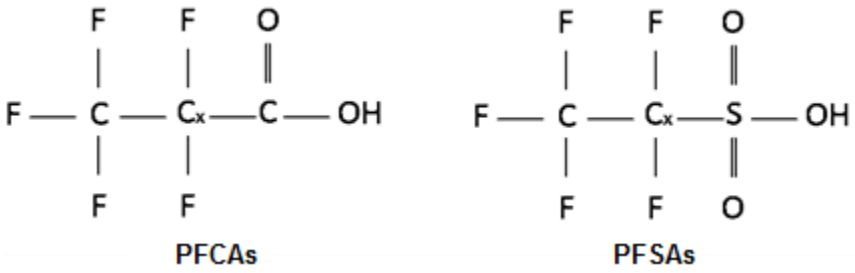
According to Buck et al. [131] terminology, PFASs are highly fluorinated aliphatic substances that contain one or more C atoms on which all the H substituents have been replaced by F atoms, with high chemical, thermal stability and high surface activity. The PFASs included:

- perfluoroalkyl carboxylic acids (PFCAs, Figure 19), i.e. perfluorooctanoic acid (PFOA) and compounds with carbon chain length between C4 and C18,
- perfluoroalkane sulfonic acids (PFSAs, Figure 19), i.e. perfluorooctane sulfonic acid (PFOS) and compounds with carbon chain length between C4 and C10,

- other PFAS as perfluoroalkane sulfinic acids, fluorotelomer alcohols, perfluoroalkane sulphonamides, etc.

In particular, when a hydrophilic acid group is introduced to a PFAS, the resulting perfluorinated acids such as PFCAs and PFSAs exhibit both hydrophobic and oleophobic active behaviour, behaving as surfactants [132]. The acidic head groups will be predominantly dissociated in most or all environmental and biological compartments and engineered systems. They have low vapor pressures that decrease with increasing chain length and hinder volatilization from aquatic or terrestrial systems; the hydrophobic chain length determine whether congeners partition in the organic matter. Moreover, it has shown that the branched PFASs congeners are likely to be, in general, more thermodynamically stable than their linear compounds [133, 134].

Figure 19. General structure of perfluoroalkyl carboxylic acids and perfluoroalkane sulfonic acid (Source: EFSA supporting publication, EN-572. http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2014.EN-572/epdf).



They are produced by 2 main processes: electrochemical fluorination and telomerisation. PFASs have been used since decades in a wide range of industrial applications as for paper, photo paper, packaging materials, textiles, carpets, furniture, shoes, cleaning agents, floor polishing agents, paint and varnish, wax, fire-extinguishing liquids and insecticides [135].

PFOA and PFOS are the most discussed molecules among PFASs. PFOS and its salts were included in 2010 in Annex B of the Stockholm Convention amongst the other POPs [125]. Several adverse health effects e.g. hepatotoxicity, immunotoxicity, neurobehavioral toxicity, developmental toxicity, reproductive toxicity, lung toxicity, hormonal effects, genotoxic and carcinogenic potential have been proved in animal

experiments [136-138]. In humans, the possible interference of PFASs with metabolism of fatty acids, as a possible risk factor for metabolic disorders and/or cardiovascular diseases need further investigation. Certain chronic diseases with an inflammatory component, including heart disease, diabetes and stroke, have been described to be preeinent amongst European populations; however, these evidence aretoo inconsistent for concrete conclusions [138, 139].

Food intake is the main exposure route to PFASs in humans. Fish and seafood were considered the most contaminated (50 to 80 %) followed by fruits and fruit products (8 to 27 %) and meat and derivates (5 to 8 %), but high deviation in contribution was detected across food analyses and age classes reflecting differences in dietary patterns [131] (Figures 20 and 21). Food can be PFASs contributor by accumulation from the environment or by contact with packaging materials and cookware containing [140-142]. Also drinking water is one of the main sources, because purification systems of wastewaters can't remove the shortest PFASs chains [133]. Finally also inhalation of air may contribute to the overall exposure.

In the last EFSA report [131] several adverse effects, as well as toxicity and epidemiology data have recently been published, based on studies in vitro in experimental animals and in humans. In particular was demonstrated that PFOA and PFOS are well absorbed in humans [143] and detected in 100 % of maternal serum and umbilical cord serum samples taken from the general population of Seoul, South Korea [144, 145], and concentrations between paired maternal and cord sera were significantly correlated. PFOS was also the predominant compound (0.06 ng/mL) in human milk, followed by PFOA (0.05 ng/mL) although the levels were several orders of magnitude lower than those in serum [146]. It was found that levels of PFOS and PFOA in milk decreased as lactation progressed and, in parallel, maternal PFOA levels decreased but rose when feeding ceased [147].

Based on further results from a toxicological point of view, the significance of various PFASs to human health could be better evaluated and allow the designation of a set of priority PFASs for future monitoring. The development of analytical methods with improved sensitivity is required to monitor such priority PFASs in order to increase the proportion of quantified results and thereby the reliability of exposure assessments.

Figure 20. Percentage contribution of different food to PFOS exposure per age group (Source: EFSA supporting publication, EN-572. http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2014.EN-572/epdf).

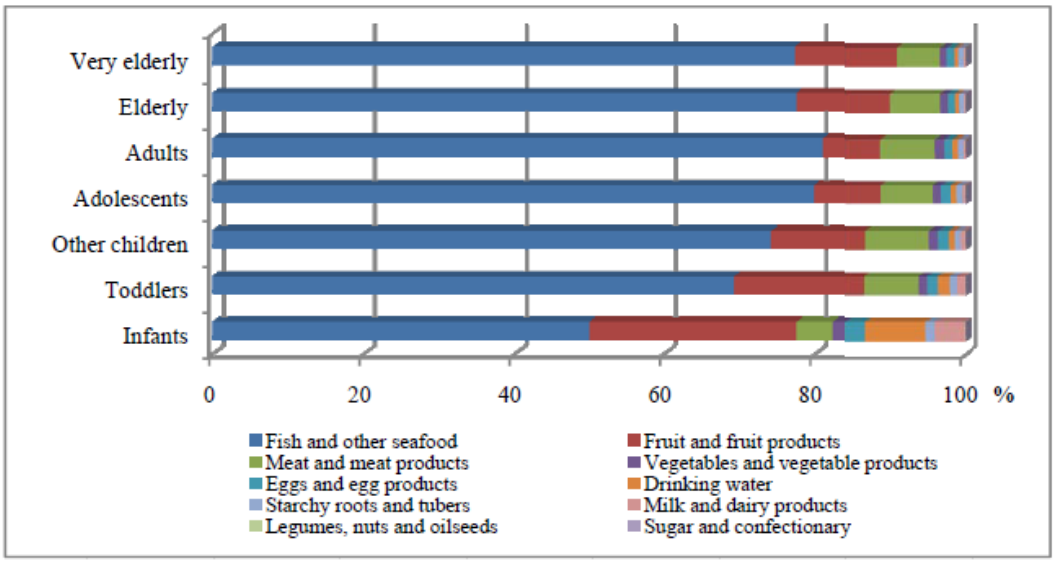
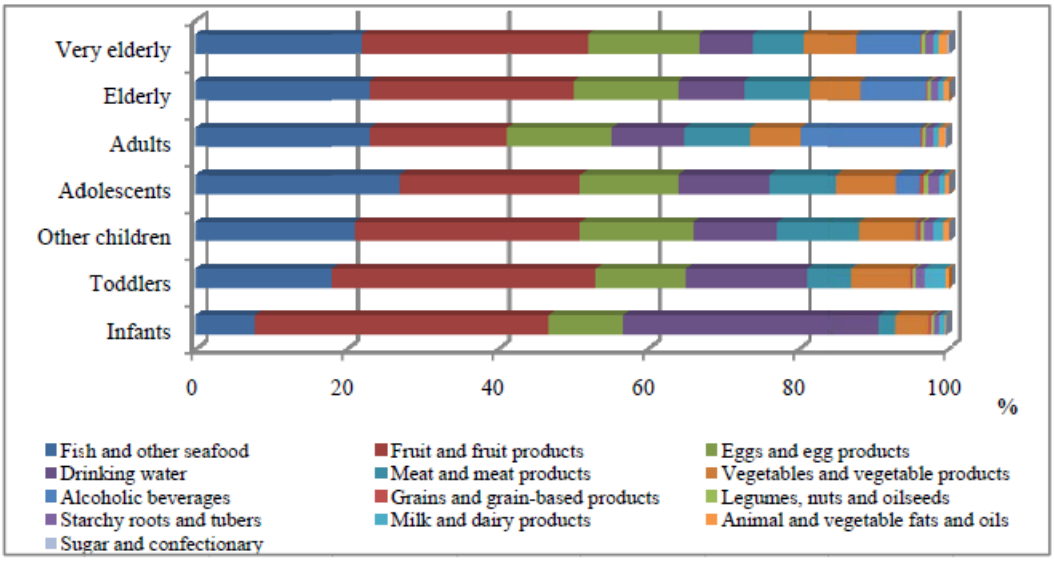


Figure 21. Percentage contribution of different food to PFOA exposure per age group (Source: EFSA supporting publication, EN-572. http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2014.EN-572/epdf).



1.7.1. Perfluoroalkyl substances and legislations

In 2008, the EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) performed a risk assessment for PFOS and PFOA concluding that it is doubtful that adverse effects of PFOS and PFOA are occurring in the European population. However, due to lack of data only a limited exposure assessment was possible [129].

Based on the limited data available for fish and beverage from four European countries, the CONTAM Panel determined an indicative dietary exposure to PFOS of 60 ng/kg b.w. per day for average consumers and 200 ng/kg b.w. per day for high consumers of fish. Indicative dietary exposure to PFOA for the same consumer categories was estimates at 2 ng/kg b.w. per day and 6 ng/kg b.w. per, respectively. The CONTAM Panel has also established a tolerable daily intake (TDI) of 150 ng/kg b.w. per day for PFOS and of 1500 ng/kg b.w. per day for PFOA [129].

No MRLs are set for these compounds in food, but Commission Recommendation 2010/161 recommended, at the beginning of this project, limits of quantification (LOQ) of 1 µg/kg for the monitoring of PFASs in food as left-censoring limits. In a preliminary analysis it appeared that the detection capabilities varied with the type of matrix and substance analysed. Particularly lower LODs/LOQs were reported for water and alcoholic beverages while higher LODs/LOQs were frequently reported for offal, mainly liver. It appears that analysis of PFASs is still challenging, especially in certain matrices as e.g. liver. In order to reduce the impact of the high left-censoring limits on the occurrence data analysis and on the dietary exposure estimations, but also not to exclude data on certain foods, the following upper limits were applied by EFSA in its report [129]:

• 0.02 µg/kg for water and alcoholic beverages;
• 5 µg/kg for edible offal of mammals and fish;
• 3 µg/kg for all other foods.

Nevertheless, as mentioned above, it is recommended to use higher sensitive analytical methods to increase the amount of quantifiable data for a reliable risk assessment.

1.7.2. Analytical approaches for perfluoroalkyl substances

The rapidly expanding field of perfluorinated alkyl substances research has resulted in a wide range of analytical strategy for pretreatment and analysis of different matrices.

Initial studies focused on PFOA and PFOS. In recent years, a range of other PFASs receive increasing attention because they are produced as alternatives for PFOA and PFOS, as intermediates in PFAS production, as by-products or as (bio)degradation products.

Substantial attentions are necessary and a lot of suggestions are present in literature to avoid contamination of PFASs losses during the analysis of the samples. Storage and conservation of samples for PFASs analysis is critical [148]. Several authors pre-cleaned the sampling bottles prior to sampling by rinsing with (semi-)polar solvents such as de-ionised water, methanol, acetone, or MTBE [149, 150]. In one study, it was shown that polypropylene sample bottles contained traces of PFOA [151] and pre-cleaning is therefore important especially when the target analytes are present in traces.

Moreover, PFASs adsorb to glass surfaces [152, 153]. Although this may happen at low concentrations in analytical standards [154], it is expected that this will not happen in samples which contain large amounts of matrix components (such as biota, serum and blood) that can shield the active sites at the glass surface. On the other hand, for water samples, irreversible adsorption of PFASs to the sample container surface was reported for long chain PFCAs (>C10), and PFOS and PFOA in acidified water [155].

Sampling equipment should preferably be made of non-fluoropolymer-containing materials, and this also holds for any tubing involved.

Due to their different polarities, the PFASs usually require different extraction strategies and a certain degree of sample pre-treatment to facilitate extraction or to remove matrix constituents that will disturb the instrumental analysis.

For complex and solid matrices trichloroacetic acid, formic acid or acetonitrile need to be added to the sample for precipitation of the protein in order to prevent clogging of the SPE columns.

Initially, extraction methods were based on ion pairing of the ionic PFASs with tetra-n-butylammonium hydrogensulfate (TBA), followed by a liquid–solid extraction (LSE) with methyl-tert-butylether (MTBE), filtration of the extract and instrumental determination by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) [153]. However, several limitations have been recognised: co-extraction of lipids and other interferences, the absence of a clean-up step to overcome the effects of matrix compounds and the wide variety of recoveries typically ranging from <50% to >200%. The method was also laborious, taking to much time and difficult to automate [148].

Without further clean-up there is enhancement or suppression of the electrospray ionization, resulting in inaccuracies. Proteins and lipids can be destroyed by KOH digestion of the sample prior to SPE sample enrichment [156].

Abiotic matrices (soil, sediment, sewage sludge) can be cleaned-up by addition of Envi-carb (graphitized carbon) and glacial acetic acid [157].

Regards purification, extraction using Oasis HLB (hydrophilic–lipophilic balance) and Oasis WAX (weak anionic exchange) cartridges was examined and compared in the work of Taniyasu et al. [157] SPE WAX resulted the best strategy, in terms of recovery especially for short chain PFASs, whose recoveries were less than 30% by SPE HLB.

Briefly, as confirmed also Leeuwen and de Boer [148], the ionic PFCAs and PFSAs require moderately polar media (Oasis WAX SPE or methanol and acetonitrile) for efficiently trapping of water soluble short-chain (C4–C6) compounds. For longer chains, less polar or non-polar SPE phases (C18 and Oasis HLB) may be applied. Non-ionic PFASs may be extracted from the matrix by non-polar media (C18 SPE or hexane) or moderate polar media (Oasis HLB and Oasis WAX SPE, a hexane–acetone mixture or acetonitrile).

Analysis of PFASs requires sensitive methods, due to the occurrence of these compounds at parts-per-trillion (ng/L) or lower levels. A reliable, sensitive and selective instrument is the liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). The high resolution power and fast scan speed are the two essential factorsfor the excellent performance of this instrumentation, both for qualitative and quantitative analysis. Usually, the dissociated acid (pseudo-molecular) ion $(M\text{-}H)^-$ is observed, and can be used for quantitative purposes or as the parent ion for multiple ion reactions in LC-MS^2. Anyway, the use of more than one transition for monitoring PFASs is an important recommendation [158]. We can conclude that the analytical problems associated with the determination of PFASs are multiple, including all the different aspects mentioned in this paragraph. As a result, more studies should be done before the analysis of this group of analytes in vogue will be fully understood and controlled.

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CHAPTER 2

Aims

2. AIMS

On the light of the different matters discussed in the Introduction, the specific aim of this research study was to look for new unconventional matrices that could accumulate veterinary drugs, or have a preferential way for their excretion, either in their unchanged or metabolised form. The development of new reliable protocols of analysis was based on the fact that there is a low percentage of non-conformity in the final reports of the National Residues Plan in recent years, although the threat of a disproportionate use of these substances is increasingly on the rise.

Where possible, a comparison with the conventional matrices was carried out to confirm our hypothesis through the analyses on real and unknown samples. One of the main objectives was therefore to place controls within well-defined stages of the food chain in order to find evidence of treatment or clarify the question of pseudoendogeneous nature of certain substances.

The approach to test the hypothesis was based on an extensive literature study, based on national and community issues, regulations on substance residues of actual interest (i.e growth promoters, antibiotics, new environmental contaminants) and input from public and private entities; search new unconventional accumulation matrices, on the basis of detected issues and molecules to search, able to detect analytes in the long term because substances from illegal treatment are hardly detectable and have not long persistence in conventional matrices, as well as urine, liver and muscle. The analytes in the different matrices required different approaches for sample pretreatment, extraction, clean up and fractionation before the analysis with liquid chromatography–tandem mass spectrometry (LC-MS/MS) or -high resolution mass spectrometry (LC-HRMS). The approach of analytical-instrumental nature has provided for the optimisation of instrumental performances as well as of the steps of sample pretreatment, in order to achieve good levels of sensitivity, specificity and robustness of the method to then make considerations of qualitative, quantitative and statistical nature for the comparison between the unconventional matrices and the classical ones. So the trials planning, optimisation and validation of the methods were performed according to Commission Decision 2002/657/EC [1]. All that, has allowed us to study the suitability of bile in the framework of control, together with the opportunity to obtain new information about the

nature of some substances and their pseudoendogenous concentrations in an innovative matrix not considered for corticosteroid and anabolic routine controls at the slaughterhouse. Bile was one of the slaughterhouse matrices provided for the first year of my project, compared with the conventional one, urine. Bile was used until the early nineties of the last century in the United Kingdom for the control on the administration to cattle of stilbenes, zeranol and trenbolone [2]. Moreover, O'Connor in a United States Patent of 1997 positively evaluated some points in routine collection of bile samples.

Bile, although it is recoverable only to the slaughterhouse, is an innovative and interesting matrix especially because avoids the above mentioned problems related to stress, as it happens before sampling of urine at the farm. It is readily obtainable with a syringe after slaughter during evisceration of the animal and can be rapidly handled following collection. It is a non-disruptive sample collection method from individual carcasses and needs few treatments before analysis. It also avoids the problem of fecal contamination and so the false positives caused by bacterial transformation of pseudoendogenous substances. Moreover, the gathering within bile provokes a time-lag in the metabolism of steroids and, consequently, their long-term retention; the majority of veterinary drugs occur at a high concentration in this fluid and reflects residue concentrations of in the liver very accurately [3].

For the detection of corticosteroids and anabolics we propose also bovine teeth, as another innovative and not invasive matrix taken at the slaughterhouse, inspirated from human forensic tossicology studies [4, 5]. The hypothesis of esterified form detection of some drugs such as prednisolone acetate, estradiol benzoate, etc. in teeth, after a bovine treatment plan and slaughter of the animals, was an important goal for the discrimination of the pharmaceutical, esterified form from the active principle of the drug molecule, for the confirmation of the illicit administration of a drug in the case of components of pseudoendogenous nature.

We propose also milk replacers used in the farm for animal feeding, to control the illegal administration of both corticosteroids and anabolics and then antibiotics at the beginning of the food chain. The inclusion of veterinary drugs in calf milk replacers is a matter of concern, particularly as their administration is not fully regulated and especially legislation varies across the Countries [6-8]. In fact, milk replacers are

commonly used for the daily feeding of calves as an adequate alternative to the mother's dairy milk and could be a simple route to illicit treatments. It should be emphasised that the presence of steroid hormones in feedstuffs can be also unintentional, due to cross-contamination or owing to the appearance of pseudo-endogenous substances.

Regards antibiotics, we propose also a multiclass method for the control of bovine urine, which could be a usefull tool to the antibiotic monitoring both at the farm and slaughterous, relying on the great results and high percentages of positives found in this simple, but not recommended, matrix. European Union, in fact, has set maximum residue limits (MRLs) for antibiotics residues in food of animal origin but not for urine [9]. With the same purpose, we propose a multiclass antibiotic method in swine urine, for the monitoring of their intensive breeding. In this case we compared the unconventional matrix, urine to the conventional one, muscle, confirming once again a more complete picture of urine information than the matrix indicated by the European authorities.

Due to the increasing phenomenon of antibiotic resistance, we also propose a multiclass method for antibiotic in mussels and clams, as filter feeders and suitable bioindicators of environmental pollution. Moreover, antibiotics use in breeding and aquaculture is a well known major cause of concern. The two types of shellfish were carefully selected for a comparison, considering that mussels tend to grow on the surface of wave-washed rocks, while clams live in shallow water. Moreover, the collection from distinct FAO areas could expand the knowledge from the point of view of food safety, relatively also to environmental contamination.

With the same purpose, we performed the analysis of the new emergent contaminants, perfluoroalkyl substances (PFASs), on the same samples of mussels and clams, trying to define how they can accumulate in the different marine layers and their distribution in edible matrixes consumed all over the world, conducting also a reliable risk assessment on the basis of EFSA’s requestes [10, 11]. In this context, the use of LC-HRMS Orbitrap has contributed to obtaining more quantifiable data thanks to the high sensitivity and specificity associated with the high resolution power of this instrumentation. The same study was also performed for another kind of edible matrix, the eels of Lake Garda (Nothern Italy) to evaluate the distribution and bioaccumulation of PFASs in this

species, facilitated by their morphological characteristics, e.g. length and body composition. Moreover, Lake Garda is a semi-enclosed environment, which has shown an increasing pollution level in recent years, in which the majority of plastic particles have been found [12].

Several collaborations with other Italian and European research groups have allowed the development of other small issues related to this project, as well as the period of 3 months abroad at RIKILT, the reference laboratory for food safety, in Wageningen (Netherlands), which contributed to increase my knoledge in analytical and instrumental field.

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CHAPTER 3

Research papers

All papers were reported keeping the reference style indicated by the guidelines of each Journal.

3.1. Detection of boldenone, its conjugates and androstadienedione, as well as five corticosteroids in bovine bile through a unique immunoaffinity column clean-up and two validated liquid chromatography–tandem mass spectrometry analyses.

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In this study I contributed to the experimental work planning, the execution of the practical work and analysis of samples, data processing and writing of the article.

Highlights

β-boldenone use in food producing animals is banned in the European Union.
Prednisolone is regulated only for therapeutic purposes.
Two validated analytical methods, after a unique clean-up, are proposed on an unusual biological matrix, such as bile.
α- and β-boldenone sulphate and glucuronide, their free forms, androstadienedione and fivecorticosteroids were determined .
The methods demonstrated good performances both for research and control purposes.

Abstract

The presence of β-boldenone II phase metabolites and prednisolone in urine samples, owing to endogenous or natural origin or illicit treatment, is under debate within the European Union. The detection of β-boldenone conjugates, α-boldenone conjugates at a concentrations higher than 2 ng mL^{-1} and prednisolone above the cut-off level of 5 ng mL^{-1} in urine have been, until now, critical in deciding if illegal drug use has occurred. The use of urine sometimes is not entirely satisfactory, especially when the drug is administrated at low doses or when its metabolic conversion is very fast. This subsequently would hamper its detection in urine. The introduction of new, advantageous matrix where the illicit treatment can be investigated would be highly appreciated. In this study, we have developed and validated a simple and unique immunoaffinity clean-up procedure, which was applied to bovine bile samples, followed by two different analytical liquid chromatography, electrospray, tandem mass spectrometry methods. The first method tests androstadienedione, α- and β-boldenone sulphate, glucuronate and related free forms, while the other method assays prednisolone, prednisone, dexamethasone, cortisone and cortisol. The methods were validated according to the European Commission Decision 2002/657/EC. The evaluated parameters were linearity, specificity, precision (repeatability and intra-laboratory reproducibility), recovery, decision limit and detection capability. The decision limits (CCα) were between 0.38 and 0.45 ng mL^{-1} for anabolic steroids, and 0.13 and 0.15 ng mL^{-1} as far as corticosteroids are concerned. Intra- and inter-day repeatability was below 15.8 and 19.9% for all analytes, respectively. The methods were applied to the

analysis of some bile samples collected from untreated young bulls in order to investigate the presence of the studied steroids in this matrix.

Keywords: boldenone sulphate, boldenone glucuronide, prednisolone, dexamethasone, bovine bile, food safety

3.1.1. Introduction

The use of growth promoters in food-producing animals allows animal performances to be improved, such as a better transformation rate, a higher meat yield at slaughter, an increase in milk production or a decrease in muscle fat. The use of growth promoters is prohibited, as detailed in Council Directives 96/22/EC and 96/23/EC [1, 2], which contain guidelines for controlling veterinary drug residues in animals and their products, with all the necessary information to set up national monitoring plans [3]. The ban of any growth-promoter was accomplished on 1 January 2006 with the prohibition of the last four antimicrobial agents [4].

Regulations on substance residues with hormonal activity in food of animal origin is essential to safeguard animal welfare, to avoid consumer health risks derived from the exposure and to ascertain commercial frauds.

Nevertheless, the simple detection of some steroids in bovine urine is currently considered to provide insufficient evidence of illicit treatment. Parameters such as cut-off levels, presence of metabolites, or both, must be accounted for. As an example, the α-epimer of boldenone was proposed, in 2003, as a naturally occurring steroid in bovine animals by experts within the EU, who set the "natural threshold" for the α-boldenone conjugates in urine at 2 ng mL^{-1}; a concentration above this could come from illicit treatment [5]. The authorities responsible for the control of residues in food must, therefore, consider either the possible endogenous production of these molecules or the existence of natural feed ingredients, such as phytosterols, as possible precursors to boldenone [6-9]. The faecal contamination of urine can also generate false positives for boldenone presence [10-11]. An analogous explanation considers the *in vitro* formation of prednisolone from cortisol in bovine [12] and human urine [13]. Moreover, cattle that are under stress conditions [14-16] could produce prednisolone. Based on recent

findings and on a study that has been carried out on 100 bovine urine samples, de Rijke et al. [17], have suggested a threshold level of 5 ng mL^{-1} for regulatory purposes, which was based on the following calculation: average level in non-treated animals + (3 x the standard deviation). From all considerations, it emerges that ultimate answer concerning the topics of boldenone and prednisolone in bovine urine, has not been accomplished yet. We, therefore, suggest a different biological matrix, such as bile, which represents a fairly complex matrix containing a lot of information. Many substances undergo, through the biliary tract, entero-hepatic recycling. Until now, scientific reports have indicated urine, liver, faeces and hair as the major biological matrices for the detection of such important analytes, whereas data are scarce for bile. Utilisation of bile as alternative matrix is convenient, due to its possibility to accumulate the steroid structures. An advantage could arise from the fact that gathering within bile provokes time-lag of steroids metabolism and, consequently their long-term retention. Therefore, it is reasonable to assume that the presence of active principles could be demonstrated in bile, but not in urine. Moreover, analysis performed in urine or plasma can be affected by the stress conditions that animals are subjected during the samples. The fact that bile can be taken only after slaughter, despite being a limitation, keep away the sampling stress. This is really crucial to consider especially when “false” positive results for prednisolone and boldenone occur, or when extremely high level of cortisol appears.

Particular research in this area includes the study of trenbolone in bovine bile and faeces [18], the analysis of hormonal steroids in fish plasma and bile [19, 20], and an automated multi-immunoaffinity chromatography screening to detect anabolic agents, including boldenone, in bile and urine [21]. However, these methods imply multistep clean-up procedure, resulting in complicated and expensive sample processing.

In this paper, we describe two methods based on a unique immunoaffinity column (IAC) clean-up and two liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses of bile, which are validated according the technical guidelines on the analytical performance criteria for confirmatory and validation procedures, as described in the Commission Decision (2002/657/EC) [22].

Anabolic steroids 17α- and 17β-boldenone, their glucuronate and sulphate conjugates, and their precursor androstadienedione (ADD) (Figure 1) are the analytes that were investigated by the first method. Second method was employed for analysis of following corticosteroids: prednisolone, prednisone, dexamethasone, cortisone and cortisol (Figure 2).

Both methods demonstrated a good performance, allowing for the detection and identification of the analytes at levels lower than 0.5 ng mL^{-1}. These validated methods were ultimately applied to the analysis of bile samples collected from untreated young bulls in order to investigate the presence of the studied steroids in this matrix.

Figure 1. Chemical structures of α-boldenone and β-boldenone free and conjugated forms and ADD.

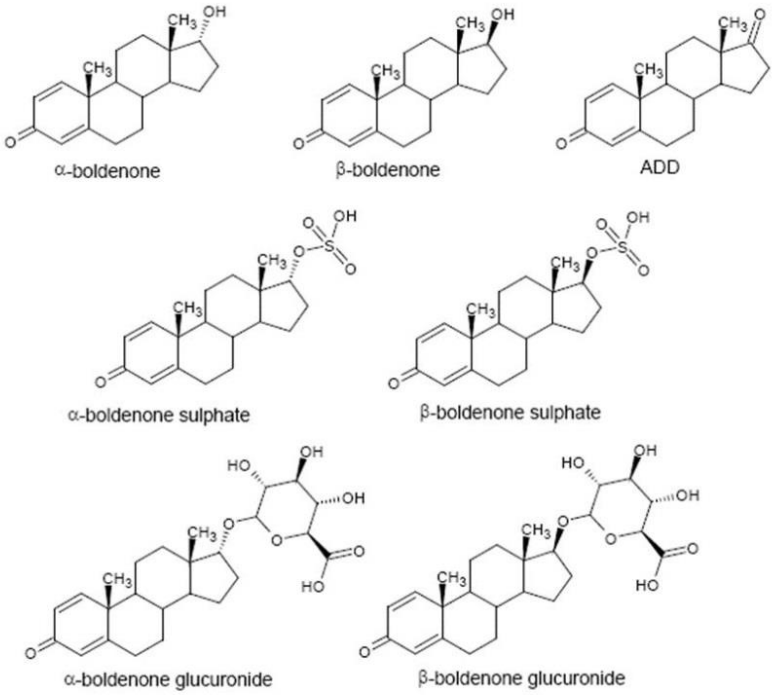
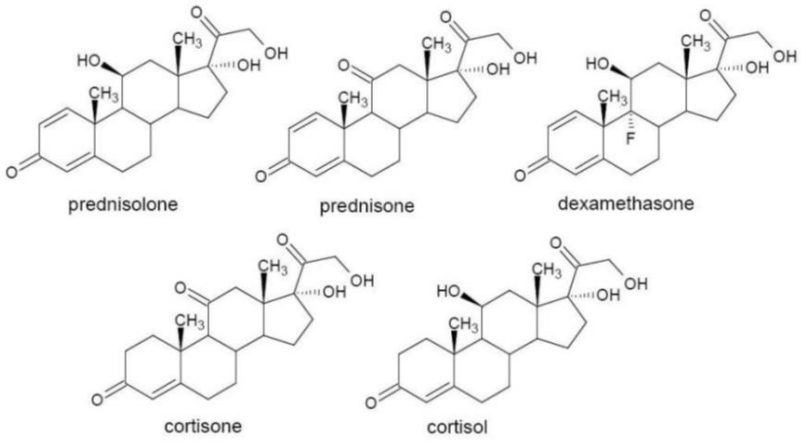


Figure 2. Chemical structures of the five studied corticosteroids.



3.1.2. Materials and methods

3.1.2.1. Sample collection

Bile samples were collected after slaughtering untreated young Charolaise bulls (14–17 months old); following collection they were immediately frozen, taken to the laboratory and stored at −40°C until the analysis was performed.

3.1.2.2. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). Formic acid 98–100% was obtained from Riedel-de Haën (Sigma-Aldrich, St.Louis, MO, USA). Water was purified by a Milli-Q System. The immunoaffinity columns (IAC) were provided by Randox (DM 2185, Randox Laboratories, Antrim, UK). Concentrated wash and storage buffers, which were diluted following the manufacturer's instructions before use, were supplied with the columns. ADD and β-boldenone were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA); β-boldenone sulphate (triethylamine salt), β-boldenone glucuronide, and α-boldenone were obtained from LGC Standards (Teddington, UK). The internal standards were β-boldenone sulphate-d3 for the sulphate forms, β-boldenone-d3 for the free forms (LGC Standards, Teddington, UK) and epitestosterone (EpiT) glucuronide-d3 for the glucuronate forms (National Measurement Institute, Pymble, NSW, Australia). The

sulphate and glucuronate forms of α-boldenone, provided by research partners, were prepared by a two-step synthesis procedure, in which β-boldenone (Steroid SpA, Cologno Monzese, Milan, Italy) was epimerised using a modified Mitsunobu protocol, according to Dodge and Lugar [23, 24], which was followed by sulphation, according to Sanaullah and Bowers [25], or glucuronation, according to Casati et al. [26]. Cortisone, cortisol, prednisone, prednisolone and dexamethasone were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA) and their internal standard, prednisolone-d6, was obtained from C/D/N Isotopes Inc (Pointe-Claire, Quebec, Canada).

3.1.2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and kept at -40°C. Working solutions, containing each of the studied analytes at the concentrations of 10 and 100 ng mL^{-1}, were prepared daily. Each working solution was maintained at 4°C during the method validation procedures.

3.1.2.4. Sample extraction

Samples of bovine bile (5 mL) were centrifuged, spiked with the internal standards to the final concentration of 2 ng mL^{-1} and then purified by using the IAC. The column was previously 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). The pH value of the bile samples was measured resulting within the operative range (7.5–8.5) of the column. The samples were loaded by gravity flow. Wash buffer (2 x 5 mL) and water (1 x 5 mL) were used to wash the column. The elution of the bound analytes was then performed by the application of 4 mL ethanol:water (70:30 v/v) (flow rate ≤ 3 mL min^{-1}), which was collected in a 15 mL polypropylene tube. The eluate was evaporated in a rotary vacuum evaporator. The dried extract was reconstructed in 200 µL of methanol:water (50:50 v/v), transferred in vial which was placed in auto-sampler. The injection volume was 10µL. The IAC could be used again (up to eight runs), starting from the equilibration described above, after a wash step with 2 x 5 mL ethanol:water (70:30 v/v).

3.1.2.5. LC-MS/MS analyses

LC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), constituted by a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with 20 µL loop. Chromatographic separation was achieved using a Synergi Hydro RP reverse-phase HPLC column (150 x 2.0 mm ID, 4 µm particle size), with a C18 (4 x 3.0 mm) guard column (Phenomenex, Torrance, CA, USA), which was kept at 30°C. The mobile phase consisted of methanol (solvent A) and 0.1% aqueous formic acid (solvent B). The gradient program for boldenone and its conjugates began at 60% A for 1 min, changing to 95% A in 11 min, which was then held for 2 min. Then, it returned to 60% A in 2 min and equilibrated for another 7 min. The flow rate was 200 µL min^{-1} and the overall run time was 22 min.

The gradient profile for corticosteroids began at 75% B, changing to 30% B in 18 min and then to 5% B in 1 min, which was held for 2 min. Finally, it returned to 75% B in 2 min and equilibrated for another 6 min. The flow rate was 250 µL min^{-1} and the overall run time was 29 min.

The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher, San Jose, CA, USA) equipped with an electrospray interface (ESI) set both in the positive (ESI+) and in the negative (ESI-) electrospray ionisation modes. Acquisition parameters were optimised in the electrospray mode by direct continuous pump-syringe infusion of the standard solutions of analytes at a concentration of 1 µg mL^{-1}, a flow rate of 20 µL min^{-1} and a MS pump rate of 100 µL min^{-1}. The following conditions were used: capillary voltage 3.5 kV, ion-transfer capillary temperature 340°C; nitrogen as sheath and auxiliary gases at 30 and 10 arbitrary units, respectively, argon as the collision gas at 1.5 mTorr and peak resolution 0.70 Da at full width half maximum (FWHM). The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. Three diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in multiple reaction monitoring (MRM). The selected diagnostic ions, one of which was chosen for the quantification, and the collision energies are reported in Table 1 for boldenone and its conjugates; those results for the

corticosteroids are reported in Table 2. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1. MS/MS conditions for the MRM acquisitions of α-boldenone and β-boldenone free and conjugated forms and ADD, as well as the relative internal standards. Ions for quantification are in boldenone. CE: collision energy, expressed in Volts.

Analyte	Precursor ion $[M-H]^-$ or $[M-H]^+$ (*m/z*)	Product ions$_{CE}$ (*m/z*)	ESI
α-boldenone sulphate	365	177_{39}, 349_{40}, **350_{30}**	(-)
β-boldenone sulphate	365	177_{39}, 349_{40}, **350_{30}**	(-)
β-boldenone sulphate-d3	368	180_{41}, 352_{40}, **353_{31}**	(-)
α-boldenone glucuronide	463	135_{21}, **269_{13}**, 287_{12}	(+)
β-boldenone glucuronide	463	135_{21}, **269_{13}**, 287_{12}	(+)
epitestosterone glucuronide-d3	468	256_{23}, 274_{16}, **292_{11}**	(+)
ADD	285	**121_{22}**, 151_{14}, 267_{11}	(+)
α-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone d3	290	**121_{27}**, 138_{14}, 272_{10}	(+)

Table 2. MS/MS conditions for the MRM acquisitions of the corticosteroids and internal standards. Ions for quantification are in bold. CE: collision energy, expressed in Volts.

Analyte	Precursor ion $[M-H]^-$ or $[M-H]^+$ (*m/z*)	Product ions$_{CE}$ (*m/z*)	ESI
prednisolone	405	187_{30}, **280_{35}**, 329_{19}	(-)
prednisone	403	**299_{21}**, 327_{19}, 357_{12}	(-)
dexamethasone	437	307_{33}, **361_{20}**, 391_{14}	(-)
cortisone	405	**301_{21}**, 329_{20}, 359_{12}	(-)
cortisol	407	282_{37}, **297_{33}**, 331_{20}	(-)
prednisolone-d6	411	284_{37}, 299_{32}, **333_{19}**	(-)

3.1.2.6. Method validation

The validation was performed according to the criteria and recommendations of the European Commission Decision 2002/657/EC [22]. All bile samples that were previously tested contained residues of α-boldenone glucuronide, cortisone and cortisol at considerable concentrations, in some cases, even higher than 3 ng mL^{-1}. Therefore, we were able to utilize pooled-bile blank samples from untreated young bulls for the validation of all steroids, except for the three mentioned. The method for these last analytes was validated in water adjusted to pH 8 with NaOH 0.1 N as a surrogate matrix of the bile, following the directions of van de Merbel [27].

For each analyte, the method performance was assessed through its qualitative parameters, such as the analyte specificity, molecular identification in terms of retention time (RT) and transition ion ratios, through its quantitative parameters, such as the linearity, recovery, accuracy in term of trueness and of precision expressed as the intra- and inter-day repeatability, and through the analytical limits [decision limit (CCα) and detection capability (CCβ)].

Specificity identification includes detecting any extra peaks in the blank matrix chromatograms as well as checking the matching of the relative retention time observed for the spiked analytes, compared to standard analytes in methanol, with a tolerance of ±2.5%. No evaluation of the specificity could be made for the validated analysis of the three chemicals in the surrogate matrix.

The instrumental linearity was evaluated by drawing five-point calibration curves in the solvent containing a fixed amount of the internal standards (2 ng mL^{-1} each), with analyte concentrations corresponding to 0.3, 1.0, 2.0, 3.0 and 5.0 ng mL^{-1} for ADD and the different forms of boldenone, and to 0.1, 0.5, 1.0, 3.0 and 5.0 ng mL^{-1} for the five corticosteroids.

Matrix calibration curves were obtained by spiking bile samples with each of the analytes (except the three validated in water), resulting in three analytical series; each series had three concentration levels (0.1, 0.2 and 0.3 ng mL^{-1} for corticosteroids and 0.3, 0.6 and 0.9 ng mL^{-1} for boldenone and its conjugate) in six replicates. Analogue curves, in water adjusted to pH 8, were obtained for α-boldenone glucuronide, cortisone and cortisol.

The trueness was assessed through recovery and was evaluated using the matrix curve results from the three analytical series, expressed in terms of a percentage of the measured concentration with respect to the spiked concentration.

The precision in terms of intra- and inter-day repeatability was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at three concentration levels of the three analytical series. The data from the matrix calibration curve were used to calculate the decision limit (CCα) and the detection capability (CCβ), according to the matrix calibration curve procedure described in the Commission Decision 2002/657/EC [22], as clarified in the document SANCO/2004/2726 revision 4 [28].

3.1.3. Results and discussion

The pseudo-endogenous nature of boldenone and prednisolone has so far hampered the control of the presence of their residues in conventional matrices, such as urine.This has led to the search of alternative indicators of treatment and different biomarkers have already been considered, along with establishment of cut-off levels, as already stated in Section 1.

A procedure that uses bovine bile as the biological matrix to detect and distinguish boldenone epimers, their phase II metabolites, ADD and five corticosteroids is described herein and two LC-MS/MS methods are validated with the aim to be used as a tool to carry out research on the origin of these steroids and their conjugated forms.

The two developed methods use a unique IAC clean-up step in bovine bile, which is suitable for both anabolic steroids and corticosteroids, and one of two LC-MS/MS analyses steps. One of the LC-MS/MS analyses can detect ADD, α- and β-boldenone sulphate, glucuronate and free forms, and, in the other LC-MS/MS method, prednisolone, prednisone, dexamethasone, cortisone and cortisol, at concentration levels suitable for research and control purposes. The two LC-MS/MS methods were developed to provide confirmatory data for the analysis of bovine bile. After preliminary trials, in full-scan mode from 50 to 500 *m/z,* the three product ions with the higher signal-to-noise ratio (s/n), for each analyte and internal standard were chosen for identification. The collision energy (CE) and the de-clustering potential (DP) were

adjusted in the MRM mode for each transition monitored, in order to reach the highest sensitivity for all analytes.

The LC–MS/MS chromatograms for the anabolic steroids and corticosteroids in a bile sample spiked with each analyte at the lowest concentration level of the validation are shown, together with the MS spectra, in Figure 3 and Figure 4, respectively; in addition, on the right-hand side of each figure, internal standards (2 ng mL^{-1}) are presented.

For a method to be deemed confirmatory under Commission Decision 2002/657/CE [22], it must yield four identification points (IPs). Each one of the three product ions is equal to 1.5 IPs, making a total of 4.5 IPs. The three diagnostic product ions, among which is the ion for the quantification, the relative intensities and the CEs are reported in Table 1 (for anabolic steroids) and Table 2 (for corticosteroids).

Figure 3. LC–MS/MS chromatograms and related MS spectra of α-boldenone and β-boldenone free and conjugated forms and ADD in a bile sample spiked at the validation lowest concentration level (0.3 ng mL^{-1}). Right-hand side: internal standards (concentration = 2 ng mL^{-1}).

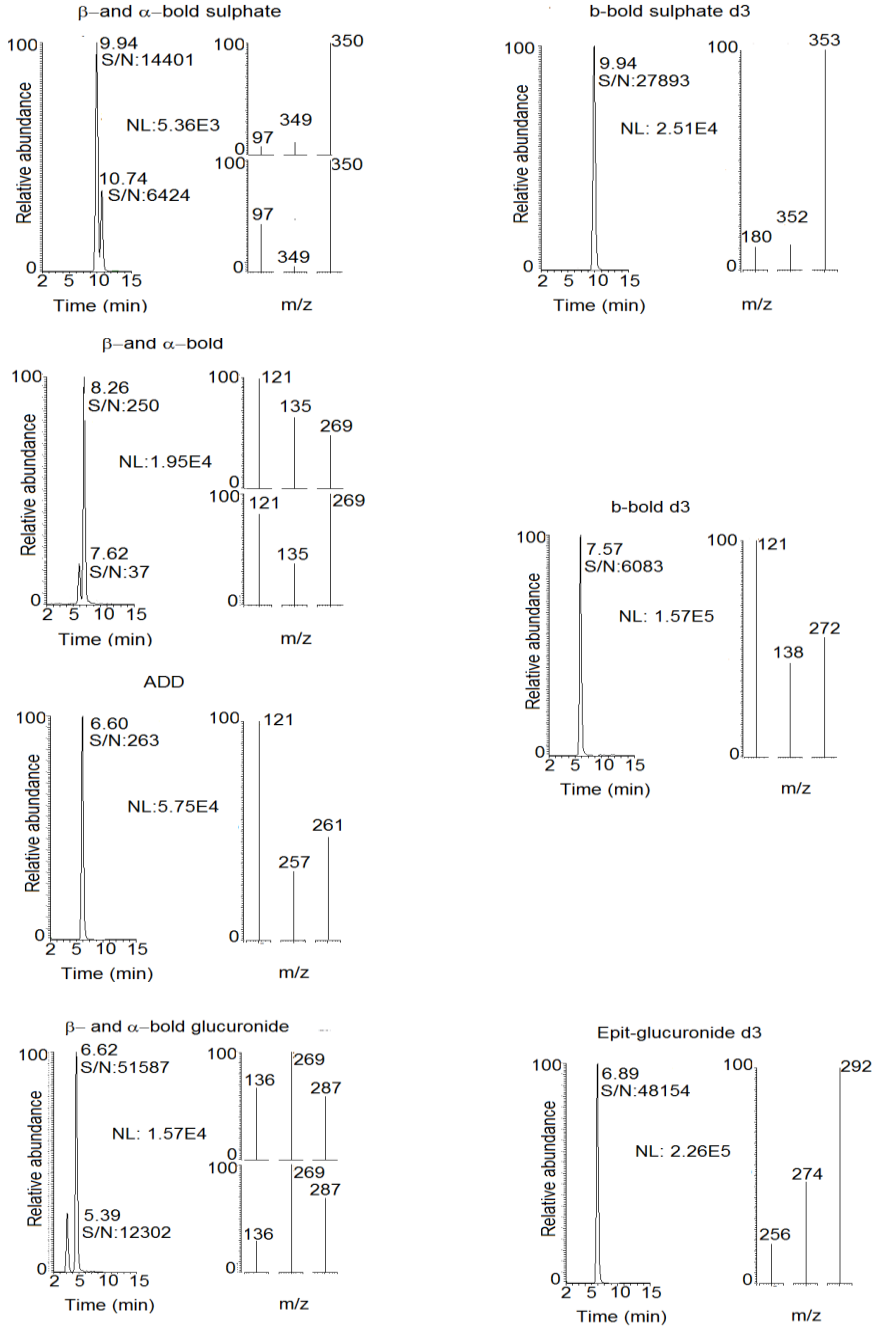
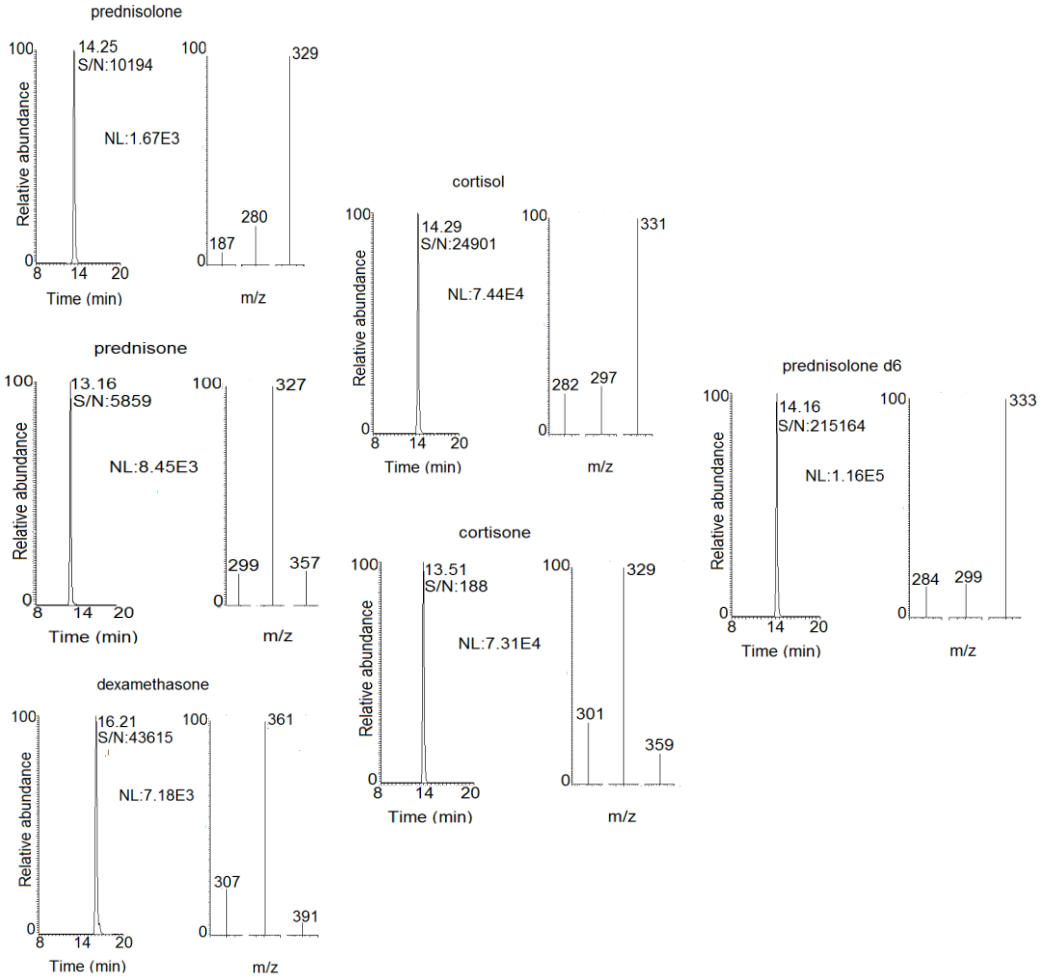


Figure 4. LC–MS/MS chromatograms and related MS spectra of the five corticosteroids in a bile sample spiked at the validation lowest concentration level (0.1 ng mL^{-1}). Right-hand side: internal standard (concentration = 2 ng mL^{-1}).



3.1.3.1. Performance characteristics of the methods

The instrumental linearity was evaluated over a concentration range of 0.3– 5.0 ng mL^{-1} for the anabolic steroids and 0.1–5.0 ng mL^{-1} for the five corticosteroids, using solutions of the analytes in methanol:water (50:50 v/v), containing a fixed amount of the internal standards (2.0 ng mL^{-1} each). Correlation coefficients of the curves were higher than 0.9970 for all compounds, indicating a good fit.

The matrix calibration curves built for the validation of each analyte were demonstrated to be linear in the range 0.3–0.9 ng mL^{-1} for the anabolic steroids and 0.1–0.3 ng mL^{-1} for the corticosteroids. The regression lines, obtained using the least-square method,

demonstrated a good fit for all analytes with had correlation coefficient > 0.99, except α-boldenone sulfate (0.9860).

Specificity and matrix effect were evaluated for all analytes except the three validated in the surrogate matrix. Blank and spiked samples were analysed and did not show any interferences (signals, peaks, ion traces) in the region of interest, where the target analytes were expected [22]. The matrix effect was evaluated as deviation obtained from concentrations of fortified bile and corresponding water solutions, multiplied with 100. For all compounds this value was minimum, less than 4%. The purification step using immunoaffinity approach turned to decisive for this achievement. Specificity and matrix effect were not evaluated for α-boldenone glucuronide, cortisone and cortisol, as the validation of the method for these three steroids was made in water adjusted to pH 8, as stated in Section 2.6.

The precision, calculated by applying the one-way analysis of variance (ANOVA), was expressed as coefficient of variation (CV), in terms of intra- and inter-day repeatability. The reported results show that the intra- and inter-day repeatability for all analytes was below 15.8 and 19.9%, respectively. These CVs were lower than 22%, as proposed by Thompson [29]. The high values were probably due to the low concentrations used for the method validation. The levels chosen were, however, addressed to subsequent research on the natural or endogenous origin of conjugated boldenone in bovine bile. The use of these methods for control purposes could consider higher concentration ranges for validation.

The mean recoveries ranged between 94 and 106% for α-boldenone sulphate, 91 and 109% for β-boldenone sulphate, 96 and 104% for α-boldenone glucuronide, 99 and 101% for β-boldenone glucuronide, 94 and 106% for ADD, 98 and 103 % for α-boldenone and 99 and 101% for β-boldenone. The mean recoveries for the corticosteroids ranged between 98 and 102% for prednisolone, 98 and 102% for prednisone, 94 and 106% for dexamethasone, 93 and 107% for cortisone and were about 100% for cortisol.

The data for the anabolic steroids and corticosteroids are reported in Table 3 and Table 4, respectively. CCα was calculated, as described in SANCO/2004/2726 revision 4 [28],

using parallel extrapolation to the *x*-axis at the lowest experimental concentration. CCα and CCβ values are reported in Table 5 and Table 6.

Table 3. Method precision for α-boldenone and β-boldenone free and conjugated forms and ADD.

Analyte	Concentration level (ng mL^{-1})	Recovery % (*n*=18)	Repeatability intra-day (CV; *n*=6)	Repeatability inter-day (CV; *n*=18)
α-boldenone sulphate	0.3	105.8	15.6	17.5
	0.6	94.2	15.8	19.5
	0.9	101.9	14.6	19.8
β-boldenone sulphate	0.3	109.4	15.2	19.9
	0.6	90.5	12.9	16.5
	0.9	103.1	5.0	6.1
α-boldenone glucuronide	0.3	96.3	15.2	9.7
	0.6	103.7	10.1	6.8
	0.9	98.8	11.0	8.8
β-boldenone glucuronide	0.3	99.2	14.4	19.3
	0.6	100.8	15.8	19.2
	0.9	99.7	15.8	16.5
ADD	0.3	106.4	11.2	14.7
	0.6	93.6	10.3	13.9
	0.9	102.1	9.0	9.5
α-boldenonone	0.3	101.9	15.8	18.0
	0.6	98.1	10.5	12.2
	0.9	103.1	15.3	15.5
β-boldenone	0.3	99.1	15.0	19.0
	0.6	100.9	8.1	15.4
	0.9	99.6	10.3	11.2

Table 4. Method precision for the five corticosteroids.

Analyte	Concentration level (ng mL^{-1})	Recovery % (*n*=18)	Repeatability intra-day (CV; *n*=6)	Repeatability inter-day (CV; *n*=18)
prednisolone	0.1	102.0	14.7	14.9
	0.2	98.0	12.2	12.4
	0.3	100.1	9.0	9.0
prednisone	0.1	101.9	15.7	19.8
	0.2	98.1	15.0	19.6
	0.3	101.	12.6	13.3
dexamethasone	0.1	93.8	11.0	19.4
	0.2	106.2	12.8	19.4
	0.3	97.0	14.0	15.0
cortisone	0.1	93.0	15.2	11.2
	0.2	107.0	10.7	18.3
	0.3	97.7	7.5	9.0
cortisol	0.1	99.9	15.4	19.4
	0.2	100.1	10.9	15.0
	0.3	100.0	15.0	19.3

Table 5. CCα and CCβ for α-boldenone and β-boldenone free and conjugated forms and ADD.

Analyte	CCα (ng mL^{-1})	CCβ (ng mL^{-1})
α-boldenone sulphate	0.42	0.55
β-boldenone sulphate	0.45	0.58
α-boldenone glucuronide	0.38	0.45
β-boldenone glucuronide	0.44	0.59
ADD	0.40	0.50
α-boldenone	0.43	0.69
β-boldenone	0.44	0.57

Table 6. CCα and CCβ for the five corticosteroids.

Analyte	CCα (ng mL^{-1})	CCβ (ng mL^{-1})
prednisolone	0.13	0.16
prednisone	0.15	0.21
dexamethasone	0.14	0.19
cortisone	0.15	0.20
cortisol	0.14	0.19

3.1.3.2. Application of the methods

In order to verify the developed methods in actual conditions, 20 bile samples, randomly collected from untreated young bulls (14–17 months old) under veterinary control, were subjected to the analysis for the detection of the studied molecules.

All bile samples showed α-boldenone glucuronide residues at a concentration interval from 8.3 to 258.2 ng mL^{-1} (average concentration 68.9 ng mL^{-1}) and evidenced traces of β-boldenone sulphate (concentration < CCα); α-boldenone was found in seven samples at a concentration interval from 0.6 to 1.3 ng mL^{-1} (average concentration 0.9 ng mL^{-1}). Only three samples showed ADD at a concentration interval from 0.7 to 2.3 ng mL^{-1} (average concentration 1.3 ng mL^{-1}). β-boldenone glucuronate, α-boldenone sulphate and β- boldenone were not detected.

Our findings evidenced the presence of boldenone and some phase II metabolites in a matrix without faecal contamination.

Regarding corticosteroids, the concentration values found in all samples ranged from 0.3 to 13.5 ng mL^{-1} for cortisone (average concentration 5.0 ng mL^{-1}) and from 0.3 to 6.8 ng mL^{-1} for cortisol (average concentration 2.3 ng mL^{-1}). Eight samples showed prednisolone at a concentration interval from 0.2 to 0.4 ng mL^{-1} (average concentration 0.3 ng mL^{-1}) and six samples evidenced prednisone from 0.2 to 0.3 ng mL^{-1} (average concentration 0.2 ng mL^{-1}). Dexamethasone was not detected.

3.1.4. Conclusion

We presented two LC-MS/MS methods for the analysis of bile samples. The first included 17α and 17β-boldenone, their precursor androstadienedione (ADD) as well as their glucuronides and sulphates, whereas the second one reported the detection of prednisolone, prednisone, dexamethasone, cortisone and cortisol. All analytes were extracted with a common and simple immunoaffinity chromatographic procedure. The performance characteristics of the two methods were evaluated in accordance with the criteria of the Commission Decision 2002/657/CE [22].

We found α-boldenone glucuronate at high concentrations as well as traces of β-boldenone sulphate, ADD, α-boldenone, prednisolone, prednisone, cortisone and cortisol in bile samples of untreated young bulls.

Further studies are ongoing in order to verify whether or not bile is an effective matrix for investigating the endogenous nature of boldenone phase II metabolites to unambiguously discriminate illicit treatments from their natural presence.

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3.2. Suitability of bovine bile compared to urine for detection of free, sulfate and glucuronate boldenone, androstadienedione, cortisol, cortisone, prednisolone, prednisone and dexamethasone by LC-MS/MS.

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

Abstract

The administration of boldenone and androstadienedione to cattle is forbidden in the European Union, while prednisolone is permitted for therapeutic purposes. They are pseudoendogenous substances (endogenously produced under certain circumstances). The commonly used matrices in control analyses are urine or liver. With the aim of improving the residue controls, we previously validated a method for steroid analysis in bile. We now compare urine (a 'classic' matrix) to bile, both collected at the slaughterhouse, to understand whether the detection of steroids in the latter is easier. With the aim of having clearer results, we tested the presence of the synthetic corticosteroid dexamethasone. The results show that bile does not substantially improve the detection of boldenone, or its conjugates, prednisolone and prednisone. Dexamethasone, instead, was found in 10 out of 53 bovine bile samples, but only in one urine sample from the same animals. Bile could constitute a novel matrix for the analysis of residues in food-producing animals, and possibly not only of synthetic corticosteroids.

Keywords: Bile; urine; boldenone, prednisolone; dexamethasone.

Chemical compounds studied in this article

Boldenone (PubChem CID: 13308); Androstadienedione (PubChem CID: 13102100); Prednisolone (PubChem CID: 5755); Prednisone (PubChem CID: 5865); Dexamethasone (PubChem CID: 5743); Cortisol (PubChem CID: 5754); Cortisone (PubChem CID: 222786);

3.2.1. Introduction

Regulations on substance residues with anabolic activity in food of animal origin is essential to safeguard animal welfare, to avoid consumer health risks derived from exposure and to ascertain commercial frauds. The debate about the presence of β-boldenone II phase metabolites and prednisolone in urine samples, owing to endogenous or natural origin, or illicit treatment, is currently ongoing within the European Union.

The use of urine as a matrix for the control of illegal treatments is not, however, entirely satisfactory. Parameters such as cut-off levels, the presence of metabolites, or both, must be accounted for.

As an example, α-boldenone was proposed in 2003 by experts within the EU as a naturally occurring steroid in bovine animals. They set the "natural threshold" for α-boldenone conjugates in veal calf urine at 2 ng mL^{-1}; a concentration above this could come from illicit treatment (European Union, 2003). The authorities responsible for the control of residues in food must therefore consider the possible endogenous production of boldenone e.g. as a product of the metabolism of natural feed ingredients, such as phytosterols (Song, Jim, & Park, 2000). Le Bizec et al. (2006) and Destrez et al. (2009) suggested, in a study carried out on a limited number of calves, that the presence in urine of the sulpho-conjugate fraction could be useful to distinguish between natural situations and the illegal use of β-boldenone in cattle. The faecal contamination of urine can also generate false positives for the presence of boldenone (Pompa et al., 2006, Arioli et al., 2008).

Analogous explanations are given regarding the in vitro formation of prednisolone from cortisol in bovine (Arioli, Fidani, Casati, Fracchiolla, & Pompa, 2010) and human urine (Bredehöft, Baginski, Parr, Thevis, & Schänzer, 2012). Moreover, cattle that are under stress conditions could produce prednisolone (Pompa, Arioli, Casati, Fidani, Bertocchi, & Dusi, 2011), as well as α- and β-nortestosterone, as shown by Glenn Kennedy et al. (2009) in a study on injured male cattle. A recent study, carried out on 100 bovine urine samples by de Rijke, Zoontjes, Samson, Oostra, Sterk, & van Ginkel (2014), suggests a threshold level of 5 ng mL^{-1} of urinary prednisolone for regulatory purposes.

These considerations suggest that the topic of the endogenous production of boldenone and prednisolone in bovine animals needs to be further explored.

With the aim of improving our knowledge about this subject, we therefore propose a comparison between two matrices, bile and urine, both analysed with two methods based on a unique immunoaffinity column (IAC) clean-up and two liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses, validated according to the Commission Decision (2002/657/EC). Anabolic steroids 17α- and 17β-

boldenone, their glucuronate and sulfate conjugates, and their precursor androstadienedione (ADD) are the analytes that were investigated in the first method. Corticosteroids cortisol, cortisone, prednisolone, prednisone and dexamethasone are the molecules searched for with the second method.

Dexamethasone is permitted in the European Union for therapeutic use in food-producing animals, but its use as a growth promoter, habitually in combination with beta-agonists, is forbidden. Several researchers have devised methods for the detection of dexamethasone in different conventional matrices (Friedrich, & Schulz, 1992, Courtheyn et al., 1994, Van den hauwe, Dumoulin, Elliott, & Van Peteghem, 2005), but we could find only one study conducted in bile and urine for steroidal compounds (Fodey, Elliott, Crooks, & McCaughey, 1996). This work used the multi-immunoaffinity chromatography (MIAC) followed by ELISA procedure on bile and urine samples that previously underwent deconjugation with *Helix pomatia* juice. Finally, we chose dexamethasone, because, unlike the above-mentioned steroids, it is a strictly synthetic molecule and its detection in urine and/or bile could provide clearer information about the suitability of the compared biological matrices studied. To the described aims, we therefore analysed a total of 53 bile and urine paired samples collected from male veal calves, young bulls and cows.

3.2.2. Materials and methods

3.2.2.1. Sample collection

Bile and urine paired samples from Friesian male veal calves (aged 9 to 12 months), Limousine young bulls (aged 16 to 21 months) and Friesian dairy cows (aged 45 to 94 months) of the food chain were collected in different slaughterhouses in Lombardy, immediately frozen, taken to the laboratory and stored at −40°C until analysis was performed.

3.2.2.2. Chemicals and reagents

All solvents were of LC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). Formic acid 98–100% was obtained from Riedel-de Haën (Sigma-Aldrich, St.Louis, MO, USA). Water was purified by a Milli-Q System. The IAC

was provided by Randox; concentrated wash and storage buffers, which were diluted following the manufacturer's instructions before use, were supplied with the columns (DM 2185, Randox Laboratories, Antrim, UK). ADD and β-boldenone were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA), while β-boldenone sulfate (triethylamine salt), β-boldenone glucuronide, and α-boldenone were obtained from LGC Standards (Teddington, UK). The internal standards were β-boldenone sulfate-d3 for the sulfate forms, β-boldenone-d3 for the free forms (LGC Standards, Teddington, UK) and epitestosterone (EpiT) glucuronide-d3 for the glucuronate forms (National Measurement Institute, Pymble, NSW, Australia). The sulfate and glucuronate forms of α-boldenone, provided by research partners, were prepared by a two-step synthesis procedure, in which β-boldenone (Steroid SpA, Cologno Monzese, Milan, Italy) was epimerised using a modified Mitsunobu protocol, according to Dodge and Lugar (Fabregat, Pozo, Marcos, Segura, & Ventura, 2013, Dodge, & Lugar, 1996) which was followed by sulphation according to Sanaullah & Bowers (1996), or glucuronation according to Casati, Ottria, & Ciuffreda (2009). Cortisone, cortisol, prednisone, prednisolone and dexamethasone were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA) and their internal standard, prednisolone-d6, was obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada).

3.2.2.3. Sample extraction and LC-MS/MS analysis

The bile and urine extractions and analyses were performed as previously described by Chiesa, Nobile, Panseri, Sgoifo Rossi, Pavlovic, & Arioli (2014) and Chiesa et al. (2015). Briefly, a 5 mL centrifuged bile or urine sample spiked with internal standards (2 ng mL^{-1}), was adjusted, when necessary, to pH=7.5-8.8 with NaOH 0.1 N; the sample was loaded into an IAC column that was previously washed (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). Elution was achieved with 4 mL ethanol:water (70:30, v/v) (all the flow rates were ≤ 3 mL min-1). The eluate was evaporated until dry in a rotary evaporator, reconstituted in 200 (bile) or 500 (urine) µL of methanol:water (50:50; v/v) and transferred in an autosampler vial. A volume of 10µL was analyzed by LC-MS/MS. The LC apparatus and chromatographic conditions were: Surveyor AS

autosampler and Surveyor MS quaternary pump (Thermo Fisher Scientific, San Jose, CA, USA), reverse-phase LC column Synergi Hydro RP 150 x 2.0 mm, i.d. 4µm, with a C18 4 x 3.0 mm guard column (Phenomenex, Torrance, CA, USA), and a temperature of 30°C.

The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and methanol (solvent B). The gradient program for boldenone and its conjugates began at 40% A for 1 min, changing to 5% A in 10 min, which was then held for 2 min. Then, it returned to 40% A in 2 min and was left to equilibrate for another 7 min. The flow rate was 200 µL min^{-1} and the overall run time was 22 min. The gradient profile for corticosteroids began at 75% A, changing to 30% A in 18 min and then to 5% A in 1 min, which was held for 2 min. Finally, it returned to 75% A in 2 min and equilibrated for another 6 min. The flow rate was 250 µL min^{-1} and the overall run time was 29 min.

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 the positive and in negative ionisation modes; 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; scan time for each monitored transition was 0.1 s and scan width was 0.5 amu. The acquisition was made in the multiple reaction monitoring (MRM) after selecting, for each analyte and internal standard, three diagnostic product ions, one of which was used for the quantification (Table 1). The injection volumes were 10 µL (bile) or 20 µL (urine). Data were acquired and elaborated by Xcalibur™ software from Thermo.

Table 1. MS/MS condition for the MRM acquisitions of the studied analytes, and relative internal standards. Ions for quantification are in boldenone. CE: collision energy, expressed in Volts, applied to break the precursor into the product ions.

Analyte	Precursor ion $[M-H]^-$ or $[M-H]^+$ (m/z)	Product ions$_{CE}$ (m/z)	ESI
α-boldenone sulfate	365	177_{39}, 349_{40}, **350_{30}**	(-)
β-boldenone sulfate	365	177_{39}, 349_{40}, **350_{30}**	(-)
β-boldenone sulfate-d3	368	180_{41}, 352_{40}, **353_{31}**	(-)
α-boldenone glucuronide	463	135_{21}, **269_{13}**, 287_{12}	(+)
β-boldenone glucuronide	463	135_{21}, **269_{13}**, 287_{12}	(+)
epitestosterone glucuronide-d3	468	256_{23}, 274_{16}, **292_{11}**	(+)
ADD	285	**121_{22}**, 151_{14}, 267_{11}	(+)
α-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone d3	290	**121_{27}**, 138_{14}, 272_{10}	(+)
prednisolone	405	187_{30}, **280_{35}**, 329_{19}	(-)
prednisone	403	**299_{21}**, 327_{19}, 357_{12}	(-)
dexamethasone	437	307_{33}, **361_{20}**, 391_{14}	(-)
cortisone	405	**301_{21}**, 329_{20}, 359_{12}	(-)
cortisol	407	282_{37}, **297_{33}**, 331_{20}	(-)
prednisolone-d6	411	284_{37}, 299_{32}, **333_{19}**	(-)

3.2.2.4. Methods Validation

The validation protocols, made accordingly 2002/657/EC, and the obtained parameters are described in detail in previous studies (Chiesa et al., 2014, 2015) and reported here in a summarised way: linear matrix calibration curves built for each analyte (0.05-0.1-0.2

ng mL^{-1} for all analytes in urine, and 0.3-0.6-0.9 ng ml^{-1} for anabolic steroids and 0.1-0.2-0.3 ng mL^{-1} for corticosteroids in bile) (6 samples×3 concentration levels×3 series = 54 analyses for each matrix). Intra-day and inter-day repeatability (Thompson, 2000), representing precision, were calculated using one-way analysis of variance (ANOVA), expressed as CVs, and were below 15.8 and 19.9% in bile and 17.2% and 21.8% in urine, for all analytes.

In bile, CCα values calculated as described in the document SANCO/2004/2726 revision 4 (European Union, 2008) ranged from 0.38-0.45 ng mL^{-1} and 0.13-0.15 ng mL^{-1} for anabolic steroids and corticosteroids, respectively. CCβ values ranged from 0.45-0.69 ng mL^{-1} and 0.16-0.21 ng mL^{-1} for anabolic steroids and corticosteroids, respectively.

In urine, CCα values (European Union, 2008) ranged from 0.07-0.08 ng mL^{-1} and 0.06-0.07 ng mL^{-1} for anabolic steroids and corticosteroids, respectively. CCβ values ranged from 0.08-0.10 ng mL^{-1} and 0.07-0.09 ng mL^{-1} for anabolic steroids and corticosteroids, respectively.

The mean recoveries for all analytes ranged between 92 and 110%, considering both matrices and all analytes.

3.2.2.5. Statistical analysis

The descriptive statistics was performed taking into account only the samples in which the studied analytes were found. The Kolmogorov–Smirnov test was performed to check the normality of the results related to α-boldenone glucuronide, cortisol and cortisone in bile and urine: the Pearson correlation or the non-parametric Spearman correlation test were calculated to verify the effective pairing of the datasets, depending on whether the Kolmogorov–Smirnov normality test was positive or negative. Then, the differences in the results obtained from the analysis of urine or bile were checked using different tests depending on the normality of each dataset and the correlation between the considered datasets. The unpaired t-test was used when there was no correlation and both passed the normality test. The Wilcoxon matched-pairs signed-ranks test was used when a correlation, but not the normality, was found between datasets. The Mann-

Whitney test was used when there was no correlation and at least one dataset did not pass the normality 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.

3.2.3. Results

3.2.3.1. Boldenone, Boldenone conjugates and ADD

The overall results related to boldenone, its conjugates and ADD are reported in Table 2.

Table 2. Concentrations, expressed as means ± SDs in ng mL^{-1}, number and percentage of positives of the studied analytes in bovine bile and urine samples.

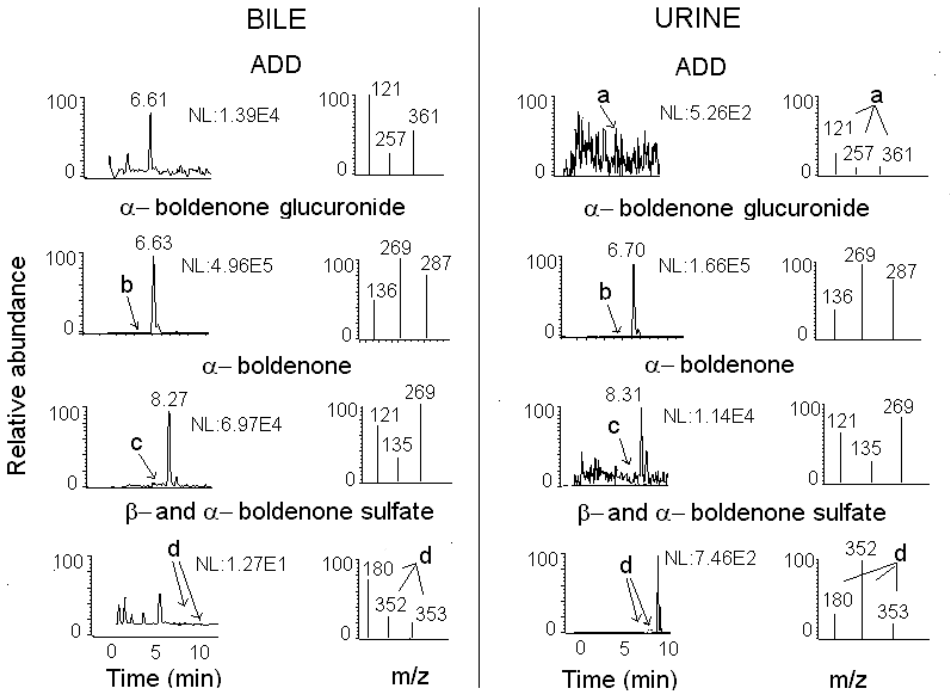
		BILE			URINE		
		Male Veal Calves (n=16)	**Young Bulls (n=18)**	**Cows (n =19)**	**Male Veal Calves (n=16)**	**Young Bulls (n=18)**	**Cows (n =19)**
α-boldenone glucuronide	Mean±SD	76.0±53.6	23.2±16.2	15.8±16.6	2.01±3.06	0.75±0.73	1.76±2.93
	Positives	16	18	18	15	18	19
	%	100	100	95	94	100	100
β-boldenone glucuronide	Mean±SD	nd	nd	nd	nd	nd	nd
	Positives	0	0	0	0	0	0
	%	0	0	0	0	0	0
α-boldenone sulfate	Mean±SD	nd	nd	nd	nd	0.39±0.05	0.34±0.02
	Positives	0	0	0	0	7	2
	%	0	0	0	0	39	11
β-boldenone sulfate	Mean±SD	0.39	nd	nd	0.07±0.01	0.08	0.07±0.01
	Positives	1	0	0	7	1	4
	%	6	0	0	44	6	21
α-boldenone	Mean±SD	2.14±3.34	0.59±0.04	1.44±1.47	1.17±0.85	0.54±0.01	0.54±0.06
	Positives	6	6	9	2	8	14
	%	38	33	47	13	44	74
β-boldenone	Mean±SD	nd	nd	0.48±0.07	nd	0.45±0.03	0.44±0.01
	Positives	0	0	3	0	10	6
	%	0	0	16	0	56	32
ADD	Mean±SD	1.12±0.85	0.57±0.12	0.80	0.79±0.25	0.60±0.09	0.51±0.01
	Positives	4	6	1	3	14	4
	%	25	33	5	19	78	21
Cortisol	Mean±SD	2.40±1.86	3.50±1.64	5.94±9.28	17.9±14.7	14.4±11.8	22.0±17.5
	Positives	16	18	19	16	18	19

	%	100	100	100	100	100	100
Cortisone	Mean±SD	2.77±1.67	2.03±1.28	2.78±1.92	6.46±3.31	5.70±4.42	6.54±5.04
	Positives	16	17	19	16	18	19
	%	100	94	100	100	100	100
Prednisolone	Mean±SD	nd	nd	0.53±0.26	0.79±0.65	0.97±0.59	0.38±0.27
	Positives	0	0	2	6	2	10
	%	0	0	11	38	11	53
Prednisone	Mean±SD	0.47±0.28	nd	nd	0.72±0.64	0.16	0.13±0.01
	Positives	2	0	0	6	1	2
	%	13	0	0	38	6	11
Dexamethasone	Mean±SD	0.14±0.01	0.51±0.54	47.5±66.5	nd	nd	10.6
	Positives	3	5	2	0	0	1
	%	19	28	11	0	0	5

nd: not detected

Illustrative chromatograms with the corresponding mass spectra of one bile and one urine sample are shown in Fig. 1. The presence of α-boldenone glucuronide in almost all samples in bile and urine has to be noted.

Fig.1 Illustrative chromatograms with the relative ion spectra obtained through the LC-MS/MS analysis of one bile and one urine sample for the presence of free sulfate and glucuronate, α- and β-boldenone and ADD.



a: ADD not detected in urine and ion ratios not corresponding to the calibration standard.
b: β– boldenone glucuronide not detected both in bile and urine.
c: β– boldenone not detected both in bile and urine.
d: β– and α– boldenone sulfate not detected both in bile and urine and ion ratios not corresponding to the calibration standard.

This datum is confirmed by the results concerning α-boldenone free form that, although at a much lower frequency, is the only other form of boldenone found at least once in bile and urine from male veal calves, young bulls and cows, which can also be asserted for ADD. These observations agree with previous studies showing the role of ADD as a precursor of boldenone (De Brabander et al., 2004, Ferretti et al., 2007), the higher concentrations of α-boldenone in urine than β-boldenone (Nielen, Rutgers, van Bennekom, Lasaroms, & van Rhijn, 2004), the prevalence of 17α- to 17β-steroid epimers in bile, indicating that epimerisation mainly takes place in the liver (Rico, 1983) and the preponderance of the glucuro- over sulpho-conjugation of α-boldenone in bovine (Le Bizec et al., 2006). With regard to the comparison between bile and urine, the results are shown in Table 3. We compared the detected concentration of α-boldenone glucuronide in bile and urine because this conjugated steroid was almost

always found in both matrices, such as the adrenal hormone cortisol and its direct metabolite cortisone. The similar frequencies once more indicate, although indirectly, the endogenous origin of boldenone. A significant correlation for α-boldenone glucuronide was found in young bulls and cows, not in male veal calves, which, however, showed a higher average concentration of this steroid. We cannot explain this finding, even if involvement of the achievement of sexual maturity could be hypothesized.

We occasionally detected α-boldenone glucuronide in urine at concentrations higher than the cut-off level of 2 ng mL^{-1}. A draft proposal of European Union in fact states: "If only α-boldenone conjugates are found at levels of 2 ppb or higher in urine of veal calves, additional investigations, in order to prove the abuse of boldenone, are strongly recommended" (European Union, 2003). The concentrations in these samples ranged from 2.60 to 12.2 ng mL^{-1} in veal calves (n=4), and from 2.33 to 11.1 ng mL^{-1} in cows (n=4), while only one detection was made in young bulls (3.33 ng mL^{-1}). In these samples, α-boldenone sulfate was never found. On the contrary, when it was detected together with the glucuronate conjugate (n=9), the total concentration was ≤ 1.13 ng mL^{-1}, which is lower than the recommended cut-off level. As for β-boldenone, the glucuronate metabolite was never found, in both bile and urine; β-boldenone sulfate was detected in the urine of 7 veal calves, 1 young bull and 4 cows at concentrations ≤0.08 ng mL^{-1}. These findings suggest treatment with boldenone or ADD, as stated by Destrez et al. (2009). The differences in the analytical limits must, however, be accounted for: the CCα indicated by those Authors was 0.2 ng mL^{-1} and the CCβ was 0.4 ng mL^{-1}, obtained by LC–MS/MS (negative ESI, SRM acquisition, triple quadrupole). The lower CCβ of our method would seem to re-open the possibility of considering β-boldenone sulfate a biomarker of treatment as proposed by Le Bizec et al. (2006) and Destrez et al. (2009); therefore, much more than a direct or indirect inter-conversion with α-boldenone sulfate could be hypothesised, given the presence of this conjugate epimer in some samples. The simple detection of β-boldenone sulfate could be not a satisfactory condition, making the determination of other parameters like a cut-off level, other metabolites or concentration ratios necessary.

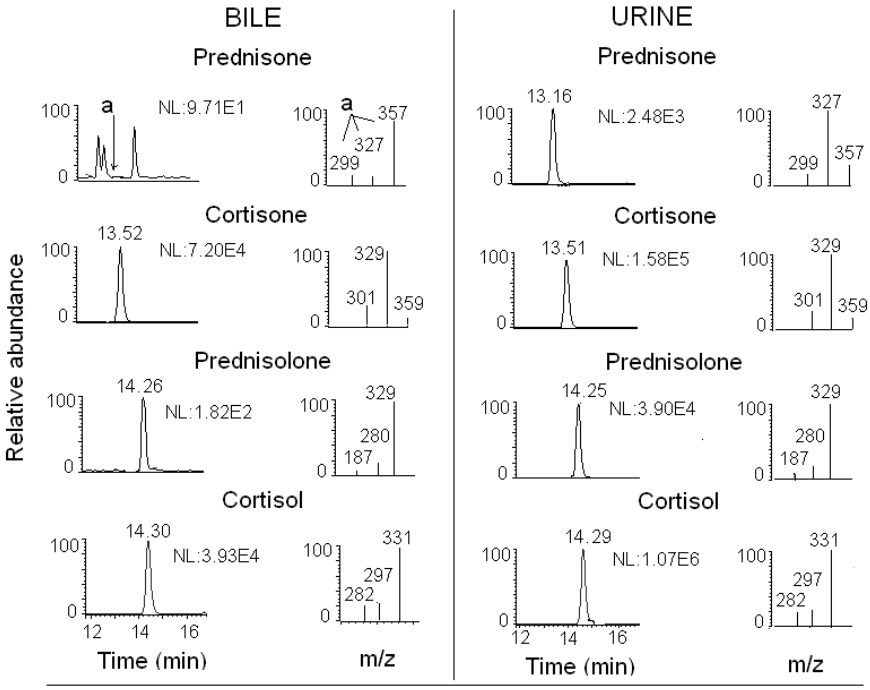
With regard to the comparison between bile and urine, the very lower analytical limits in urine do not indicate bile as an alternative matrix for the detection of α- and β-boldenone, their Phase II metabolites and ADD. Only α-boldenone glucuronide allowed correlation tests between the two matrices to be performed (Table 3), as the detection of other analytes often did not overlap and their frequencies were too low or too different in urine and bile.

3.2.3.2 Cortisol, cortisone, prednisolone and prednisone

The overall results regarding cortisol, cortisone, prednisolone and prednisone are reported in Table 2.

Illustrative chromatograms with the corresponding mass spectra of one bile and one urine sample are shown in Fig. 2.

Fig.2 Illustrative chromatograms with the relative ion spectra obtained through the LC-MS/MS analysis of one bile and one urine sample for the presence of prednisone, cortisone, prednisolone and cortisol.



a: prednisone not detected in bile and ion ratios not corresponding to the calibration standard.

Due to their endogenous nature and therefore their constant presence, cortisol and cortisone were used to compare the two matrices (Table 3); a correlation was only

found for cortisol in veal calves, thus demonstrating again a general lack of superimposability for these two matrices. This lack is moreover confirmed by the very low detection frequency of prednisolone and prednisone in bile, which resulted in a value lower than 4% for both corticosteroids if the total number of sampled animals (n=53) was considered. In urine, prednisolone was found at a frequency of 34% and prednisone of 17% considering all of the animals. An influence of gender on the presence in urine of prednisolone and consequently of its metabolite prednisone could be suggested. The higher frequencies were found in cows and male veal calves. Considering that the latter have not yet reached full sexual maturity, then adult males, i.e. the young bulls, show a frequency that is always lower than those seen in other animals. Finally, the average concentration of prednisolone was lower than the cut-off level of 5 ng mL^{-1} indicated by the European Union for urine. The highest concentration of prednisolone was 1.97 ng mL^{-1}, which was found in a male veal calf.

Table 3. Statistical analyses performed on male veal calves, young bulls and cows: normality, correlation and comparison of the results related to α-boldenone glucuronide, cortisol and cortisone in bile and urine samples.

	Matrix	Analyte	Normality	Correlation	Mean or median comparison
Male veal calves	Bile	α-boldenone glucuronide	yes	Spearman r= 0.37 P>0.05, NS	Mann-Whitney test P<0.0001, S
	Urine	α-boldenone glucuronide	no		
	Bile	Cortisol	no	Spearman r= 0.60 P<0.05, S	Wilcoxon test P<0.0001, S
	Urine	Cortisol	no		
	Bile	Cortisone	no	Spearman r= 0.48 P>0.05, NS	Mann-Whitney test P<0.001, S
	Urine	Cortisone	yes		
Young bulls	Bile	α-boldenone glucuronide	no	Spearman r= 0.70 P<0.01, S	Wilcoxon test P<0.0001, S
	Urine	α-boldenone glucuronide	no		
	Bile	Cortisol	yes	Pearson r= 0.30 P>0.05, NS	Unpaired t-test P<0.001, S
	Urine	Cortisol	yes		
	Bile	Cortisone	yes	Pearson r= 0.12 P>0.05, NS	Unpaired t-test P<0.01, S
	Urine	Cortisone	yes		
Cows	Bile	α-boldenone glucuronide	no	Spearman r= 0.58 P<0.05, S	Wilcoxon test P<0.0001, S
	Urine	α-boldenone	no		

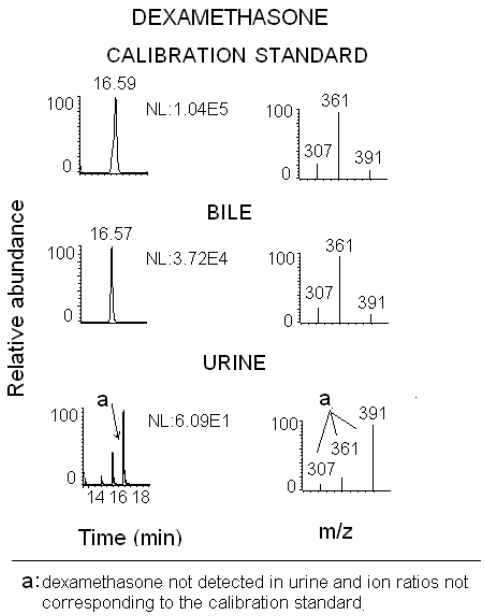
		glucuronide			
	Bile	Cortisol	no	Spearman r= 0.05 P>0.05, NS	Mann-Whitney test P<0.0001, S
	Urine	Cortisol	no		
	Bile	Cortisone	no	Spearman r=- 0.04 P>0.05, NS	Mann-Whitney test P=0.001, S
	Urine	Cortisone	yes		

S: significant; NS: non-significant

3.2.3.3 Dexamethasone

Dexamethasone is worth of a separate argument. It is a merely exogenous corticosteroid, so its detection is undoubtedly the evidence of treatment. It has to be stressed that the animals came from the food chain, that the samples were completely anonymous and not used for official controls on residue presence. Belonging to the food chain indicates bile as a better matrix than urine when dexamethasone is searched. This corticosteroid was found in one only urine sample but in 10 bile samples that were collected from the same 53 animals, i.e. a 10-fold higher frequency in bile than urine. The chromatograms with the corresponding mass spectra of one bile and urine sample, belonging to the same animal, are shown in Fig. 3.

Fig.3 Illustrative chromatograms with the relative ion spectra obtained through the LC-MS/MS analysis of a solution of dexamethasone in methanol (10 ng mL^{-1}), one bile and one urine sample for the presence of dexamethasone.



As confirmation of this result, the range of concentration in 9 bile samples was 0.14-1.5 ng mL^{-1}, while in the tenth sample, the level of dexamethasone was 94.7 ng mL^{-1}. This last bile sample came from the only cow whose urine tested positive: the concentration found in urine was 10.5 ng mL^{-1}, a relatively high value, but much lower than that found in bile, thus indicating a better suitability of bile for the determination of dexamethasone. Some considerations must anyway be made. First, the 2013 final report from the Italian national residue plan (PNR), (Italian Ministry of Health, 2013), states that only two non-compliant bovines to corticosteroids were found, not specifying if the matrix was urine or liver. This corresponds to the 0.16% of the whole number of bovine samples analysed for these drugs. It is a very lower percentage than the 19% we found. In a study by Vincenti et al. (2009) the excretion kinetics of dexamethasone in young bulls, when administered as sodium phosphate salt, showed a very fast urinary excretion after both an intra-muscular therapeutic protocol or an oral growth promoting schedule: “namely a couple of days after drug withdrawal, after which the drug rapidly disappears from the urine”. The high difference between our results, and the data from the 2013 Italian PNR could be therefore due to slower excretion through bile. Second, synthetic corticosteroids are now becoming the most commonly used growth promoters, administered both alone or in combination with anabolizing agents (steroids with hormonal activity, β-agonists), as their detection is not *per se* a proof of a non-therapeutic treatment (Gottardo et al., 2008). If our data on urine were used for official controls, the non-compliant samples to α-boldenone conjugate (glucuronide + sulfate forms concentration higher than 2ng mL^{-1}) would be 10, to presence of β boldenone conjugate would be 12 (European Union, 2003), and just one positive for dexamethasone, not corresponding to any “non-compliant“ sample for boldenone. When the 10 bile samples positive for dexamethasone are considered with respect to urinary conjugated boldenone, the simultaneous presence of α-boldenone glucuronide was observed in one animal and of α- and β- boldenone in two animals. The use of dexamethasone alone or in cocktails, as above reported, could explain these data, while the low frequency of dexamethasone detection in bile concomitant to “non compliant” boldenone presence in urine from the same animal, could confirm the likely pseudoendogenous nature of boldenone found in our samples. Furthermore it is well

documented that, even if the illegal use of growth promoters seems to decrease, “the results obtained on samples of preparations (vials, syringes, needles, etc.), however, are in high contrast with these reassuring figures” (Courtheyn et al., 2002). The use of the method described in this study, could therefore give new information about the illicit use of the synthetic corticosteroids.

3.2.4. Conclusions

The results shown in this study indicate that some of the considered steroids, 17α-boldenone glucuronide, 17α-boldenone, and dexamethasone are generally found at higher concentrations in bile than in urine. The levels of 17β-boldenone sulfate, 17β-boldenone, and ADD are comparable, while the endogenous cortisol and cortisone, and pseudoendogenous prednisolone and prednisone, are present at higher concentrations in the urine. We found a general lack of correlation for the presence of the natural steroids in bile or urine. Because of the dissimilarity of the two matrices, the most noticeable observation relates to the detection of dexamethasone, the only purely exogenous steroid. For this substance, the results show a greater possibility of detection in bile than in urine: this finding could lead to a substantial improvement in the control of residues in food-producing animals. It is moreover conceivable that other substances may also be more easily detectable in bile than urine. Further studies therefore have to be carried out on food chain animals, possibly analysing bile and urine by LC-HRMS techniques, to identify residues in bile that are not found in urine or on animals treated purposely with known pharmacologically active principles.

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3.3. Detection of selected corticosteroids and anabolic steroids in calf milk replacers by liquid chromatography–electrospray ionisation – tandem mass spectrometry

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

Abstract

The use of corticosteroids and anabolic steroids in food producing animals is regulated or banned in the European Union (EU). However, their use as growth promoters cannot be excluded. Milk replacers, considered by EU legislation as feeds, may be a good way of administration of these compounds. In order to improve the control of growth promoter utilization in animal husbandry and preventing possible consequences to animal welfare, we developed a method for multiresidue analysis of prednisolone, prednisone, dexamethasone, cortisone, cortisol, 17α- and 17β-boldenone and their precursor androstadienedione (ADD), testosterone, epitestosterone, 17α- and 17β-nandrolone, and trenbolone in powdered milk for calves. All analytes were extracted, after a common deproteinization and defatting sample pre-treatment, by a unique immunoaffinity column and analysed by liquid chromatography tandem mass spectrometry (LC–MS/MS) in both positive and negative electrospray ionization (ESI) modes. The method was validated according to the criteria of the Commission Decision 2002/657/CE. The analytical limits were from 0.39 to 0.73 ng mL^{-1} for the decision limit (CCα) and 0.46 to 0.99 ng mL^{-1} for detection capability (CCβ). The analysis of 50 samples of milk replacers for calves, always revealed the presence of cortisol and cortisone (average concentrations 2.56 and 1.06 ng mL^{-1}, respectively), frequently testosterone and epitestosterone (1.24 and 0.63 ng mL^{-1}, respectively), occasionally β-nandrolone (0.82 ng mL^{-1}) and prednisolone (0.41 ng mL^{-1}). The other anabolic steroids were never found.

Keywords: calf milk replacers, corticosteroids, anabolic steroids, immunoaffinity columns, liquid chromatography tandem mass spectrometry.

Chemical compounds studied in this article:

Cortisol (PubChem CID: 5754); Cortisone (PubChem CID: 222786); Prednisolone (PubChem CID: 5755); Prednisone (PubChem CID: 5865); Dexamethasone (PubChem CID: 5743); Testosterone (PubChem CID: 6013); Epitestosterone (PubChem CID: 10204); Nandrolone (PubChem CID: 9904); Trenbolone (PubChem CID: 25015); Boldenone (PubChem CID: 13308).

3.3.1. Introduction

Successful calf growth depends on a combination of many factors related to health, management and nutrition (Heinrichs, Wells, & Losinger , 1995). From the alimentary aspect, natural milk, as a wholesome food, is the most important source of nutrition for young mammals before they are able to digest other types of food. Powdered milk is commonly used for the daily feeding of calves, as it is an adequate alternative to the mother's dairy milk and an economically feasible source of all essential nutriments. Feeding with high quality milk replacers allows healthy growth in calves equal to that attainable with whole milk (Jorgensen, Hoffman, & Nytes, 2006). Since manufacturing powdered milk directly from whole milk is an expensive process, the bulk of the constituents of commercial calf milk replacers are either by-products of dairy processing or non-dairy products. Powdered milk replacers are generally made up of ingredients such as skim milk powder, vegetable or animal fat, whey protein, soy lecithin and vitamin-mineral premix (Geiger et al., 2014). Protein levels in dry milk replacers range from 18% to 30% with an average value of approximately 20–22%, preferably of diary origin, but can also include soy protein, soy flour, wheat proteins, potato and animal plasma protein. Fat levels range from 10% to 28–30%, with 18% to 22% being the most common fat levels, mainly added as tallow, lard or coconut oil (Bamn, 2014 and Ontario veal association, 2015).

The inclusion of veterinary drugs in calf milk replacers is a matter of concern, particularly if their administration is not fully regulated and especially when legislation varies across the Countries. For example, in the USA medications (decoquinate, lasalocid, oxytetracycline, chlortetracycline, and neomycin) are approved for inclusion in milk replacers, but the U.S. Food and Drug Administration (FDA, 2013) recommended a three-year judicious period (starting from December 2013) during which utilisation of antibiotics in animal husbandry has to be reduced. European legislation does not treat milk replacers individually, but sets out the conditions under which medicated animal feeds may be prepared, placed on the market and used within the Community (European Union, 1990 and European Union, 2010 a).

The use of steroids in food-producing animals for therapeutic purposes is regulated (corticosteroids) or banned (anabolic steroids) in the European Union; however, their use as growth promoters cannot be excluded (Pavlovic et al., 2012). Cortisol, cortisone, testosterone and epitestosterone are endogenous, prednisolone (Bertocchi et al., 2013) , boldenone (Chiesa et al., 2014 a) and nandrolone (Glenn Kennedy et al., 2009) are considered pseudoendogenous steroids while dexamethasone and trenbolone are well-known synthetic steroids. A faster feed conversion rate, improvement of the carcass with improved meat quality, fat reduction, and increase in milk production are some of the notable features that could be achieved by treatment with these substances. Thus, regulations on steroid residues with hormonal activity in food of animal origin are essential in order to safeguard animal welfare and ascertain any fraud. In the case of therapeutic use of regulated substances, a prescription by a veterinarian is needed and a suspension period has to be respected between the end of treatment and slaughter or milk marketing. The European Commission has established the maximum residue limits (MRLs) for four corticosteroids in several matrices such as muscle, liver, kidney and milk from different animal species (European Union, 2010 b)

On the basis of the regular protocol applied, there are a few principal techniques by which medication can be introduced into an animal: oral administration, intramuscular, subcutaneous and intravenous injection or implantation under the skin (Courtheyn et al., 2002). Unfortunately, some illegal growth-promoting agents are suspected of being administered with feed, despite the fact that they are not licensed as additives (Courtheyn et al., 1993 and European Union, 2004). Therefore, in order to achieve comprehensive surveillance and have insight into how a medication was delivered to an animal, analysis of the feed for the presence of steroids should be included as well. It should be emphasized that the presence of steroid hormones in feedstuffs can be also unintentional, due to cross-contamination or owing to the appearance of pseudo-endogenous substances such as prednisolone (Chiesa et al.; 2014 b). Among feedstuffs used in animal husbandry, powdered milk replacers are perhaps most suitable for illegal tampering as drug distribution via this route is very simple: during the reconstitution of milk replacers, immediately before feeding. As hormones and steroids migrate to milk from the cow bloodstream, we need additional information about their

physiological levels in milk related to milk replacers (Jouan, Sylvie, Gauthier, & Laforest, 2006). To the best of our knowledge, there has been neither a preliminary assessment of the status of endogenous or exogenous steroids, nor a fully validated method for their screening in powdered milk used in calf breeding.

Taking into account all the above mentioned factors, with the intention of improving residue control and preventing possible consequences for animal welfare and the consumer, our aim was to develop a liquid chromatography–tandem mass spectrometry (LC-MS/MS) analytical method for evaluating selected glucocorticosteroids and anabolic steroids in milk replacers used as dairy feed replacement in calf rearing.

Nowadays, LC-MS/MS is the most suitable technique for detecting veterinary drugs in feedstuffs because it provides unambiguous identification and a reliable confirmation. On the other hand, milk replacers are complex matrices, containing many solutes with different physico-chemical properties: fatty acids, proteins, neutral lipids (glycerides, phospholipids and sterols), glycides, vitamins and minerals, which may interfere with analyses. The removal of these compounds is necessary in LC-MS/MS methods, especially if low ng mL^{-1} of steroid levels are to be screened for. Applying adequate and efficient purification, ion suppression can be successfully avoided together with improvements in overall method performances such as the detection limit and reproducibility.

There have been just a few studies in the literature on powdered milk – infant formulas for human use (Romero-González, Aguilera-Luiz, Plaza-Bolaños, Frenich, & Vidal, 2011 and Zhan et al., 2013) and only one that described a multi-residue method for detecting 17 selected veterinary hormones in six different powdered ingredients derived from bovine milk used modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation (Ehling, & Reddy, 2013). Other researchers devised a method for the detection of eight corticosteroids in milk replacers, through C18 SPE, but with relatively high detection limits (Fiori, Pierdominici, Longo, & Brambilla, 1998). Immunology-based pre-treatment techniques have been introduced recently, but have not yet been used in powdered milk analysis. For other matrices (urine, bile) this kind of purification in general has exhibited better selectivity than those obtained with common

procedures (Chiesa et al., 2014a and 2015). This is the reason we decided to take advantage of an immunoaffinity sample cleaning approach in the multi-drug method presented in this paper.

To this end, the main objective of this study was the establishment of a LC-MS/MS method able to identify steroids such as corticosteroids (prednisolone, prednisone, dexamethasone, cortisone and cortisol) and anabolic steroids (17α- and 17β-boldenone, their precursor androstadienedione (ADD), testosterone, epitestosterone, 17α- and 17β-nandrolone and trenbolone) in calf milk powder. All analytes were investigated after a common pretreatment step of deproteinization and defatting followed by immunoaffinity column (IAC) clean-up and LC–MS/MS analysis, validated according to Commission Decision 2002/657/EC (European Union, 2002). Finally, we applied the validated method to the analysis of 50 samples of commercially available powdered bovine milk.

3.3.2. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). Formic acid 98–100% was obtained from Riedel-de Haën (Sigma-Aldrich, St.Louis, MO, USA). Water was purified by a Milli-Q System. The IACs were provided by Randox (DM 2185, Randox Laboratories, Antrim, UK). Concentrated wash and storage buffers, diluted following the manufacturer's instructions before use, were supplied with the columns. ADD and β-boldenone were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA); α-boldenone was obtained from LGC Standards (Teddington, UK). Their internal standard was β-boldenone-d3 (LGC Standards, Teddington, UK). Cortisol, prednisone, prednisolone and dexamethasone were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA) and their internal standard, prednisolone-d6, was obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Testosterone, epitestosterone, 17α- and 17β-nandrolone, trenbolone and their internal standard 17β-nandrolone-d3 were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA).

3.3.2.1 Powdered milk replacer used for method validation and application

For the method validation we used a commercially available powdered calf milk. It contained partially skimmed milk whey, whey protein, fat, wheat gluten, vegetable oil, pregelatinized wheat flour, pea fibre and potato protein. The analytical constituents were: crude protein 22.50%, oils and fats 22.50%, crude fibre 0.30%, crude ash 8%, calcium 0.65%, sodium 0.50% and phosphorus 0.65%. Vitamin A, vitamin D_3 and vitamin E were present as additives in all complementary milk (25000 UI kg^{-1}, 3700 UI kg^{-1} and 75 mg kg^{-1}, respectively). The formulation also contained the following quantities per kg: choline 5 mg, copper pentahydrate sulfate 5 mg, manganese sulfate 45 mg, zinc sulfate 135 mg, potassium iodide 1 mg and sodium selenite 0.32 mg. All the information about the feedstuff composition came from the manufacturer's certificates.

With the aim to check the method effectiveness, we used 50 samples of powdered calf milk, collected in farms from North Italy. No information about their composition was available.

3.3.2.2. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and kept at -40°C. Working solutions containing each of the studied analytes at concentrations of 10 and 100 ng mL^{-1} were prepared daily. Each working solution was maintained at 4°C during the method validation procedures.

3.3.2.3. Sample extraction

The sample was initially deproteinized and defatted following the Wang et al. protocol (Wang, Zhou, & Jiang, 2011) slightly modified as regard the relative amounts of matrix and reagents, and acetonitrile substituted by methanol. Briefly, samples of powdered milk (1 g) were spiked with the internal standards to a final concentration of 2 ng mL^{-1} and reconstituted in water (10 mL). The mixture was vortexed and then sonicated for 10 min, followed sequentially by through the addition of methanol (10 mL), 2 min of shaking, and 10 min of sonication. After the addition of sodium chloride (2 g), 2 min of shaking, and 10 min of centrifugation 4500×g were carried out. The supernatant was transferred into a 50 mL polytetrafluoroethylene centrifuge tube and defatted with 2 × 7 mL of n-hexane extraction. Each time, after centrifugation at 2500×g, the n-hexane layer

was removed. The methanol/water layer was evaporated and reconstituted in 5 mL of water for further purification and extraction by using the IAC. The column was previously 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). The samples were loaded by 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 performed by the application of 4 mL ethanol:water (70:30 v/v) (flow rate ≤ 3 mL min^{-1}), which was collected in a 15 mL polypropylene tube. The IAC could be used again, starting from the equilibration described above, after a washing step with 2 x 5 mL ethanol:water (70:30 v/v). We also checked the number of runs sustainable by a column and the results were similar to the ones already shown for urine (Chiesa et al., 2015): using a column for 10 cycles before discarding it is advisable.The eluate was evaporated in a rotary vacuum evaporator. The dried extract was reconstituted in 200 µL of methanol:water (50:50 v/v) and transferred into an auto-sampler vial. The injection volume was 10 µL.

3.3.2.4. LC-MS/MS analyses

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 auto-sampler with a column oven and a Rheodyne valve with a 20 µL loop. Chromatographic separation was achieved using a Synergi Hydro RP reverse-phase HPLC column (150 x 2.0 mm, 4 µm internal diameter) with a C18 (4 x 3.0 mm) guard column (Phenomenex, Torrance, CA, USA), which was kept at 30°C.The mobile phase consisted of methanol (solvent A) and 0.1% aqueous formic acid (solvent B). The gradient program began at 60% A for 1 min, changing to 95% A in 10 min, which was then held for 2 min. Then it returned to 60% A in 2 min and equilibrated for another 7 min.

The flow rate was 200 µL min^{-1} and the overall run time was 22 min. The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher, San Jose, CA, USA) equipped with an electrospray interface (ESI) set in both positive (ESI+) and negative (ESI-) electrospray ionization modes. Acquisition parameters were optimized in the ion-spray mode by direct continuous pump-syringe infusion of standard solutions of

the analytes at a concentration of 1 µg mL^{-1}, a flow rate of 20 µL min^{-1} and a MS pump rate of 100 µL min^{-1}. The following conditions were used: 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 the collision gas at 1.5 mTorr and peak resolution 0.70 Da at full width half maximum (FWHM). The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. Three diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in multiple reaction monitoring (MRM). The selected diagnostic ions, one of which was chosen for quantification, and the collision energies are reported in Table 1. Data were acquired and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1. MS/MS conditions for MRM acquisitions of all analytes and relative internal standards. Ions for quantification are in bold characters. CE: collision energy, expressed in Volts.

Analyte	**Precursor ion $[M-H]^-$ or $[M-H]^+$ (m/z)**	**Product ions$_{CE}$ (m/z)**	**ESI**
cortisol	407	282_{37},**297_{33}**, 331_{20}	(-)
cortisone	405	**301_{21}**, 329_{20}, 359_{12}	(-)
prednisolone	405	187_{30},**280_{35}**, 329_{19}	(-)
prednisone	403	**299_{21}**, 327_{19}, 357_{12}	(-)
dexamethasone	437	307_{33},**361_{20}**, 391_{14}	(-)
prednisolone-d6	411	284_{37}, 299_{32},**333_{19}**	(-)
Testosterone and epitestosterone	289	97_{21}, **109_{23}**, 253_{16}	(+)
ADD	285	**121_{22}**, 151_{14}, 267_{11}	(+)
α- and β-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone d3	290	**121_{27}**, 138_{14}, 272_{10}	(+)
α- and β-nandrolone	275	91_{40}, **109_{27}**, 239_{16}	(-)
trenbolone	271	165_{56}, **199_{23}**, 253_{19}	(+)
β-nandrolone–d3	278	**109_{19}**, 242_{16}, 260_{15}	(+)

3.3.2.5. Method validation

Validation was performed according to the criteria and recommendations of European Commission Decision 2002/657/EC (European Union, 2002). After a preliminary screening of some samples of reconstituted milk as described in the 'Sample extraction' section, it was observed that all of them contained cortisol, cortisone, testosterone and epitestosterone.

We therefore made preliminary trials using a milk replacer batch, containing the lowest amount of these analytes, diluted 1:10 with water: no analyte was so detected. Then we compared the 2 six-point standard curves (0.0, 0.5, 1.0, 2.0, 5.0 and 10.0 ng mL^{-1}) performed both in milk replacer reconstituted following the manufacturer indication and in the diluted one. The slopes and the Y-intercepts for each analyte of each curve were then compared with the unpaired t-test. No significant difference was observed in slopes of all analytes. The Y-intercepts of cortisol, cortisone, testosterone and epitestosterone were slightly different ($P<0.05$) in the two standard curves, due to the presence of these endogenous hormones in milk. Therefore, the validation was performed on diluted 1:10 calf milk replacer.

For each analyte, the method's performance was assessed through its qualitative parameters [molecular identification in terms of retention time (RT) and transition ion ratios that characterize selectivity and specificity], through its quantitative parameters (linearity, recovery, accuracy in terms of trueness and precision expressed as intra- and inter-day repeatability) and through the analytical limits [decision limit (CCα) and detection capability (CCβ)]. At the end, we also tested the robustness of the method and the stability of the standard solutions in the solvent and in the matrix.

Specificity, for analytical methods, is the power to discriminate between the analyte and closely related substances (isomers, metabolites, degradation products, endogenous substances, matrix constituents, etc.). Therefore, potentially interfering substances should be chosen and relevant blank samples should be analysed to detect the presence of possible interference and estimate the impact of the interference. We analysed an appropriate number of representative blank samples (n= 20) and checked for any interference (signals, peaks, ion traces) in the region of interest where the target

analyte was expected to elute. Selectivity was evaluated in the same samples. Selectivity requires compliance with the relative retention times for each analyte. Moreover, three transitions from the analyte molecular peak were monitored with a signal-to-noise ratio greater than 3.

Instrumental linearity was evaluated by drawing five-point calibration curves in the solvent containing a fixed amount of the internal standards (2 ng mL^{-1} each) with analyte concentrations corresponding to 0.5, 1.0, 2.0, 5.0 and 10.0 ng mL^{-1}. Matrix validation curves were obtained by spiking milk samples with each of the analytes, resulting in three analytical series; each series comprised of six replicates for three concentration levels (0.3, 0.6 and 0.9 ng mL^{-1} for all analytes except for 17α- and 17β-nandrolone, positivized at 0.5, 1.0 and 1.5 ng mL^{-1}). Trueness was assessed through recovery and was evaluated using the matrix curve results from the three analytical series, expressed in terms of the percentage of the measured concentration with respect to the spiked concentration.

The precision in terms of intra- and inter-day repeatability was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at three concentration levels of the three analytical series. The data from the matrix validation curves were used to calculate the decision limit (CCα) using parallel extrapolation to the x-axis at the lowest experimental concentration and detection capability (CCβ) according to the guideline described in Commission Decision 2002/657/EC (European Union, 2002) and clarified in document SANCO/2004/2726 revision 4 (European Union, 2008). Experiments to evaluate matrix effects corresponded to Matuszewski's strategy (Matuszewski, Constanzer, *&* Chavez-Eng, 2003), that requires sample extracts with the analyte of interest added postextraction compared with pure solutions prepared in mobile phase containing equivalent amounts of the analyte of interest. The difference in response between the postextraction sample and the pure solution divided by the pure solution response determines the extent of the matrix effect occurring for the analyte in question under chromatographic conditions.

Stability was evaluated by testing spiked samples and standard solutions over time from one week to one month under defined storage conditions (-20° C), and quantitation of

components was determined by comparison to freshly prepared standards. If the concentration variations were lower than 2% the stability was considered acceptable.

Finally, we evaluated robustness using the approach of Youden (European Union, 2002), a fractional factorial design. Eight experiments were carried out, fortifying eight samples at the lowest validation level, changing the nominal values reported in the 'Sample extraction' section slightly (± 10%) of seven factors that may influence the outcome of the analysis. The seven factors were: the initial reconstitution volume in water, the volume of methanol during the deproteinization step, the sonication time, the volume of exane during the defatting step, the percentage ethanol in the elution solution of the IAC column, the elution volume of the IAC column and the resuspension volume of the dry extract. Finally, the eight samples were quantified using a calibration curve constructed in conjunction with the Youden experiment.

3.3.3. Results and discussion

3.3.3.1 Method development

During method development, different options were evaluated in order to optimize chromatographic separation and detection parameters as well as sample extraction.

3.3.3.2. Optimization of LC–MS/MS conditions

An LC–MS/MS method was developed to perform analyses of selected synthetic and natural corticosteroids and anabolic steroids in calf milk powder. Initially, in order to achieve high sensitivity of the target analytes, optimisation of the liquid chromatography and mass spectrometry conditions was performed by injecting standard solutions of a mixture of all the analytes. After preliminary trials, in full-scan mode from 50 to 500 m/z, the three product ions with the highest signal-to-noise ratio (s/n) for each analyte and the internal standards were chosen for identification. The collision energy (CE) and de-clustering potential (DP) for each transition were adjusted in multiple reaction monitoring (MRM) mode in order to reach the highest sensitivity. For a method to be deemed confirmatory under Commission Decision 2002/657/CE, (European Union, 2002) it must yield a minimum of four identification points (IPs) for Group A (forbidden substances) of Directive 1996/23/CE or three IPs for substances listed in Group B (permitted

substances) (European Union, 1996). Each one of the three product ions is equal to 1.5 IPs, making a total of 4.5 IPs. The three diagnostic product ions, among which is the ion chosen for quantification, their CEs are reported in Table 1.

Special attention was paid to the separation of stereoisomeric compounds (α-boldenone from β-boldenone, α-nandrolone from β-nandrolone) (Fig. 2) that exhibited the same (or similar) fragmentation pattern. Additionally, the composition of the mobile phase was shown to be adequate for both types of ionisation (positive and negative), further promoting the remarkable sensitivity to the target compounds. Under our LC–MS/MS conditions, it was possible to individuate all compounds of interest at concentration levels suitable for research aimed for control purposes (Fig. 1 and 2).

Fig. 1. LC–MS/MS chromatograms and related MS spectra of the five corticosteroids in a powdered milk sample spiked at the lowest concentration level for validation. Right-hand side: internal standard (concentration = 2 ng mL^{-1}).

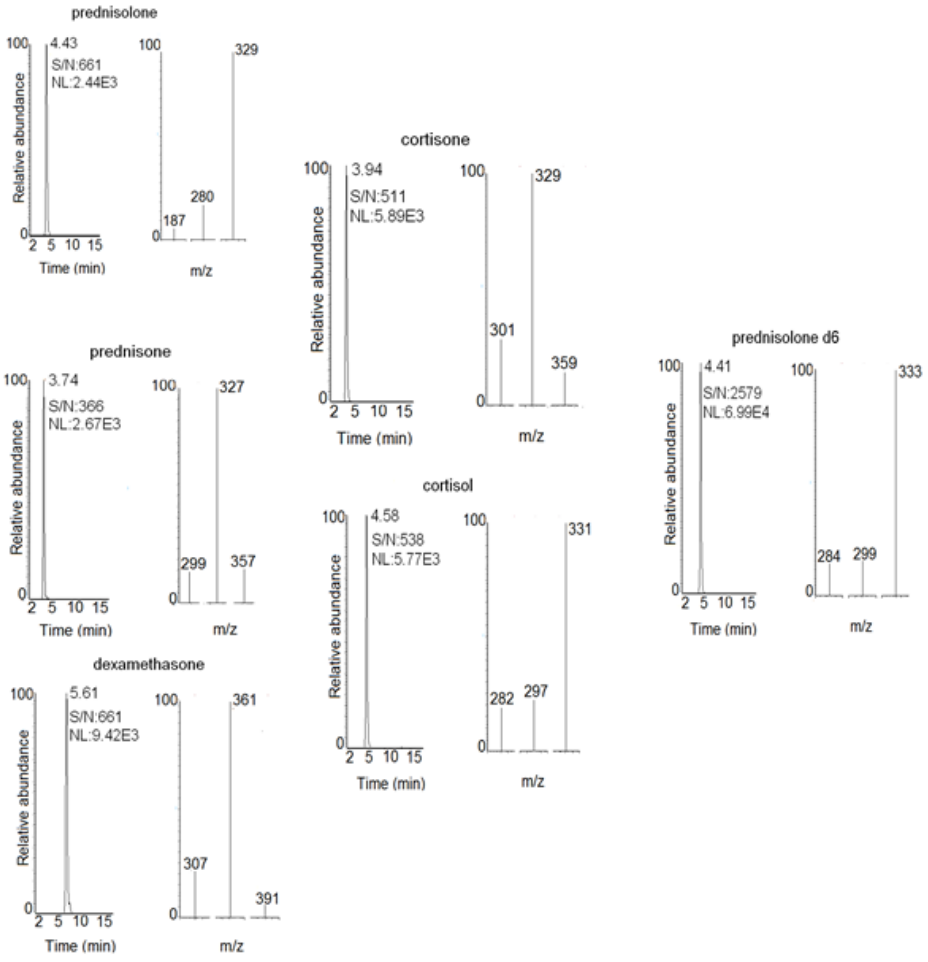
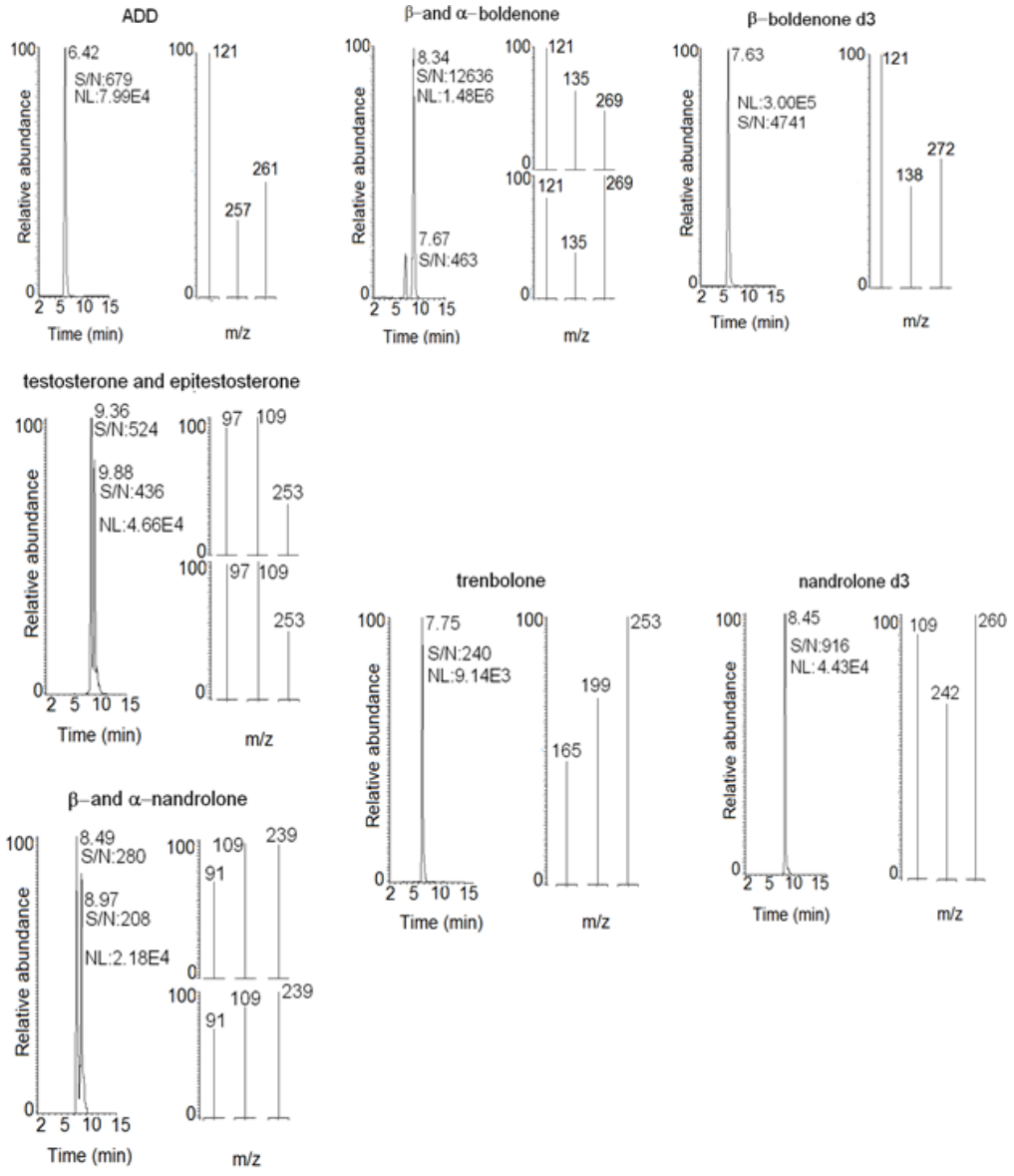


Fig. 2. LC–MS/MS chromatograms and related MS spectra of the selected anabolic steroids in a powdered milk sample spiked at the lowest concentration level for validation. Right-hand side: internal standards (concentration = 2 ng mL^{-1}). Chromatographic peaks and ion spectra of stereoisomers are in the sequence listed in their respective headings.



3.3.3.3. Optimization of sample purification and IAC extraction

The critical step in the method setup is the sample preparation procedure, owing to the high percentages of proteins and lipids and the overall complexity of the matrix. There are a number of potential pitfalls associated with corticosteroid and anabolic steroid analysis of milk samples. A major problem with some currently available methods is

interference with steroid determination by a significant number of other lipids. The purification procedure using an immunoaffinity approach could eliminate most of these interferences. Additionally, in our previous studies we demonstrated the high efficiency of IAC purification of bile (Chiesa et al., 2014 a) and urine samples (Chiesa et al., 2015). IAC also turned out to be a good strategy in this study, as selected analytes were retained and extracted with satisfactory efficiency: overall method recoveries ranged between 99.6 and 105.4 % for all analytes investigated (Table 2).

Table 2. Validation parameters for all analytes.

Analyte	Concentration (ng mL^{-1})	Recovery % (n= 18)	Repeatability %		CCα (ng mL^{-1})	CCβ (ng mL^{-1})
			intra-day	inter-day		
cortisol	0.3	95.1	10.2	20.1	0.51	0.73
	0.6	105.0	10,0	20.0		
	0.9	98.4	9.1	17.3		
cortisone	0.3	95.0	14.8	16.0	0.41	0.51
	0.6	105.0	10.0	11.8		
	0.9	98.3	7.7	7.9		
prednisolone	0.3	101.5	15.3	16.1	0.41	0.51
	0.6	98.5	13.1	14.0		
	0.9	100.5	9.0	9.2		
prednisone	0.3	98.1	12.2	13.1	0.39	0.46
	0.6	101.9	7.7	8.9		
	0.9	99.4	10.8	10.9		
dexamethasone	0.3	105.4	10.8	14.7	0.40	0.49
	0.6	94.6	7.6	12.6		
	0.9	101.8	9.5	10.1		
testosterone	0.3	98.9	7.9	13.0	0.39	0.48
	0.6	101.1	10.7	14.9		
	0.9	99.6	9.1	9.7		
epitestosterone	0.3	97.0	15.4	19.2	0.43	0.54
	0.6	99.0	10.0	10.2		
	0.9	102.9	8.0	9.8		
ADD	0.3	100.2	14.8	15.4	0.41	0.49
	0.6	99.8	6.8	8.1		
	0.9	100.0	9.2	9.3		
α-boldenone	0.3	101.3	13.2	14.4	0.40	0.49
	0.6	99.0	9.4	11.0		
	0.9	100.4	8.1	8.4		
β-boldenone	0.3	102.7	9.7	18.6	0.43	0.57
	0.6	97.3	15.1	20.9		

	0.9	101.0	8.0	9.6		
	0.5	102.4	12.8	13.6		
α-nandrolone	1.0	97.6	8.9	9.5	0.65	0.78
	1.5	100.8	13.9	14.0		
	0.5	96.2	12.2	20.1		
β-nandrolone	1.0	103.7	14.2	20.2	0.73	0.99
	1.5	98.8	10.6	11.9		
	0.3	96.7	15.4	20.3		
trenbolone	0.6	103.3	16.2	20.2	0.45	0.61
	0.9	98.8	12.0	12.3		

3.3.3.4. Performance characteristics of the methods

The curves prepared to check instrumental linearity showed correlation coefficients greater than 0.99 for all compounds, indicating a good fit.

Selectivity showed compliance with the relative retention times for each analyte, which in our case were found to be within a tolerance of 2.5% when compared with standards. Moreover all ion ratios of analytes in the samples were within recommended tolerances as required by Commission Decision 2002/657/EC (European Union, 2002) when compared with standards.

Blank and spiked samples were analysed and did not show any interference (signals, peaks, ion traces) in the region of interest where the target analytes were expected to be (European Union, 2002).

The matrix validation curves were demonstrated to be linear in the range 0.3–0.9 ng mL^{-1} for all analytes except 17α- and 17β-nandrolone, which spiked in the range between 0.5 and 1.5 ng mL^{-1}. A linear regression, obtained using the least-square method, demonstrated a good fit for all analytes with a correlation coefficient > 0.99.

The matrix effect value calculated according to Matuszewski et al., (2003) was approximately 100% for each compound, indicating the absence of ion suppression. It is likely the clean up performed by IAC tends to eliminate the matrix effect.

The precision, calculated by applying one-way analysis of variance (ANOVA), was expressed as the coefficient of variability (CV) in terms of intra- and inter-day repeatability. The reported results showed that the intra- and inter-day repeatability for

all analytes was less than 16.2 and 20.9 %, respectively. These CVs were lower than 22%, as proposed by Thompson (Thompson, 2000), representing good method repeatability.

CCα and CCβ were calculated as described in SANCO/2004/2726 revision 4 (European Union, 2008) using parallel extrapolation to the *x*-axis at the lowest experimental concentration (Table 2).

Standard solutions and samples showed an acceptable stability with less than 2% change after one month.

Finally, according to Youden's experiment (European union, 2002), none of the seven changed factors showed significant variation in the concentration measurements, confirming the good robustness of the method.

3.3.3.5. Application of the method

In order to monitor selected corticosteroids and anabolic steroid residues in bovine powdered milk, 50 samples were subjected to analysis. All samples analysed revealed the presence of cortisol and cortisone; testosterone was found in 45, epitestosterone in 34, prednisolone in 2 and β-nandrolone in 7 samples (Table 3), quantified by extrapolation from calibration curves which were built specifically of 6 points: 0, 0.5, 1.0, 2.0, 5.0, 10.0 ng mL^{-1} for all analytes. Very good, satisfactory linearity was obtained for all curves (R^2>0.99).

The average concentration is expressed as ng mL^{-1} of reconstituted milk (1 g/10 mL, considering that the dilution generally recommended by the manufacturers is approximately 100 g of powdered milk in 1 L of water).

Table 3. Survey of the steroids detected in the 50 samples of reconstituted calf milk replacers (1 g/10 mL). Concentration is expressed in ng mL^{-1}.

	cortisol	cortisone	prednisolone	testosterone	epitestosterone	β-nandrolone
Positives	50	50	2	45	34	7
Concentration mean±SD	2.56±0.89	1.06±0.37	0.41±0.00	1.24±0.68	0.63±0.23	0.82±0.13
Maximum concentration	3.81	1.64	0.41	3.72	1.33	1.03
Minimum concentration	0.76	0.42	0.41	0.48	0.43	0.73

The physiological concentrations of cortisol and cortisone in milk vary from 0 to 50 ng mL^{-1} (Jouan, Sylvie, Gauthier, & Laforest, 2006), a range that includes the concentrations shown in Table 3. As regards testosterone and epitestosterone in milk, very high variability is reported in the literature. The concentration of testosterone increases proportionally during pregnancy from 20 to 120 pg mL^{-1} (Gaiani, Chiesa, Mattioli, Nannetti, & Galeati, 1984), varying from undetectable to 50 pg mL^{-1} in milk at oestrus and 150 pg mL^{-1} during the luteal phase (Hoffman, & Rattenberger, 1977). These steroids are probably found in lower concentrations or are even absent after the process of milk skimming, but they are present in tallow and lard (Ontario veal association, 2015), often added to raise the lipid concentrations of milk replacers. However, in bovine fat and boar backfat, testosterone is present at concentrations of up to 10.95 ± 8.68 µg kg^{-1} and 20.34 µg kg^{-1}, respectively (Hartmann, Lacorn, & Steinhart, 1998). In light of the above, available information does not allow defining the “natural” values of testosterone and epitestosterone concentrations in powdered milk. We occasionally detected in the milk replacers the pseudoendogenous steroids prednisolone and β-nandrolone. To our knowledge, no data are available in the scientific literature about their presence in cow milk so we are not able to hypothesize if their origin is endogenous or due to administration.

3.3.4. Conclusions

The validation parameters of this method demonstrate its effectiveness for the analysis of selected corticosteroids and anabolic steroids in milk replacers. As already stated, this feed could be the vehicle for the administration of regulated or forbidden substances. Moreover, no studies are available on the presence in cow milk of substances with a pseudoendogenous origin. This method could therefore be a useful tool both for research purposes aimed to the improvement of control of feedstuff.

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3.4. Bovine teeth as a novel matrix for the control of food chain: liquid chromatography–tandem mass spectrometry detection of treatments with prednisolone, dexamethasone, estradiol, nandrolone and seven β2-agonists

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

Abstract

Veterinary drugs usually have rapid clearance rates in the liver and kidney, hampering their detection in conventional matrices like liver or urine. Pharmacological principles may be esterified to facilitate their administration and increase drug half-life. Prednisolone, whose therapeutic administration is regulated for food producing animals in the European Union, is available in its acetate form as well as nandrolone, a banned anabolic steroid, may be obtained as nandrolone phenylpropionate and estradiol as benzoyl ester. While the distribution and accumulation of lipophilic and hydrophilic substances in human teeth has been well documented, studies on residues in bovine teeth are lacking. We hypothesised that analysis of bovine teeth could be used to detect both regulated and banned veterinary drugs. Steroids may be illegally used as growth promoters in food producing animals, alone or combined with β2-agonists; therefore, we developed, and validated, in accordance with the Commission Decision 2002/657/EC, two analytical confirmatory HPLC-MS/MS methods to detect these classes of compounds following a unique liquid extraction procedure. We finally analysed teeth from three male Friesian veal calves treated with intramuscular estradiol benzoate, oral prednisolone acetate or intramuscular nandrolone phenylpropionate in combination with oral ractopamine, respectively, and from seven bovines from the food chain. Teeth from treated animals were positive for their respective drugs, with the exception of nandrolone phenylpropionate. One sample from a food chain bovine was positive for isoxsuprine, one of the seven β2-agonists studied. Non-esterified forms of the steroids were not found. These results demonstrate that bovine teeth are a suitable matrix for the determination of pseudoendogenous substances or illicit administration of veterinary drugs.

Keywords: bovine teeth; residues; steroids; β2-agonists; HPLC-MS/MS

3.4.1. Introduction

The illicit administration of veterinary drugs for growth promoting purposes in cattle breeding has been banned from European Union (EU) since 1988 (European Union 2003) and detailed in Council Directives 96/22/EC and 96/23/EC (European Union 1996a, 1996b). Veterinary drugs generally show high clearance rates in conventional biological matrices such as urine, blood, liver and muscle, hampering the detection of many active compounds. This is true for the active compounds of synthetic, natural or pseudoendogenous origin depending on the commercial formulation.

β_2-adrenoceptor agonists have powerful bronchodilator and tocolytic actions, but may also be administered as growth promoters to improve the production of lean meat by lowering fat levels through increased lipolytic activity. Although the European Union (EU), China and other Asian countries have banned the use of β_2-agonists for growth promoting purposes, the United States of America (USA) authorised ractopamine as a feed additive for swine in 1999, cattle in 2003 and turkey in 2008 (Flynn 2014).The EU has set the maximum residue levels (MRL) for clenbuterol in muscle, liver and kidney of bovine and horses as well as in cow milk (European Union 2010). Its only approved usage is as a tocolytic to parturient cows (EMEA 2000). and cannot be used under any circumstances on other categories of cattle. Isoxsuprine administration is similarly regulated, but MRLs are not indicated (European Union 2010) due to its infrequent use; moreover it is rapidly absorbed, distributed and excreted making it unlikely the animal will be sent for slaughter during or immediately after the treatment (EMEA 1996). The metabolic cycle of ractopamine is also very short and its degradation pathway varies among animals making it hard to test for ractopamine during the withdrawal period (Wu et al. 2014).

Estradiol benzoate is often used in combination with a progestin to induce oestrus, ovulation and increase the number of embryos in domestic livestock. Estradiol also stimulates the somatotropic axis to produce growth hormone and increase carcass weight and feed efficiency. As it is a potential carcinogen, its use in food producing animals has been banned since 2008 in the EU (European Union 1999, 2008). The possibility of widespread abuse of hormonal substances by unscrupulous farmers and

veterinary professionals in some parts of Europe has been reported, due to economic benefits that these illicit substances provide in animal husbandry and the possibility to get them in non-European countries where they are authorized (Stephany 2001).

Nandrolone phenylpropionate can be utilised in medical veterinary practices to slow degenerative processes and to promote tissue repair. Its use in cattle breeding is prohibited in the EU (European Union 1996a), yet it is one of the most frequently applied illegal anabolic steroids. The controls by the official organisms are complicated by the pseudoendogenous nature of nandrolone in bovine. The presence of its metabolites in untreated, injured males and pregnant females is detectable (Kennedy et al. 2009).

The same applies to prednisolone (Bertocchi et al. 2013), a corticosteroid commercially available as an acetate that is allowed for therapeutic purposes as an anti-inflammatory drug or for treatment of ketosis (Aiello 2014; McSherryt et al. 1960). Apart from its legal usage, prednisolone could also be administered at low doses, either alone or in combination with other steroids or β_2-agonists, to promote growth.

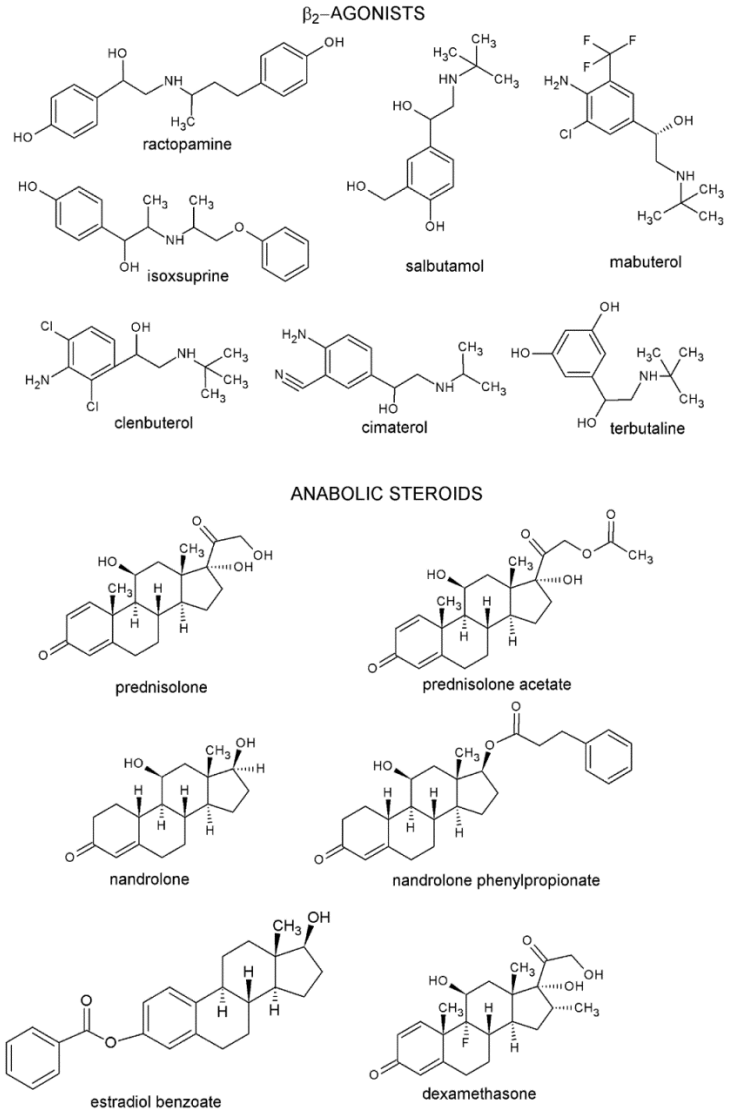
The natural or pseudoendogenous origin, rapid excretion and low dosages (especially if cocktails are used) of illegally applied anabolic substances make it challenging to concretely verify their use. Thus, it is necessary to identify a matrix in which these drugs accumulate and persist in their administered chemical form to undoubtedly demonstrate that treatment occurred, discriminate between exogenous or endogenous origin and improve the framework of controls.

In recent years, many studies have proposed hair analysis as a useful strategy to detect drug residues in food producing animals (Nielen et al. 2006; Rambaud et al. 2005; Gaillard et al. 1999) Although hair provides a long retention window, it is limited to a monthly time scale (Hinners et al. 2012) in teeth, exposure or administration markers remain stable enabling a longer detection window (Gulson et al. 1997). Human teeth have been used to assess exposure to inorganic chemicals since the 1960s (Altshuller et al. 1962) and to determine prenatal exposure to environmental organic chemicals in the early 2000s (Andra et al. 2015). Particularly, ^{14}C-labeled substances have been shown to penetrate into the calcified tissues and pulp of deciduous and permanent teeth

in humans, rendering this biological matrix a potentially important deposit of exogenous substances (Haustein et al. 1994). The studies that to date use teeth as a matrix, however, are almost exclusively limited to human teeth and have been used to detect drugs of abuse (opiates, cocaine, nicotine, etc.) or environmental contaminants. To our knowledge, only Spinner et al. (2014) used artificially loaded bovine dentine as an experimental model to demonstrate the possibility to detect common drugs of abuse for toxicological-forensic purposes. However, bovine teeth have not yet been demonstrated as a useful matrix to determine illicit anabolic treatment in veterinary medicine.

We propose that the analysis of bovine teeth, a non-disruptive matrix when collected at the slaughterhouse, is a powerful strategy to demonstrate the administration of growth promoters in livestock. To test whether teeth could be used for detection of drugs in food producing animals, we developed a simple and unique liquid extraction procedure from teeth followed by two HPLC-MS/MS analyses. The first one dealt with seven β_2-agonists (cimaterol, clenbuterol, isoxsuprine, mabuterol, ractopamine and terbutaline) and the second one with selected steroids (prednisolone acetate, prednisolone, dexamethasone, estradiol benzoate, nandrolone phenylpropionate and nandrolone). Their structures are shown in Figure 1.

Figure 1. Chemical structures of the seven β_2-agonists and the selected steroids.



The developed methods were validated according to Commission Decision 657/2002/CE (European Union 2002) to demonstrate the power of this novel, unconventional matrix to uncover illicit administration of these drugs. Finally, these methods were used to analyse teeth from experimentally treated veal calves collected at the slaughterhouse and from bovines from the food chain.

3.4.2. Materials & Methods

3.4.2.1. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). Formic acid 98-100% was obtained from Riedel-de Haën (Sigma-Aldrich, St.Louis, MO, USA). Water was purified by a Milli-Q System (Millipore, Merck KGaA, Darmstadt, Germany). Ractopamine, isoxsuprine, clenbuterol, salbutamol, terbutaline, mabuterol, cimaterol, estradiol benzoate, prednisolone acetate, prednisolone, dexamethasone and nandrolone were purchased from Sigma-Aldrich (St. Louis, MO, USA). The veterinary medicament Nandrosol (AST Farma B.V. Oudewater, the Netherlands) consisting of nandrolone phenylpropionate 50 mg mL^{-1} was used. The internal standards were ractopamine-d6 and testosterone benzoate-d3 (RIKILT laboratory, Wageningen, Netherlands), prednisolone-d6 (C/D/N Isotopes Inc, Pointe-Claire, Quebec, Canada) and testosterone-d3 (LGC Standards, Teddington, UK).

3.4.2.2.Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and stored at -20°C. Working solutions at concentrations of 10 and 100 ng mL^{-1} were prepared daily. Each working solution was maintained at -20°C during the method validation procedure.

3.4.2.3. Sample collection

The teeth used for the analyses included 20 blank samples used for validation of the methods, taken at the slaughterhouse from the food chain bovines, gathered in a pool (two molars or premolars and two incisors for each animal), and teeth samples used for application of the methods. In the last case, the samples (two molars or premolars and two incisors) were collected from seven bovines from the food chain and from three male Friesian veal calves aged three months with known treatments. The first treated animal was intramuscularly given once a week 5 mg estradiol benzoate for six weeks and slaughtered one week after the last treatment. The second calf was orally treated with 15 mg day^{-1} prednisolone acetate for 32 days and slaughtered three days after the last treatment. The third calf was intramuscularly treated with 150 mg of nandrolone phenylpropionate every two weeks for six weeks in combination with 80 mg day^{-1} oral ractopamine, starting from the twenty-first day for 32 days, and slaughtered four days after the last treatment with ractopamine. All teeth were collected at the slaughterhouse and stored at −20°C until the analysis was performed.

3.4.2.4. Sample extraction

Prior to analysis, teeth samples were cleaned by immersing them in distilled boiling water for 10 minutes to remove residual blood and, subsequently, the adherent tissue was removed with a scalpel. Once dry, teeth were cleaved and reduced in size with a hammer. After scraping out the pulp, teeth were pulverised by a ball mill (30 freq sec^{-1}, 40 sec). One gram of teeth was spiked with internal standards at a concentration of 2 ng g^{-1} and, after the addition of 3 mL of ethyl acetate:tert-butyl methyl ether (4:1, v/v) mixture, was sonicated for 1 hour. After centrifugation at room temperature at 2500 x g for 5 min, the supernatant was collected and evaporated in a rotary vacuum evaporator at 37°C. The dried extract was reconstituted in 200 µL of methanol:aqueous formic acid 0.1% (50:50 v/v) and then transferred to an auto-sampler vial. The injection volume was 10 µL.

3.4.2.5. HPLC-MS/MS analyses

Chromatographic separations were carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) made up of a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven kept at 30°C and a Rheodyne valve with a 20 µL loop equipped with a Synergi Hydro RP reverse-phase HPLC column (150 x 2.0 mm, internal diameter 4 µm) and a C18 guard column (4 x 3.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of a binary mixture of solvents A (aqueous formic acid 0.1%), and B (methanol). The following gradient program was used for β2-agonists: solvent A was decreased from 95% to 45% over 10 min, decreased to 10% over 1 min, held for 6 min at 10%, increased to 95% over 5 min and equilibrated for another 8 min. The flow rate was 0.2 mL min^{-1} and the overall run time was 30 min. Anabolic steroids were separated with the following gradient program: solvent A was maintained at 60% for 1 min, decreased to 40% over 1 min, decreased to 5% over 11 min, held at 5% for 1 min, increased to 40% over 2 min, increased 60% over 5 min and equilibrated for another 8 min. The flow rate was 0.2 mL min^{-1} and the overall run time was 29 min.

The mass spectrometer was a triple-quadrupole TSQ Quantum mass spectrometer (MS) (Thermo Fisher) equipped with an electrospray ionisation (ESI) interface that was

set in both positive (ESI+) and negative (ESI-) mode. Acquisition parameters were optimised in the electrospray mode by direct continuous pump-syringe infusion of standard 1 µg mL^{-1} solutions of analytes at a flow rate of 20 µL min^{-1} and a MS pump rate of 100 µL min^{-1}. The following conditions were used: capillary voltage 3.5 kV; ion-transfer capillary temperature 340°C; nitrogen as sheath and auxiliary gases at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr; and peak resolution 0.70 Da at full-width half-maximum (FWHM). Three diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in multiple reaction-monitoring (MRM) mode. The selected diagnostic ions (one of which was chosen for the quantification), their relative intensities and the collision energies are reported in Tables 1 and 2 for β2-agonists and anabolic steroids, respectively. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1. MS/MS conditions for the MRM acquisitions of the seven β_2-agonists, as well as for the internal standard. Ions used for quantification are in bold. The values in parentheses represent the relative intensities (%). CE: collision energy, subscripted and expressed in volts.

Analyte	Precursor ion (*m/z*)	Product ions(%)$_{CE}$ (*m/z*)	ESI
cimaterol	220	143(55)$_{23}$, **160(60)$_{16}$**, 202(100)$_{7}$	(+)
terbutaline	226	125(30)$_{24}$, **152(100)$_{16}$**, 170(20)$_{11}$	(+)
salbutamol	240	130(35)$_{29}$, **148(50)$_{18}$**, 222(100)$_{10}$	(+)
clenbuterol	277	158(18)$_{29}$, **203(50)$_{18}$,** 259(100)$_{10}$	(+)
ractopamine	302	107(53)$_{30}$, 121(62)$_{22}$, **164(100)$_{15}$**	(+)
isoxsuprine	302	107(25)$_{29}$, 150(20)$_{21}$, **284(100)$_{14}$**	(+)
mabuterol	311	217(50)$_{26}$, **237(100)$_{17}$**, 293(45)$_{11}$	(+)
ractopamine-d6	308	121(58)$_{23}$, **168(95)$_{16}$**, 290(100)$_{12}$	(+)

Table 2. MS/MS conditions for the MRM acquisitions of the selected steroids, as well as for the internal standards. Ions used for quantification are in bold. The values in parentheses represent the relative intensities (%). CE: collision energy, subscripted and expressed in volts.

Analyte	Precursor ion (*m/z*)	Product ions(%)$_{CE}$ (*m/z*)	ESI
prednisolone acetate	403	**307(78)$_{13}$**, 325(40)$_{11}$, 385(100)$_{9}$	(+)
prednisolone	405	187(7)$_{30}$, **280(18)$_{35}$**, 329(100)$_{19}$	(-)
dexamethasone	437	307(24)$_{33}$, **361(100)$_{20}$**, 391(7)$_{14}$	(-)
prednisolone-d6	411	284(20)$_{37}$, **299(18)$_{32}$**, 333(100)$_{19}$	(-)
estradiol benzoate	377	**105(100)$_{26}$,** 135(20)$_{15}$, 359(32)$_{11}$	(+)
testosterone benzoate-d3	396	**105(100)$_{25}$**, 256(36)$_{20}$, 274(40)$_{16}$	(+)
nandrolone phenylpropionate	407	**105(100)$_{31}$**, 239(82)$_{18}$, 257(94)$_{16}$	(+)
nandrolone	275	91(75)$_{40}$, **109(98)$_{27}$**, 239(100)$_{16}$	(-)
testosterone-d3	292	**109(100)$_{25}$**, 123(20)$_{27}$, 256(19)$_{18}$	(+)

3.4.2.6.Method validation

The unique pre-treatment method followed by two different HPLC-MS/MS analyses was fully validated for all analytes according to the criteria of Commission Decision 657/2002/CE (European Union 2002). The following performance parameters were assessed for each analyte: specificity, selectivity, linearity, trueness, recovery, precision, decision limit (CCα), detection capability (CCβ), ruggedness and matrix effect.

To confirm specificity of these methods, 20 bovine tooth blank samples previously checked for the absence of the analytes were tested to ensure the absence of possible interferences at the retention time where the target analyte was expected to elute.

Selectivity was tested by verifying a signal-to-noise ratio greater than three at the expected retention time of the analyte and the ion abundance ratio for all MRM transitions.

Matrix validation curves were performed by spiking the pooled blank samples with known concentrations of each analyte resulting in three analytical series. Each series had six replicates for three concentration levels C_0, $2xC_0$ and $3xC_0$, where C_0 was the minimum concentration detectable with our instrumentation. The β_2-agonists, estradiol benzoate, nandrolone, nandrolone phenylpropionate and dexamethasone were tested at 0.1, 0.2 and 0.3 ng g^{-1}. Prednisolone was tested at 0.2, 0.4, 0.6 ng g^{-1} and prednisolone acetate was tested at 0.5, 1.0, 1.5 ng g^{-1}. The chromatograms and MS/MS spectra of the β_2-agonists and of the steroids in the matrix spiked with each analyte at the lowest validation level (C_0) are shown with their related internal standards (2 ng g^{-1}) in Figures 2 and 3, respectively.

Figure 2. HPLC–MS/MS chromatograms and related MS spectra of the seven β_2-agonists in teeth sample spiked at the lowest validation concentration level (0.1 ng g^{-1}) and the internal standard ractopamine-d6 (2 ng g^{-1}).

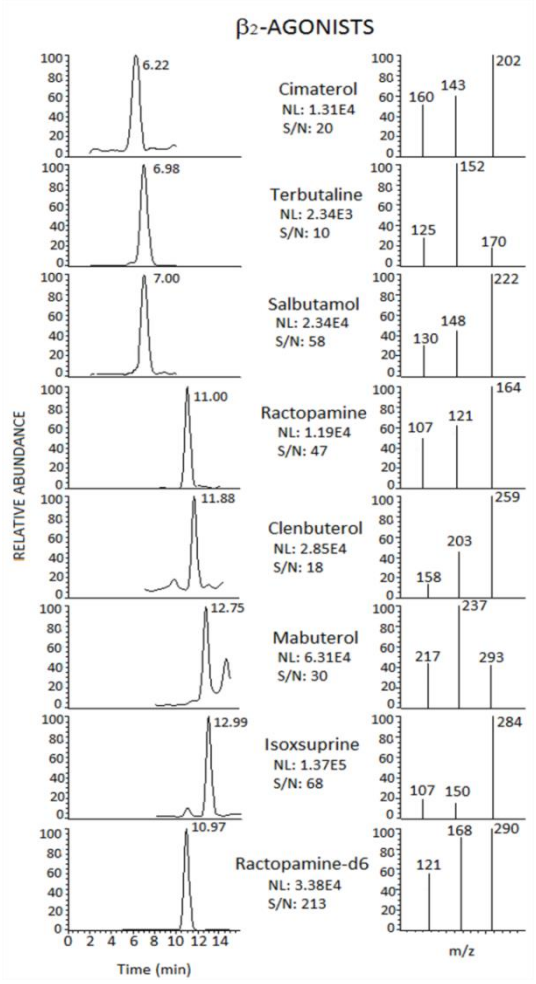
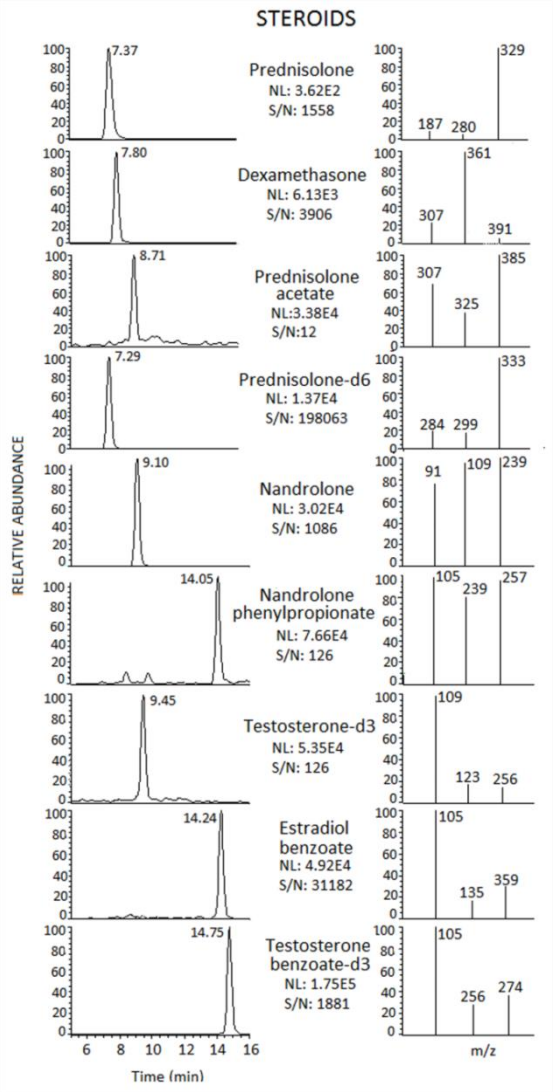


Figure 3. HPLC–MS/MS chromatograms and related MS spectra of the selected steroids in teeth sample spiked at the lowest validation concentration level (0.1 ng g^{-1} for estradiol benzoate, nandrolone, nandrolone phenylpropionate and dexamethasone; 0.2 ng g^{-1} for prednisolone and 0.5 ng g^{-1} for prednisolone acetate). Each group of analytes is followed by the related internal standard (2 ng g^{-1}).



Instrumental linearity was evaluated on pure standard solutions at six concentration levels in two replicates, from the minimum concentration detectable with our instrumentation up to 15 ng mL^{-1}, in order to estimate if method's quantification range overlaid the instrumental linear range. From this data, slope and intercept were determined by the least squares regression method and the linear fit was verified using squared correlation coefficient (R^2).

Matrix calibration curves, applied for the quantitation of the real samples, were similarly built by analysing two replicates of blank tooth samples spiked with working solutions at the same concentration range used to evaluate instrumental linearity. The following deuterated standards were used for quantitation: ractopamine-d6 for β_2-agonists; testosterone benzoate-d3 for estradiol benzoate; prednisolone-d6 for prednisolone acetate, prednisolone and dexamethasone; testosterone-d3 for nandrolone phenylpropionate and nandrolone.

The trueness estimated through recovery was evaluated using the data from the validation points of the three analytical series and expressed in terms of percentage of the measured concentration with respect to the spiked concentration.

The precision in terms of intra- and inter-day repeatability, estimated as the percent coefficient of variation (CV%), was evaluated by calculating the relative standard deviation of the results obtained from the 54 validation replicates and applying analysis of variance test (ANOVA).

CCα and CCβ values were calculated from the validation curves using the x-axis extrapolation method as clarified in the document SANCO/2004/2726 revision 4 (European Union 2008b).

Ruggedness was evaluated using the fractional factorial design of Youden as described in the Commission Decision 2002/657/EC (European Union 2002). This test was conducted by introducing slight variations (±10%) to seven potentially critical analytical parameters in eight different trials by fortifying eight blank teeth samples at the lowest validation concentration. The parameters selected included the frequency of the ball mill during pulverization, the time of pulverization, the extraction mixture volume, the extraction mixture percentage composition, the sonication time, the centrifugation time and the evaporation temperature of the extract. The Fisher test was applied to compare the standard deviation of the differences obtained from the high- and the low-value setting for each experimental parameter with the standard deviation of the method carried out under within-laboratory reproducibility condition.

The Matuszewski et al. strategy (Matuszewski et al. 2003) was used to evaluate matrix effects. Sample extracts spiked with the analyte of interest before analysis were compared to pure solutions prepared in the mobile phase containing equivalent amounts of the analyte. The percentage ratio between the corresponding peak area for the standard spiked after extraction and the peak area obtained in standard solution was used to determine the extent of the matrix effect occurring for the analyte in question under chromatographic conditions.

3.4.3. Results & Discussion

The proposed analytical protocol was entirely validated as confirmation method, in agreement with the European guidelines (European Union 2002, 2008b) for all steroids and β_2-agonists tested. Previous trials were made to develop a unique procedure, i.e. one extraction followed by just one analysis for all studied molecules. However, in a unique chromatographic run, the separation of the steroids hampered the retention of some β_2-agonists (cimaterol, terbutaline and salbutamol) that instantly eluted, making their detection impossible. As the purpose of this study was to demonstrate the suitability of bovine teeth as a matrix for the detection of veterinary drugs, we preferred to perform two chromatographic analyses having optimal conditions.

The chromatographic profiles obtained from blank tooth samples did not show the presence of any interference signals at the relative retention time expected for our compounds, demonstrating the method specificity. The methods also displayed selectivity with signal to noise ratios greater than three and the expected ion ratio abundances in correspondence of retention time for each analyte.

The least squares regression method was used to confirm instrumental linearity of standard solutions, with a R^2 greater than 0.997. Similarly, the R^2 was greater than 0.991 for the calibration curves for quantitation of the real samples, indicating a good fit of the curves on the experimental points.

The validation parameters presented in Tables 3 and 4 confirm that the extraction method and analyses are compliant with European guidelines. In particular, the recoveries ranged from 95 to 106% for the β_2-agonists and from 94 to 105% for the

steroids, demonstrating that a simple liquid extraction without incubation in acidic or alkaline solution, as some Authors perform (Andra et al. 2015), was able to extract all analytes from the complex structure and composition of the tooth.

Table 3. Validation parameters for the seven β2-agonists.

Analyte	CCα (ng g^{-1})	CCβ (ng g^{-1})	Concentration level (ng g^{-1})	Recovery % (*n*=18)	Repeatability	
					intra-day (CV; *n*=6)	inter-day (CV;*n*=18)
cimaterol	0.16	0.20	0.1	102	18	20
			0.2	97	14	17
			0.3	101	13	14
terbutaline	0.16	0.19	0.1	97	17	19
			0.2	99	13	15
			0.3	100	12	13
salbutamol	0.17	0.21	0.1	104	17	20
			0.2	96	16	18
			0.3	101	13	15
clenbuterol	0.13	0.16	0.1	97	13	14
			0.2	103	13	14
			0.3	99	10	10
ractopamine	0.15	0.19	0.1	106	17	18
			0.2	97	12	14
			0.3	102	12	12
isoxsuprine	0.15	0.19	0.1	97	17	18
			0.2	102	12	15
			0.3	95	10	11
mabuterol	0.14	0.18	0.1	95	16	19
			0.2	104	14	17
			0.3	98	12	12

Table 4. Validation parameters for the selected steroids.

Analyte	CCα (ng g^{-1})	CCβ (ng g^{-1})	Concentration level (ng g^{-1})	Recovery % (*n*=18)	Repeatability	
					intra-day (CV; *n*=6)	inter-day (CV;*n*=18)
prednisolone acetate	0.76	0.87	0.5	104	18	21
			1.0	96	16	17
			1.5	101	13	14
prednisolone	0.25	0.37	0.2	101	19	20
			0.4	96	13	15
			0.6	100	11	13
dexamethasone	0.16	0.23	0.1	105	16	17
			0.2	94	13	15
			0.3	101	11	12
estradiol benzoate	0.20	0.35	0.1	99	19	21
			0.2	101	19	20
			0.3	100	19	20
nandrolone phenylpropionate	0.25	0.38	0.1	95	14	17
			0.2	105	15	16
			0.3	98	11	12
nandrolone	0.17	0.25	0.1	103	11	15
			0.2	100	9	13
			0.3	100	10	11

The precision, in terms of intra- and inter-day repeatability, calculated by applying the one-way analysis of variance (ANOVA) and expressed as the percent coefficient of variation (CV%),ranged from 10 to 20% for β_2-agonists and from 9 to 21% for steroids. These values were lower than 23%, as proposed by Thompson (2000). and considered satisfactory according to the international guidelines.

Calculated CCα values ranged from 0.13 to 0.17 ng g^{-1} and from 0.16 to 0.76 ng g^{-1}, for β_2-agonists and steroids, respectively. The CCβ values ranged from 0.16 to 0.21 ng g^{-1} and from 0.23 to 0.87 ng g^{-1} for β_2-agonists and steroids, respectively. These experimentally determined levels were slightly higher than the lowest levels of validation

chosen through the minimum concentration detectable with our instrumentation, ensuring compliance with all the identification criteria.

The sample quantitation planned to carry out the Youden (European Union 2002) approach for ruggedness evaluation, was interpreted through both Student and Fisher tests. No significant variation was found by these analyses even when slight alterations of the seven potentially critical analytical parameters were introduced in the sample preparation and extraction steps.

The modest matrix effect gave values ranging from 88 to 106% for the β_2-agonists and from 84 to 109% for the anabolic steroids.

3.4.3.1. Application of the methods to real samples

We next applied our method consisting of a common liquid extraction followed by two analyses to test our hypothesis of accumulation of veterinary drugs in teeth. Samples from three veal calves subjected to treatment and from seven anonymous bovines were analysed. Importantly, all of the analytes from the treatment protocols were detectable in their respective tooth samples (ractopamine 8.90 ng g^{-1}, estradiol benzoate 8.78 ng g^{-1}, prednisolone acetate 2.90 ng g^{-1}) except nandrolone phenylpropionate. A major observation is that we did not find the free form where the hydrophilic pharmaceutical esterified form was detected, likely due to the nature of the matrix, constituted for the most part by hydroxyapatite, for which the esterified form should have a much higher affinity. The detection of the pharmaceutical form could therefore be a valid proof of illegal treatment, particularly in the case of endogenous (estradiol) or pseudoendogeneous substances (nandrolone and prednisolone) (Kennedy et al. 2009 and Bertocchi et al. 2013). The data collected do not allow us to explain the absence of nandrolone ester. Some suggestion could however be given: the gap between the last administration and the slaughtering was two weeks long, different from all the time intervals of other drugs. Nandrolone phenylpropionate could not accumulate into teeth, as well as need a longer time to reach the teeth and/or the cocktail with ractopamine could affect the distribution of the steroid.

Finally, we detected isoxsuprine in one of the unknown teeth samples at a concentration of 13.67 ng g^{-1}, a further evidence of the effectiveness of this method. None of the other analytes in this study was ever found.

3.4.4. Conclusion

Two HPLC–MS/MS methods for the analysis of bovine teeth with a common liquid extraction was validated and applied to samples from treated and anonymous bovines. The analytes included seven β_2-agonists (cimaterol, clenbuterol, isoxsuprine, mabuterol, ractopamine, salbutamol and terbutaline), and four steroids in free or esterified forms (prednisolone acetate, prednisolone, dexamethasone, estradiol benzoate, nandrolone phenylpropionate and nandrolone). The methods were validated in accordance with the criteria of the European Commission Decision (2002/657/CE) (European Union 2002) and SANCO/2004/2726 revision 4 (European Union 2008b).

The application of the methods to teeth from animals with known anabolic treatment lead to effective detection of ractopamine, prednisolone acetate and estradiol benzoate. However, nandrolone phenylpropionate was not found in teeth. The detection of isoxsuprine in one unknown sample confirms the suitability of this method for detection of β_2-agonists use.

The utilization of teeth as an accumulation matrix will be the subject of further studies dealing with different tooth groups and animals of varying ages.

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3.5. Determination of endogenous and exogenous corticosteroids in bovine urine and effect of fighting stress during the "Batailles des Reines" on their biosynthesis

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In this study I contributed to the experimental work planning, the execution of practical work, analysis of samples and data processing.

Highlights

• The fighting stress is considerably lower than that induced by slaughtering.

• Prednisolone presence in one urine sample is correlated with high level of stress.

• Concurrence of prednisolone with high cortisol level rules out its exogenous origin.

Abstract

Natural corticosteroids include two families of substances: mineralocorticoids and glucocorticoids. Several drugs of similar structure and biological activity have been synthesized and are currently used in the clinical practice. Beside legal pharmacological treatments, these drugs have been consistently misused in animal breeding. One of the most abused corticosteroids is prednisolone. For many years, prednisolone has been considered of exclusive synthetic origin, but nowadays a debate about its possible endogenous production is under way. Several studies have been addressed to ascertain the potential relationship between stressful conditions, such as transportation and slaughtering, and endogenous production of prednisolone. In order to verify further the effect of stressful conditions, our laboratory analysed urine samples collected from the cows participating to the “Batailles des Reines” (a traditional contest based on ritual and spontaneous fights of pregnant cows), to verify if an endogenous prednisolone production may occur in these animals. We developed and validated a LC-MS/MS method for the simultaneous determination of cortisol, cortisone, prednisolone and five of its metabolites. The method was applied to the analysis of urine samples collected from “Batailles des Reines” competitions in 2012 and 2013. All these samples had been previously analysed within an anti-doping control program and tested compliant to all screenings.

Keywords Prednisolone; Stress; Bovine urine; Cortisol; Cortisone

3.5.1. Introduction

Natural corticosteroids are a class of steroid hormones synthesized in the adrenal cortex from cholesterol and include two families of substances: mineralocorticoids and

glucocorticoids. Mineralocorticoids influence the electrolyte-water balance, while glucocorticoids act on carbohydrate and protein metabolism (Courtheyn et al., 2002; Savu et al., 1996). Several further synthetic corticosteroids have been produced so far to be used in the clinical practice. In synthetic corticosteroids an increased pharmacological activity is obtained by introducing small modifications in the chemical backbone of physiological glucocorticoids. Such modifications result in both prolonged therapeutic effects and a several-fold increase in pharmacological potency, particularly in the anti-inflammatory action. These features, combined with the absence of a parallel increase of sodium-retaining effects, enhance the suitability of synthetic glucocorticoids for therapeutic purposes (Ferguson and Hoenig, 1995). A number of commercial preparations are currently available for administration to cattle, covering a wide range of therapeutic applications, including primary ketosis, disorders of tendons and the musculoskeletal system, allergic reactions, skin diseases, and shock (McDonald et al., 2007). Besides legal treatments, glucocorticoids may be illicitly administered shortly before the animals' sale, to mask various pathologies, as in the case of old cows at the end of their productive cycle. Another common law infringement is the administration of intra-mammary glucocorticoid infusion without applying an appropriate withdrawal time.

One of the most utilized corticosteroids is prednisolone (Chiesa et al., 2016, 2014), which has been considered for years as an exclusively exogenous substance. In 2008, the official veterinary controls of Regione Lombardia, a high positivity rate for prednisolone (82% of all non-compliances) in the urine samples collected in slaughterhouses, whereas the urine samples collected in farms from living animals did not give any positivity to either prednisolone or prednisone (Regione Lombardia, Unità Organizzativa Veterinaria-Struttura Controllo degli Alimenti di Origine Animale, 2008). The European Commission reported that 0.14% of the bovine urine samples officially tested in 2010 were non-compliant for prednisolone (De Clercq et al., 2013). From these results, it was hypothesised that the stress evoked by handling the animals before their slaughter resulted in the endogenous production of prednisolone and/or prednisone to a level that could be detected using the current analytical methods. In 2011, Pompa and coworkers treated three dairy cows with tetracosactide hexaacetate, a synthetic analogue of adrenocorticotropic hormone, able to simulate stress. The animals were

slaughtered at the end of the study and the results indicated that prednisolone could be only occasionally detected in the non-treated animals, but was consistently found in the urine of pharmacologically stressed cows (the concentrations ranged from 1.01 to 4.08 ng/mL). The stress condition was also confirmed by unusually high urinary cortisol and cortisone levels in urine, typically detected at concentrations of hundreds ng/mL. The results of this preliminary study did not reveal the specific metabolic pathway responsible for prednisolone biosynthesis, but suggested that a mechanism of endogenous production exists (Bertocchi et al., 2013). Still in 2011 and 2013, other studies found prednisolone residues in urine samples collected from control bovines especially at the slaughterhouse, together with high levels of hydrocortisone and cortisone (Ferranti et al., 2013, 2011).

In 2012, Vincenti et al. conducted a field survey on urine samples collected from 131 guaranteed untreated cows and analysed for to verify the possible occurrence of prednisolone and prednisone, and also for determined cortisol concentrations. None of the examined samples exhibited prednisolone or prednisone levels higher than the CCα limit (0.70 ng/mL and 0.66 ng/mL respectively), therefore resulting officially compliant for both analytes. Trace amounts of prednisolone, estimated in the range 0.1–0.3 ng/mL, were found in only 7 samples from cows also showing high urinary cortisol level, possibly resulting from stressful conditions (Vincenti et al., 2012).

In accordance with the European Union Reference Laboratory (de Rijke et al., 2014), the Italian Ministry of Health enacted a new disposition that considers a bovine urine sample noncompliant only when the prednisolone concentration exceeds 5.0 ng/mL (Department of Public and Veterinary Health, 2012). This threshold appears to be largely conservative in avoiding false non-compliant results: a study from our laboratory demonstrated that the urine of beef cattle treated with low doses of prednisolone acetate for extended periods of time, as occurs in growth-promoting illegal treatments, may contain prednisolone at 1 ng/mL or even below even during the administration period (Cannizzo et al., 2011).

In order to obtain better insight into the metabolic fate of prednisolone, our laboratory evaluated the possible presence of prednisolone metabolites in the urine of treated and

untreated beef cattle (Leporati et al., 2013). We found that 20β-dihydroprednisolone is a major urinary prednisolone metabolite in beef cattle experimentally treated with low dosages of prednisolone acetate according to a growth-promoting schedule. The complete metabolic urinary excretion profile of prednisolone was also characterized after intramuscular (i.m.) administration to healthy finishing bulls and cows using a therapeutic schedule, in which three other prednisolone metabolites and one prednisone metabolite were found (Nebbia et al., 2014). All these results indicate that the merely quantitative testing currently adopted for prednisolone (5 ng/mL cut-off for the parent drug in urine) is not adequate to ascertain the illicit administration of prednisolone to cattle, according to both a growth-promoting schedule and a single high-dose treatment. Similar conclusions were obtained by Famele et al. (Famele et al., 2015). Besides the effect of stress, the hypothesis of a conversion of the natural cortisol into prednisolone during inappropriate sample storage conditions, i.e., in the presence of faecal microbiota, was investigated through studies on long-term stability of natural and synthetic glucocorticoids in livestock urine (De Clercq et al., 2013) and feaces (Arioli et al., 2010; De Clercq et al., 2014). Recently, a metabolic fingerprinting approach proved to be a powerful tool to classify unknown bovine urine samples that tested positive for prednisolone, while providing information about the stress status of the animal (De Clercq et al., 2015).

Whereas stressful conditions (i.e. transport and slaughter imminence) proved to generate a dramatic increase of cortisol and cortisone urinary concentrations (Bertocchi et al., 2013; De Clercq et al., 2015; Capra, 2016), we decided to evaluate the effects of a different source of stress, namely the fight between cows participating to the “Batailles des Reines”, on the endogenous production of cortisol, cortisone, and, possibly, prednisolone, in these animals.

The “Batailles des Reines” (Association Régionale Amis des Batailles de Reines, 2017) is a traditional event, typically taking place in the alpine regions of Valle d'Aosta (Italy), Valais (Switzerland), and Haute-Savois (France), in which couples of pregnant cows spontaneously fight against each other, by joining their head and pushing, until one of the two backs down from the contest and is eliminated. Each “bataille” takes place

between two “Reines”, namely the most combative representatives of the herd, who struggles for dominance, especially during the summer period when they instinctively compete for the best mountain pastures. During the course of the “Bataille de Reines”, the cows are not forced to struggle by their breeders, who remains mere spectators. Competition is fair and totally bloodless: the animal instinctively fight against an equal opponent.

3.5.2. Materials and methods

3.5.2.1. Chemicals, reagents and standard solutions

Acetonitrile, methanol, ethyl acetate, acetic acid glacial, ammonium acetate, prednisolone, prednisone, cortisol and cortisone were supplied by Sigma Aldrich Srl (Milan, Italy) and were all of analytical grade. The analytical standards 20α-dihydroprednisolone, 20β-dihydroprednisolone, 6β-hydroxyprednisolone and 20β-dihydroprednisone were from Steraloids (Newport, RI, USA). The internal standards (ISTDs) prednisolone D6, cortisol D2 and cortisone D2 were from CDN Isotopes (Pointe-Claire, QC, Canada). β-glucuronidase/aryl-sulfatase was from Roche Diagnostics (Mannheim, Germany). Sodium hydroxide and hydrochloric acid were from Carlo Erba Reagenti (Milan, Italy). Ultrapure water was obtained by a Milli-Q Millipore system (Bedford, MA, U.S.A.). Stock standard solutions of analytes and ISTDs were prepared in acetonitrile at a concentration of 200 ng/mL and stored at − 20 °C in the dark.

3.5.2.2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Chromatographic separations were performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), including a vacuum degasser, a binary pump, an autosampler and a column thermostat. The liquid chromatograph was equipped with a Waters (Milford, MA, USA) X-Select HSS T3 (2.5 µm, 3.0 × 100 mm) column and a Phenomenex (Castel Maggiore, BO, Italy) SecurityGuard 4.0 mm × 2.0 mm precolumn. The column was kept in a column oven at 23 °C. The chromatographic run was carried out by a binary mobile phase of a 0.1% v/v aqueous acetic acid solution and acetonitrile, using the following program: isocratic with

20% acetonitrile for 2 min; linear gradient from 20% to 40% in 10 min; isocratic with 40% acetonitrile for 6 min; linear gradient from 40% to 70% in 1 min; isocratic with 70% acetonitrile for 2 min; total run time 21 min. The injection volume was 10 µL, while the flow-rate was 0.2 mL/min. The LC was interfaced to an Applied Biosystems API 4000 triple–quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in electrospray ionization (ESI) – negative ionization mode. The other MS parameters were set as follows: curtain gas: 30 psi; nebulizer gas: 40 psi; probe temperature: 300 °C; IS voltage: − 4200 V; gas for collisional activation: N2 at 2 psi. Ion acquisition was operated at unit mass resolution in the selected reaction monitoring (SRM) mode, using the transitions from the acetate adduct ion of each analyte (precursor ion) to the fragment ions indicated in Supplementary Material.

3.5.2.3. Sample preparation

After centrifugation at 3500 rpm for 5 min, 5.0 mL of urine was transferred into 30 mL glass tubes and 100 µL of the ISTDs solution at 0.1 µg/mL was added. 1.0 mL of aqueous ammonium acetate solution 1.1 M pH = 4.8 was added and the pH was checked out and adjusted to 5.0 with HCl 1 M, if required. Then 20 µL of β-glucuronidase/arylsulfatase solution, obtained by 1:20 dilution of the enzyme in deionized water, was added and the enzymatic deconjugation was carried out for 2 h at 37 °C. The sample mixture was cooled at room temperature and loaded onto a Strata-X 33 µm, 60 mg × 3 mL SPE column (Phenomenex, Castel Maggiore (BO), Italy), previously conditioned with 3 mL of methanol and 3 mL of aqueous ammonium acetate solution 0.15 M pH = 4.8. After sample loading, the column was washed with 3 mL of deionized water, 6 mL of a methanol:sodium hydroxide 0.02 M (30:70 v/v) mixture and 3 mL of deionized water. The analytes were eluted with 4 mL of ethyl acetate. The resulting solution was evaporated to dryness under a gentle stream of nitrogen and mild heating (50 °C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was dissolved in 100 µL of 0.1% acetic acid aqueous solution and acetonitrile (80:20 v/v) mixture and transferred into the analytical vials for LC–MS/MS analysis (injection volume = 10 µL).

3.5.2.4. Analytical method validation

A first set of experiments on prednisolone, prednisone, 20α-dihydroprednisolone, 20β-dihydroprednisolone, 20β-dihydroprednisone and 6β-hydroxyprednisolone used blank bovine urine preliminarily tested as negative to these molecules. A pool of 20 urines of female animals of “Valdostana” breed, aged between one and three years, were used. Since cortisol and cortisone are endogenous corticosteroids, they are commonly present in blank urine samples, therefore their validation experiments were conducted on a second set of samples, collected from animals treated with prednisolone acetate. A pool was obtained from the urines of six Friesian non-lactating cows at the end of their productive cycle. These samples were available from another work (Nebbia et al., 2014) and they were obtained in adherence to Italian regulations and guidelines for the care and use of experimental animals. The study was approved by the Ministry of Health and the local Committee for Animal Welfare. The treatment with this synthetic corticosteroid suppressed the production of endogenous glucocorticoids, making the collected urine negative for cortisol and cortisone. Recently published practical guidelines were followed for reporting analytical calibration results (Olivieri, 2015). Also the guiding principles expressed in the Commission Decision 2002/657/EC were considered (European Commission, 2002). Positive identification of the analytes was expressed by the recognition of 4 identification points, namely the SRM transitions and retention times listed in Supplementary material. Specificity, linearity, precision, trueness, limit of detection (LOD), limit of quantification (LOQ) and ruggedness were evaluated.

3.5.2.5. Creatinine detection

Urinary creatinine was measured using a creatinine assay by ARCHITECT C8000 System (Abbott, Abbott Park, IL, USA). The creatinine assay is based upon the reaction between creatinine and sodium picrate to form a creatinine-picrate complex. The rate of increase in absorbance at 500 nm due to the formation of this complex is directly proportional to the concentration of creatinine in the sample. Since the creatinine kit is proposed for the quantitation of creatinine in human serum, plasma or urine and not for bovine urine, in a previous experimentation the creatinine level was measured in ten samples of bovine urine by means of both the creatinine kit and a quantitative LC-MS/MS method. Strong correlation (Pearson correlation r = 0.9755) and no significant

differences were found between the two sets of results. A p-value of 0.00236 was obtained, allowing us to reject the null hypothesis and accept the alternative hypothesis (i.e. there is actually a positive correlation between the different methods).

3.5.2.6. Chemical analysis of real samples

The analytical method was applied to the urine samples collected for anti-doping control from the winner cows (three weight categories) for each eliminating round and finals during the 2012 and 2013 “Batailles des Reines” tournaments (about 20 eliminating rounds plus one final event each year). Since at the final round participated the winners of the eliminatory rounds, for three animals each year urine samples were collected in two distinct events resulting in a total of 114 samples for 108 animals. Unfortunately, for 24 samples the urine volume was not sufficient for the creatinine analysis. Therefore, creatinine was measured only on 90 samples. Animals arrived on the site of competition around 9:00 a.m. from their locations on the mountains (eliminating rounds) in order to undergo the registration operations. The cows taking part in the “Bataille des Reines” events were brought to the contest field and were allowed to rest before and after each fight. In the eliminating round, from which most of the urine samples arose, the cows generally walked to the contest field coming from the mountain pastures where they spend the summer season. Before each of the matches, and between them, the cows were separated from one another and allowed to rest. On average, the three best-classified cows, from which urine samples were collected, competed in about 3–5 matches. Only in the final event, more complex operations were necessary to transport the animals (about 120–140 cows competed in each category in the final event, but only the winners were sampled). In general, sampled animals equally competed for 3–5 matches each. In general, the competitions started around 1:30 p.m. and went on until late-afternoon. Urine sampling was performed with care to prevent faecal contamination by a licensed veterinarian under conditions of natural micturition at the end of the challenge after watering. After collection, the urine specimens were immediately stored at − 20 °C pending their analysis, which were carried out within one week. Beside the present method, all samples had been previously analysed within an anti-doping control

program and tested negative to all target analytes. The samples underwent two freeze-thaw cycles, one for antidoping analysis and one for corticosteroids analysis.

3.5.2.7. Data analysis

In Table 2 descriptive statistics data relevant to creatinine, cortisol and cortisone concentration are summarized. Prednisolone was found in one sample only, while no prednisone metabolites were detected in the analysed samples. Results on the 90 urine samples collected during “Batailles des Reines” tournaments (group A) are compared with those collected in previous works investigating 6 tethered cows reared in traditional farms, without traces of prednisolone, in the following reproductive status: early pregnancy (4 months) n = 2, late pregnancy (8 months) n = 2, oestral phase n = 1, anoestrus n = 1 (group B) (Vincenti et al., 2012), and with 6 cows, aged between 4 and 6 years, whose urines were collected after slaughter (group C) (Capra, 2016). While urine samples from group A were necessarily collected in the afternoon, after competition, those from groups B and C, i.e. under programmed experiments, were collected in the morning (group B) or at the slaughterhouse (group C). The lack of uniformity in the urine withdrawal timing represents an unavoidable constraint, that did not allowed us to evaluate the changes of cortisol and cortisone levels due to the circadian rhythm.

Missing values, corresponding to concentration levels below the limit of detection (LOD) of the analytical method, were replaced with a value equal to half of the validated LOD, as a rule-of-thumb useful for statistical purposes. Cortisol and cortisone concentrations for each sample were standardized according to the creatinine levels, to compensate for the degree of urine dilution. In particular, cortisol and cortisone values were multiplied by a correction factor equal to the ratio between the average creatinine level (calculated from all animals participating to the “Batailles des Reines” tournaments) and the creatinine value for each cow. Then, base-10 logarithm transformation was performed on the collected data. The creatinine, cortisol, and cortisone data values for all animals are reported in the Supplementary Material. Boxplots, Gaussian and Kernel density estimation (KDE) plots were evaluated to identify possible outliers and compare the data distributions. Then, t-tests together with one-way ANOVA and Tukey's tests were

performed to compare the corrected cortisol and cortisone mean values for the different groups of animals. All calculations were performed with R software version 3.2.2 (R Core Team, 2015).

3.5.3. Results

3.5.3.1. Method development

In the course of method development, liquid-liquid extraction was compared with SPE extraction, the latter yielding cleaner extracts and consequent better sensitivity. Three solvents were tested for liquid-liquid extraction: ethyl acetate, tert-butyl-methyl ether and diethyl ether. The best recovery results were obtained with ethyl acetate (46%), but much lower than those obtained with SPE (87%). The chromatographic run was optimized so as to assure adequate separation of the 20α- and 20β-dihydroprednisolone isomers (see Fig. 2). Calculated resolution is 1.0933 (Snyder et al., 2010). No other compounds with the same MS-transitions was affected by separation problems. Positive and negative ionization modes were compared in ESI-MS setting: the latter proved to provide better sensitivity for the entire set of target analytes. The ion [M + acetate]− ion was selected as the precursor for all the analytes.

3.5.3.2. Validation

Occurrence of possible interferences from endogenous substances was tested by the analysis of twenty blank urine samples as described above; no interfering substances were found. Linear matrix calibration curves were built for each analyte (0, 0.4, 0.8, 1.2, 2.5, 5 ng/mL for prednisolone, cortisol and cortisone, 0, 0.3, 0.5, 1, 2, 5 ng/mL for 6β-hydroxyprednisolone and prednisone and 0, 1, 2, 5, 8, 10 ng/mL for 20β-dihydroprednisolone, 20α-dihydroprednisolone and 20β-dihydroprednisone), with two replicates for each level. Since IUPAC discourages the correlation coefficient (R) as an indicator of linearity in the correlation between concentration and signal (Danzer and Curriet, 1998), we reported the experimental F value, corresponding to the ratio of residual variance to squared pure error, and the tabulated critical F for comparison in order to test linearity (lack-of-fit test). This test is the best linearity indicator, as recommended by IUPAC. The lack-of-fit test was passed for all the analytes in the

concentration ranges considered (see Table 1), with F-values below 1.4 for the main analytes (cortisol, cortisone, prednisone, prednisolone). LOD and LOQ were calculated as reported by Olivieri (Olivieri, 2015) and ranged between 0.13 ng/mL to 0.69 ng/mL and to 0.39 ng/mL to 2.08 ng/mL respectively (see Table 1).

Table 1. Calibration curves obtained from spiked urine samples (quantitative determinations) with corresponding linearity test results, LOD and LOQ values (where F_{tab} and F_{exp} are the tabulated critical and the experimental F values, respectively, LOD is the limit of detection and LOQ is the limit of quantitation).

Analyte	Spiked urine			LOD (ng/mL)	LOQ (ng/mL)
	Linearity range (ng/mL)	F_{tab}	F_{exp}		
6β-Hydroxyprednisolone	0–5.0	4.060	0.800	0.17	0.51
20α-Dihydroprednisolone	0–10		3.230	0.57	1.72
20β-Dihydroprednisolone	0–10		3.230	0.69	2.08
20β-Dihydroprednisone	0–7.6		2.345	0.67	2.04
Prednisolone	0–5.0		0.800	0.14	0.44
Prednisone	0–5.0		0.772	0.16	0.49
Cortisol	0–5.0		1.327	0.14	0.42
Cortisone	0–5.0		1.254	0.13	0.39

Precision and trueness were evaluated at three levels and were estimated both intra-day (n = 6) and inter-day (n = 18). An ANOVA test was set for each validation level. Intra-day precision, expressed by the experimental coefficients of variation, ranged between 2.9% and 16%, while inter-day precision was between 5.3% and 19%. Quite similarly, limited bias from true values were recorded (from − 6.2% to + 7.8% for intra-day trueness and from − 5.9% to + 6.7% for inter-day trueness).

Ruggedness test was conducted by introducing slight variations (± 10% maximum) to previously selected analytical parameters and observing the resulting changes in term of quantitative response on blank urine samples spiked at the lowest validation level (see Supplementary material). A Youden approach was used, in order to minimize the number of experiments required. It was found that some factors influenced the final results for 6β-hydroxyprednisolone and the critical factors were identified using a t-test.

They are the methanol percentage in SPE washing mixture - methanol:sodium hydroxide 0.02 M (30:70 v/v) - and its volume.

The urine extracts were stable for six days at least, if stored under appropriate conditions, as proved by the negligible differences (i.e., lower than the limit of repeatability of the method) in the absolute concentration values determined at day 0 and day 6 for all the target analytes. Complete validation data are reported in the Supplementary Materials.

3.5.3.3. Real samples

Cortisol and cortisone were found above the LOQ value in all the urine samples, with only one exception. In 2012 and 2013 sample collections, the average concentrations measured for cortisol were 8.65 ± 5.24 ng/mL and 8.00 ± 5.11 ng/mL respectively, and for cortisone 4.90 ± 3.09 ng/mL and 4.96 ± 3.14 ng/mL, respectively. It was observed no significant difference in the comparison between the two years (two-tailed t-test, $p < 0.01$).

Prednisolone was found in only one sample, at a concentration of 1.45 ng/mL. In this sample, both cortisol and cortisone were found at the highest concentration among all 114 urines, i.e. 35.5 and 18.1 ng/mL, respectively. Trace amounts of prednisolone were also found in three other samples (at estimated concentrations of 0.35, 0.12 and 0.10 ng/mL, respectively, all values were under the LOQ). In the same samples, cortisol and cortisone concentrations were above the average, but not exceedingly high (14.3 and 7.78 ng/mL, 11.6 and 10.8 ng/mL, 10.3 and 9.55 ng/mL, respectively). Some other samples exhibited higher cortisol and cortisone concentrations, but no prednisolone was found. In no samples, any of prednisolone metabolites were detected, not even at trace level, unlike what was observed in the urine samples of bovines treated with prednisolone (Leporati et al., 2013; Nebbia et al., 2014).

3.5.3.4. Descriptive statistics

Minimum, maximum, quartiles and the median values of creatinine, cortisol and cortisone values were calculated for the 3 groups of cows (A–C). Data are reported in

Table 2(a–c). Creatinine detection was possible only on 90 samples out of 114 because insufficient volume was available for the remaining 24 urines.

Table 2. Descriptive statistics relevant to the urinary creatinine (2a), cortisol (2b) and cortisone (2c) values of cows from “Batailles des Reines” tournaments (A), the tethered cows reared in traditional farms, without traces of prednisolone (B) and the slaughtered ones (C). Cortisol and cortisone values were multiplied by a corrected factor according to the creatinine data in order to standardize the different dilution degrees of the urine samples.

Cows	Minimum	1st Quartile	Median	3rd Quartile	Maximum
2a					
A	61	151	214	291	750
B	19	34	60	111	168
C	14	62	107	198	248
2b					
A	1.23	4.38	9.05	14.1	48.8
B	0.80	1.03	1.63	3.93	128
C	9.11	14.8	44.0	112	291
2c					
A	0.90	2.54	4.86	8.61	32.6
B	0.59	0.75	0.84	2.31	58.9
C	6.42	10.3	19.4	41.2	141

Boxplots and distribution plots relevant to cortisol (Fig. 1a–b) and cortisone (Fig. 1c–d) values were calculated. In particular, Fig. 1(b–d) shows both Gaussian and KDE distribution plots (red and green lines, respectively) for the 90 urine samples of the cows participating to the “Batailles des Reines” tournaments (group A, blue histograms), together with their mean, standard deviation and CV% values. Cortisol and cortisone levels of the 6 tethered cows reared in traditional farms (group B) and of slaughtered animals (group C), are reported too. Although boxplots show overlaps among the groups, appreciable difference exists among the central quartiles for the three groups, whose significance was tested to evaluate whether the populations can be distinguished by the target analytes. Furthermore, 5 out of 6 animals of groups B and C showed

largely increased cortisol and cortisone values when they were transported to the slaughterhouse and had to wait before their slaughter (i.e. animals 2, 3, 4, 5 and 6). Conversely, animal #1 showed an opposite trend.

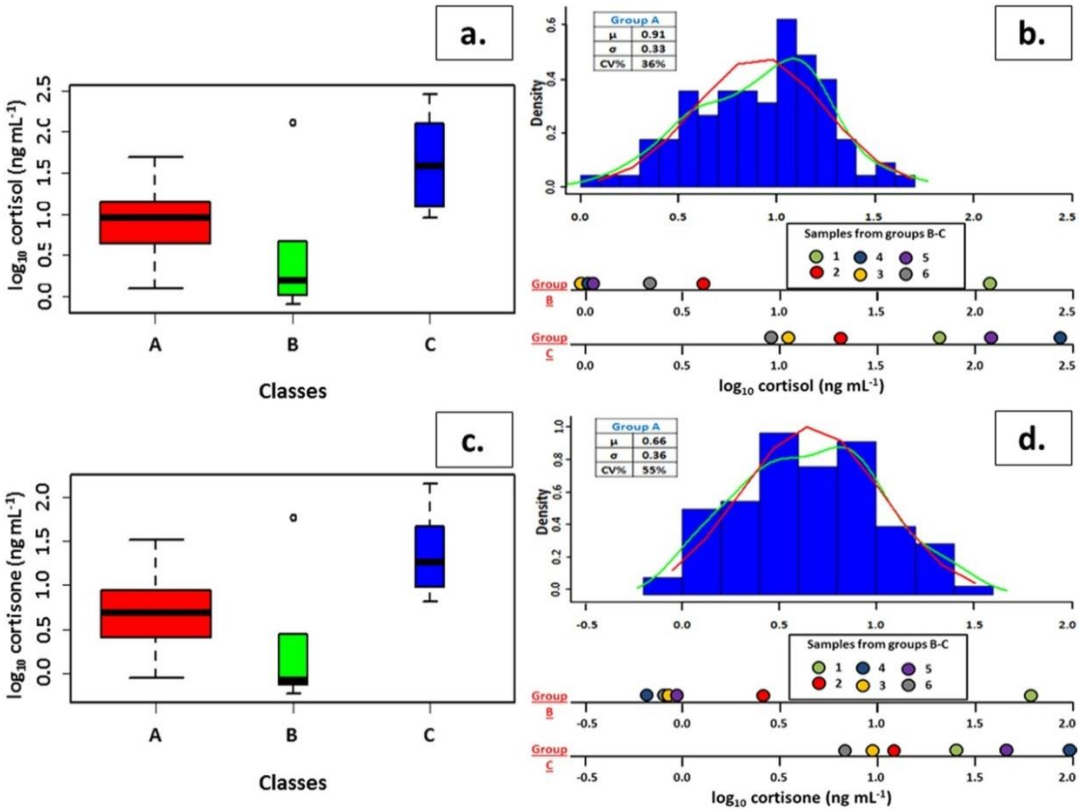

Fig. 1. Boxplots, histograms (only for group A) Gaussian (red line, only for group A), KDE (green line, only for group A) plots relevant to cortisol (a–b) and cortisone (c–d) values of the different groups of cows, where (A) represents the animals from “Batailles des Reines” tournaments (n = 90), (B) indicates the tethered cows reared in traditional farms, without traces of prednisolone (n = 6) (Vincenti et al., 2012) and the slaughtered ones (C), whose urines were collected after the death (n = 6) (Capra, 2016). Cortisol and cortisone values of the 6 animals from groups B and C are indicated by circles and arranged below the plots relevant to the urine samples from group A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

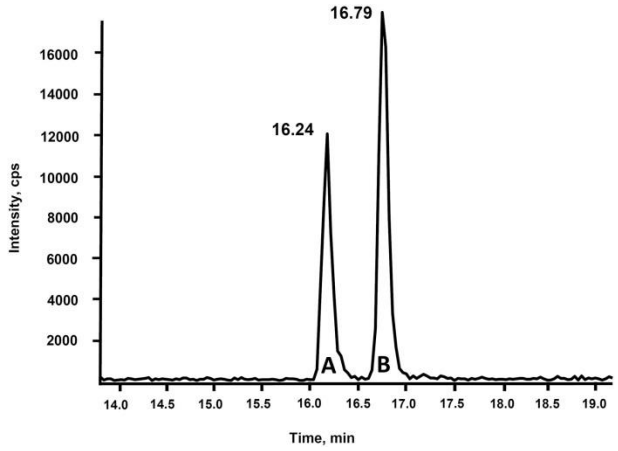

Fig. 2. Chromatographic separation of the two isomers 20α- (peak B) and 20β- (peak A) dihydroprednisolone.

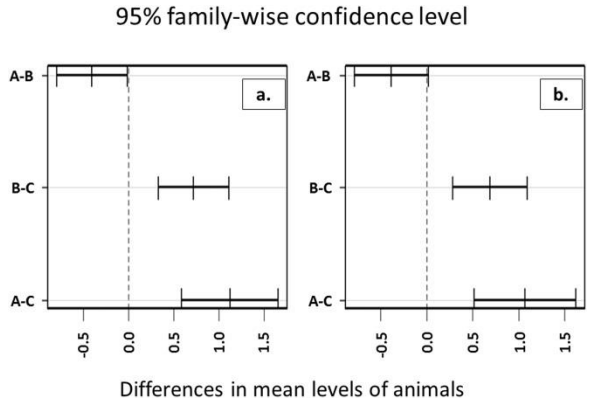

Fig. 3. Tukey's test plot relevant to the 95% family-wise confidence level of all the pairwise comparisons (reported on the Y-axis) relevant to the cortisol (a) and cortisone (b) concentration of the different groups of cows, where (A) represents the animals from “Batailles des Reines” tournaments (n = 90), (B) indicates the tethered cows reared in traditional farms, without traces of prednisolone (n = 6) (Vincenti et al., 2012) and the slaughtered ones (C), whose urines were collected after the death (n = 6) (Capra, 2016).

3.5.4. Discussion

The appraisal of cortisol, cortisone, and prednisolone as potential biomarkers for assessing the occurrence of fighting-generated stress requires the consideration under which the cows taking part in the various “Bataille des Reines” events are brought to the contest field and are allowed to rest before and after each fight (see “Material and methods – Chemical analysis of real samples”). The natural conditions under which the cows walk to the contest field and rest before competing made us conclude that

transport and environmental conditions are not likely to add a significant contribution (bias) to the stress originated by the fight itself, at least for the eliminating rounds.

By comparing the global analytical results of the present investigation with those collected in a previous study (Vincenti et al., 2012), it can be observed that the stress generated by the struggle during each “Bataille”, and measured by means of cortisol and cortisone concentrations, is significantly higher (t-test, p value < 0.05) than that commonly experienced by the cows living in a farm (Fig. 1, groups A and B).

On the other hand, the comparison of the same data with those collected in another more recent study (Capra, 2016), show that the fighting stress produced in the “Batailles” is significantly lower (t-test, p value < 0.05) than that experienced by the animals that are transported to the slaughterhouse and wait before their killing (Fig. 1, groups A and C). Furthermore, one-way ANOVA and Tukey's tests were performed on the collected data with the aim of comparing the analytical results of this study with those from the previous studies, involving groups of cows living in a farm or slaughtered. At first, one-way ANOVA test was performed and p-values equal to 7.39×10^{-6} and 3.36×10^{-5} for cortisol and cortisone, respectively, were observed, thus revealing that there was at least one group significantly different from the other ones. Secondly, Tukey's test was employed with the aim of investigating such differences more accurately. As it is reported in Fig. 3(a–b), all groups proved to be significantly different as no confidence intervals including the zero value were observed. In this case, Tukey's test proved that all the tested groups of cows were significantly different as p-values lower than 0.05 were obtained for all the pairwise comparisons of both cortisol (Fig. 3a) and cortisone (Fig. 3b), with the unique exception of the cortisone levels from samples collected during “Batailles des Reines” tournaments (A) and those from the cows reared in loose housing farms (B), that shows a p-value slightly higher than 0.05 (i.e. 0.067), possibly because cortisone is a somewhat less efficient stress biomarker than cortisol.

3.5.5.Conclusions

The repeated fighting and environmental conditions produced in the “Batailles des Reines” events assuredly generates relatively high concentrations of cortisol and cortisone in the urines of the struggling cows, together with occasional presence of traces of prednisolone. On this basis, it is apparent that a significant stress is induced in the “Batailles des Reines” fighting cows, with respect to the general living conditions of the cows reared in loose housing farms. However, the fighting stress of the “Batailles des Reines” is considerably lower than that induced in the bovines transported to the slaughterhouse, which is extremely high indeed (De Clercq et al., 2015).

While the occasional presence of prednisolone in the urine of the competing cows can be somehow associated with the fighting stress, its rare occurrence (3%) make prednisolone an unreliable biomarker for stress. The differentiated and random production of prednisolone in stressed animals might be attributed to both the intensity and the source of stressful conditions.

The presence of prednisolone in the urine of the fighting cows was in no cases associated with the concurrent presence of its metabolites, particularly 20β-dihydroprednisolone, which was by contrast observed at concentrations often exceeding the parent compound when exogenous prednisolone was administered, both at high (therapeutic) and low (mimicking illicit purposes) dose (Leporati et al., 2013; Nebbia et al., 2014). Simultaneously, suppression of cortisol and cortisone was documented in these studies.

While the official cut-off of 5.0 ng/mL for prednisolone avoids false positive results due to endogenous production under stressful conditions, it does not appear to be adequate to ascertain the illicit repeated low-dose administration of prednisolone typical of a growth–promoting schedule.

For these reasons, a more biologically-oriented strategy involving the simultaneous determination of urinary cortisol, cortisone, prednisolone, prednisone, and 20β-dihydroprednisolone is likely to represent an effective approach for surveillance purposes and consumer's protection.

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3.6. Determination of veterinary antibiotics in bovine urine by liquid chromatography–tandem mass spectrometry

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

Highlights

Analysis of antibiotics in bovine urine, as a non-invasive matrix, is convenient.
Antibiotics presence in bovine urine might be considered as an environmental risk.
Multiclass HPLC–MS/MS method for antibiotics assay in bovine urine was validated.
Majority of samples enrolled in this study were positive on tetracyclines.
It is crucial to screen antibiotics in bovine urine before processing in food industry.

Abstract

A follow-up of antibiotics (tetracyclines, fluoroquinolones, cephalosporins, penicillins and amphenicols) in the bovine urine is important for two reasons: to understand if they are still present in organism, and whether their occurrence in urine might be considered as an environmental risk. A validated HPLC–MS/MS method (Decision 2002/657/EC) for antibiotics determination in bovine urine was developed. CCα and CCβ were in the range of 0.58–0.83 and 0.55–1.1 ng mL^{-1}, respectively. Recoveries were 92–108%, with inter-day repeatability below 12%. Analysis of bovine urine revealed frequent presence of tetracyclines, which was related with animal's age. The cause, most presumably, might be found in different therapeutic protocols applied for veal calves and young bulls enrolled in this study. Most abundant was oxytetracycline with highest level in veal calves (1718 ng mL^{-1}) vs. young bulls (2.8 ng mL^{-1}). Our results indicate the necessity of antibiotics monitoring in bovine urine before animals undergo further processing in the food industry.

Keywords: Tetracyclines; Fluoroquinolones; Cephalosporins; Penicillins; Amphenicols; Bovine urine; LC–MS/MS

3.6.1. Introduction

Antibiotics constitute an important group of pharmaceuticals that have been widely used in veterinary medicinal practices to treat a wide range of diseases. Only 20% of antibiotics are used to medicate sick animals, while 80% are used as production tools: either to prevent diseases that arise from the way animals are treated during breeding (so-called "production diseases"), or for growth-promotion purposes. The widespread

exploitation of antibiotics in the past has favoured the growth of resistant microorganisms, resulting in ever widening antimicrobial resistance, an important human health issue. On Dec. 11th, 2013, the U.S. Food and Drug Administration (FDA) announced important steps to ensure the judicious use of antibiotics in food animals, as one approach to addressing antimicrobial resistance in human medicine (FDA, implementing plan). European Union (EU) national and international authorities emphasise the need for environmental and health risk assessment for chemicals with antimicrobial effects (Kools, Moltmann, & Knacker, 2008; Serratosa et al., 2006). As a result, new strategies to reduce antibiotic utilisation in animal husbandry have been proposed (Trevisi et al., 2014). The increasing awareness of food safety with respect to antimicrobial resistance (European Community, 2005a) has resulted in the banning of any antibiotic with growth-promoting activity: antibiotics are only allowed to be added to animal feed for therapeutic purposes (European Community, Regulation 1831/2003/EC). This decision was based on opinions from the Scientific Steering Committee, which recommended the progressive phasing-out of antibiotics used for growth stimulation, while still preserving animal health and animal welfare (European Community, 2005b).

Generally, the food animal industry has grown into an integrated production system where large quantities of antibiotics are administered to the animals for therapeutic or sub-therapeutic purposes. This may lead to accumulation of residues in food matrices as milk (Zhan et al., 2012) or meat (Stubbings & Bigwood, 2009). These residues may include the non-altered parent compounds as well as metabolites, and may have direct or indirect toxic effects on consumers. Logically, these compounds are excreted by the animals and end up in the urine and faeces. This, consequently, carries substantial environmental problems, as during the maturation process, the animal dejections become manure, which is frequently used in agriculture.

To minimise the exposure of antibiotics to humans and safeguard public health, European legislation (Commission Regulation ECC/2377/90 and 37/2010) has established corresponding tolerance levels, termed as maximum residual limits or levels (MRLs), for controlling the use of antibiotics in food-producing animals. Also, the Italian National Residue Control Plan (NRCP, 2014) is very precise: samples taken at

slaughterhouse are screened for the presence of residues/metabolites on the bases of MRLs. Analysis of positive screening tests for these residues in animal products must adhere to legislation laid out in Council Directive 96/23/EC and Commission Decision 2002/657/EC, whereby suitable confirmatory methods are based on chromatographic analysis and mass spectrometric detection.

Despite the above mentioned research that deals with the determination of antibiotics in food, manure (Panseri et al., 2013), soil (Carballo, Barreiro, Scharf, & Gans, 2007) and waste water (Babic, Asperger, Mutavdzic Horvat, & Kastelan-Macan, 2006), the data on the animal's urine content of the most frequently exploited antibiotics in veterinary medicine are sporadic. To the best of our knowledge, no method has previously been reported for simultaneous screening of major antibiotics groups in bovine urine as a starting matrix, although reports on detection of antibiotics in general in bovine urine are already available (Heller, Smith, & Chiesa, 2006; Kondo, Morikawa, & Tateyama, 1989). In addition, reports on multiclass analysis of antibiotics in human urine has been published recently (Cazorla-Reyes, Romero-González, Frenich, Rodríguez Maresca, & Martínez Vidal, 2014; Wang, Wang, Zhou, & Jiang, 2014) as well as determination of some individual groups e.g. tetracyclines (Jin et al., 2010).

Urine analysis is a useful alternative to improve the effectiveness of surveillance plans, as it offers several advantages compared to the analysis of other biological samples (liver, kidney, blood, muscles, etc.). Urine collection, similarly to hair sampling (Fernández et al., 2014) is a non-invasive procedure and offers a possibility to evaluate drug withdrawal time after eventual inevitable treatment of sick animals.

The antibiotics considered in this investigation were selected using the following criteria: documented frequent utilisation, lower degree of metabolism in animals' bodies, and environmental traits. Additionally, the exemplification of different classes of antibiotics was aimed at covering a wide-ranging assortment of substances with antimicrobial activity used in Italian animal husbandry. Therefore, our antibiotics of interest were amoxicillin and ampicillin (penicillins), chlortetracycline, doxycycline, oxytetracycline, tetracyclines (tetracyclines), ciprofloxacin, enrofloxacin, lomefloxacin, marbofloxacin (fluoroquinolones), cephalexin, cefquinome (cephalosporins), florfenicol, florfenicol amine (amphenicols APHs) and streptomycin (aminoglycoside).

The simultaneous determination of these compounds is especially difficult because of large differences in their physicochemical properties, such as polarity, solubility, pKa, and stability. Many liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods have been employed for a multiclass determination of antibiotics in various matrices including foodstuffs and environmental samples, relying on different purification strategies (Boix et al., 2014). There are various factors which need to be taken into consideration during development of a method that would be capable of analysing the wide range of compounds to the required level (e.g. pH, extraction methods, mobile phase composition, mass spectrometry acquisition parameters).

This paper reports the results of our work on multi-residue analysis using LC–MS/MS to determine the concentrations of target antibiotics, with a single SPE pre-treatment, chromatographic separation and mass detection method. The method was developed in order to test eventual presence of antibiotics residues in bovine urines collected at a slaughterhouse.

3.6.2. Materials and methods

3.6.2.1. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid 98–100% and hydrochloric acid 37% were obtained from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA). Water was purified by a Milli-Q system. The chemicals for the preparation of artificial urine were from Sigma-Aldrich (St. Louis, MO, USA). The extraction cartridges (Oasis HLB 3cc, 60mg) were provided by Waters (Milford, MA, USA). Amoxicillin, ampicillin, cefalexin, cefquinome sulphate, florfenicol, florfenicol amine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, streptomycin solution (1mg mL^{-1} in 1 mM EDTA) and sulfameter (internal standard IS) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Oxytetracycline was obtained from European Pharmacopoeia Reference Standard.

3.6.2.2 Artificial urine preparation

Artificial urine was prepared in our laboratory for the validation studies, as described by Fabregat et al. (2013). 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 aschloride·$2H_2O$, 5.2 g of sodium chloride, 0.0012 g of iron II sulfate·$7H_2O$, 0.49 g of magnesium sulfate·$7H_2O$, 3.2 g of sodium sulfate·$10H_2O$, 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.

3.6.2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and kept at –40°C. Working solutions, containing each of the studied analytes at the concentrations of 10 and 100 ng mL^{-1}, were prepared daily. Each working solution was maintained at 4°C during the method validation procedures.

3.6.2.4. Sample collection

In order to verify the developed method in actual conditions, 39 urine samples were collected at the slaughterhouse before processing. The samples arrived from three different slaughterhouses and were randomly collected from Friesian veal calves (6 and 11 months old) and Limousine young bulls (18 months old). Following collection they were immediately frozen (−20 °C) and taken to laboratory. During the transportation, the samples remained frozen using the dry ice. Upon the arrival in laboratory the samples were finally stored at −40 °C until the analysis was performed.

3.6.2.5. Sample extraction

Urine samples (5.5 mL) were centrifuged 5 min at 2500g at 4 °C. Five mL of supernatant was spiked with the internal standard to the final concentration of 2 ng mL^{-1}. The compounds of interest were extracted by using the Oasis HLB Cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5 M HCl and 3 mL of Milli-Q water. The samples were loaded, and after all the urines passed through the SPE, the cartridges were washed with 3 mL of water and 3 mL of methanol: water (20:80, v/v). Finally, samples were eluted using 5 mL of methanol and

collected in a 15 mL polypropylene tube. The eluate was evaporated in a rotary vacuum evaporator. The dried extract was reconstructed in 200 µL of methanol:water (10:90 v/v), then transferred to a vial which was placed in auto-sampler. The injection volume was 10 µL.

3.6.2.6. LC-MS/MS analyses

LC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), constituted by a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with 20 µL loop. During method development, attempts to accomplish satisfactory analytical separation were made employing Synergi Hydro RP reverse-phase HPLC column 150 × 2.0 mm, internal diameter 4 µm, with a C18 guard column, 4 × 3.0 mm (Phenomenex, Torrance, CA, USA). As this column did not give satisfactory results, final chromatographic separation was achieved using a Raptor (Restek) biphenyl column (150 × 2.1 mm, internal diameter 2.7 µm with a Raptor (Restek) biphenyl 2.7 (5 × 2.1 mm) guard column, which was kept at 30 °C. Simultaneous separation of studied pharmaceuticals were achieved at the following flow rate and mobile phase gradient program. The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (aqueous formic acid 0.1%)) and B (MeOH). The elution started with 98% of A, which was maintained for 5 min, followed by a linear gradient toward 50% up to 22th min. Subsequently, the mobile phase B gradually increased on 95% (at 24th min), which remained constant up to 29th min. The initial conditions were reached at 31st min, with equilibration time that included interval from 31st to 40th min. In order to provide good separation of all antibiotics, avoiding the peaks overlapping, we applied two flow rates during the chromatographic run: first 5 min and equilibration period (both with 98% of A) were set at 0.1 mL/min, while rest of the run was performed at 0.2 mL/min.

The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher, San Jose, CA, USA) equipped with an electrospray interface (ESI) set for all analytes in the positive (ESI+) electrospray ionisation modes and in the negative (ESI−) only for florfenicol. Acquisition parameters were optimised in the electrospray mode by direct continuous pump-syringe infusion of the standard solutions of analytes at a

concentration of 1 μg mL^{-1}, a flow rate of 20 μL min^{-1} and an MS pump rate of 100 μL min^{-1}. The following conditions were used: capillary voltage 3.5 kV; ion-transfer capillary temperature 340 °C; nitrogen as sheath and auxiliary gases at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr; and peak resolution 0.70 Da at full width half maximum (FWHM). The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. Three diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in multiple reaction monitoring (MRM). The selected diagnostic ions, one of which was chosen for the quantification, and the collision energies are reported in Table 1. The LC–MS/MS chromatograms for the antibiotics at the lowest concentration level of the validation are shown, together with the ion spectra, in Fig. 1; in addition, at the end, there is the internal standard (2 ng mL^{-1}). Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

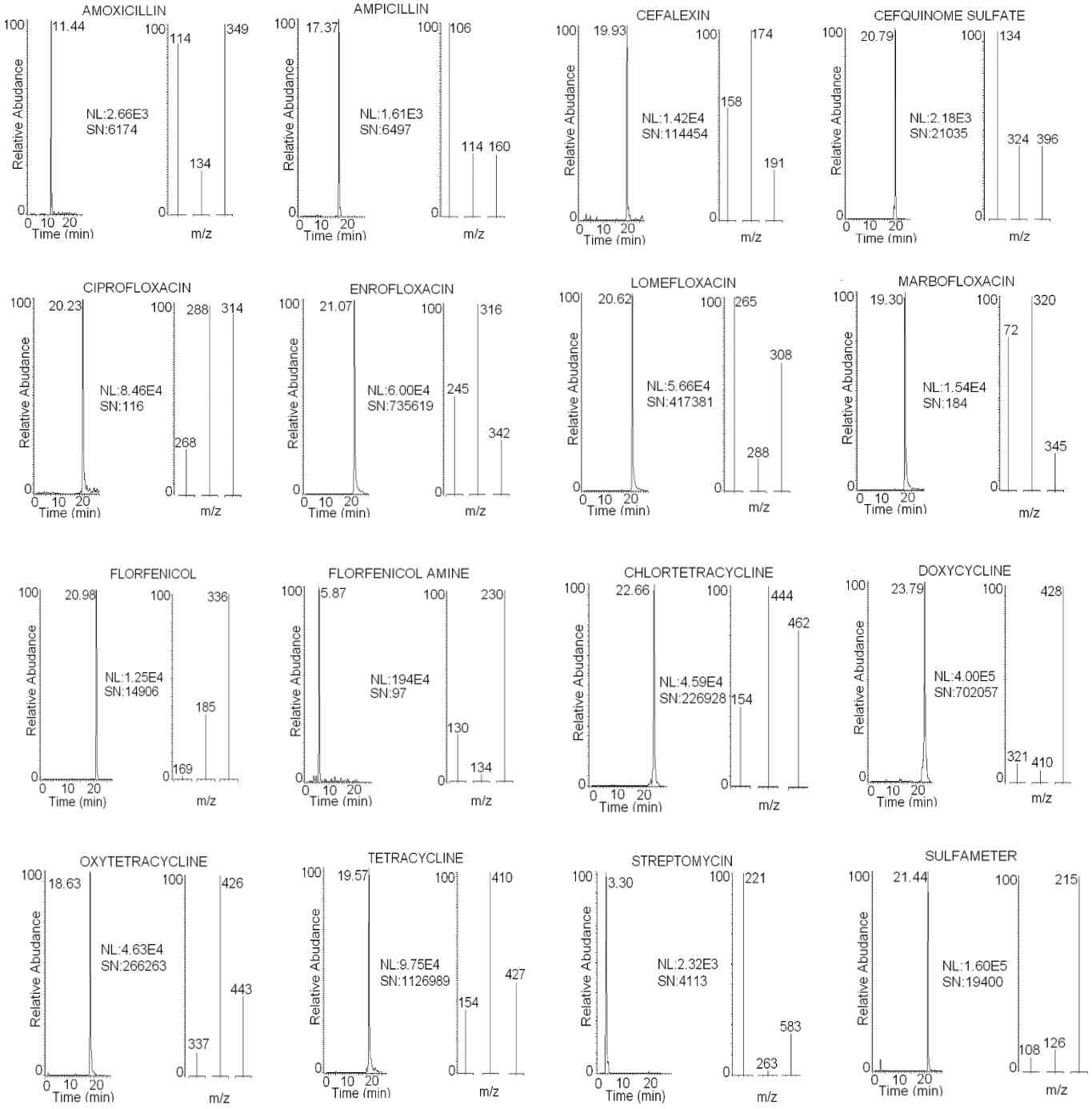
Table 1. MS/MS conditions for the MRM acquisitions of investigated antibiotics. Ions for quantification are in bold. The values in brackets represent the relative intensities (%). CE: collision energy, subscripted and expressed in volts.

Analyte	Precursor ion* (*m/z*)	Product ions$_{CE}$ (*m/z*)	ESI
Amoxicillin	366	**114(80)$_{20}$**, 134(21)$_{31}$, 349(100)$_{7}$	(+)
Ampicillin	350	**106(100)$_{18}$**, 114(14)$_{29}$**,** 160(14)$_{14}$	(+)
Cefalexin	348	158(63)$_{5}$, **174(100)$_{15}$,** 191(23)$_{6}$	(+)
Cefquinome	529	**134(100)$_{15}$**, 324(43)$_{15}$, 396(44)$_{10}$	(+)
Ciprofloxacin	332	268(16)$_{22}$, **288(100)$_{17}$**, 314(94)$_{21}$	(+)
Enrofloxacin	360	245(49)$_{26}$, **316(100)$_{18}$**, 342(29)$_{21}$	(+)
Lomefloxacin	352	**265(100)$_{23}$**, 288(16)$_{19}$, 308(63)$_{16}$	(+)
Marbofloxacin	363	72(83)$_{23}$, **320(100)$_{15}$**, 345(18)$_{21}$	(+)
Florfenicol	356	169(1)$_{39}$, 185(35)$_{21}$, **336(100)$_{12}$**	(-)
Florfenicol amine	248	130(24)$_{23}$, 134(8)$_{28}$, **230(100)$_{11}$**	(+)
Chlortetracycline	479	154(39)$_{27}$, **444(100)$_{21}$**, 462(69)$_{16}$	(+)

	Doxycycline	445	$321(10)_{31}$, $410(8)_{24}$, **$428(100)_{19}$**	(+)
	Oxytetracycline	461	$337(26)_{29}$, **$426(100)_{19}$**, $443(52)_{12}$	(+)
	Tetracycline	445	$154(38)_{30}$, **$410(100)_{19}$**, $427(43)_{14}$	(+)
	Streptomycin	614	**$221(100)_{42}$**, $263(1)_{35}$, $583(20)_{19}$	(+)
	Sulfameter	281	$108(6)_{26}$, $126(11)_{25}$, **$215(100)_{17}$**	(+)

* $[M+H]^+$ for all compounds except: $[M-H]^-$ - florfenicol and $[M+H+CH_3OH]^+$ - streptomycin

Figure 1. LC–MS/MS chromatograms and relative ion spectra of analytes in artificial urine spiked at the lowest concentration level validated (0.5 ng mL^{-1}). At the end relative internal standard (concentration = 2 ng mL^{-1}).



3.6.2.7. Method validation

The validation was performed according to the criteria and recommendations of the European Commission Decision 2002/657/EC. Starting with method validation, we –

completely unexpectedly faced with a substantial number of positive results (generally tetracyclines). This, along with the fact that bovine urines were collected from few slaughterhouses, and that they differed among each other considerably as far as the presence of common interferences were concerned, we decided to use artificial urine as a surrogate matrix for method validation, following the directions of Van de Merbel (2008).

For each analyte, the method performance was assessed through its qualitative parameters, such as molecular identification in terms of retention time (RT) and transition ion ratios; through its quantitative parameters, such as the linearity, recovery, accuracy in terms of trueness and of precision expressed as the intra- and inter-day repeatability; and through the analytical limits [decision limit (CCα) and detection capability (CCβ)].

The instrumental linearity was evaluated by drawing five-point calibration curves in the solvent containing a fixed amount of the internal standards (2 ng mL^{-1} each), with analyte concentrations corresponding to 0.5, 1.0, 2.0, 3.0 and 5.0 ng mL^{-1}. Also, six-point calibration curves (0–10 ppb) both in blank and artificial urine were prepared in order to verify the artificial urine suitability.

Matrix calibration curves were obtained by spiking urine samples with each of the analytes, resulting in three analytical series; each series had three concentration levels (0.5, 1.0 and 1.5 ng mL^{-1}) in six replicates. The precision in terms of intra-day repeatability was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at three concentration levels. For inter-day repeatability the same procedure was repeated in three analytical series executed in three different days. The data from the matrix calibration curves were used to calculate the decision limit (CCα) and the 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.

Whenever the concentrations found in the real samples (namely of tetracyclines) were above the highest point of matrix calibration curve used for validation, corresponding quantification calibration curves were constructed, as well.

The trueness was assessed through recovery and was evaluated using the matrix curve results from the three analytical series, expressed in terms of a percentage of the measured concentration with respect to the spiked concentration.

We evaluated robustness using the approach of Youden, (Commission Decision 2002/657/EC) that is a fractional factorial design.

Selectivity and specificity were tested for all analytes, except for tetracyclines that were almost always present in our real bovine samples.

3.6.2.8. Statistical analysis

The statistical analysis was performed only when the antibiotics (exclusively tetracyclines) were found. The Kolmogorov–Smirnov test was used to check the normality of results. Non-parametric Kruskal–Wallis one way analysis of variance by ranks and, subsequently, all pairwise multiple comparison procedures (Dunn's Method) were used to check the differences between the medians of three datasets. The statistical analysis was performed using Microsoft Excel spreadsheets and Sigma Stat (Statistical Analysis System, version 2.03) statistical software package (Jandel Scientific GmbH, Herckrath, Germany). A *p*-value of <0.05 was defined as the level of statistical significance.

3.6.3. Results and discussion

3.6.3.1. Method development

During method development, different options were evaluated to optimise sample extraction, chromatographic separation and detection parameters.

The critical step in method setup is the sample preparation procedure, especially when compounds of interest possess amphoteric properties. Hydrophilic–lipophilic balance cartridges (HLB) have been commonly used for this purpose as referred to in the literature (Moreno-Bondi, Marazuela, & Rodriguez, 2009). In this study, this turned out to be a good strategy as selected antibiotics were retained and extracted with satisfactory efficiency: the mean method recoveries ranged between 92% and 108% for all antibiotics investigated (Table 2). However, due to the complexity of the matrix, in most cases an extraction step for sample clean-up requires pH adjustment in order to

achieve the optimal recovery. Regardless, we did not notice significant improvement in extraction recovery (*p*-value <0.05), bringing the pH of the sample to the weak acidic conditions, pH-4 (data not shown), as was shown for other types of sample (Tong, Li, Wang, & Zhu, 2009). As a matter of fact urine exhibits weak basic properties that were favourable to the satisfactory extraction of amoxicillin and ampicillin, which were proven to be susceptible to degradation in acidic conditions (Khuroo, Monif, Verma, & Gurule, 2008).

Table 2. Validation data for the 15 studied antibiotics in artificial urine.

Analyte	CCα	CCβ	Concentration level (ng mL^{-1})	Recovery % (*n*=18)	Repeatability intra-day (CV; *n*=6)	Repeatability inter-day (CV; *n*=18)
Amoxicillin	0.63	0.74	0.5	98	6	11
			1.0	102	5	11
			1.5	99	6	7
Ampicillin	0.64	0.77	0.5	101	10	12
			1.0	99	8	11
			1.5	100	8	9
Cefalexin	0.61	0.71	0.5	106	6	10
			1.0	94	4	9
			1.5	102	10	10
Cefquinome	0.65	0.83	0.5	100	10	13
			1.0	100	12	20
			1.5	100	12	12
Ciprofloxacin	0.67	0.87	0.5	103	11	15
			1.0	97	12	20
			1.5	101	9	10
Enrofloxacin	0.62	0.73	0.5	102	6	11
			1.0	98	6	11
			1.5	101	7	7
Lomefloxacin	0.70	0.90	0.5	104	8	17
			1.0	96	9	17
			1.5	101	12	13
			0.5	97	5	8

Marbofloxacin	0.53	0.55	1.0	103	6	6
			1.5	99	5	7
Florfenicol	0.73	0.97	0.5	102	10	20
			1.0	98	9	19
			1.5	101	12	14
Florfenicol amine	0.63	0.75	0.5	102	10	11
			1.0	98	11	12
			1.5	101	10	10
Chlortetracycline	0.63	0.74	0.5	93	6	11
			1.0	107	5	10
			1.5	98	5	6
Doxycycline	0.58	0.68	0.5	108	7	7
			1.0	92	12	12
			1.5	103	8	8
Oxytetracycline	0.70	0.89	0.5	108	15	17
			1.0	92	13	15
			1.5	103	10	11
Tetracycline	0.63	0.74	0.5	108	8	11
			1.0	92	7	10
			1.5	103	7	7
Streptomycin	0.83	1.11	0.5	107	12	17
			1.0	93	4	8
			1.5	102	12	12

To obtain satisfactory separation and high sensitivity of the target analytes, an optimisation of the liquid chromatography and mass spectrometer conditions was performed by the injection of standard solutions of a mixture of all analytes. The column chosen was the Raptor (Restek) biphenyl column (150 × 2.1 mm, 2.7 µm), which achieved superior resolution and sensitivity as compared with the Synergi Hydro RP reverse-phase HPLC column. The Synergi column was our first choice regarding its potential to retain polar compounds, and it showed good performances for the majority of compounds involved in this study. Nevertheless, it turned out to be problematic where streptomycin was concerned, which had practically been lost in all attempts to prolong its retention time. On the contrary, the biphenyl stationary phase from Raptor proved to be particularly suitable for this purpose as this column was potentially compatible with

98% of the aqueous mobile phase. Previously, it was suggested that hydrophilic interaction liquid chromatography (HILIC) or volatile ion pair approach were the most convenient for aminoglycoside analysis (Jadhav et al., 2013). Moreover, the unique composition of Raptor biphenyl groups in sterically favourable positioning enhances the interactions with tetracyclines fused-ring moieties, resulting in complete separation and satisfactory selectivity. On another hand, amoxicillin and fluoroquinolones, preferably analysed by the HILIC technique (Rossmann, Schubert, Gurke, Oertel, & Kirch, 2014), in our conditions express considerable retention with very good peak shape. Also, the gradient elution program with suitable flow rates was carefully optimised until it permitted the best separation ability for all the analytes investigated.

Sulfameter is an antibiotic that is chemically distinct from antibiotics included in the assay, and initially was used as the internal standard just to control eventual shifts in retention time as it was suggested by others (Dias et al., 2013). However, we observed that its extraction recovery correlated with all antibiotics analysed. Also we noticed that the chromatographic properties and MS/MS fragmentation of sulfameter were always stabile and reproductive even when different Oasis SPE preliminary trials were tried out. Therefore, it was concluded that this substance can be used as an internal standard in the quantitative purposes.

The LC–MS/MS analyses can detect all antibiotics at concentration levels suitable for research and control purposes and was developed to provide confirmatory data for the analysis of bovine urine. After preliminary trials, in full-scan mode from 50 to 500 *m*/*z*, the three product ions with the higher signal-to-noise ratio (s/n) for each analyte and internal standard were chosen for identification. The collision energy (CE) and the de-clustering potential (DP) were adjusted in the MRM mode for each transition monitored, in order to reach the highest sensitivity for all analytes. The *m*/*z*-values of the selected parent and daughter ions used for MS detection are generally in agreement with those previously reported in the literature (Rossmann et al., 2014; Stubbings & Bigwood, 2009; Tong et al., 2009).

For a method to be deemed confirmatory under Commission Decision 2002/657/EC, it must yield four identification points (IPs). Each one of the three product ions is equal to

1.5 IPs, making a total of 4.5 IPs. The three diagnostic product ions, among which is the ion for the quantification, the relative intensities and the CEs are reported in Table 1.

3.6.3.2. Performance characteristics of the methods

The suitability of artificial urine was evaluated according to procedures explained elsewhere (Chiesa et al., 2015). Addition experiments were performed by comparing the slopes of six-point standard curves (0–10 ng mL^{-1}) performed both in blank and artificial urine (Table 3). Differences in percentage for all compounds ranged from 0.02% up to 1.3%.

Table 3. Calibration curves and linearity in both blank and artificial urine spiked with standard solutions at six concentrations (0, 0.5, 1, 3, 5 and 10 ng mL^{-1}).

Antibiotics	Blank urine	R^2	Artificial urine	R^2
Florfenicol	$y = 0.0839x + 0.0014$	0.9989	$y = 0.0833x + 0.0019$	0.9997
Florfenicol amine	$y = 0.1923x + 0.0052$	0.9921	$y = 0.1984x + 0.0036$	0.9914
Ciprofloxacin	$y = 0.8736x + 0.0164$	0.9951	$y = 0.8776x - 0.022$	0.9936
Cefalexin	$y = 0.0886x - 0.0175$	0.9906	$y = 0.0896x + 0.0057$	0.9987
Ampicilline	$y = 0.0976x - 0.0439$	0.9864	$y = 0.0987x - 0.0347$	0.9811
Lomefloxacin	$y = 10.531x + 0.0553$	0.9972	$y = 10.443x - 0.5319$	0.9977
Enrofloxacin	$y = 2.8914x + 0.2809$	0.9922	$y = 2.9044x - 0.0449$	0.9912
Marbofloxacin	$y = 0.7877x - 0.0483$	0.9939	$y = 0.8007x - 0.1071$	0.9959
Amoxicilline	$y = 0.0092x + 0.0016$	0.9911	$y = 0.009x + 0.0029$	0.9902
Tetracycline	$y = 1.4699x - 0.3115$	0.9936	$y = 1.4541x + 0.296$	0.9968
Doxycycline	$y = 0.9613x - 0.2145$	0.9906	$y = 0.9673x - 0.044$	0.9968
Oxytetracycline	$y = 1.2634x - 0.1871$	0.9956	$y = 1.2681x - 0.1633$	0.9972
Chlorotetracycline	$y = 0.245x - 0.0383$	0.9966	$y = 0.2476x - 0.021$	0.9933

Antibiotics	Blank urine	R^2	Artificial urine	R^2
Cefquinome	$y = 0.0423x - 0.0077$	0.9961	$y = 0.0434x - 0.0092$	0.9965
Streptomycin	$y = 0.0057x + 0.0005$	0.9931	$y = 0.0056x + 0.0006$	0.9914

The instrumental linearity was evaluated over a concentration range of 0.5–5.0 ng mL^{-1} for all antibiotics, using solutions of the analytes in methanol:water (10:90 v/v), containing a fixed amount of the internal standards (2.0 ng mL^{-1} each). Correlation coefficients of the curves were higher than 0.9980 for all compounds, indicating a good fit.

Very high levels of tetracyclines (tetracycline, oxytetracycline, doxycycline) found in almost all samples were extrapolated from quantification calibration curves which were built specifically of 6 points that cover the whole range of concentration: 0–100 ng mL^{-1} for tetracycline and doxycycline and 0–2000 ng mL^{-1} as far as oxytetracycline is concerned. Very good, satisfactory linearity was obtained for all three curves ($R^2 > 0.990$). The matrix calibration curves built for the validation of each analyte were demonstrated to be linear in the range 0.5–1.5 ng mL^{-1} for the detected antibiotics. The regression lines, obtained using the least-square method, demonstrated a good fit for all analytes with a correlation coefficient >0.99.

MRLs for antibiotic in bovine urine have been not defined, up to now. Therefore, the lowest points of calibration curves were chosen on the bases of minimum required performance limits (MRPL) of analytical methods for the compounds for which permitted limit has not been established, as it was suggested by Commission Decision 2002/657/CE.

Specificity was evaluated for all antibiotics, except tetracyclines, as those compounds were presented in samples that we had available, as stated in Section 2.6. Blank urine, artificial urine and spiked samples were analysed and did not show any interference (signals, peaks, ion traces) in the region of interest, where the target analytes were expected to be (Commission Decision 2002/657/CE). Selectivity requires compliance

with the relative retention times for each analyte, which in our case were found to be within 2.5% tolerance when compared with standards. Moreover three transition from the analyte molecular peak were monitored with a signal-to-noise ratio greater than 3. All ion ratios of samples were within the recommended tolerances as required by the 2002/657/EC when compared with standards.

The precision, calculated by applying the one-way analysis of variance (ANOVA), was expressed as coefficient of variability (CV), in terms of intra- and inter-day repeatability. The reported results show that the intra- and inter-day repeatability for all analytes was below 12% and 20%, respectively (Table 2). These CVs were lower than 22%, as proposed by Thompson (2000), representing good method performance.

CCα and CCβ was calculated, as described in SANCO/2004/2726 revision 4, using parallel extrapolation to the *x*-axis at the lowest experimental concentration (Table 2). These results are obtained as the first of their kind for this type of matrix. The acquired levels might, however, be used in subsequent research on the presence of antibiotics in bovine urine, especially when MPLs for these compounds in urine remain to be established.

Samples and standards were tested at a distance of one week to one month under defined storage conditions (−20 °C), and quantification of components was determined by comparison to freshly prepared standards with an acceptable stability (CV = 2%). The special attention was paid on cefquinome and amoxicillin that were expressed lower stability compared to others.

Robustness was observed in eight different trials, fortifying eight blank urine samples at a concentration of 10 ng mL^{-1}, changing slightly (±10%) the nominal values, reported in the Section 2.5, of seven factors that may influence the outcome of the analysis. The factor were: centrifugation time of urine, HCl concentration for the conditioning of the SPE columns, % methanol in the washing solution of the SPE columns, the volume of the methanolic solution of washing SPE columns, the elution volume of the SPE columns, evaporation temperature of the extract, resuspension volume of the dry

extract. None of the factors showed a significant variation in the concentration measurements.

3.6.3.3. Application of the methods

In order to monitor antibiotic residues in bovine urine, 39 samples, collected from male veal calves and young bulls, were subjected to analysis. All samples were taken from live animals at the slaughterhouse before processing and were divided into free groups according to their age.

Only one sample showed florfenicol amine at a concentration level of 0.75 ng mL^{-1}, while the other two revealed traces of florfenicol and amoxicillin (concentration < CCα) respectively. The great majority of samples were positive for tetracyclines (Table 4, Fig. 2), which can be explained by the fact that those four members of this antibiotic group are commonly used in the treatment of animal diseases because of their high activity and low production costs. Only 3 from the oldest group (group C) were completely negative, while the remaining 6 exposed an oxytetracycline presence. Astonishing results were found with regard to oxytetraycline, with an overwhelming level observed in the calves' samples (group A). As a matter of fact, oxytetracycline is poorly metabolised in target animals and is excreted practically in its parent form, probably due to its high water solubility (log $K_{ow} = -1.12$) (Slana & Dolenc, 2013). This datum is of particular concern as after administering to target animals, this compound can be excreted into the environment. Calves' urine samples were also positive for the presence of tetracycline and doxycycline, which, along with oxytetracycline outcomes, implies that those animals are more susceptible to infection and, most presumably, treated with higher drug doses as a result. The other two groups also tested positive for tetracycline presence, but to a lesser extent. Thoughtful statistical analysis indicates the differences between groups with regard to each of the tetracyclines analysed.

Table 4. Urinary concentrations of tetracyclines in bovine urines at slaughterhouse. Data are reported as median (ng mL^{-1}) with corresponding 25th–75th percentile.

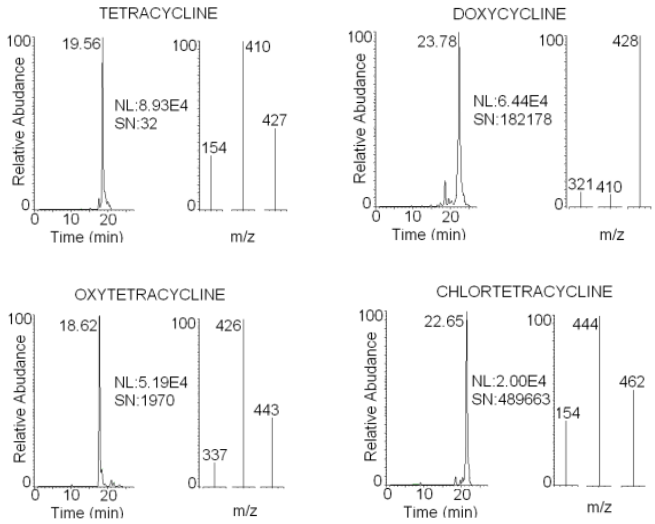
	Tetracycline	Doxycycline	Oxytetracycline	Chlortetracycline
Veal calves 6 months old n-20				
Positive (%)	100% **32.0** (21.0 – 51.3)	100% **34.6** (18.7-45.9)	100% **1718.0** (1220.9-2180.3)	0% **ND**
Veal calves 11 months old n-15				
Positive (%)	47% **0**[#] (0 – 1.4)	100% **19.4** (10.0-25.5)	100% **6.7**[#] 3.5-11.30	93% **2.1**[#, £] 1.3-4.0
Young bulls 18 months old n-9				
Positive (%)	0% **ND**[§]	0% **ND**[§,£]	33% **2.8**[§] 0-5.7	0% **ND**

ND = not detected

Non-parametric Kruskal-Wallis one-way analysis of variance by ranks was used to test the significance between the groups: there is a statistically significant difference within groups for all compounds ($p<0.001$)

To isolate the group that differs from the others, all pairwise multiple comparison procedures (Dunn's Method) were applied:
[#] Group A vs group B ($p<0.05$)
[§] Group A vs group C ($p<0.05$)
[£] Group B vs group C ($p<0.05$)

Figure 2. Reconstructed LC-MS/MS chromatograms and respective ion spectra of the analytes detected in a urine sample from male Friesian veal calf (0.7, 14.5, 6.4 and 2.3 ng mL^{-1} for tetracycline, doxycycline, oxytetracycline and chlortetracycline, respectively).



In this paper, we presented the results obtained at the slaughterhouse, having no information about eventual treatment, urinary metabolic rate or excretion profile of these pharmaceuticals in bovines. The available data in literature are limited; for instance, the degree of metabolism for tetracyclines and β-lactams antibiotics is lower than 20%, whereas it is higher than 80% for sulphonamides (Boxall et al., 2004). Therefore, further and more profound study in this area is necessary.

Antibiotics administered to food-producing animals close to the time of slaughter often result in prohibited antimicrobial residues in the animal tissues at slaughter, while only one report has been published as far as evaluation of its presence in urine is concerned (Omeiza, Ajayi, & Ode, 2012). Nevertheless, urine analysis can be appropriate even for correction of the so-called “withdrawal period”. Generally, as defined in Article 1, Point 9 of Directive 2001/82/EC, the withdrawal period is the time interval necessary between the last administration of a veterinary medicinal product to animals, and the production of foodstuffs from such animals, to protect public health. The withdrawal period estimated by urine analysis is much more convenient as it can be systematically performed at the farm during breeding. In this manner, animals legally treated with antibiotics cannot be processed before the complete elimination of antibiotic residues.

Consequently, it should be evaluated if control systems applied for other drugs (group A, i.e. anabolic hormones and corticosteroids) (NRCP, 2014) that are based on the analysis of urine samples could be applied for antibiotic (group B). This matrix has the advantage of being one of the few matrices available while the animals are still alive (Chiesa et al., 2015). However, it remains to be established as to whether high concentrations of antibiotics in urine correlate with their content in meat and dairy products produced from those animals.

3.6.4. Conclusion

We developed and validated a LC–MS/MS method for the multiclass analysis of antibiotics in bovine urine samples. All analytes were extracted with a unique clean-up step using SPE cartridges. The performance characteristics of the method were evaluated in accordance with the criteria of the Commission Decision 2002/657/CE. We found tetracycline, doxycycline, oxytetracycline and chlortetracycline in all urine samples of male veal calves, while only a small quantity of oxytetracycline was detected in young bulls. Our research pointed out the possibility to use a non-invasive sample such as urine to identify antibiotic residues. It remains to be established what the effects and significance are of tetracycline levels found in bovine urine samples enrolled in this study: that is, whether these levels could be a warning sign for administration of sub-therapeutic doses (prevention of diseases or growth-promotion purposes), or if they are indicative of legal therapeutic use, which necessitates adequate safety (withdrawal) periods during which the animals cannot be used for human consumption.

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3.7. A Liquid Chromatography-Tandem Mass Spectrometry Method for the Detection of Antimicrobial Agents from Seven Classes in Calf Milk Replacers: Validation and Application.

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

ABSTRACT

Calf milk replacers are low-cost feeds that contain available, digestible protein. During their reconstitution, however, the addition of drugs, like antibiotics, could make them a very simple route for illicit treatment for therapeutic, preventive or growth promoting purposes. We developed an HPLC-MS/MS method, preceded by a unique extraction step, able to identify 17 antibiotics from seven classes (penicillins, tetracyclines, fluoroquinolones, sulphonamides, cephalosporins, amphenicols and lincosamides) in this matrix. Prior to solid phase extraction (SPE), the sample underwent deproteinization and defatting. The method was fully validated according to Commission Decision 2002/657/EC. Decision limits (CCα) ranged from 0.13-1.26 ng/mL, and detection capability (CCβ) from 0.15-1.47 ng/mL, respectively, for sulfadimidine and chlortetracycline. Thirty-eight samples were finally analyzed, showing the occasional presence of marbofloxacin (six samples) and amoxicillin (one sample).

Keywords: calf, milk replacers, antibiotics, HPLC-MS/MS.

3.7.1. INTRODUCTION

Milk replacers can be an economical and valid source of calf nutrition. Their formulation is carried out in order to: increase the protein level to ensure maximum muscle growth of the calves; enhance the palatability and consequently, the ingestion to facilitate the weaning phase; make energy from diverse sources (starch, soluble fibre and digestible fat) for maximum growth without digestive problems (acidosis, intestinal problems, etc.); increase the range of supply, both from the point of view of the nutritional characteristics and from the point of view of prices; and simplify the management of calves' food, due to the availability of products used at each stage (starter, weaning, breeding until 6 months). Since milk replacers were introduced, there have been two primary objectives in formulating them: first, to find ingredients containing available, digestible protein, and second, to reduce the cost of feeding calves. Balancing these two objectives is not easy. Historically, as the price of ingredients has changed, milk-replacer manufacturers have changed their formulations to reduce the cost of their products.[1]

Powdered milk replacers are usually made up of skimmed-milk powder, whey protein, vegetable or animal fat, soy lecithin and vitamin-mineral premix.[2] Fat levels range from 10%-30%, with 18-22% being the most common fat levels, which are mainly added as tallow, lard or coconut oil. Protein levels in dry milk replacers range from 18%-30% with an average value of approximately 20-22%, preferably of diary origin, but can also include soy protein, soy flour, potato, wheat proteins and animal plasma protein.[3, 4]

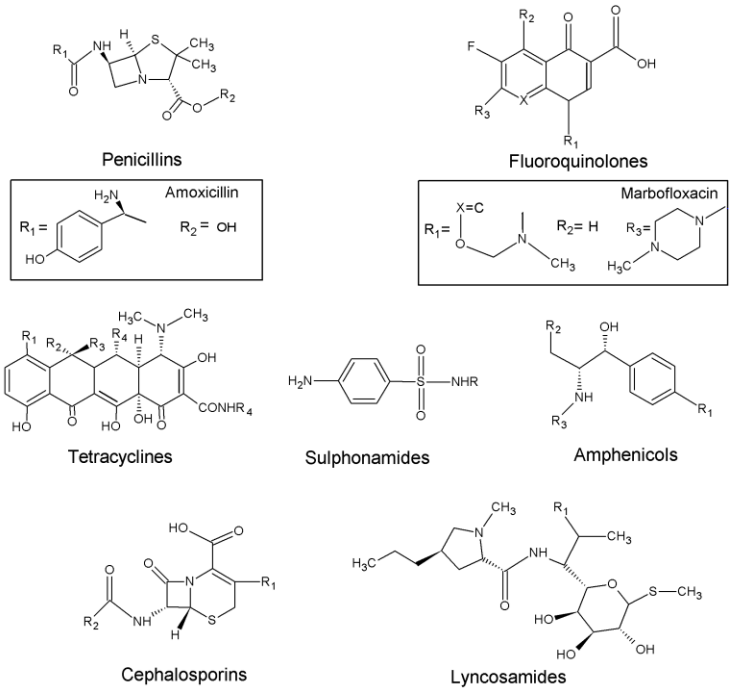
Antibiotics are usually added to preweaned calf milk replacer to: treat bacterial infections; decrease the incidence of scours, morbidity and mortality; improve calf growth; improve feed consumption; and increase average daily gains.[5-7] Among feedstuffs used in animal husbandry, powdered milk replacers are perhaps the most suitable for illegal treatments and, for example, drug administration via this route is very simple, i.e. during the reconstitution of milk replacers or immediately before feeding. It should be considered that the antibiotics found in milk replacers might result from prior treatment of cows producing milk, what the milk replacer was made with, or they may have intentionally been added directly to the feed.[8] Whatever the reason, the overuse of antibiotics in animal husbandry may affect the antibiotic resistance of pathogens [9-11] and the consequent risk of human infection with resistant zoonotic bacteria.[12, 13]

The inclusion of veterinary drugs in calf milk replacers has become an increasingly important public health concern, particularly as their regulation varies between countries. For example, inclusion in milk replacers of decoquinate, lasalocid, oxytetracycline, chlortetracycline and neomycin-based medications is approved in the USA. The Food and Drug Administration (FDA) in 2013, however, recommended a 3-year “judicious period” during which utilisation of antibiotics should be reduced. Therefore, the presence of these antibiotics in feedstuff can be authorised for therapeutic and prophylactic purposes but is not authorised as a growth promoter. [14]

European legislation does not treat milk replacers individually, but sets out the conditions under which feedstuffs may be prepared, marketed and employed within the European Community.[15, 16] From 2006, the use of antibiotics (other than coccidiostats and histomonostats) as feed additives is no longer allowed.[17] Currently, the use of

antibiotics for auxinic purposes has been abolished and is illegal. The EU has established Maximum Residue Limits (MRLs) for several classes of antibiotics in milk and edible tissues with the aim of minimising the risk to human health.[18] However until now, no legislation has been published regarding maximum levels of antibiotics either in feedstuff in general or in milk replacers.

Figure 1. Structures of the seven classes of antimicrobial agents. The substituents of the two antibiotics found in the samples are shown in the boxes.



The analytical methods for monitoring the presence of undesirable pharmacological principles must have high sensitivity and selectivity. As feedstuffs are heterogeneous matrices with different protein, fatty acid, neutral lipid (phospholipids, glycerides and sterols), glucide, vitamin and mineral contents, the occurrence of interferences is a major issue in the analysis. The methods used for the detection of drug residues in these matrices often need intensive steps for the preparation and extraction of samples in order to improve the analysis performances. Studies in the literature on antibiotics in powdered calf milk or feed in general include a rapid multi-residue and multi-class screening method for 50 antimicrobials in feed[19] , some about detection of chloramphenicol and florfenicol in powdered milk, [20-22] one about a specific class of antibiotics, as well as fluoroquinolone residues, but in powdered infant formulae [23] and many more on milk for human consumption.[24] The purpose of this study was the development of a unique extraction and HPLC MS/MS analysis method that is able to identify antibiotics from different classes in calf powdered milk with the aim of improving residue control and preventing possible consequences for animal and consumer welfare. The considered antibiotics included: amoxicillin and ampicillin (penicillins);

chlortetracycline, doxycycline, oxytetracycline, and tetracycline (tetracyclines); ciprofloxacin, enrofloxacin, lomefloxacin and marbofloxacin (fluoroquinolones); sulfadimidine and sulfathiazole (sulphonamides); cephalexin and cefquinome (cephalosporins); florfenicol and its metabolite florfenicol amine (amphenicols); and lincomycin (lincosamides). The chemical structures of the seven antimicrobial classes are shown in Figure 1. The method validation was made according to Commission Decision 2002/657/EC[25] , and the application of the analysis to 38 real samples of powdered bovine milk was performed.

3.7.2. MATERIALS AND METHODS

3.7.2.1. Chemicals and reagents. All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO). Formic acid (98–100%) and hydrochloric acid (37%) were obtained from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges (Oasis HLB 3 mL, 60 mg) were provided by Waters (Milford, MA). Amoxicillin, ampicillin, cefalexin, cefquinome sulphate, florfenicol, florfenicol amine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, oxytetracycline, lincomycin, sulfathiazole sulfadimidine and enrofloxacin d5 as the internal standard (IS) were purchased from Fluka.

3.7.2.2. Composition of powdered milk replacer. For the method validation, we used a commercially available complete milk replacer for calves being weaned. The chosen milk replacer, which was proven free of antibiotics, contained whey powder, vegetable oils (palm and coconut oil), hydrolysed wheat protein, soy protein concentrate, calcium carbonate and magnesium sulphate. The analytical constituents were: crude protein 21%, oils and fats 16%, crude fibre 0.3%, crude ash 9%, calcium 0.8%, sodium 0.8%, phosphorus 0.7%, lysine 1.5%, methionine 0.4% and cysteine 0.4%. Additives included vitamin A (40,000 IU/kg), vitamin D3 (5000 IU/kg), vitamin E (200 mg/kg), vitamin B1 (6 mg/kg), vitamin B6 (4 mg/kg), vitamin C (158 mg/kg), vitamin K (4 mg/kg) and niacin (40 mg/kg). The formulation also contained the following quantities per kg: iron

monohydrate sulphate 273.6 mg, copper pentahydrate sulphate 39.3 mg, manganese monohydrate sulphate 141.7 mg, zinc monohydrate sulphate 230.2 mg, potassium iodide 2.62 mg and sodium selenite 0.66 mg. All the information about the feedstuff compositions came from the manufacturer's certificates.

3.7.2.3. Standard solutions. Stock solutions (1 mg/mL) for each standard were prepared in methanol and kept at -20 °C. Working solutions in methanol, containing each of the studied analytes at concentrations of 10 and 100 ng/mL, were prepared daily. Each working solution was maintained at -20 °C during the method validation procedures.

3.7.2.4. Sample extraction. The preliminary deproteinization and defatting steps were performed using the protocol of Wang et al.,[26] which was slightly modified with regard to the relative amounts of matrix and reagents, and acetonitrile was substituted by methanol. The whole procedure was as follows: 1 g of powdered milk was reconstituted in 10 mL of water, and 1 mL of this solution was spiked with the internal standard to a final concentration of 2 ng/mL. The sample was vortexed and then sonicated for 10 min, followed sequentially by deproteinization through the addition of methanol (10 mL), 2 min of shaking, and 10 min of sonication. After the addition of sodium chloride (2 g), samples underwent 2 min of shaking and 10 min of centrifugation at 4500×g. The supernatant was transferred into a 50-mL polytetrafluoroethylene centrifuge tube and defatted with 2 × 7 mL of *n*-hexane extraction. Each time, after centrifugation at 2500×g, the *n*-hexane layer was removed. The methanol/water layer was evaporated by a vacuum rotary evaporator at 40 °C and was then reconstituted in 5 mL of water for further purification and extraction using the Oasis HLB Cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5 M HCl and 3 mL of Milli-Q water. The samples were loaded, and then the cartridges were washed with 3 mL of water and 3 mL of methanol:water (20:80, v/v). Finally, samples were eluted using 5 mL of methanol and were collected in a 15 mL polypropylene tube. The solvent was evaporated w a rotary vacuum evaporator. The dried extract was reconstituted in 200 µL of methanol:water (10:90 v/v), and then transferred to an auto-sampler vial. The injection volume was 10 µL. [27]

3.7.2.5. HPLC-MS/MS analyses. HPLC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA) that was made up of a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with a 20-µL loop. Analytical separation was carried out using a reverse-phase HPLC column 150 mm x 2 mm i.d., 4 µm, Synergi Hydro RP, with a 4 mm x 3 mm i.d. C18 guard column (Phenomenex, Torrance, CA). The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (0.1%aqueous formic acid), and B (MeOH). The elution started with 98% A, which was maintained for 5 min, followed by a linear gradient to 50% A at 22 min. Subsequently, the mobile phase was gradually increased to 95% B at 24 min, then held constant until 29 min. The mobile phase was returned to initial conditions at 31 min, with equilibration time that included the interval from 31–40 min. The run was performed at 0.2 mL/min. The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher) equipped with an electrospray interface (ESI) that was set in the positive (ESI+) electrospray ionisation mode for all analytes except for florfenicol in which it was set in the negative (ESI-) mode. Acquisition parameters were optimized in the electrospray mode by direct continuous pump-syringe infusion of the standard solutions of analytes at a concentration of 1 µg/mL, a flow rate of 20 µL/min and a MS pump rate of 100 µL/min. The following conditions were used: capillary voltage 3.5 kV; ion-transfer capillary temperature 340 °C; nitrogen as sheath and auxiliary gases at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr; and peak resolution 0.70 Da at full-width half-maximum (FWHM). Three diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in multiple reaction-monitoring (MRM) mode. The selected diagnostic ions, one of which was chosen for the quantitation, and the collision energies are reported in Table 1. Acquisition data were recorded and elaborated using Xcalibur software from Thermo Fisher.

Table 1. MS/MS Conditions for the MRM Acquisitions of Investigated Antibiotics.

analyte	precursor ion* (*m/z*)	product ions$_{CE}$ (*m/z*)	ESI
amoxicillin	366	**114(80)$_{20}$**, 134(21)$_{31}$, 349(100)$_{7}$	(+)
ampicillin	350	**106(100)$_{18}$**, 114(14)$_{29}$**,** 160(14)$_{14}$	(+)
cefalexin	348	158(63)$_{5}$, **174(100)$_{15}$,** 191(23)$_{6}$	(+)
cefquinome	529	**134(100)$_{15}$**, 324(43)$_{15}$, 396(44)$_{10}$	(+)
ciprofloxacin	332	268(16)$_{22}$, **288(100)$_{17}$**, 314(94)$_{21}$	(+)
enrofloxacin	360	245(49)$_{26}$, **316(100)$_{18}$**, 342(29)$_{21}$	(+)
lomefloxacin	352	**265(100)$_{23}$**, 288(16)$_{19}$, 308(63)$_{16}$	(+)
marbofloxacin	363	72(83)$_{23}$, **320(100)$_{15}$**, 345(18)$_{21}$	(+)
florfenicol	356	169(1)$_{39}$, 185(35)$_{21}$, **336(100)$_{12}$**	(-)
florfenicol amine	248	130(24)$_{23}$, 134(8)$_{28}$, **230(100)$_{11}$**	(+)
chlortetracycline	479	154(39)$_{27}$, **444(100)$_{21}$**, 462(69)$_{16}$	(+)
doxycycline	445	321(10)$_{31}$, 410(8)$_{24}$, **428(100)$_{19}$**	(+)
oxytetracycline	461	337(26)$_{29}$, **426(100)$_{19}$**, 443(52)$_{12}$	(+)
tetracycline	445	154(38)$_{30}$, **410(100)$_{19}$**, 427(43)$_{14}$	(+)
lyncomicin	407	**126(100)$_{16}$**, 359(10)$_{18}$, 389(5)$_{28}$	(+)
sulfathiazole	256	92(50)$_{27}$, 108(45)$_{25}$, **156(100)$_{15}$**	(+)
sulfadimidine	279	108(32)$_{26}$, 124(39)$_{265}$, **186(100)$_{18}$**	(+)
enrofloxacin-d5 (IS)	365	245(49)$_{32}$, **321(100)$_{27}$**, 347(46)$_{19}$	(+)

* $[M+H]^+$ for all compounds except $[M-H]^-$ for florfenicol. Ions for quantitation are in bold. The values in brackets represent the relative intensities (%). CE: Collision Energy, subscripted and expressed in Volts.

3.7.2.6. Method validation. After a preliminary screening of a few samples of reconstituted milk to search the “blank” milk, the validation was performed according to the criteria of the European Commission Decision 2002/657/EC.[25] For each analyte, the method performance was assessed as follows: through its qualitative parameters, as

well as molecular identification in terms of retention time (RT) and transition ion ratios, specificity and selectivity; through its quantitative parameters, such as linearity, recovery, accuracy in terms of trueness, and precision expressed as the intra- and inter-day repeatability; and through the analytical limits, i.e. decision limit (CCα) and detection capability (CCβ), as clarified in the document SANCO/2004/2726 revision 4. [28]

Finally we evaluated robustness, matrix effect and stability of antibiotics in the standard solutions and in the spiked samples.

3.7.3. RESULTS AND DISCUSSION

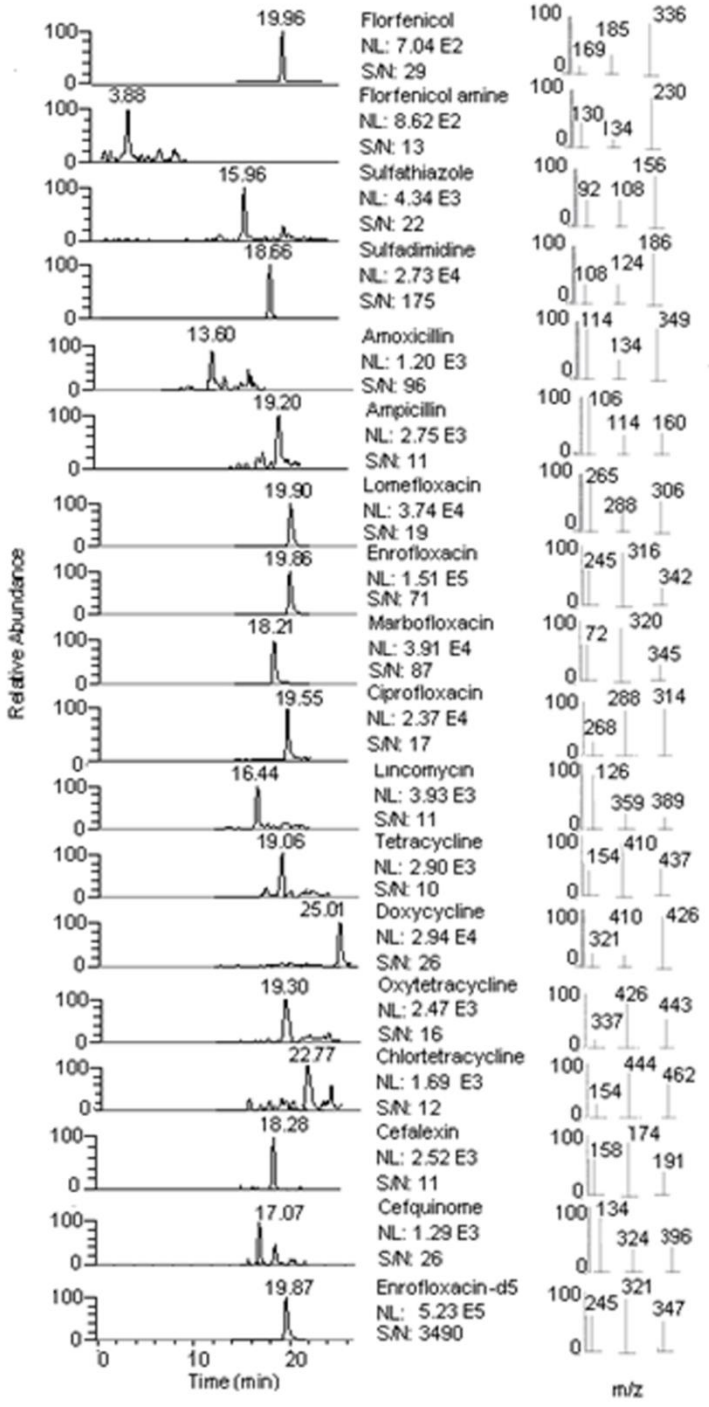
3.7.3.1. Performance characteristics of the methods. The specificity was good as an appropriate number of representative blank samples (n≥20) was analyzed and no interference were found (signals, peaks, ion traces) in the region of interest where the target analyte was expected to elute.

Selectivity showed a good compliance with the relative retention times for each analyte, which in our case were found to be within 2.5% tolerance, when compared with the standards. Moreover, the three chosen transitions showed an ion ratio within the recommended tolerances,[25] when compared with the standards.

Validation was performed by spiking milk samples with each of the analytes, resulting in three analytical series (matrix validation curves). Each series had six replicates for three concentration levels that were previously chosen according to the minimum concentration detectable with our instrumentation: 0.1, 0.2 and 0.3 ng/mL for lomefloxacin, lyncomicin, sulfathiazole and sulfadimidine; 0.3, 0.6 and 0.9 ng/mL for ampicillin, cefalexin, florfenicol amine, ciprofloxacin, marbofloxacin, tetracycline, doxycycline and oxytetracycline; 0.5, 1.0 and 1.5 ng/mL for amoxicillin, cefquinome and florfenicol; and 1.0, 2.0 and 3.0 ng/mL for chlortetracycline. The matrix validation curves demonstrated a good fit for all analytes with a correlation coefficient of >0.99.

The HPLC–MS/MS chromatograms for the antibiotics at the lowest concentration level of the validation and of the internal standard (2 ng/mL) are shown, together with the ion spectra, in Figure 2.

Figure 2. Chromatograms performed in matrix and related ion spectra of antibiotics at the lowest concentration level of validation (0.1 ng/mL for lomefloxacin, lyncomicin, sulfathiazole and sulfadimidine; 0.3 ng/mL for ampicillin, cefalexin, florfenicol amine, ciprofloxacin, marbofloxacin, tetracycline, doxycycline, and oxytetracycline; 0.5 ng/mL for amoxicillin, cefquinome and, florfenicol, 1.0 ng/mL for chlortetracycline) and of the Internal Standard (2 ng/mL).



The instrumental linearity was also evaluated by drawing six-point calibration curves in the solvent containing a fixed amount of the internal standards (2 ng/mL), with the initial analyte concentration corresponding to the minimum detectable for each group up to 10 ng/mL.

The correlation coefficients of these curves were >0.99 for all compounds, this indicating a good fit.

Calibration curves were made in matrix to quantitate the analytes eventually detected during the application of the method. The levels were the same used for the assessment of the instrumental linearity. Also these curves demonstrated a good fit for all analytes with a correlation coefficient of >0.99.

The trueness was assessed through recovery and was evaluated using the data from the validation points of the three analytical series, expressed in terms of a percentage of the measured concentration with respect to the spiked concentration. The recoveries ranged between 89% and 111%.

The precision was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at three concentration levels of the three analytical series, performed in three different days. It was expressed as the coefficient of variability (CV) in terms of intra- and inter-day repeatability, which never exceeded 20% and 22%, respectively. These CVs were ≤22%, as proposed by Thompson,[29] and thus represent good method performance.

In Table 2, the analytical limits, recoveries and precision are shown. CCα and CCβ, which were calculated as described in SANCO/2004/2726 revision 4 [28] using parallel extrapolation to the x-axis at the lowest experimental concentration, were in the orders of magnitude 0.1–1.0 ng/mL.

Table 2. Validation Parameters for Antibiotics.

analyte	CCα (ng/mL)	CCβ (ng/mL)	concentration levels (ng/mL)	recovery % (*n*=18)	repeatability	
					intra-day (CV; *n*=6)	inter-day (CV;*n*=18)
amoxicillin	0.78	1.04	0.5-1.0-1.5	104-96-101	18-8-7	22-17-9
ampicillin	0.47	0.62	0.3-0.6-0.9	101-99-100	20-13-9	21-15-9
cefalexin	0.53	0.80	0.3-0.6-0.9	102-97-101	19-19-16	22-21-18
cefquinome	0.75	0.93	0.5-1.0-1.5	103-91-109	19-11-8	20-12-9
ciprofloxacin	0.41	0.52	0.3-0.6-0.9	95-105-98	14-15-11	16-16-12
enrofloxacin	0.13	0.17	0.1-0.2-0.3	100-100-100	8-8-7	15-15-8
lomefloxacin	0.18	0.27	0.1-0.2-0.3	97-103-98	20-14-16	22-21-18
marbofloxacin	0.45	0.58	0.3-0.6-0.9	103-97-101	17-11-8	20-16-9
florfenicol	0.69	0.88	0.5-1.0-1.5	98-101-100	11-12-8	16-17-9
florfenicol amine	0.38	0.47	0.3-0.6-0.9	93-107-98	6-11-10	12-15-11
chlortetracycline	1.26	1.47	0.1-0.2-0.3	92-103-98	7-5-7	11-11-10
doxycycline	0.46	0.64	0.3-0.6-0.9	104-96-101	18-16-12	22-21-13
oxytetracycline	0.41	0.52	0.3-0.6-0.9	102-98-101	10-8-8	16-15-9
tetracycline	0.48	0.63	0.3-0.6-0.9	89-111-96	20-9-10	21-12-10
lyncomicin	0.15	0.19	0.1-0.2-0.3	101-99-100	17-13-11	20-17-12
sulfathiazole	0.16	0.21	0.1-0.2-0.3	104-96-101	20-10-9	21-17-10
sulfadimidine	0.13	0.15	0.1-0.2-0.3	101-99-100	8-4-6	11-9-7

The recovery, intra- and inter-day repeatability values follow one another in reference to the three validation levels.

We evaluated robustness using the approach of Youden,[25] which has a fractional factorial design. It was observed in eight different trials by fortifying eight blank milk-replacer samples at the lowest validation concentration, changing slightly (±10%) the nominal values of seven factors that may influence the outcome of the analysis. The factors included: the methanol volume for the deproteinization milk step; HCl

concentration for the conditioning of the SPE columns; percentage methanol in the washing solution of the SPE columns; the volume of the methanolic solution used for washing the SPE columns; the elution volume of the SPE columns; evaporation temperature of the extract; and resuspension volume of the dry extract. Applying the Fisher test to compare the standard deviation of the differences between high- and low-value settings for each experimental parameter with the standard deviation of the method carried out under within-laboratory reproducibility condition,[25] none of the parameters showed a significant variation in the concentration measurements, so demonstrating the method robustness.

Experiments to evaluate matrix effects corresponded to the strategy applied by Matuszewski et al.[30] that compares sample extracts plus the analyte of interest added post-extraction with pure solutions prepared in the mobile phase containing equivalent amounts of the analyte of interest. The percentage ratio between the corresponding peak areas for standards spiked after extraction and the peak areas obtained in neat solution standards determines the extent of the matrix effect occurring for the analyte in question under chromatographic conditions. It ranged from 93%-114% for each compound, indicating a very low ion suppression and sometimes a low ion enhancement.

Stability was evaluated by testing spiked samples and standard solutions over time from one week to one month under defined storage conditions (-20 °C), and quantitation of components was determined by comparison to freshly prepared standards.

All the analytes in the working solutions and in the samples showed an acceptable stability (CV≤ 2%) until one month storage at -20 °C, except amoxicillin and cefquinome. After one week, the concentration of these two antibiotics decreased more than the acceptable value. We therefore decided to daily prepare the working solutions.

This paper describes a sensitive, selective and robust multi-class method for antimicrobials in calf milk replacers. A number of studies is retrievable in the literature on the determination of single classes of antibiotics as well as β-lactam antibiotics in milk for human use[31, 32] and fluoroquinolones both in dairy milk[33] and in powdered milk

for infants.[23] The only multiclass method for antibiotics regarding feedstuffs is a qualitative screening method on feed different from milk replacers. The validation level are usually 5 to 100 times higher than our analytical limits, except florfenicol and doxycycline.[19]

3.7.3.2. Application of the method. The use of medicated feeds is most common in intensive production.[16] Although the studied antimicrobials are authorized, they must be absent in non-medicated feed. In order to fully demonstrate the applicability of the proposed method, 38 anonymous samples collected from different farms in Lombardy were subjected to analysis. Only one sample was positive for amoxicillin at a concentration of 1.26 ng/mL, while six samples contained marbofloxacin residues ranging from 0.52-0.91 ng/mL, with an average concentration of 0.74±0.15 ng/mL. In Figure 1 the substituents of these two antimicrobial agents are itemized in the boxes.

Tetracyclines were not found, in contrast to our previous work on urine,[27] in which this class of antibiotics was present in almost all samples. These data could be a demonstration of the good quality of the milk replacers analyzed as milk is the major tetracycline excretion route after urine (50–80%) and bile (10-20%).[34] Considering that calves eat from 400 to 2500 g of powdered milk per day, the control of residues in this non-invasive matrix is, however, still of concern due to the practicality of illicit administration of drugs via this route.

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3.8. Antibiotic use in heavy pigs: comparison between urine and muscle samples from food chain animals analysed by HPLC-MS/MS

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

Abstract

The antibiotic overuse in zoothechnics, due to prophylactic and therapeutic treatments, or to their growth-promoting activity, is a major cause for the onset of widespread antibiotic resistance. Of particular relevance to this study, is the antibiotic abuse in pig breeding. Despite the comprehensive literature on residue controls in pig muscle, data on pig urine, a non-invasive, on-farm collectable matrix, are lacking. Therefore, we validated an HPLC-MS/MS method to detect 29 antimicrobials from eight classes and applied it to 43 anonymous pig urine and muscle paired samples and fulfilled the parameters in agreement with the Commission Decision 2002/657/UE. The analytical limits were moreover much lower than the maximum residue limits (MRLs) required by the Commission Regulation 37/2010/UE. In the samples, antibiotics were usually detected at higher frequencies and concentrations in urine than muscle. Urine proved a useful tool to detect antibiotic administration and their excessive use in pig farming is depicted.

Keywords: antibiotics, HPLC-MS/MS, muscle tissue, swine, urine

Highlights

Antibiotic overuse in pig breeding is of concern, due to antibiotic resistance onset.

Despite being invasive, muscle is usually the matrix used for the residue control.

Urine could allow improved monitoring, as on-farm sample collection is feasible.

A multiclass method for antibiotics detection in urine and muscle was validated.

Its application to 43 paired urine and muscle samples was effective.

Chemical compounds studied in this article

Amoxicillin (PubChem CID: 33613), ampicillin (PubChem CID: 6249), cloxacillin (PubChem CID: 6098), dicloxacillin (PubChem CID: 18381), benzylpenicillin (PubChem CID: 5904), oxolinic acid (PubChem CID: 4628), nalidixic acid (PubChem CID: 4421), cefquinome sulphate (PubChem CID: 9577261), cefalexin (PubChem CID: 27447), florfenicol (PubChem CID: 114811), florfenicol amine (PubChem CID: 156406),

chloramphenicol (PubChem CID:5959), flumequine (PubChem CID: 3374), lomefloxacin hydrochloride (PubChem CID: 68624), ciprofloxacin (PubChem CID: 2764), enrofloxacin (PubChem CID: 71188), marbofloxacin (PubChem CID: 60651), tetracycline hydrochloride (PubChem CID: 54704426), doxycycline hyclate (PubChem CID: 54686183), chlortetracycline (PubChem CID: 54737570), oxytetracycline (PubChem CID: 54675779), lincomycin (PubChem CID: 3000540), sulfathiazole (PubChem CID: 5340), sulfadimidine (PubChem CID: 5327), sulfadiazine (PubChem CID: 441244), sulfadimethoxine (PubChem CID: 5323), trimethoprim (PubChem CID: 5578), erythromycin (PubChem CID: 12560), tylosin (PubChem CID: 5280440).

3.8.1. Introduction

Over the last decade, the overuse of antimicrobial agents as growth promoters in food-producing animals have caused favourable conditions for the threat of bacterial resistance. It is well-established that multiantibiotic-resistant microorganisms results from chromosomal mutations or the exchange of mobile genetic elements, such as plasmids and transposons (Neu, 1992). The presence of antimicrobial residues in food, their environmental accumulation via the application of manure to soil as organic fertiliser or sludge storage, and the direct contamination of illicitly additivated water and feed, represents a threat to consumers.

It seems reasonable to hypothesise that an increase in the antibiotics concentrations in natural ecosystems may not only influence antibiotic resistance but also affect the broader microbial population dynamics in various natural environments (Martínez, 2008). The swine and poultry industries are the main users of antimicrobials (Castanon, 2007; der Fels-Klerx, Puister-Jansen, van Asselt, & Burgers, 2011). In an attempt to decrease their environmental and health risk, the use of antibiotics as animal growth promoters has been banned in EU countries and their monitoring is regulated in Council Directive 96/23/EC (European Union, 1996). In order to ensure food safety, the European Union (2010) has also set maximum residue limits (MRLs) for antibiotic residues in food of animal origin.

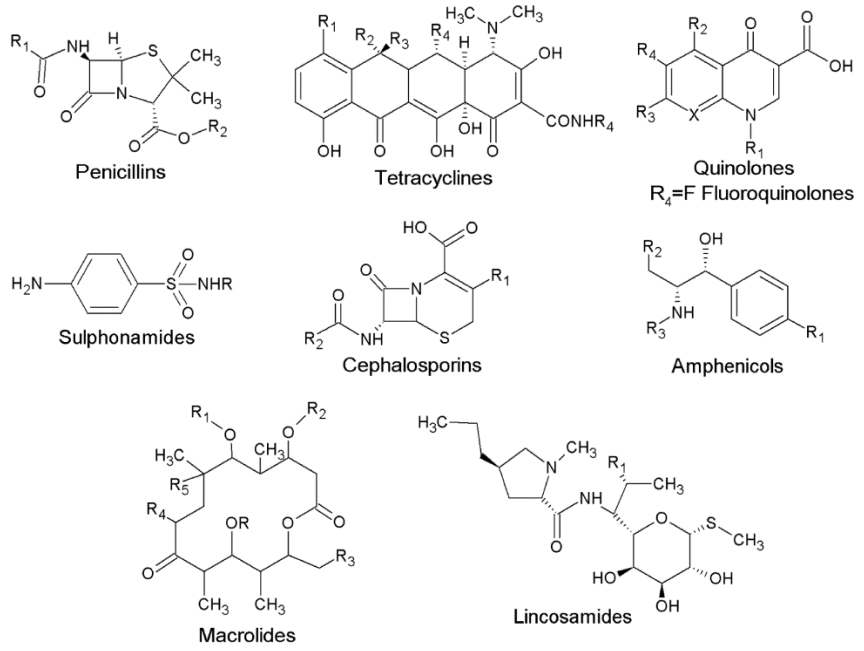
In the United States of America, in the year 2000, the Food and Drug Administration (FDA) approved 17 antimicrobial agents in swine feed (Cromwell, 2002). Some are

permitted in combination like chlortetracycline plus penicillin plus sulphamethazine, or sulphathiazole, neomycin plus oxytetracycline. Most pigs receive antimicrobials in their feed. The chemical composition and mode of action of antibiotics are variable and heterogeneous, but all antimicrobials that are used in swine production should have one common goal. Namely, the capacity to inhibit or decrease the growth of systemic pathogens, even if these characteristics are less readily associated with the ability of a given antimicrobial agent to stimulate growth. The efficacy of antibiotics in improving the rate and efficiency of growth in pigs is well-documented in the scientific literature (Cromwell, 1991; Hays, 1981). Furthermore, antibiotics in breeding and during lactation provide reproductive benefits and improve lactational performance in sows. In this context, the economic benefits are several times greater than the cost of the antibiotic (Cromwell, 2002). Even if these economic benefits are greater in the short terms, there is a growing awareness that antibiotics should be used with more care or avoided, due to the development of antibiotic resistant bacteria, that is the real and potential threat to human health. Different approach, which includes good husbandry practices based on prevention of microbial exposure, infection controls through vaccination, optimizing hygiene to separate potential pathogens from the target animal, the isolation of sick animals, etc., will probably be most effective in the long term (Wierup, 2000).

Despite researches that deal with the multiclass determination of antibiotics in soils and pig slurry (Blackwell, Lützhøft, Ma, Halling-Sørensen, Boxall, & Kay, 2004), swine wastewater (Tong, Li, Wang, & Zhu, 2009), in pig muscle and kidney (Granelli & Branzell, 2007) and nitrofurans in the retina of pigs (Cooper & Kennedy, 2005), the data on pig urine are lacking, except a work regarding only one antibiotic, chloramphenicol, in swine urine and muscle (Gantverg, Shishani, & Hoffman, 2003). To the best of our knowledge, no method has previously been reported for simultaneous screening of major antibiotics groups in swine urine. Urine analysis could be a useful alternative to tissues to improve the effectiveness of surveillance controls, as it offers several advantages compared to the analysis of other biological samples as well as muscle tissue. In particular, urine analysis is non-invasive, thus, it could permit the controls at farms and slaughterhouses. We previously reported the multi-residual screening of

antibiotics in bovine urine by LC–MS/MS analyses that detected (Chiesa et al., 2015) 29 antimicrobial agents from eight different classes, as shown in Figure 1.

Fig. 1. General structures of the nine classes of studied antimicrobial agents.



In the current study, we used these detected antibiotics to develop a new method for the analysis of pig urine, enabling a direct comparison between the suitability of urine (not contemplated in most EU countries as a conventional matrix) and swine muscle tissue by analysing 43 paired urine and muscle samples. The multi-residual antibiotic strategies for the two matrices were developed and validated according to the Commission Decision 657/2002/CE (European Union, 2002), clarified by SANCO/2004/2726 revision 4. (European Union, 2008).

3.8.2. Materials and methods

3.8.2.1. Chemicals and reagents

All HPLC or analytical grade solvents were from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Formic (98–100%) and hydrochloric acid (37%) were from Riedel-de Haën (Sigma–Aldrich, St. Louis, MO, USA). Purified water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). Amoxicillin, ampicillin, cloxacillin, dicloxacillin, benzylpenicillin, oxolinic acid, nalidixic acid, cefquinome sulphate,

cefalexin, florfenicol, florfenicol amine, chloramphenicol, flumequine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, oxytetracycline, lincomycin, sulphathiazole, sulphadimidine, sulphadiazine, sulphadimethoxine, trimethoprim, erythromycin, tylosin and enrofloxacin d5 were used as the internal standard (IS) and purchased from Fluka.

For the preparation of EDTA-McIlvaine buffer solution (pH 4.0), 15 g of disodium hydrogen phosphate dihydrate, 13 g of citric acid monohydrate and 3.72 g of EDTA were dissolved in water and made up to 1 L with distilled water. Trichloroacetic acid 20% (w/v) aqueous solution was also prepared. All these reagents were purchased from Fluka.

3.8.2.2. Sample collection

Paired urine and muscle samples from 27 male and 16 female heavy pigs (160–170 kg weight) derived from ten different farms, were collected from the food chain in different slaughterhouses of Lombardy, Italy. The samples were immediately frozen, taken to the laboratory and stored at -20°C until analysis.

3.8.2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and kept at -20°C. Working solutions containing each of the studied analytes at 10 and 100 ng mL^{-1} were prepared daily. Each working solution was maintained at 4°C during the method validation procedures.

3.8.2.4. Sample extraction

3.8.2.4.1. Urine

Each urine sample (5 mL) was centrifuged at 2500 x g at 4°C for 5 min, then spiked with the IS to give a final concentration of 2 ng mL^{-1}. The compounds of interest were extracted by using Oasis HLB cartridges (3 mL, 60 mg, Waters, Milford, MA, USA) under vacuum. The cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5 M HCl and 3 mL of Milli-Q water. The sample was loaded, and then the cartridges were

washed with 3 mL of water and 3 mL of methanol:water (20:80, v/v). Finally, the analytes were eluted with 5 mL of methanol and collected in a 15-mL polypropylene tube. The eluate was evaporated by rotary vacuum evaporation. The dried extract was reconstituted in 200 µL of methanol:water (10:90 v/v), then transferred to an HPLC vial.

3.8.2.4.2. Muscle tissue

Each minced muscle sample (1 g) was spiked with the IS to give a final concentration of 2 ng g^{-1}. The analytes were then extracted by adding 5 mL of McIlvaine buffer (pH 4.0). Trichloroacetic acid (100 µl, 20% w/v) was added for protein precipitation and the sample then vortexed followed by sonication for 10 min. After centrifugation (2500 × g, 4°C, 10 min), the supernatant was transferred into a new polytetrafluoroethylene centrifuge tube and defatted with 2 × 3 mL of n-hexane. After each centrifugation (2500 × g, 5 min), the n-hexane layer was removed. The sample was further purified and extracted using Oasis HLB cartridges under vacuum. SPE cartridges were preconditioned with 3 mL of methanol and 3 mL of Milli-Q water. The sample was loaded, and then the cartridge was washed with 2 x 3 mL methanol:water (5:95 v/v). Finally, the compounds were eluted with 5 mL of methanol and were collected in a 15-mL polypropylene tube. The eluate was evaporated using a rotary vacuum evaporator Hei-VAP (Heidolph, Germany). The dried extract was reconstituted in 200 µL of methanol:water (10:90 v/v) and then transferred to an HPLC vial.

3.8.2.5. HPLC-MS/MS analyses

The HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was equipped with a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with a 20-µL loop. Analytical separation was carried out using a Synergi Hydro-RP reverse-phase HPLC column (150 x 2.0 mm, internal diameter 4 µm), with a C18 guard column (4 x 3.0 mm, Phenomenex, Torrance, CA, USA). The injection volume was 10 µL. The flow rate was 0.2 mL min^{-1}. The mobile phase consisted of a binary mixture of solvents A (0.1% aqueous formic acid) and B (MeOH). The elution started with 98% A, which was maintained for 5 min, followed by a linear gradient to 50% A at 22 min. Mobile phase B was then gradually increased to

95% at 24 min, which remained constant up to 29 min. The initial conditions were reached at 31 min, with an equilibration time that included the interval from 31–40 min. The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher) equipped with an electrospray interface (ESI) that was set in both the positive (ESI+) and negative (ESI-) modes. Acquisition parameters were optimised in the ESI mode by direct continuous pump-syringe infusion of the standard analyte solutions at 1 μg mL^{-1}, a flow rate of 20 μL min^{-1} and an MS pump rate of 100 μL min^{-1}. The following conditions were used: 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 the collision gas at 1.5 mTorr; and peak resolution 0.70 Da at full-width half-maximum (FWHM). Three diagnostic product ions were chosen for each analyte and IS. The multiple reaction-monitoring (MRM) mode was used for all data acquisition. The selected diagnostic ions, one of which was chosen for the quantification, the collision energies and the relative intensities are reported in Table 1. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1 MS/MS conditions for the MRM acquisitions of investigated antibiotics. Ions for quantification are in bold. The values in brackets represent the relative intensities (%). The collision energy (CE) is subscripted and expressed in volts.

Analyte	Precursor ion (m/z)	Product ions CE (m/z)	ESI
Amoxi	366	**$114(80)_{20}$**, $134(21)_{31}$, $349(100)_{7}$	(+)
Ampic	350	**$106(100)_{18}$**, $114(14)_{29}$**,** $160(14)_{14}$	(+)
Cloxac	436	$160(48)_{13}$, $178(35)_{33}$, **$277(100)_{14}$**	(-)
Dicloxa	468	$291(100)_{21}$, **$327(63)_{16}$**, $424(32)_{12}$	(-)
Benzylpe	335	$114(61)_{32}$, $160(92)_{12}$, **$176(100)_{14}$**	(+)
Oxolinic	262	$160(5)_{35}$, **$216(10)_{29}$,** $244(100)_{18}$	(+)
Nalidixic	233	$159(22)_{33}$, $187(69)_{26}$, **$215(100)_{16}$**	(+)
Cefale	348	$158(63)_{5}$, **$174(100)_{15}$,** $191(23)_{6}$	(+)
Cefquin	529	**$134(100)_{15}$**, $324(43)_{15}$, $396(44)_{10}$	(+)

Ciproflo	332	268(16)$_{22}$, **288(100)$_{17}$**, 314(94)$_{21}$	(+)
Enroflox	360	245(49)$_{26}$, **316(100)$_{18}$**, 342(29)$_{21}$	(+)
lomeflox	352	**265(100)$_{23}$**, 288(16)$_{19}$, 308(63)$_{16}$	(+)
Marbofloc	363	72(83)$_{23}$, **320(100)$_{15}$**, 345(18)$_{21}$	(+)
Florfer	356	169(1)$_{39}$, 185(35)$_{21}$, **336(100)$_{12}$**	(-)
Florfenico	248	130(24)$_{23}$, 134(8)$_{28}$, **230(100)$_{11}$**	(+)
Chloramp	321	152(65)$_{20}$, 194(35)$_{16}$, **257(100)$_{14}$**	(-)
Flumeq	262	174(13)$_{39}$, **202(54)$_{32}$,** 244(100)$_{19}$	(+)
Chlortetra	479	154(39)$_{27}$, **444(100)$_{21}$**, 462(69)$_{16}$	(+)
Doxycy	445	321(10)$_{31}$, 410(8)$_{24}$, **428(100)$_{19}$**	(+)
Oxytetrac	461	337(26)$_{29}$, **426(100)$_{19}$**, 443(52)$_{12}$	(+)
Tetracy	445	154(38)$_{30}$, **410(100)$_{19}$**, 427(43)$_{14}$	(+)
Lyncon	407	**126(100)$_{16}$**, 359(10)$_{18}$, 389(5)$_{28}$	(+)
Sulphath	256	92(50)$_{27}$, 108(45)$_{25}$, **156(100)$_{15}$**	(+)
Sulphadi	279	108(32)$_{26}$, 124(39)$_{265}$, **186(100)$_{18}$**	(+)
Sulphad	251	92(58)$_{27}$, **108(62)$_{23}$**, 156(100)$_{16}$	(+)
Sulphadim	311	92(30)$_{31}$, 108(34)$_{28}$, **156(100)$_{20}$**	(+)
Trimeth	291	230(100)$_{22}$, **261(75)$_{24}$**, 275(47)$_{21}$	(+)
Erythror	735	116(32)$_{36}$, **158(100)$_{30}$**, 576(37)$_{19}$	(+)
Tylos	817	156(12)$_{42}$, **174(100)$_{37}$**, 772(38)$_{29}$	(+)
Enrofloxaci	365	245(49)$_{32}$, **321(100)$_{27}$**, 347(46)$_{19}$	(+)

3.8.2.6. Methods validation

After the preliminary screening of a few samples of urine and muscle tissue, to identify "blank" samples, the validation was performed according to the criteria of the Commission Decision 2002/657/EC (European Union, 2002). For each analyte, the method performance was evaluated through its qualitative parameters, as well as molecular identification by retention time (RT) and transition ion ratios; through its quantitative parameters, such as linearity, recovery, accuracy in terms of trueness, and

precision expressed as the intra- and inter-day repeatability; and through the analytical limits, i.e. decision limit (CCα) and detection capability (CCβ), as clarified in SANCO/2004/2726 revision 4. (European Union, 2008).

Validation was performed by spiking the samples with each of the analytes, resulting in three analytical series (matrix validation curves). Each series had six replicates for three concentration levels that were previously chosen according to the minimum concentration detectable with our instrumentation (C0), which was 0.5 ng mL^{-1} for all analytes in urine and ranged from 1.0–10 ng mL^{-1} for the different analytes in the muscle samples (Table 2 and 3).

The instrumental linearity was also evaluated by drawing six-point calibration curves in the solvent containing a fixed amount of the IS (2 ng mL^{-1}), with the initial analyte concentration corresponding to the minimum detectable concentration for each group up to 100 ng mL^{-1}.

We evaluated robustness using the fractional factorial Youden design (European Union, 2002).

The experiments to evaluate matrix effects corresponded to the strategy used by Matuszewski, Constanzer and Chavez-Eng (2003).

Table 2. Validation parameters of all investigated antibiotics in pig urine samples.

Analyte	CCα (ng mL^{-1})	CCβ (ng mL^{-1})	Concentration level (ng mL^{-1})	Recovery % (*n*=18)	Repeatability intra-day (CV; *n*=6)	Repeatability inter-day (CV;*n*=18)
Amoxicillin	0.54	0.71	0.50	95	13	20
			1.00	93	11	14
			1.50	103	9	11
Ampicillin	0.51	0.69	0.50	98	12	20
			1.00	99	11	11
			1.50	99	10	9
Cloxacillin	0.57	0.73	0.50	90	15	19
			1.00	92	10	12
			1.50	93	10	11
			0.50	95	13	18

Dicloxacillin	0.60	0.75	1.00	99	10	15
			1.50	99	10	12
Benzylpenicillin	0.86	1.10	0.50	91	15	18
			1.00	91	12	15
			1.50	92	11	15
Oxolinic acid	0.55	0.63	0.50	98	9	12
			1.00	99	9	8
			1.50	99	7	8
Nalidixic acid	0.54	0.60	0.50	99	10	13
			1.00	100	7	9
			1.50	101	7	7
Cefalexin	0.62	0.73	0.50	105	7	10
			1.00	100	5	9
			1.50	102	5	9
Cefquinome	0.66	0.81	0.50	99	10	20
			1.00	99	11	13
			1.50	100	11	13
Ciprofloxacin	0.63	0.84	0.50	102	12	20
			1.00	100	12	16
			1.50	101	8	10
Enrofloxacin	0.60	0.71	0.50	101	8	11
			1.00	100	7	10
			1.50	101	7	8
Lomefloxacin	0.72	0.91	0.50	104	10	15
			1.00	102	8	15
			1.50	102	8	13
Marbofloxacin	0.55	0.62	0.50	104	6	11
			1.00	101	5	7
			1.50	100	5	7
Florfenicol	0.70	0.90	0.50	103	12	19
			1.00	102	10	19
			1.50	106	9	14
Florfenicol amine	0.65	0.76	0.50	100	11	12
			1.00	100	10	11
			1.50	100	10	11
Chloramphenicol	0.54	0.62	0.50	97	10	15
			1.00	97	10	12
			1.50	98	8	11

Flumequine	0.58	0.67	0.50	94	15	15
			1.00	93	11	15
			1.50	95	8	13
Chlortetracycline	0.56	0.68	0.50	99	7	11
			1.00	98	5	8
			1.50	98	5	6
Doxycycline	0.55	0.65	0.50	102	8	12
			1.00	103	8	10
			1.50	103	8	7
Oxytetracycline	0.69	0.85	0.50	100	10	13
			1.00	99	9	11
			1.50	100	8	10
Tetracycline	0.65	0.76	0.50	105	9	11
			1.00	105	8	9
			1.50	101	8	8
Lyncomicin	0.53	0.62	0.50	100	10	12
			1.00	101	7	9
			1.50	100	6	7
Sulfathiazole	0.55	0.71	0.50	98	12	13
			1.00	98	10	11
			1.50	97	10	10
Sulfadimidine	0.57	0.74	0.50	99	8	10
			1.00	99	8	8
			1.50	100	7	8
Sulfadiazine	0.55	0.73	0.50	100	10	15
			1.00	98	10	11
			1.50	100	9	11
Sulfadimethoxine	0.58	0.78	0.50	97	13	18
			1.00	98	9	15
			1.50	98	9	13
Trimethoprim	0.60	0.79	0.50	92	11	17
			1.00	95	11	13
			1.50	96	10	11
Erythromycin	0.63	0.80	0.50	92	15	19
			1.00	95	9	10
			1.50	95	9	10
Tylosin	0.54	0.76	0.50	96	14	19
			1.00	95	13	18
			1.50	96	8	12

Table 3. Validation parameters of all investigated antibiotics in pig muscle samples

Analyte	CCα (ng mL^{-1})	CCβ (ng mL^{-1})	Concentration level (ng mL^{-1})	Recovery % (*n*=18)	Repeatability	
					intra-day (CV; *n*=6)	inter-day (CV;*n*=18)
Amoxicillin	10.02	10.51	10.00	90	11	21
			20.00	91	9	16
			30.00	101	9	10
Ampicillin	5.11	5.63	5.00	90	15	20
			10.00	98	13	14
			15.00	101	10	9
Cloxacillin	10.05	10.54	10.00	96	15	18
			20.00	97	11	13
			30.00	99	9	10
Dicloxacillin	5.10	5.68	5.00	93	14	19
			10.00	97	12	17
			15.00	99	11	11
Benzylpenicillin	5.32	5.89	5.00	90	14	20
			10.00	92	14	16
			15.00	93	13	14
Oxolinic acid	1.10	1.63	1.00	90	17	22
			2.00	93	15	17
			3.00	95	12	12
Nalidixic acid	1.14	1.67	1.00	93	14	18
			2.00	93	10	14
			3.00	95	9	11
Cefalexin	5.51	5.82	5.00	101	15	21
			10.00	99	13	20
			15.00	102	11	17
Cefquinome	10.09	11.02	10.00	90	14	22
			20.00	91	13	16
			30.00	103	9	10
Ciprofloxacin	1.40	1.52	1.00	95	14	16
			2.00	105	14	16
			3.00	98	11	12

Enrofloxacin	0.95	1.13	1.00	101	11	19
			2.00	100	9	19
			3.00	101	7	9
Lomefloxacin	1.17	1.29	1.00	97	15	20
			2.00	102	14	20
			3.00	99	13	17
Marbofloxacin	1.42	1.57	1.00	103	13	20
			2.00	99	13	14
			3.00	100	8	10
Florfenicol	1.32	1.84	1.00	97	14	20
			2.00	99	12	17
			3.00	100	9	9
Florfenicol amine	1.30	1.45	1.00	92	11	16
			2.00	95	11	15
			3.00	97	10	11
Chloramphenicol	0.97	1.20	1.00	90	15	18
			2.00	91	15	15
			3.00	91	11	12
Flumequine	0.96	1.23	1.00	90	13	17
			2.00	93	10	16
			3.00	95	9	11
Chlortetracycline	1.22	1.49	1.00	92	7	11
			2.00	103	5	11
			3.00	98	7	10
Doxycycline	0.97	1.74	1.00	106	14	19
			2.00	98	12	19
			3.00	101	12	13
Oxytetracycline	1.21	1.52	1.00	102	11	18
			2.00	99	9	14
			3.00	101	9	9
Tetracycline	1.33	1.65	1.00	99	14	19
			2.00	102	11	13
			3.00	98	9	10
Lyncomicin	1.15	1.29	1.00	101	14	20
			2.00	99	13	17
			3.00	100	11	12
Sulfathiazole	1.08	1.26	1.00	95	15	19
			2.00	93	12	16
			3.00	97	9	11

Sulfadimidine	1.13	1.25	1.00	98	10	13
			2.00	99	8	11
			3.00	100	7	8
Sulfadiazine	1.09	1.28	1.00	101	12	17
			2.00	98	10	14
			3.00	103	9	11
Sulfadimethoxine	1.19	1.42	1.00	90	14	20
			2.00	90	11	16
			3.00	93	11	11
Trimethoprim	1.15	1.41	1.00	90	12	19
			2.00	91	11	16
			3.00	91	8	11
Erythromycin	5.23	5.55	5.00	90	14	18
			10.00	91	10	12
			15.00	92	11	11
Tylosin	1.06	1.24	1.00	91	13	19
			2.00	95	11	15
			3.00	95	9	13

3.8.3. Results and discussion

3.8.3.1 Validation performances

The mean recoveries for all analytes ranged between 90–107%, considering both matrices and all analytes. The 20 urine and muscle blank swine samples analysed to evaluate specificity, did not show any interference (signals, peaks, ion traces) in the region of interest, i.e. where the target analytes were expected to elute. The selectivity showed a good compliance with the relative RTs for each analyte, which was found to be within the 2.5% tolerance, with a signal-to-noise ratio >3 when compared with the standards. Moreover, the three chosen transitions showed an ion ratio within the recommended tolerances (European Union, 2002), when compared with the standards. The matrix validation curves constructed for each analyte demonstrated a good fit for all the analytes with a correlation coefficient >0.99 in both matrices. The intra- and inter-day repeatability (Thompson, 2000), representing precision, were calculated using one-way analysis of variance (ANOVA) and expressed as coefficients of variation (CVs) For all analytes, the intra-day repeatability values were below 15 and 17% in the urine and

muscle tissue samples, respectively, while the corresponding inter-day repeatability values were below 20 and 22%.

Based on the methods described in SANCO/2004/2726 revision 4 (European Union, 2008), the CCα ranged from 0.54–0.86 and 0.95–10.09 ng mL^{-1} in the urine and muscle tissue, respectively, while the CCβ values ranged from 0.60–1.10 ng mL^{-1} in urine and 1.13–11.02 ng mL^{-1} in muscle tissue (Table 2 and 3).

The method ruggedness, evaluated using the fractional factorial Youden design (European Union, 2002), was good in both matrices. Using the strategy of Matuszewski et al. (2003), a modest matrix effect was obtained, with values ranging from 89–104 and 82–109% for the different compounds in the urine and muscle tissue samples, respectively.

As we proved in other previous works (Chiesa et al., 2015 and Chiesa et al., 2016), all analytes in the working solutions and in the spiked samples showed an acceptable stability (CV ≤ 2%) until 1 month of storage at −20 °C, except amoxicillin and cefquinome. After 1 week, the concentration of these two antibiotics decreased more than the acceptable value. We therefore decided to daily prepare the working solutions.

3.8.3.2 Application of the method

The developed and validated methods were applied to the analyses of 43 urine and muscle paired samples from male and female heavy pigs, collected from different slaughterhouses. The samples were completely anonymous and represented official controls for monitoring residues of antibiotics within the food chain. A comparison of the average concentrations ± SD and the medians for the analytes detected in the paired urine and muscle tissue samples (Table 4), revealed the suitability of urine samples for the majority of detected antibiotics and for the antibiotics present at higher concentration in the urine than muscle tissue.

Table 4 Average concentration ±SD, number and percentage of positives and median of the analytes detected in urine and muscle samples.

	Urine samples				muscle samples			
Analyte	**Average conc.±SD (ng mL^{-1})**	**Positives**	**% Positives**	**Median**	**Average conc.±SD (ng g^{-1})**	**Positives**	**% Positives**	**Median**
Chloramphenicol	7.83±2.07	4	9	0	1.42±0.67	5	12	0
Florfenicol	7.27±9.92	5	12	0	0	0	0	0
Florfenicol amine	7.68±5.60	17	40	0	0	0	0	0
Doxycycline	46.59±74.89	34	79	4.55	6.12±6.53	15	35	0
Tetracycline	1.71±1.27	10	23	0	0	0	0	0
Oxytetracycline	17.06±16.55	12	28	0	1.89	1	2	0
Chlortetracyclin	0.86±0.38	7	16	0	0	0	0	0
Lyncomicin	0.86±0.38	17	40	0	2.66±1.34	3	7	0
Tylosin	110.27±112.09	8	19	0	0	0	0	0
Sulphadiazine	86.47±66.66	5	12	0	0	0	0	0
Trimethoprim	14.59±5.81	5	12	0	0	0	0	0
Enrofloxacin	2.36±1.20	6	14	0	0	0	0	0

These data provide evidence that supports urine as the preferential elimination route of most antibiotics in their unchanged form, as observed previously in bovine urine (Chiesa et al., 2015). The possibility to do on-farm controls using this matrix is controversial, considering that most illegal treatments could be detected in the instance of illicit drug administration or non-recorded administration of regulated drugs. Even if most of the antibiotics found are below the MRLs set by the European Regulation (European Union, 2010), the results reveal the contemporary presence of different classes of antibiotics in the urine of the analysed sample. Moreover, identical combinations of antibiotics were found in the samples belonging to the same farm, but distinct from those obtained from different slaughterhouses. In particular, chloramphenicol, a strictly forbidden antibiotic, was detected both in urine and muscle tissue samples coming from the same farm (on four occasions in urine and five occasions in muscle). In this instance, their urine concentrations were more than four-fold higher than in muscle tissue.

Regarding the other antimicrobial agents studied, when they were found in muscle, the MRLs were never exceeded. For all the antimicrobials studied, the MRL value was 100 μg kg^{-1}, except for florfenicol, whose MRL is 200 μg kg^{-1} because it represents the sum of the antimicrobial and its metabolite, florfenicol amine (European Union, 2010).

Doxycycline was one of the antibiotics most frequently found in urine (in 37 samples, at a maximum of 339.45 μg L^{-1}). In the muscle tissues, doxycycline was found 15 times and the maximum recorded was 21.05 μg kg^{-1}. When it was present in the muscle, it was always detected in the urine but not vice versa. However, when the concentration in the urine was > 13.28 μg L^{-1} it was also detected in the muscle. When compared with muscle tissue, the urinary doxycycline concentration was 1.3–50 times higher. In the muscle tissue, its concentration never exceeded 100 μg L^{-1}. In contrast, this value was exceeded in six urine samples, four of which belonged to the same farm. However, all the samples were from anonymous animals. Consequently, we could neither discern if the animals had been illicitly treated, or if they had undergone a prophylactic or therapeutic treatment nor identify the route of administration, so it is not possible to evaluate pharmacokinetic considerations.

For the other antimicrobial agents evaluated, oxytetracycline was detected in some urine samples from four different farms, but only once in a muscle sample (1.89 μg kg^{-1}) with its paired urine sample showing the highest concentration, which was 52.14 μg L^{-1}. Tetracycline was found in all urine samples derived from one farm and sporadically in other instances, but was never detected in the muscle. Its highest concentration was 4.28 μg L^{-1}. Chlortetracycline was found in all urine samples from one farm, and in one another urine sample derived from a different farm (maximum value 1.27 μg L^{-1}), but was never detected in the muscle. Lincomycin was in 17 urine samples at a maximum of 120.69 μg L^{-1} and only three times in the muscle tissue. Its urine-to-muscle concentration ratio ranged from 0.2–12.88. Florfenicol and florfenicol amine, alone or combined, were detected in 17 urine samples at a maximum of 40.28 μg L^{-1}. Neither florfenicol nor its metabolite was found in the corresponding muscle samples. Tylosin was detected seven times, mostly in urine samples from two different farms but never in the muscle samples. The highest concentration detected was 267.74 μg L^{-1}. Sulphadiazine was found at a maximum 180.42 μg L^{-1} in five samples from one farm and concomitantly with trimethoprim, but never in the muscle. Enrofloxacin was detected in almost all the urine samples originating from two farms and in one other sample from a different farm (3.72 μg L^{-1} maximum), but never in the muscle.

3.8.4. Conclusion

The results shown in this study indicate that different classes of antibiotics are contemporary used for swine breeding and are generally found at a higher frequency and at higher concentrations in urine than in muscle tissue. We did not find a strict correlation between the two matrices, but usually, when the concentration in urine was very high, the analyte was detected in the paired muscle sample. The detection in urine depicts an overall framework of an excessive use of the antibiotics in pig farming. The use of urine as a control matrix may, therefore, be useful to ascertain any illicit treatment or to monitor the antibiotic concentrations after therapeutic uses, at the farm through a non-invasive collection method or, alternatively, at the slaughterhouse for the higher concentrations detectable in urine than in muscle. Consequently, withdrawal periods should be accurately scrutinised to ensure the safety of meat in the supply chain before it is presented to consumers. Moreover, it highlights the overuse of antibiotics associated with the increasingly urgent threat of antibiotic resistance.

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3.9. Occurrence of antibiotics in mussels and clams from various FAO areas

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

ABSTRACT

Filter feeders, like mussels and clams, are suitable bioindicators of environmental pollution. These shellfish, when destined for human consumption, undergo a depuration step that aims to nullify their pathogenic microorganism load and decrease chemical contamination. Nevertheless, the lack of contamination by drugs may not be guaranteed. Antimicrobials are a class of drugs of particular concern due to the increasing phenomenon of antibiotic resistance. Their use in breeding and aquaculture is a major cause of this. We developed a multiclass method for the HPLC-MS/MS analysis of 29 antimicrobials, validated according to the Commission Decision 2002/657/UE guidelines, and applied it to 50 mussel and 50 clam samples derived from various Food and Agricultural Organisation marine zones. The results obtained, indicate a negligible presence of antibiotics. Just one clam sample showed the presence of oxytetracycline at a concentration slightly higher than the European Union Maximum residue limit set for fish.
***Keywords**:* Antibiotics, Clam, HPLC-MS/MS, Mussel

Highlights

A multiclass LC-MS/MS method for 29 antibiotics was developed and validated.
Our detection limits were much lower than the maximum residue limits.
Pool of mussels and clams from different FAO zones were analysed.
Antibiotic presence in the analysed shellfish is negligible.

Chemical compounds studied in this article
Amoxicillin (PubChem CID: 33613); Ampicillin (PubChem CID: 6249); Benzylpenicillin (PubChem CID: 5904); Cefalexin (PubChem CID: 27447); Cefquinome sulphate (PubChem CID: 9577261); Chloramphenicol (PubChem CID:5959); Chlortetracycline (PubChem CID: 54737570); Ciprofloxacin (PubChem CID: 2764); Cloxacillin (PubChem CID: 6098); Dicloxacillin (PubChem CID: 18381); Doxycycline hyclate (PubChem CID: 54686183); Enrofloxacin (PubChem CID: 71188); Erythromycin (PubChem CID: 12560); Florfenicol (PubChem CID: 114811); Florfenicol amine (PubChem CID: 156406);

Flumequine (PubChem CID: 3374); Lincomycin (PubChem CID: 3000540); Lomefloxacin hydrochloride (PubChem CID: 68624); Marbofloxacin (PubChem CID: 60651); Nalidixic acid (PubChem CID: 4421); Oxolinic acid (PubChem CID: 4628); Oxytetracycline (PubChem CID: 54675779); Sulphadiazine (PubChem CID: 441244); Sulphadimethoxine (PubChem CID: 5323); Sulphadimidine (PubChem CID: 5327); Sulphathiazole (PubChem CID: 5340); Tetracycline hydrochloride (PubChem CID: 54704426); Trimethoprim (PubChem CID: 5578); Tylosin (PubChem CID: 5280440).

3.9.1. Introduction

Antibiotics are among the most frequently detected group of potentially toxic pharmaceuticals; this underscores the following ecotoxicological concerns: 1) the cumulative toxic effects of antibiotics on aquatic animals are not well understood, 2) their continuous presence leads to the development of antibiotic-resistant bacteria, and 3) antibiotics can act, at very low concentrations, as signalling agents and change the natural microbial diversity in aquatic ecosystems (Fatta-Kassinos, Meric, & Nikolaou, 2011).An unknown amount of these drugs ends up either indirectly in the receiving waters, through sewer plants and land-fields, or directly as a result of intensive fish farming. For these reasons, organisms could also be exposed to a variety of compounds present in the environment at low concentrations. In recent years, pharmacological substances in the aquatic environment have become an increasing concern. In this respect, municipal wastewater effluents represent the main source of pharmaceuticals in the environment (Kolpin et al., 2002).

Bivalves and the blue mussel (*Mytilus edulis*), in particular, are successfully used as indicator organisms for marine pollution monitoring (Baumard Budzinski, & Garrigues, 1998; O'Connor, 1998; Widdows et al., 1995). The general assumption is that mussel appears to be an appropriate sentinel organism because of its global distribution of large and accessible populations, its large size and sedentary adulthood, its tolerance to diverse environmental conditions, the ventilation of large volumes of water for nutrition, respiration and excretion (Krieger, Gee, & Lim, 1981), and its ability to accumulate numerous contaminants (Moy & Walday, 1996).

Hence, an increasing demand for biological studies of aquatic organisms has become a major impetus for the development and validation of high-performing analytical techniques capable of determining various antibiotics. Zouiten, Beltifa, Van Loco, Mansour and Reyns (2016) demonstrated the usefulness of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to detect certain antibiotic residues in *Mytilus galloprovincialis* exposed to pharmaceutical wastewater in Tunisia. Li, Shi, Gao, Liu and Cai (2012) reported 22 antibiotics in molluscs obtained from the Bohai sea (China), based on accelerated solvent extraction pressurised liquid extraction, followed by a solid-phase extraction (SPE) clean-up. An enzymatic-microwave assisted extraction method with subsequent high-performance liquid chromatography (HPLC) was developed for the determination of 11 antibiotics in fish tissue and mussels of Spain (Fernandez-Torres, Lopez, Consentino, Mochon, & Payan, 2011). Conversely, Le Bris and Pouliquen (2004) studied the bioaccumulation of two antibiotics, oxytetracycline and oxolinic acid, by the blue mussel, and stated that most veterinary and human antibiotics, such as tetracyclines and sulphonamides, should weakly accumulate in mussel.

In this context, the current study aimed to develop and validate (European Community, 2002; European Union, 2008) a sensitive, specific and robust HPLC-MS/MS multiclass method, for the determination of 29 antibiotics belonging to eight different chemical classes (penicillin, quinolones, tetracyclines, sulphonamides, macrolides, lincosamides, cephalosporins, amphenicols), in mussels and clams, both wild and farmed, collected from various geographic areas of the world and, particularly, Italy. The two types of shellfish were carefully selected for a comparison, considering that mussels tend to grow on the surface of wave-washed rocks, while clams live in shallow water. Hence, the development of a high sensitive multiclass method for antibiotics in this two edible organisms located from distinct areas and marine layers, and the differences in bioaccumulation between these organisms could be achieved to expand the knowledge from the point of view of food safety, relatively also to environmental contamination, to increase the proportion of quantified data and accurately monitor the presence of antibiotics due to the antibiotic resistance matter.

3.9.2. Material and methods

3.9.2.1. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100%) was obtained from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA). Trichloroacetic acid (TCA) crystals and the ingredients required to prepare EDTA-McIlvaine buffer solution, pH 4 (disodium hydrogen phosphate dihydrate, citric acid monohydrate and EDTA) were purchased from Fluka. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges (Oasis HLB 3 mL, 60 mg) were provided by Waters (Milford, MA, USA). Amoxicillin, ampicillin, cloxacillin, dicloxacillin, benzylpenicillin, oxolinic acid, nalidixic acid, cefquinome sulphate, cefalexin, florfenicol, florfenicol amine, chloramphenicol, flumequine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, oxytetracycline, lincomycin, sulphathiazole, sulphadimidine, sulphadiazine, sulphadimethoxine, trimethoprim, erythromycin, tylosin and enrofloxacin d5 as the internal standards (IS) were purchased from Fluka.

3.9.2.2. Standard solutions

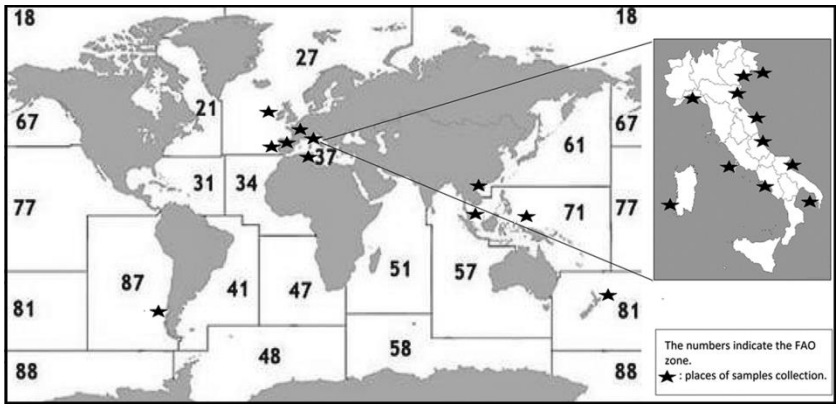
For each standard, stock solutions were prepared (1 mg mL^{-1}) in methanol and kept at -20 °C. Working solutions at 10 and 100 ng mL^{-1}, were prepared daily to spike the samples during the validation and to construct the calibration curves for the quantification of the real samples. Each working solution was maintained at 4 °C during the method validation procedures.

3.9.2.3. Sample collection

We collected a total 100 samples (500 g each one), and we created 100 pools obtained by dispersing 200 g of shellfish edible parts pooled by using an Ultraturrax (IKA®-Werke GmbH and Co. KG, Staufen, Germany) at 13500 rpm for 4 minutes. Mussels (a total of 50 pool samples of three species*: M. galloprovincialis*, *Mytilus edulis* and *Mytilus chilensis*) and clams (a total of 50 pool samples of six species*: Meretrix lyrata*, *Venerupis decussata*, *Venerupis philippinarum*, *Meretrix meretrix*, *Paphia textile* and

Venus gallina), half wild and half farmed to evaluate the presence of antibiotics due to eventual antibiotic treatments in farms and/or the presence of these drugs due the environmental pollution in case of wild shellfish. Moreover they were collected from various Food and Agricultural Organisation (FAO) marine zones (Fig. 1) to evaluate the antibiotic detection relatively to the different geographical location. The samples were also collected from different marine layers because mussels tend to grow on the surface of wave-washed rocks, while clams live in shallow water so in depth. The samples were immediately frozen, transported to the laboratory and stored at -20 °C, until further analysis.

Figure 1. Map of sample collection sites and magnification of Italy (inset).



3.9.2.4. Sample extraction

An aliquot (1 g wet weight) of homogenised shelled mussel or clam, spiked with the IS at a final 2 ng mL^{-1}, 100 µl of 20% TCA for protein precipitation, and 5 mL McIlvaine buffer (pH 4.0), were combined. The samples were vortexed and sonicated for 15 min. After centrifugation (2500*g*, 4 °C, 10 min), the supernatant was transferred to a clean polytetrafluoroethylene centrifuge tube and defatted with 2 × 3 mL n-hexane. Each time, the n-hexane layer was discarded after centrifugation at 2500*g*, 4 °C for 5 min. The obtained extracts were purified by SPE Oasis HLB cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL methanol and 3 mL Milli-Q water. The samples were loaded, and then washed with 2 x 3 mL methanol:water (5:95 v/v). Finally, the analytes were eluted with 5 mL methanol and collected in a 15-mL glass tube. The eluate was evaporated in a rotary vacuum evaporator at 40 °C. The dried

extract was reconstituted in 200 µL methanol:water (10:90 v/v), and then transferred to an auto-sampler vial. The injection volume was 10 µL.

3.9.2.5. HPLC-MS/MS analyses

The chromatographic separation was performed by a Surveyor MS quaternary pump with a degasser, a Rheodyne valve with a 20-µL loop and a Surveyor AS autosampler with a column oven (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation of the compounds was obtained using a Synergi Hydro-RP reverse-phase HPLC column (150 x 2.0 mm, internal diameter 4 µm), with a C18 guard column (4 x 3.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase was a binary mixture of solvents A (aqueous formic acid 0.1%) and B (methanol). The run (0.2 mL min^{-1}) started with 98% A (5 min), which was then increased linearly to 50% (at 22 min). Next, mobile phase B was gradually increased to 95% (at 24 min) and remained constant for 5 min. The initial conditions were reached at 31 min, with an equilibration time that included the interval from 31–40 min. A triple-quadrupole TSQ Quantum MS (Thermo Fisher) equipped with an electrospray interface (ESI) set in the positive (ESI+) mode was used to detect all analytes, except isoxazolyl penicillins and amphenicols, which were detected in the negative (ESI-) mode. Acquisition parameters were optimised by direct continuous pump-syringe infusion of the standard analyte solutions at 1 µg mL^{-1}. The flow rate was set at 20 µL min^{-1} flow rate, and the MS pump rate at 100 µL min^{-1}. The following conditions were used: capillary voltage 3.5 kV; ion transfer capillary temperature 340 °C; nitrogen as the sheath and auxiliary gases at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr, and peak resolution 0.70 Da at full-width half-maximum (FWHM) (Chiesa et al., 2016). Three diagnostic product ions were chosen for each analyte and IS, as carried out in an our previous styudy about antibiotics in bovine urine (Chiesa et al., 2015). The acquisition was performed in multiple reaction-monitoring (MRM) mode. The selected diagnostic ions, one of which was chosen for the quantification, the collision energies and the relative intensities are reported in Table 1. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1. MS/MS conditions for the MRM acquisitions of investigated antibiotics.

Analyte	Precursor ion (m/z)	Product ions CE (m/z)	ESI
amoxicillin	366	**114(80)$_{20}$**, 134(21)$_{31}$, 349(100)$_{7}$	(+)
ampicillin	350	**106(100)$_{18}$**, 114(14)$_{29}$, 160(14)$_{14}$	(+)
cloxacillin	436	160(48)$_{13}$, 178(35)$_{33}$, **277(100)$_{14}$**	(-)
dicloxacillin	468	291(100)$_{21}$, **327(63)$_{16}$**, 424(32)$_{12}$	(-)
benzylpenicillin	335	114(61)$_{32}$, 160(92)$_{12}$, **176(100)$_{14}$**	(+)
oxolinic acid	262	160(5)$_{35}$, **216(10)$_{29}$**, 244(100)$_{18}$	(+)
nalidixic acid	233	159(22)$_{33}$, 187(69)$_{26}$, **215(100)$_{16}$**	(+)
cefalexin	348	158(63)$_{5}$, **174(100)$_{15}$**, 191(23)$_{6}$	(+)
cefquinome	529	**134(100)$_{15}$**, 324(43)$_{15}$, 396(44)$_{10}$	(+)
ciprofloxacin	332	268(16)$_{22}$, **288(100)$_{17}$**, 314(94)$_{21}$	(+)
enrofloxacin	360	245(49)$_{26}$, **316(100)$_{18}$**, 342(29)$_{21}$	(+)
lomefloxacin	352	**265(100)$_{23}$**, 288(16)$_{19}$, 308(63)$_{16}$	(+)
marbofloxacin	363	72(83)$_{23}$, **320(100)$_{15}$**, 345(18)$_{21}$	(+)
florfenicol	356	169(1)$_{39}$, 185(35)$_{21}$, **336(100)$_{12}$**	(-)
florfenicol amine	248	130(24)$_{23}$, 134(8)$_{28}$, **230(100)$_{11}$**	(+)
chloramphenicol	321	152(65)$_{20}$, 194(35)$_{16}$, **257(100)$_{14}$**	(-)
flumequine	262	174(13)$_{39}$, **202(54)$_{32}$**, 244(100)$_{19}$	(+)
chlortetracycline	479	154(39)$_{27}$, **444(100)$_{21}$**, 462(69)$_{16}$	(+)
doxycycline	445	321(10)$_{31}$, 410(10)$_{24}$, **428(100)$_{19}$**	(+)
oxytetracycline	461	337(26)$_{29}$, **426(100)$_{19}$**, 443(52)$_{12}$	(+)
tetracycline	445	154(38)$_{30}$, **410(100)$_{19}$**, 427(43)$_{14}$	(+)
lincomycin	407	**126(100)$_{16}$**, 359(10)$_{18}$, 389(5)$_{28}$	(+)
sulphathiazole	256	92(50)$_{27}$, 108(45)$_{25}$, **156(100)$_{15}$**	(+)
sulphadimidine	279	108(32)$_{26}$, 124(39)$_{265}$, **186(100)$_{18}$**	(+)
sulphadiazine	251	92(58)$_{27}$, **108(62)$_{23}$**, 156(100)$_{16}$	(+)

sulphadimethoxine	311	$92(30)_{31}$, $108(34)_{28}$, **$156(100)_{20}$**	(+)
trimethoprim	291	$230(100)_{22}$, **$261(75)_{24}$**, $275(47)_{21}$	(+)
erythromycin	735	$116(32)_{36}$, **$158(100)_{30}$**, $576(37)_{19}$	(+)
tylosin	817	$156(12)_{42}$, **$174(100)_{37}$**, $772(38)_{29}$	(+)
enrofloxacin-d5 (IS)	365	$245(49)_{32}$, **$321(100)_{27}$**, $347(46)_{19}$	(+)

Ions for quantification are in bold. The values in brackets represent the relative intensities (%). CE: collision energy, subscripted and expressed in volts.

3.9.2.6. Method validation

After the identification of samples in which we checked the absence of antibiotics, through a preliminary screening of pooled mussel or clam samples, the method was validated according to the Commission Decision 2002/657/EC criteria (European Community, 2002).

For each analyte, the method performance was evaluated by the determination of retention time (RT), transition ion ratios, recovery, accuracy (trueness), precision (expressed as the intra- and inter-day repeatability), linearity, as well as the decision limit (CCα) and detection capability (CCβ), which were calculated as described in SANCO/2004/2726 revision 4 (European Union, 2008).

Twenty blank samples were used to evaluate the specificity and to check for any interference (signals, peaks, ion traces) in the region of interest where the target analytes were expected to elute. The selectivity was also tested by verifying a signal-to-noise ratio > 3 at the expected RT, and the ion abundance ratio associated with the different fragmentations. Validation was done by spiking the samples with all analytes at three concentration levels (C_0, 2 x C_0, 3 x C_0, validation levels Table 2) that were previously chosen according to a minimum detectable experimental concentration (C_0) in our conditions, considering that the maximum residue limits (MRLs) recommended by the Commission Regulation 37/2010 (European Union, 2010) for fish (but not for shellfish) range from 50–200 μg kg^{-1}. Each level had six replicates. The validation trials were repeated for three different days, resulting in three analytical series (matrix validation curves).

The instrumental linearity was also assessed through six-point calibration curves in the solvent containing a precise amount of IS (2 ng mL^{-1}), starting from the minimum detectable concentration for each group up to 100 ng mL^{-1}.

The recovery was calculated using the data from the validation points of the three, analytical series, expressed as a percentage of the measured concentration relative to the spiked concentration. The precision (intra- and inter-day repeatability) was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at the three concentration levels of the three, analytical series. Robustness was assessed using the approach of Youden (European Union, 2002), which is a fractional factorial design, based on minor modification (±10%) of seven experimental conditions of eight samples spiked at the minimum detectable concentrations.

Matrix effects was evaluated by Matuszewski, Constanzer and Chavez-Eng (2003) strategy, comparing the analytes of interest added post-extraction with pure solutions prepared in the mobile phase containing an equivalent amounts of the studied compounds.

Table 2. Validation parameters for all antibiotics.

Analyte	CCα (ng g^{-1})*	CCβ (ng g^{-1})*	Validation levels (ng g^{-1})*	Recovery (%) (*n*=18)	Repeatability	
					intra-day (CV; *n*=6)	inter-day (CV; *n*=18)
Amoxicillin	1.04	1.55	1.00	86	14	20
			2.00	92	9	16
			3.00	101	8	10
Ampicillin	1.10	1.62	1.00	90	14	20
			2.00	98	13	14
			3.00	100	9	9
Cloxacillin	5.05	5.56	5.00	95	14	17
			10.00	97	11	13
			15.00	98	9	10
Dicloxacillin	5.10	5.68	5.00	93	13	18
			10.00	97	12	17
			15.00	99	11	11

			5.00	90	14	19
Benzylpenicillin	5.32	5.89	10.00	92	13	17
			15.00	93	13	14
			1.00	88	14	20
Oxolinic acid	1.11	1.64	2.00	87	14	18
			3.00	92	12	13
			1.00	92	13	17
Nalidixic acid	1.17	1.70	2.00	95	11	15
			3.00	95	9	11
			5.00	102	14	20
Cefalexin	5.53	5.80	10.00	97	13	20
			15.00	101	13	18
			5.00	103	14	20
Cefquinome	5.75	5.93	10.00	91	11	15
			15.00	109	9	9
			1.00	95	14	16
Ciprofloxacin	1.40	1.52	2.00	105	14	16
			3.00	98	11	12
			1.00	100	8	15
Enrofloxacin	1.13	1.17	2.00	100	8	15
			3.00	100	7	8
			1.00	97	14	20
Lomefloxacin	1.18	1.27	2.00	103	13	20
			3.00	98	13	18
			1.00	103	14	20
Marbofloxacin	1.44	1.58	2.00	97	14	15
			3.00	101	8	10
			1.00	98	13	17
Florfenicol	1.39	1.89	2.00	101	12	17
			3.00	100	8	9
			1.00	92	6	12
Florfenicol amine	1.37	1.48	2.00	104	11	15
			3.00	97	10	11
			1.00	87	14	15
Chloramphenicol	1.03	1.34	2.00	91	11	13
			3.00	91	11	12
			0.50	89	13	17
Flumequine	0.54	0.83	1.00	89	11	15

			1.50	91	9	11
Chlortetracycline	1.26	1.48	1.00	92	7	11
			2.00	103	5	11
			3.00	98	7	10
Doxycycline	0.56	0.74	0.50	104	14	20
			1.00	96	13	20
			1.50	101	12	13
Oxytetracycline	0.51	0.72	0.50	102	10	16
			1.00	98	8	15
			1.50	101	9	9
Tetracycline	0.53	0.65	0.50	99	14	20
			1.00	113	10	12
			1.50	96	9	10
Lincomycin	1.15	1.29	1.00	101	14	20
			2.00	99	13	17
			3.00	100	11	12
Sulphathiazole	1.16	1.31	1.00	86	14	20
			2.00	96	10	17
			3.00	99	9	11
Sulphadimidine	1.13	1.25	1.00	101	8	11
			2.00	99	7	9
			3.00	100	7	7
Sulphadiazine	1.09	1.36	1.00	102	11	18
			2.00	102	9	15
			3.00	104	9	11
Sulphadimethoxine	1.14	1.45	1.00	87	12	19
			2.00	89	11	13
			3.00	93	10	11
Trimethoprim	1.11	1.39	1.00	90	12	19
			2.00	91	9	15
			3.00	91	7	12
Erythromycin	5.23	5.54	5.00	89	14	18
			10.00	87	10	11
			15.00	92	9	10
Tylosin	1.07	1.21	1.00	91	12	19
			2.00	94	11	13
			3.00	95	7	13

*The concentrations were expressed in ng g^{-1} wet weight.

3.9.3. Results and discussion

3.9.3.1 Validation performances

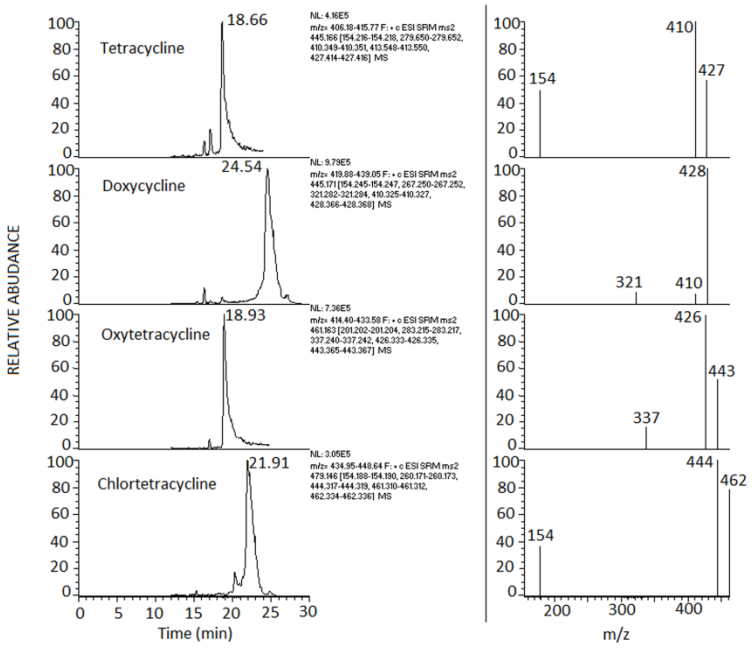
The selectivity of the method, assessed by injecting blank samples (20 mussel and 20 clam samples), did not show any interference (signals, peaks, ion traces) in the region of interest, i.e. where the target analytes were expected to be eluted. The selectivity also showed a good compliance with the relative RTs for each analyte, which were found to be within 2.5% tolerance, when compared with the standards, with peaks having a signal-to-noise ratio > 3. Moreover, the three chosen transitions showed an ion ratio within the recommended tolerances (European Union, 2002), when compared with the standards. The mean recoveries for all analytes ranged between 86–113%. The matrix validation curves also demonstrated a good fit for all analytes, with correlation coefficients > 0.99.

The intra- and inter-day repeatability values, which were calculated using one-way analysis of variance and expressed as coefficients of variation, were below 14 and 20%, respectively. These values were lower than the variability of 22% indicated by Thompson (2000). The CCα ranged from 0.51–5.76 ng g^{-1} wet weight, and CCβ values from 0.65–5.93 ng g^{-1} wet weight (Table 2). Also, the method ruggedness was good in the considered matrices. A modest matrix effect was found, with values ranging from 86–115% for the various compounds in the mussel and clam samples.

3.9.3.2 Investigation on clams and mussels from the food chain

The developed and validated method was applied to the analyses of 50 mussel and 50 clam pooled samples, both wild and farmed, collected from various FAO zones and locations within Italy. The samples were completely anonymous and randomly collected from the food chain. Four tetracyclines (49.45 ng g^{-1} tetracycline, 125.03 ng g^{-1} oxytetracycline, 60.45 ng g^{-1} doxycycline and 77.48 ng g^{-1} chlortetracycline) were detected in one pool of farmed clams obtained from the Italian side of the North Adriatic Sea (Table 3). Figure 2 presents the chromatograms and the MS spectra of the four tetracyclines detected in this pool, as an example.

Figure 2. Chromatograms and MS spectra of the clams in which the four tetracyclines were found.



In this instance, the oxytetracycline concentration was higher than the MRL of 100 ng g^{-1} (European Union, 2010) set for fish. The finding of the four tetracyclines in this pool of farmed clams should be correlated with an intentional treatment. Tetracycline was also found, at low concentration (0.55 ng g^{-1}) in a pool of farmed mussels from Atlantic Spain, depurated in a plant in North Italy. The quinolone, flumequine, was found in two other pools, one of mussels (3.59 ng g^{-1}) and one of clams (0.84 ng g^{-1}), from two different Italian farms in the North Adriatic Sea. In these instances, the detection of antibiotics concerned only farmed mussels or clams. As stated by Cabello (2006), the heavy prophylactic use of antibiotics in aquaculture is well known.

Among the various antibiotics used in fish treatments, oxytetracycline is commonly prescribed against bacterial diseases for its wide antibacterial spectrum, its potency and its low cost. Doses usually administered by fish farmers are often higher than the recommended 50–100 mg kg^{-1} fish day^{-1}, for 7–10 d (Le Bris, Pouliquen, Debernardi, Buchet, & Pinault, 1995).

In the European Union, the cultivation methods of shellfish, with some minor differences, provide the distribution of juvenile molluscs on structures located in the open sea (Baylon, 1990). The use of antibiotics in these conditions would predictably lead to a dilution of these drugs, minimising their effect. After a period of about 20

months, before they are sold, the shellfish must undergo a depuration (few hours to days) in filtered and daily renewed seawater or in natural sites that meet the requirements of the EC Regulation No 853/2004 regarding the microbiological characteristics, chemical pollution and biotoxins present in the water of the culture area (European Union, 2004). The detection of four positive samples out of 100 (just one of which was non-compliant), seemed to confirm the previous statement on the possibility of antibiotic dilution in the open sea and the efficacy of the depuration treatment. It is moreover conceivable an illicit use of antimicrobials in the depuration step, to diminish or nullify the bacterial load in shellfish. The presence of tetracycline in a pool of mussels grown in Atlantic Spain and depurated in a plant of North Italy, suggested illegal practice had occurred because the antimicrobial was only detected in the shellfish from Italy. Conversely, oxytetracycline and oxolinic acid are bioaccumulated by the blue mussels (Le Bris & Pouliquen, 2004) and this observation could provide an alternative explanation for the presence of tetracyclines in mussels. Moreover, the availability of oxytetracycline from sediment, the formation of complexes between this antibiotic and some mineral or organic components of the bivalves, and their low xenobiotic metabolism, as proved in the study of Le Bris et al. (1995) could explain the persistence of oxytetracycline in shellfish and consequently our results. The relatively stable oxytetracycline concentration in the clam *Scrobicularia plana* (up to 20 d) (Le Bris et al.,1995), supports the highest concentration of tetracyclines detected in one of our clam samples, particularly, considering they are grown “on land” between mud and sediments, a favourable environment for oxytetracycline accessibility, as above-mentioned and that the depuration of shellfish lasts around 48 h, explaining the persistence of this antibiotic. Finally, because of the scarcity of positive samples, no argumentation could be made about the differences between species and marine layer.

Low antibiotic concentrations were also reported in the study of Dodder et al. (2014), where they studied and found only few target antibiotics (lomefloxacin, enrofloxacin, sulfamethazine and erythromycin at the mean concentrations of 29, 1.3, 24 and 0.14 ng g^{-1} dry weight, respectively) but with a higher detection frequency from 17 to 94 % related to 68 mussel sampling stations of the coast of California collected from November 2009 and April 2010. Our results were reassuring if compared with the study

of Li et al. (2012), where all 22 target antibiotics of three classes, except tylosin were detected in the 190 molluscs samples of Bohai Sea of China. Their results, showed quinolones as the major compounds with concentrations of 0.71-1575.10 μg kg^{-1}, which were up to two orders of magnitude higher than those of sulphonamides (0-76.75 μg kg^{-1}) and macrolides (0-36.21 μg kg^{-1}). But in that study, they didn't discriminate the different antibiotics among the different molluscs analysed.

Finally, in the light of our results, we can say that the MRLs, are slightly exceeded only in one clam sample, as already elucidated above. However, considering the annual Per capita consumption of 0.33 Kg clams (European Commission, 2016), the daily consumption is 0.91 g; the result of the multiplication of this value by the sum of the concentrations of the four tetracyclines (312.41 ng g^{-1}) found in the clam sample of North Adriatic Sea, is 0.29 μg day^{-1}. This datum could represents a risk mainly associated with the increase of antibiotic resistance phenomenon. Instead, due to the lack of detections, we cannot estimate a potential risk for the environment.

Table 3. List of the detected samples, their provenience and antibiotic concentration expressed in ng g^{-1} wet weight.

Sample provenience	**Tetracycline (ng g^{-1})**	**Oxytetracycline (ng g^{-1})**	**Doxycycline (ng g^{-1})**	**Chlortetracycline (ng g^{-1})**	**Flumequine (ng g^{-1})**
Clams North Adriatic Sea	49.45	125.03	60.45	77.48	
Mussels Atlantic Spain	0.55				
Mussels North Adriatic Sea					3.59
Clams North Adriatic Sea					0.84

3.9.4. Conclusions

In this study we developed, optimised and validated a multiclass HPLC-MS/MS method for analysis of 29 antibiotics, belonging to eight different chemical classes, in mussel and clam samples. The aim was to monitor the eventual presence of antibiotics in various FAO marine zones, with particular attention on Italian seas, considering that antibiotic occurrence is available in wastewater. The two different matrices, mussels and clams never compared before, were chosen to study antibiotic bioaccumulation in distinct marine layers, given that the first grow, primarily, on the surface and the second in shallow. Even if the method had detection limits well lower than the MRLs, useful to increase the proportion of quantified data and accurately monitor the presence of antibiotics due to the antibiotic resistance matter, only few detections had been registered, although, in one instance, the oxytetracycline content was higher than the MRL recommended for fish.

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Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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3.10. Detection of Perfluoroalkyl Acids and Sulphonates in Italian Eel Samples by HPLC-HRMS Orbitrap

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In this study I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

Abstract

Perfluoroalkyl substances (PFASs) contain one or more carbon-bound hydrogens substituted by fluorine. Since the 1950s, these compounds have been used to manufacture fat- and water-resistant fabrics, paper and food containers, and to produce photographic films, firefighting foams, detergents and insecticides. The widespread use and global distribution of PFASs, have led to their accumulation in the environment. Food, particularly fish and other seafood, is considered the main route of human exposure to PFASs. Consequently, the European Food Safety Authority (EFSA) recommends that more data be collected, to build a database on the contamination levels of the individual PFASs in food, to evaluate a reliable chronic risk to the European consumers. This requires high-sensitivity analytical methods, to increase the number of quantifiable samples and, thereby, improve the credibility of exposure assessments. In this context, the aim of the present research is to develop and validate a sensitive and specific method based on high-performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) analysis, to monitor the presence of 16 PFASs in Italian eels (Anguilla anguilla) from the Italian Lake Garda. The detection limits (CCα) and detection capability (CCβ) in the order of pg g^{-1}, the recoveries between 80-101% and the other validation parameters fulfilled the requirements of Commission Decision 657/2002/EC. The identification and quantification of PFASs, up to 11 in the same sample, showed a similar distribution among 90 eels. Perfluorooctane sulphonic acid (PFOS) and perfluorobutanoic acid (PFBA) were the analytes more frequently found in the eel samples (94 and 82%, respectively).

Keywords Perfluoroalkyl substances; Eels; LC-HRMS; Food safety.

Highlights

Perfluoroalkyl substances (PFASs) accumulate in environment and in human through diet.

One sensitive HPLC-HRMS method for PFASs in eels is reported.

Eels from the Lake Garda in North Italy were analysed.

Up to eleven PFASs were contemporaneously detected in several eel samples.

3.10.1. Introduction

Perfluoroalkyl substances (PFASs) are molecules, in which one or more carbon-hydrogen (C-H) bonds, are replaced by carbon-fluorine (C-F) bonds (Lau et al. 2004). Fluorine is a reactive element in its ionic form and very stable in a bound form. Therefore, perfluorocarbons are stable in the environment, even at > 150 °C, are non-flammable, not subject to photolysis and not readily degraded by alkalis, strong acids or oxidising agents. These stability characteristics make them non-biodegradable and highly persistent in the environment (Lau, Butenhoff, & Rogers, 2004). Perfluoroalkyl acids have also a unique partitioning behaviour that reveals their hydrophobic and oleophobic nature when they are mixed with water and hydrocarbons, forming three immiscible phases. By attaching a charged moiety, such as carboxylic acid, sulphonic acid, or phosphate, to the perfluorinated chain, the molecule becomes more hydrophilic. All known, biologically produced, fluorinated organics contain only one fluorine atom. However, partially or fully fluorinated organic molecules are synthesised in the laboratory on a large-scale, for their many useful properties (Key, Howell, & Criddle, 1997).

PFASs are used in a lot of industrial and chemical sectors, as well as for packaging materials, fire-extinguishing fluids, textiles, carpets, paper, furniture, floor polishing agents, cleaning agents, varnish, polish, photograph paper, and insecticides (3M Company, 1999). The global utilisation and distribution of PFASs, has caused their accumulation in the environment and human body. Perfluorooctane sulphonic acid (PFOS) and perfluorooctanoic acid (PFOA) are the most common PFASs. Both cause adverse health effects and have shown immunotoxicity, hepatotoxicity, neurobehavioral toxicity, developmental toxicity, reproductive toxicity, lung toxicity, hormonal effects, weak genotoxic and carcinogenic potential (Eriksen, Raaschou-Nielsen, Sørensen, Roursgaard, Loft, & Møller, 2010; Pinkas, Slotkin, Brick-Turin, Van der Zee, & Yanai, 2010).

Food, particularly fish and other seafood, is considered the main exposure route to PFASs in the human population. However, scarce information is available in the literature about the detection of PFASs in eels. One research report focused on the detection of PFOS in the liver of three freshwater fish species (gibel carp, carp and eel),

in Belgium (Hoff et al. 2005). In another study, PFOS and PFOA residues were investigated in 51 wild eels, among other wild fish, in Germany (Schuetze, Heberer, Effkemann, & Juergensen 2010). Kwadijk et al. (2010) measured the distribution of 15 PFASs among water, sediment and eels, in The Netherlands. Furthermore, PFOA and PFOS in the organs of 35 wild eels, from two Italian locations, were analysed by Giari et al. (2015).

Food exposure can derive by accumulation from the environment or by contact with cookware or packaging materials containing PFASs (Trier, Granby, & Christensen,2011). The EFSA Panel on Contaminants in the Food Chain (CONTAM) set a tolerable daily intake (TDI) of 150 ng kg^{-1} body weight (b.w.) per day for PFOS and 1500 ng kg^{-1} b.w. per day for PFOA (EFSA, 2008). However, the scarce data allowed only a limited exposure assessment. Therefore, CONTAM recommended increasing the database, through more studies about PFASs in food, by evaluating the contamination levels, which would improve the accuracy of the chronic dietary exposure risk to the European populations (EFSA, 2012). For this purpose, high-sensitivity analytical methods that increase the proportion of quantified data and accurately monitor PFASs in food, are required, thereby, improving the reliability of the exposure assessments. Regarding the analytical strategies present in literature, the extraction of perfluorinated compounds from biological samples is usually performed through an alkaline digestion with potassium hydroxide (KOH) (So et al., 2006) or the ion-pair extraction method (Hansen et al., 2001) based on ion pairing of the ionic PFASs with tetra-nbutylammonium hydrogensulfate (TBA), followed by a liquid–solid extraction with methyl-tert-butylether (MTBE). For purification of the samples the HLB, WAX cartridges or Dispersive Envi-carb are used based on the different matrices (van Leeuwen and de Boer, 2007). Several methods based on liquid chromatography coupled with triple quadrupole mass spectrometry (MS), have been proposed in the literature for the analysis of PFASs, for several matrices. Also in the few studies on eels
reported above, the analyses were performed by LC-MS/MS system. In particular, high-resolution mass spectrometry (HRMS) represents a powerful tool for the determination of trace analysis of various compounds in complex matrices. The advantages of Orbitrap-MS, such as the high MS resolving power and mass accuracy down to 1 ppm,

combined with the rapid scan speed, results in high sensitivity, selectivity and specificity, providing new improvements for confirmatory analytical methods, in the challenge against emerging contaminants (Krauss et al.,2010).

In this context, the present research aimed to develop and validate a sensitive and specific method based on high-performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) analysis, to monitor the presence of 16 PFASs in Italian eels (*Anguilla anguilla*) from Lake Garda (Northern Italy). The choice of eel was due to the authors' assumption of potential bioaccumulation of PFASs in this species, facilitated by their length and body composition and, also, because it is an edible matrix, intended for human consumption. Moreover, Lake Garda is a semi-enclosed environment, which has shown an increasing pollution level in recent years, in which the majority of plastic particles have been found (Imhof, Ivleva, Schmid, Niessner, & Laforsch, 2013).

3.10.2. Materials and Methods

3.10.2.1. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges (Oasis HLB WAX 3 mL, 60 mg) were provided by Waters (Milford, MA, USA). Sixteen perfluorinated compounds including both perfluorinated sulphonates and perfluorinated carboxylates, were examined in this study: perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), PFOA, perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), PFOS, perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA), and perfluorooctadecanoic acid (PFODA) (see Table 1 for the formula pertaining to the individual compounds). All these compounds and the two ^{13}C-labeled internal standards (ISs) perfluoro-[1,2,3,4,5-13C5]nonanoic acid (MPFNA) and perfluoro-[1,2,3,4-13C4]octanesulfonic acid (MPFOS) were purchased

from Fluka. Ammonium formate, sodium acetate, acetic acid (99.9%) and 25% ammonia solution, were purchased from Fluka.

3.10.2.2. Sample collection

Ninety farmed eel samples (average weight 909.2 ± 434.1 g; average length 74.5 ±10.0 cm; average fat percentage 26.1 ± 5.4 %), were collected from Lake Garda (Northern Italy). The samples were immediately taken to the laboratory and eviscerated. As the high water content of many food samples previously showed to affect the extraction performance of PFASs, we lyophilised eel muscle tissues, according to other studies that used freeze-drying prior the sample clean-up (Vestergren et al., 2012). Then the samples were stored at 4°C until the analysis.

3.10.2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) of each standard, were prepared in methanol and kept at -20°C. Working solutions, containing each of the studied analytes, at 10 and 100 ng mL^{-1}, were prepared daily. Each working solution was maintained at 4°C, during the method validation procedures.

3.10.2.4. Sample extraction

A 2-g aliquot of lyophilised eel sample, was spiked with the two ISs, to obtain a final concentration of 5 ng mL^{-1}. Then, 10 mL acetonitrile was added for the protein precipitation and analytes extraction, before the sample was vortexed and sonicated for 15 min. After centrifugation (2500×g, 4 °C for 10 min), the supernatant was transferred to a glass flask and rotary evaporated to dryness at 35°C. The extract was suspended in 10 mL water and solid-phase extraction (SPE) performed using Oasis WAX-SPE cartridges under vacuum, for further purification and extraction. The SPE cartridges were preconditioned with 3 mL of 0.5% ammonium hydroxide (NH_4OH) in MeOH, 3 mL MeOH and 3 mL Milli-Q water. The sample was loaded, and, then, the cartridges were washed with 3 mL of 25 mM acetate buffer, pH 4.5, to remove interferences, as well as lipids or proteins and to improve adsorption of target anions to the cartridge, followed by 2 mL MeOH. Finally, the compounds were eluted using 3 mL of 0.5% NH_4OH in MeOH

and were collected in a 15-mL polypropylene tube. The eluate was rotary evaporated at 35°C. The dried extract was reconstituted in 100 µL of 20 mM MeOH:ammonium formiate (10:90 v/v), and, then, transferred to an auto-sampler vial. The injection volume was 10 µL. The method was developed and optimised, taking into consideration the work of Taniyasu et al. (2005), about the different effect of pH of acetate buffer, the percentage of NH_4OH in MeOH, and the influence of elution volume of NH_4OH in MeOH, on recoveries of PFASs. Moreover, considering the ubiquity of PFASs in the environment of analytical laboratories, several precautions were taken, such as washing glassware with MeOH and the execution of at least 10 procedural blanks, on the analysis days, to subtract any background contamination.

3.10.2.5. HPLC-HRMS analyses

HPLC analysis was performed by an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), equipped with a Surveyor MS quaternary pump and degasser, a Surveyor AS autosampler and column oven, and a Rheodyne valve with a 20-µL loop. The analytes were chromatographically separated, using a Synergi Hydro-RP reverse-phase HPLC column (150 × 2.0 mm, i.d. 4 µm), with a C18 guard column (4 × 3.0 mm; Phenomenex, Torrance, CA, USA). Stainless-steel tubes and peeks were used, to minimise background PFAS contamination in the system. Moreover, since PFOA and PFOS were always present in the blank of the chromatographic system, a small Megabond WR C18 column (5 cm × 4.6 mm, i.d. 10 µm) was introduced between the pump and injector, to allow delaying the target analytes by 2 min compared to those already present in the system.

The mobile phase used for the gradient, consisted of a binary mixture of solvents A (20 mM aqueous ammonium formate) and B (MeOH). The elution started with 10% B, which increased to 40% in 4 min. Subsequently, mobile phase B was gradually increased to 95% at the 12th min, which remained constant up to the 18th min. The initial conditions were reached in the 20th min, with an equilibration time of 7 min. The run was performed at 0.3 mL min^{-1}.

The detector, was a Thermo Q-Exactive Plus (Thermo Scientific, San Jose, CA, USA), equipped with a heated electrospray ionisation (HESI) source. Capillary and vaporiser

temperatures were set at 330 and 280°C, respectively, while the electrospray voltage was set at 3.50 kV, operating in negative mode. The sheath and auxiliary gas were set at 35 and 15 arbitrary units (AU). Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HPLC-HRMS system. The exact mass of the compounds was calculated, using Qual Browser in Xcalibur 3.0 software. Instrument calibration was done every analytical session, using LTQ Velos ESI negative ion calibration solution (Pierce Biotechnology Inc., Rockford, IL, USA).

The full scan (FS) acquisition was combined with a data-independent acquisition (DIA) strategy, providing the MS^2 spectra for a confirmatory response, based on an inclusion list. The FS resolution was 70,000 FWHM. On the basis of the compound list, a scan range of 200–950 m/z was chosen; the automatic gain control (AGC) was set at 1E6, and the maximum injection time was 200 ms. The DIA segment operated in negative mode at 35,000 FWHM. The AGC target was set to 5E4, with the maximum injection time of 100 ms. The precursor ions are filtered by the quadrupole, which operates at an isolation window of 2 m/z. Fragmentation of the precursors was optimised with a two-step normalised collision energy (10 and 70 eV). The mass tolerance window was set to 2 ppm. Detection of the analytes was based on the retention time (RT) of the target compounds, and on the calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment (Table 1). The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition, used to confirm the various PFASs, are reported in Table 1. The extracted parent ion chromatograms, acquired from FS analysis of each analyte in the matrix, are reported in Fig. 1. Acquisition data were recorded and elaborated using Xcalibur™ software (Thermo Fisher).

3.10.2.6. Method validation

After the identification of the “blank” eel samples, based on a preliminary screening, the validation was performed according to the criteria of the Commission Decision 657/2002/EC (European Community, 2002). For each compound, the method performance was assessed, through both qualitative and quantitative parameters, providing molecular identification in terms of RT and transition ion ratios; evaluating

recovery, linearity, accuracy in terms of trueness, precision as intra- and inter-day repeatability; and through the analytical decision limit (CCα) and detection capability (CCβ), as indicated in SANCO/2004/2726-revision 4 (European Community, 2008).

Twenty blank samples were analysed, to evaluate specificity and selectivity, check for any interference (signals, peaks, ion traces), verify the presence of analytes by a signal-to-noise (S/N) ratio of >3 at the expected RT, and to confirm the ion abundance ratio for the different fragmentations. Validation was performed, by spiking the eel samples at three concentration levels in six replicates, repeated for three independent days, resulting in three analytical series (matrix validation curves). The three concentration levels (C0, 2C0, and 3C0) were previously chosen, according to the minimum concentration detectable with the instrumentation (C0) used, for each analyte (Table 2).

The instrumental linearity was also evaluated, by drawing six-point calibration curves for the solvent containing a fixed amount of the ISs (5 ng mL^{-1}) and the initial analyte concentration, corresponding to C0 up to 100 ng mL^{-1}, for all analytes. The recovery, expressed as a percentage of the measured concentration with respect to the spiked concentration, was evaluated using the data from the validation points of the three analytical series. The precision, in terms of intra- and inter-day repeatability, was evaluated by calculating the relative standard deviation of the results obtained from the six replicates of each analyte, at the three concentration levels during the three analytical series. Robustness was evaluated, using the approach of Youden (European Community, 2002). The seven factors selected for the robustness study were: the volume of acetonitrile used for extraction and protein precipitation, the sonication time, the centrifugation time, the centrifugation temperature, the percentage and the volume of ammonium hydroxide (NH4OH) in MeOH using during the SPE purification and the temperature of the rotary evaporator.The matrix effect was assessed based on Matuszewski et al. (2003), by calculating the percentage ratio between the corresponding peak areas of the standards spiked after extraction and the peak areas of the neat standard solution.

3.10.3. Results and Discussion

3.10.3.1. Development and optimisation of sample preparation

The method was developed and optimised, taking into consideration the work of Taniyasu et al. (2005), that reported useful comparisons about the different effect of pH of acetate buffer, the percentage of NH_4OH in MeOH, and the influence of elution volume of NH_4OH in MeOH on extraction and consequently on recoveries of PFASs. In particular, the samples considered in the work were water and biota pre-treated through an alkaline digestion before the WAX SPE. We chose acetonitrile for the pretreatment step because was useful not only for the extraction but also for protein precipitation to avoid interferences during analysis. As regard WAX purification, we decreased the volumes of the solutions and solvents used during the SPE, we used a higher percentage (0.5 % instead of 0.1%) of NH4OH in MeOH and we analysed only the final eluate because it was purified by any interference and contained all the analytes we were interested in. The choices and modifications made to the sample clean-up protocol have been fundamental to obtain satisfactory validation parameters, reported and discussed in the next paragraph.

3.10.3.2. Validation performance

The method showed high specificity, without interference signals close to the RT of the analytes. Consequently, a high S/N ratio in the presence of analytes, even at concentrations in the order of pg g^{-1}, was demonstrated. Selectivity demonstrated a good compliance with the relative RTs for each analyte, which, in this instance, was within 2.5% tolerance, with an S/N ratio >3, when compared with the standard solution mix, both in FS and MS^2 chromatograms. Moreover, diagnostic fragments showed an ion ratio within the recommended tolerances (European Community, 2002). The mean recoveries for all analytes ranged between 80 and 117%, indicating the efficiency of the extraction protocol.

The matrix validation curves were linear over the working range, demonstrating a good fit for all analytes with an R^2 > 0.99. Precision, in terms of intra- and inter-day repeatability (Thompson 2000), were calculated using one-way analysis of variance (ANOVA), expressed as coefficients of variation (CVs), and were below 19 and 21%, respectively. The detection limits (CCα) ranged from 5–35 pg g^{-1} and detection

capability (CCβ) from 8–39 pg g^{-1} (Table 2). These limits indicate the potentiality of the method to detect these emergent analytes that currently, do not have established maximum residue limits in edible matrices. Our detection limits resulted lower than the ones in the few literature studies regarding the detection of PFASs in eels. In the work of Hoof et al. (2005) about PFOS and other organohalogen pollutants in liver of three freshwater fish species of Belgium, the detection limits ranged from 0.1 to 1 ng g^{-1} wet weight; in the study of Schuetze et al. (2010) LODs were 0.019 and 0.27 μg kg^{-1} fresh weight for PFOS and PFOA, respectively; in the study of Kwadijk et al. (2010) about distribution of perfluorinated compounds in aquatic systems in The Netherlands, no information regarding the detection limits is reported.The Youden approach showed a good robustness. There was a modest matrix effect, with values ranging from 84 to 109%, for the studied compounds.

3.10.3.3. Application in eel samples

The optimised and validated method, was then applied to the analysis of 90 lyophilised eel samples, farmed and collected from Lake Garda. The results showed the presence of several PFASs, up to 11 in the same eel. The average concentrations, standard deviations and the percentage of positivity, are reported in Table 3. The distribution of the various contaminants, in the order of ng g^{-1}, was mostly similar in each sample, representing the low contamination level of the lake, without any relation to the weight, length or the percentage of animal fat. Usually organic molecules tend to transfer from abiotic to biotic compartments, with persistent lipophilic compounds concentrating in the adipose tissue, but this partitioning approach cannot be applied to the bioaccumulation of perfluorinated compounds (Houde et al., 2006), for their proteinophilic nature (Jones et al. 2003). PFOS was the analyte found more frequently but the average concentrations did not appear concerning, although they were slightly higher than the average muscle concentrations (0.89 ± 0.58 ng g^{-1}wet weight) present in the eels from north Italian waters (Giari, Guerranti, Perra, Lanzoni, Fano, & Castaldelli, 2015) but considerably lower than those reported in eel liver (17 to 9031 ng g^{-1} wet weight) in Belgium (Hoff et al. 2005), in eel muscle tissue (37 to 83 ng g^{-1} wet weight) in Germany (Schuetze et al. 2010), and in eel tissue (7 to 58 ng g^{-1} wet weight) in The Netherlands

(Kwadijk, Korytár, & Koelmans 2010). PFOS was found to be the predominant compound in all eel samples of the Netherlands (Kwadijk et al., 2010), with concentrations ranging from 7 to 58 ng g^{-1} wet weight. In the same work PFHxS and PFDoA, were the PFASs detected at the next highest level, approximately 10 times lower than that of PFOS. These three PFASs were also the only compounds to be detected in all the samples.

The PFOA concentrations were also remarkably lower than the previous above-mentioned studies. In the current study, the highest concentrations found in the eel samples were associated with PFBA, with a wide standard deviation, which was observed for the sulphonate form (PFBS) in the sediment and water samples of The Netherlands that was attributed to various sources, given the presence of industries along the Rhine (Kwadijk et al. 2010). In this last work, although at some locations PFBS was not detected in the water samples, low levels of PFBS were detected in eel (0.1−2.3 ng g^{-1} of wet weight), despite the fact that PFBS is considered nonbioaccumulative (Conder et al., 2008): the absence of PFBS in our eel samples agrees with the statement of no bioaccumulation. Based on the literature and the findings of the Water Research Foundation project #4322 (Fulmer, 2016), conventional treatment at wastewater treatment plants and most drinking water treatment plants, is ineffective at removing short-chain PFASs, as well as PFBA from water. This could explain the higher level of PFBA in respect to other PFASs in our samples. In a study on distribution and sources of polyfluoroalkyl substances in the River Rhine watershed, the dominant concentration of PFBA likely originated from industrial point sources (Möller et al., 2010). In another work about the sources of polyfluoroalkyl compounds in the North Sea, Baltic Sea and Norwegian Sea, the Authors hypothesised that an additional water contamination source can be the contaminated sewage sludge applied to neighboring agricultural fields (Ahrens et al., 2010). It should be emphasised that this analyte was always present, even in the background contamination of the extractive procedure, but a maximum 4 ng g^{-1} was detected, in the analysis of a batch of 10 procedural blanks, during each analytical session.

3.10.4.Conclusion

The HPLC-HRMS Orbitrap represents a powerful technical approach, for the analysis of emerging contaminants, due to its resolving power and scanning speeds that contribute to the high selectivity, specificity and sensitivity of the instrumentation. Moreover, the effectiveness of the extraction method, facilitated the instrumental analysis, by the lack of particular interferences, considering the complexity of the studied matrix: *A. anguilla.* Application of the validated method, to the analysis of 90 farmed eel samples collected from Lake Garda, showed a homogeneous situation of modest PFASs contamination compared to eels from other European countries, despite simultaneous detection of up to 11 compounds, in each sample.

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Table 1 Main information of investigated PFASs (formula, parent, main product, polarity and retention time (RT))

Compound[a]	Formula	Parent [m/z]	Main product [m/z]	Polarity	RT (min)
PFBA	C4HF7O2	212.97920	168.98836	(-)	9.07
PFPeA	C5HF9O2	262.97601	218.98560	(-)	11.68
PFBS	C4F9HO3S	298.94299	98.95434	(-)	12.02
PFHxA	C6HF11O2	312.97281	268.98288	(-)	13.22
PFHpA	C7HF13O2	362.96962	318.97949	(-)	14.36
PFHxS	C6F13HO3S	398.93660	98.95437	(-)	14.39
PFOA	C8HF15O2	412.96643	368.97681	(-)	15.27
PFNA	C9HF17O2	462.96323	418.97385	(-)	16.03
PFOS	C8F17HO3S	498.93022	79.95598	(-)	16.00
PFDA	C10HF19O2	512.96004	468.97064	(-)	17.96
PFUnDA	C11HF21O2	562.95684	518.96729	(-)	18.48
PFDoA	C12HF23O2	612.95365	568.96387	(-)	18.98
PFTrDA	C13HF25O2	662.95046	618.96057	(-)	19.50
PFTeDA	C14HF27O2	712.94726	668.95823	(-)	20.06
PFHxDA	C16HF31O2	812.94088	768.95184	(-)	20.80
PFODA	C18HF35O2	912.93449	868.94513	(-)	21.81
MPFNA	[13]C5C4HF17O2	467.98001	422.98703	(-)	16.03
MPFOS	[13]C4C4F17HO3S	502.94364	79.95592	(-)	16.00

[a] Refer to text (materials and methods section) for full names of the abbreviated compounds

Table 2 Validation parameters[a] of the investigated perfluoroalkyl substances (PFASs)

PFAS[b]	C0, 2C0, 3C0 (pg g^{-1})[c]	CCα (pg g^{-1})	CCβ (pg g^{-1})	Recovery %	CV% Intra-day	CV% Inter-day
PFBA	5, 10, 15	10	12	80	5	18
PFPeA	10, 20, 30	12	15	117	12	12
PFBS	10, 20, 30	12	15	105	10	14
PFHxA	20, 40, 60	30	35	113	4	10
PFHpA	5, 10, 15	10	12	115	4	8
PFHxS	15, 30, 35	20	25	105	7	9
PFOA	5, 10, 15	8	10	116	3	7
PFNA	5, 10, 15	10	12	93	11	18
PFOS	5, 10, 15	5	8	80	14	20
PFDA	20, 40, 60	25	30	80	14	20
PFUnDA	20, 40, 60	30	35	82	6	16
PFDoA	20, 40, 60	35	39	88	7	12
PFTrDA	15, 30, 35	20	25	89	6	10
PFTeDA	5, 10, 15	10	13	93	17	20
PFHxDA	5, 10, 15	8	10	87	19	20
PFODA	5, 10, 15	10	12	85	19	21

[a] According to Commission Decision 657/2002/CE (European Community 2002).

[b] Refer to text (materials and methods section) for full names of the abbreviated compounds

[c] Validation was performed, by spiking the eel samples at three concentration levels (C0, 2C0, 3C0), in six replicates, repeated for three independent days, resulting in three analytical series (matrix validation curves)

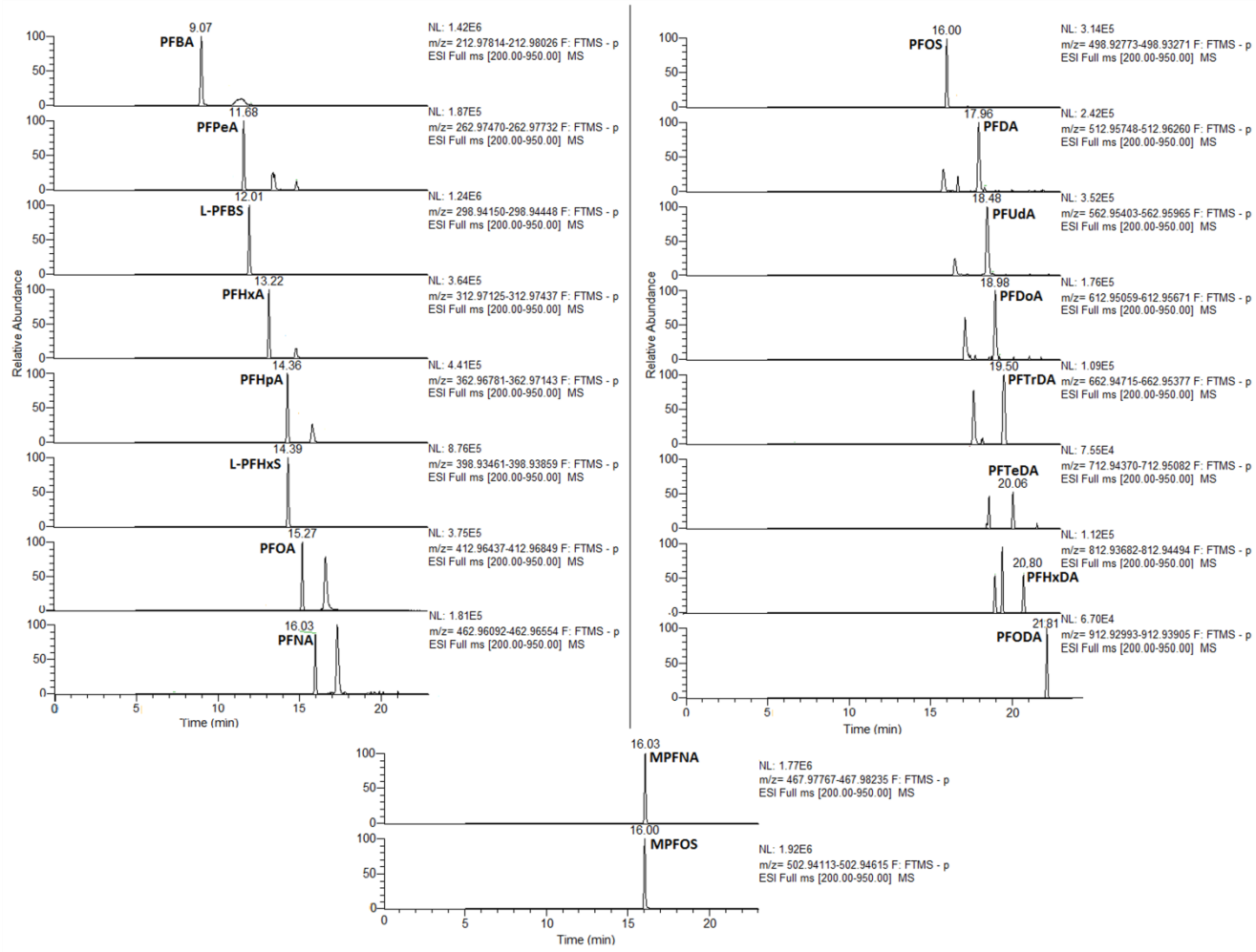
CCα: decision limit; CCβ: detection capability; CV: coefficient of variation

Table 3 Distribution of perfluoroalkyl substances in Italian eel samples from Garda lake (ng g^{-1})

Compounds[a]	Average (ng g-1)	Standard deviation (±)	Median	% Positives
PFBA	16.66	23.65	8.56	82
PFPeA	0.01	0.02	0.00	7
PFHpA	0.01	0.06	0.00	6
PFOA	0.39	0.26	0.39	77
PFNA	0.69	0.66	0.64	74
PFOS	4.74	4.06	3.75	94
PFDA	1.71	1.75	1.34	82
PFUnDA	0.14	0.57	0.00	11
PFDoA	1.11	2.13	0.04	51
PFTrDA	0.40	0.77	0.00	42
PFTeDA	0.94	3.40	0.00	23

[a] Refer to text (materials and methods section) for full names of the abbreviated compounds

Fig. 1 Extracted parent ion chromatograms from full scan HPLC-HRMS analysis of each PFAS in the eel matrix, at the lowest validation level.



3.11. Food safety traits of mussels and clams: distribution of PCBs, PBDEs, OCPs, PAHs and PFASs in sample from different areas using HRMS-Orbitrap® and modified QuEChERS extraction followed by GC-MS/MS

In press, Food Additives and Contaminants

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In this study I contributed to the experimental work planning, the execution of preliminary trials and analyses, data processing and writing of the article concerning the HPLC-HRMS part.

Abstract

Reviewing the presence of contaminant residues is important both for food safety and monitoring of environmental pollution. Here, the occurrence of 6 polychlorinated biphenyls (PCBs), 15 organochlorine pesticides (OCPs), 7 polybrominated diphenyl ethers (PBDEs), 4 polycyclic aromatic hydrocarbons (PAHs) and 17 perfluoroalkyl substances (PFASs) was evaluated in mussels and clams. A liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) and an innovative QuEChERS extraction followed by gas chromatography-tandem mass spectrometry (GC-MS/MS) methods were developed, validated and applied. We demonstrate good linearity, repeatability and accuracy of these methods, confirming that these methods are suitable for the analyses of mollusc samples. The prevalence of PCBs, OCPs and PAHs was higher in mussels than clams. For PFASs, the contamination was higher in clams than in mussels. The samples were all compliant with the regulations and, for the compounds without limit, a risk assessment confirmed that the values were lower than the tolerable intake suggested by EFSA.

Keywords: Mussels, Clams, POPs, PFASs, HPLC-HRMS, GC-MS/MS.

Highlights

One sensitive HPLC-HRMS method for PFASs in shellfish was developed and validated.
Another method for POPs in shellfish using GC-MS/MS was optimised and validated.
Innovative QuEChERS extraction was developed.
Pools of mussels and clams from multiple FAO zones were analysed.
Comparing mussels to clams, POPs were higher but PFASs lower in clams.

Chemical compounds studied in this article

PCB 28 (PubChem: CID 23448); PCB 52 (PubChem CID: 37248); PCB 101 (PubChem: CID 37807); PCB 138 (PubChem: CID 37035); PCB 153 (PubChem: CID 37034); PCB 180 (PubChem: CID 37036); PCB 209 (PubChem: CID 16318); PBDE 28 (PubChem: CID 12110098); PBDE 33 (PubChem: CID 39506); PBDE 47 (PubChem: CID 95170); PBDE 99 (PubChem: CID 36159); PBDE 100 (PubChem: CID 154083); PBDE 153 (PubChem: CID 155166); PBDE 154 (PubChem: CID 15509898); α- HCH (PubChem: CID 727); Hexachlorobenzene (PubChem: CID 8370); β-BHC (PubChem: CID 727); Lindane (PubChem: CID 727); Heptachlor (PubChem: CID 3589); Aldrin (PubChem: CID 61103); Heptachlor epoxide (PubChem: CID 13930); Trans Chlordane (PubChem: CID 45356234); 4,4'- Dichlorodiphenyldichloroethylene (PubChem: CID 3035); Endosulfan I (PubChem: CID 6433227); Endosulfan II (PubChem: CID 12309466); Endosulfan sulfate (PubChem: CID 13940); Endrin (PubChem: CID 46174049); 4,4'Dichlorodiphenyldichloroethane (PubChem: CID 6294); 2,4'-Dichlorodiphenyltrichloroethane (PubChem: CID 13089); Chrysene (PubChem: CID 9171); Benz(a)anthracene (PubChem: CID 5954); Benzo(b)fluoranthe (PubChem: CID 9153); Benzo(a)pyrene (PubChem: CID 2336); Perfluoropentanoic acid (PubChem: CID 75921); Perfluorohexanoic acid (PubChem: CID 67542); Perfluorobutane sulphonic acid (PubChem: CID 75922); Perfluoroheptanoic acid (PubChem: CID 67818); Perfluorooctanoic acid (PubChem: CID 9554); Perfluorohexane sulphonate (PubChem: CID 67734); Perfluorononanoic acid (PubChem: CID 67821); Perfluorodecanoic acid (PubChem: CID 9555); Perfluorooctane sulfonic acid (PubChem: CID 74483); Perfluorododecanoic acid (PubChem: CID 67545); Perfluoroundecanoic acid (PubChem: CID 77222); Sodium perfluoro-1-decanesulfonate (PubChem: CID 2724181); Perfluorotridecanoic acid (PubChem: CID 3018355); Perfluorotetradecanoic acid (PubChem: CID 67822); Perfluorohexadecanoic acid (PubChem: CID 106027), Perfluorooctadecanoic acid (PubChem: CID 167547); 4-nonylphenol (PubChem: CID 1752).

3.11.1. Introduction

Marine ecosystems are subjected to continuous pollution events because of increasing anthropogenic activities and the releasing of various sources of contaminants (Van De Vijver et al., 2003). Bivalve molluscs are considered good environmental contamination indicators because their tissues accumulate contaminants with little metabolic transformations (Roesijadi, Young, Drum, & Gurtisen, 1984; Sericano, 1993). In fact, mussels and clams are filter-feeding organisms. Therefore, most of the contaminants are directly bioavailable and can accumulate across gills and by ingestion of particles (Kimbrough, Johnson, Lauenstein, Christensen, & Apeti, 2008). Mussels were often used as sentinel indicator species to monitor the environmental accumulation of various persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) (Webster et al., 2008) and perfluoroalkyl substances (PFASs). Concerning PFASs, research attention has rapidly increased because of their worldwide spread in multiple environmental areas (Kannan, 2011). Global monitoring of PFAS contamination has identified perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) as the predominant compounds, ubiquitously distributed in several animal tissues (Giesy & Kannan, 2001; Van de Vijver et al., 2003). The persistence in the environment of PFOS and PFOA was demonstrated, such as their capability to bioaccumulate in the trophic chain (Valsecchi, Rusconi, & Polesello, 2013) but, as emerging contaminants, no maximum residue levels (MRLs) have yet been set. Among other POPs, PCBs, PBDEs and PAHs are contaminants commonly found in sediments, waters and wildlife (Erickson, 1997; Safe, 2002). These three classes of compounds have similar physicochemical characteristics of lipophilicity and resistance to degradation (Xua, Wanga, & Caia, 2013). Their high bioaccumulation potential added to a variety of toxic effects on humans and animals makes the evaluation of their occurrence a pivotal task (Van den Berg et al., 2006; Robertson, & Hansen, 2001). PAHs, PCBs and PBDEs produced by anthropogenic activities can undergo long-range atmospheric transport and could be, therefore, found in the marine environments (Fernandez, & Grimalt, 2003; Teil, Blanchard, & Chevreuil, 2004; Chiesa, Labella, Panseri, Pavlovic, Bonacci, & Arioli, 2016 a). PFASs have no MRLs, whereas PCBs and PAHs have maximum limits that are recommended by Commission Regulation No 1259/2011 (European Union, 2011) and

Commission Regulation No 1881/2006 (European Commission, 2006). Also, no MRLs have been established for PBDEs, but the European Commission recommended their monitoring in food, especially of animal origin (European Union, 2014). Organochlorine pesticides (OCPs) have a similar behavior to the other contaminants described. OCPs reach the marine environment from surface runoff and ground leachate but can also be found in stormwater and wastewater discharges (Clendening, Jury, & Ernst, 1990). Although several pesticides (as DDT) are prohibited, they and their metabolites are still found in coastal waters, sediment and biota (Richardson, & Zheng, 1999). Monitoring guidelines for OCPs in fish are reported by the Food and Drug Administration (2011).

Because of the very low limits reported by legislations (in the order of ng g^{-1}) and considering the large number of compounds that have to be monitored, novel analytical protocols are necessary to allow the quantification of these compounds with high sensitivity, selectivity and specificity. Among the analytical techniques available, high-pressure liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) and gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) represent the best choice for the detection of ultra-trace levels of different compounds in heterogeneous matrices. In particular, the Orbitrap HRMS resolving power, combined with the fast scan speed, results in high accuracy (lower than 1 ppm), sensitivity and specificity, providing all the characteristics for confirmatory methods, while GC-MS/MS guarantees the high performances required for the analyses of lipophilic compound, such as PBDEs and PCBs, as reported by Chiesa et al. (2016 b).

Based on the considerations discussed above, the aim of this study was to develop and validate two analytical methods, a HPLC-HRMS method for the analysis of PFASs and a GC-MS/MS method with an innovative Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction, for the analysis of PCBs, PBDEs, PAHs and OCPs. Later, the occurrence of these five classes of POPs was evaluated in mussels and clams. The two mollusk species were selected because they are the most consumed in EU (European Commission, 2016) and live at different depths: the mussel habitat is epipelagic whereas the clam habitat is benthonic, therefore possibly representing different levels of contamination.

3.11.2. Material and methods

3.11.2.1. Sampling

Mussels and clams were collected at the wholesale fish market of Milan, the most important Italian fishery market. The sample collection, randomly made, was representative of the contamination levels of mollusks available to Italian consumers. Multiple species were selected: *Mytillus Galloprovincialis, Mytillus Edulis* and *Mytillus Chilensis* for mussels, and *Venerupis philippinarum*, *Perna Canaliculus, Tapes decussatus, Tapes Semidecussatus, Meretrix Meretrix* and *Meretrix Iyrata* for clams. All molluscs were collected from June 2016 until February 2017, and the sampling areas are shown in Fig. 1. A total of 50 mussel and 39 clam samples were made: the soft tissue was separated from the shells and pools of about 50 individuals were prepared for each sample; after homogenization, the samples were stored at -20°C until analyses.

3.11.2.2. Chemicals and reagents

A mixed solution of PCB congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153 and PCB 180), PCB 209 (internal standard [IS] for PCBs and PAHs), a mixed solution of PBDEs (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154) (numbered according to IUPAC) and fluoro-bromodiphenyl ether (FBDE), IS for flame retardants, were purchased from AccuStandard (New Haven, USA). A standard solution of 15 OCPs and their metabolites (α- HCH; Hexachlorobenzene; β-BHC; Lindane; Heptachlor; Aldrin; Heptachlor epoxide; Trans Chlordane; 4,4'-Dichlorodiphenyldichloroethylene [4,4'- DDE]; Endosulfan I; Endosulfan II, Endosulfan sulfate; Endrin, 4,4'Dichlorodiphenyldichloroethane [4,4'-DDD], 2,4'-Dichlorodiphenyltrichloroethane [2, 4'-DDT]) and a standard solution of four PAH congeners (Chrysene, Benz(a)anthracene, Benzo(b)fluoranthe and Benzo(a)pyrene) were purchased from Restek (Bellefonte, PA, USA). Seventeen acid and sulfonate perfluorinated compounds were examined in this study: perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane

sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluorooctane sulfonic acid **(**PFOS), perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), Sodium perfluoro-1-decanesulfonate (PFDS), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA) and perfluorooctadecanoic acid (PFODA). All of these compounds and the two ^{13}C-labeled internal standards (ISs) MPFNA and MPFOS were purchased from Fluka (SigmaeAldrich, St. Louis, MO, USA), as well 4-nonylphenol (IS for OCs) and all GC and HPLC solvents. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). For the extraction and clean-up of POPs, QuEChERS materials were obtained from Supelco (SigmaeAldrich, St.Louis, MO, USA); Supel^{TM} QuE Citrate (EN) tubes, containing Sodium Citrate tribasic dihydrate and Sodium Citrate dibasic sesquihydrate. Magnesium Sulfate and Sodium Chloride were used for the extraction. Supel^{TM} QuE-Sep tubes were used for the clean-up step. For the extraction of PFAs, the extraction cartridges (Oasis HLB WAX 3 mL, 60 mg) were provided by Waters (Milford, MA, USA). Ammonium formate, sodium acetate, acetic acid (99.9%) and 25% ammonia solution were purchased from Fluka.

3.11.2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) of each standard used for HPLC-HRMS analyses, were prepared in methanol and stored at -20°C. Working solutions at the concentrations of 10 and 100 ng mL^{-1} were prepared during each analytical session and maintained at 4°C throughout the method validation. For GC-MS/MS analyses, working solutions were prepared daily in hexane from various stock solutions containing a mix of standards. The storage conditions of the solutions were the same as described for HPLC analyses.

3.11.2.4. Extraction procedure

For the extraction of PFASs, 2 g of sample was spiked with the two internal standards at the concentration of 5 ng mL^{-1}. After the addition of 10 mL of acetonitrile for the protein precipitation and analytes extraction, the sample was vortexed and sonicated for 15 min. After centrifugation (2500×g, 4°C for 10 min), the supernatant was collected into a glass flask and evaporated in a rotary vacuum evaporator at 35°C. The extract was suspended in 10 mL of water and underwent the SPE extraction using the Oasis WAX Cartridges under vacuum, for further purification and extraction. The SPE cartridges were preconditioned with 3 mL of 0.5% ammonium hydroxide in methanol, 3 mL of methanol, and 3 mL of Milli-Q water. The sample was loaded, and then the cartridges were washed with 3 mL of 25 mM acetate buffer pH 4.5 to remove interferences, as well as lipid or proteins, and to increase the adsorption of target anions to the cartridge, followed by 2 mL of methanol. Finally, the compounds were eluted using 3 mL of 0.5% ammonium hydroxide in methanol and were collected in a 15 mL polypropylene tube. The eluate was dried in a rotary vacuum evaporator at 35°C. The dried extract was suspended in 100 µL of methanol:ammonium formate 20 mM (10:90 v/v), and then transferred to an auto-sampler vial. The injection volume was 10 µL. The method was developed and optimized taking into consideration the work of Taniyasu et al. (2005), considering the different effect of pH of acetate buffer, the percentage of ammonium hydroxide in methanol and the influence of elution volume of ammonium hydroxide in methanol on recoveries of PFASs.

Moreover, taking into account the ubiquity of PFAS in the environment of analytical laboratories, several precautions were taken, such as washing glassware with methanol, the execution of at least 10 procedural blanks at days to subtract any background contamination.

The extraction of PCBs, PBDEs, OCPs and PAHs was performed using the QuEChERS approach. A 5 g of sample was homogenized and transferred to a QuEChERS extraction tube, then the three ISs were added. Ten milliliters of a mixture of hexane/acetone (4:1 v/v) was added as extraction solvent; the tube was shaken for 1 min using a vortex and centrifuged for 10 min at 2000×g at 4°C. Later, the supernatant was transferred to a QuEChERS clean up tube, shaken and centrifuged at the same

conditions described above. The extract was transferred in a flask and evaporated under vacuum in a centrifugal evaporator at 35°C. The residue was dissolved in 1 mL of hexane and analysed by GC/MS-MS.

3.11.2.5. HPLC-HRMS analyses

The HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), consisted of a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with a 20-µL loop. A Synergi Hydro-RP reverse-phase HPLC column (150 × 2.0 mm, 4 µm particle size), with a C18 guard column (4 × 3.0 mm) (Phenomenex, Torrance, CA, USA) was used for the chromatographic separation. Stainless steel capillary tubes were used for minimising PFAS background contamination in the system. Moreover, since PFOA and PFOS were always present in the chromatographic system, we introduced a small Megabond WR C18 column (5 cm × 4,6 mm, i.d. 10 µm) between pump and injector, allowing us to delay our analytes by 2 min relative to those already present in the system.

Solvents A (aqueous ammonium formate 20 mM) and B (MeOH) were the mobile phases used for the gradient. The elution started with 10% B, which increased to 40% at the 4th minute and more gradually to 95% at the 12th minute, then remaining constant up to the 18th minute. The initial conditions were reached at the 20th minute, with an equilibration time of 7 min. The flow was 0.3 mL min^{-1}. The detector was a Thermo Q-Exactive Plus (Thermo Scientific, San Jose,CA, USA), equipped with a heated electrospray ionization (HESI) source. Capillary temperature and vaporizer temperature were set at 330°C and 280°C, while the electrospray voltage was set at 3.50 kV operating in negative mode. The sheath and auxiliary gas were set at 35 and 15 arbitrary units. S lens RF level of 60 instrument calibration was done for every analytical session with a direct infusion of an LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The full scan acquisition was combined with a DIA Independent Data Acquisition mode, providing the MS2 spectra for the confirmatory response, based on an inclusion list. The resolving power of FS was set at

70,000 FWHM. On the basis of our compound list, a scan range of m/z 200–950 was chosen; the automatic gain control (AGC) was set at 1×10^{-6} and the maximum injection time was 200 ms. The DIA segment operated in negative mode at 35,000 FWHM. The AGC target was set to 5×10^{-4}, with the maximum injection time of 100 ms. the quadrupole filtered the precursor ions with an isolation window of 2 m/z. Fragmentation of precursors was optimised as two-stepped normalized collision energy (NCE) (10 and 70 eV). The mass tolerance window was set to 2 ppm. Detection of analytes was based on the retention time of target compounds, on calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment. The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm the different PFASs are reported in Table 1. Xcalibur™ 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HPLC-HRMS system, the exact mass of the compounds, record and elaborate data.

3.11.2.6. GC-MS/MS analysis of contaminants

The GC analysis was described in a previous study of ours (Chiesa, Labella, Panseri, Pavlovic, Bonacci, & Arioli, 2016 a). Briefly, GC-MS/MS in electronic impact (EI) mode was carried out by a GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) using a fused-silica capillary column RXi-XLB (30 m, 0.25 mm i.d., 0.25 mm film thickness, Restek, Bellefonte, PA, USA).

Selected reaction monitoring mode (SRM) was used to detect two ot three transitions per analyte according to European Commission (2015). Compound identification was performed by comparing relative retention times of samples and standard solutions and mass fragmentations obtained for each compound. All fragments are reported in Table 1. Xcalibur™ and Trace Finder™ 3.0 (Thermo Fisher Scientific) were the software used as instrument control and data processing, respectively.

3.11.2.7. Validation parameters

Validation was carried out following the European Commission (2015) SANTE/2015 guideline. The selectivity of the method was evaluated by injecting extracted blank mollusc samples. The absence of interferences was proved by the lack of peaks with a signal-to-noise ratio higher than 3 at the retention times of the target compounds. Mollusk sample, previously analysed and checked for the absence of all POPs, were used as control samples during optimization and validation steps. For mollusc fortification, 5 g of the control sample was spiked in order to cover the concentration range from 0.5 to 100 ng g^{-1} (five calibration points: 0.5, 1, 10, 50 and 100 ng g^{-1}) for PCBs and PAHs; from 0.5 to 50 ng g^{-1} (five calibration points: 0.5, 1, 10, 25, 50 ng g^{-1}) for PBDEs and from 5 to 1000 ng g^{-1} for OCs (five calibration points: 5, 50, 100, 500 and 1000 ng g^{-1}). For PFASs, 2 g of control sample was spiked to cover the concentration range from LOQ to 10 ng g^{-1} (six calibration points LOQ, 0.05, 0.1, 3, 5, 10 ng g^{-1}), except for PFBA, PFOA and PFUdA (up to 50 ng g^{-1}, six calibration points: LOQ, 0.05, 0.1, 5, 10, 50 ng g^{-1}) in order to realize the matrix-matched calibration curves. For the limit of quantification (LOQ) of the methods, we used the lowest validated spiked level meeting the requirements of recovery within the range of 70–120% and an RSD ≤ 20%, as defined by the European Commission (2015). Finally, the extraction methods were also evaluated for their repeatability, linearity and recovery. Recoveries were calculated by comparing the concentrations of the extracted compounds with those from the MMC calibration curves at LOQ for all compounds. The repeatability (evaluated as the coefficient of variation, CV%) was calculated by analysing six replicates at the same fortification level.

3.11.3. Results and discussion

3.11.3.1. Validation parameters

The methods showed high specificity, without any interferences close to the retention time where the investigated compounds were expected to elute, and consequently showed a high S/N ratio in the presence of analytes, even at the lowest detectable

concentration. The mean recoveries ranged between 70 and 120%, indicating the efficiency of the extraction protocol. Matrix validation curves demonstrated a good linearity over the working range with a good fit (R^2 > 0.985) for all compounds. Repeatability was calculated using one-way analysis of variance (ANOVA), the CV was lower or equal to 20 % for all POPs, satisfying the criteria required by European Commission (2015).

Regarding the LOQs, our satisfactory results showed high method sensitivity for the selected contaminants both for LC-HRMS and GC-MS/MS analyses. In particular, the analytes detected with GC-MS/MS showed LOQs equal or lower than those reported by Pizzini et al. (2016), for example, benzo(b)fluoranthe has an LOQ of 0.5 ng g^{-1}, which is lower than the 3.54 ng g^{-1} reported by Pizzini et al. (2016). For PFASs, the LOQs were much lower than those reported by Nania et al. (2009) and Wille et al. (2011), which have for PFOS an LOQ of 6 ng g^{-1} and 0.1 ng g^{-1} respectively, compared to our LOQ of 0.005 ng g^{-1}. All of the validation parameters for GC-MS/MS and HPLC-HRMS are reported in Table 2.

3.11.3.2. Mussel and clam sample POPs distribution

Results on the prevalence and concentration of contaminants are reported in Table 3. PCBs were found with the highest prevalence in mussels, while they were not found in clams, as showed in Fig. 2. In particular, the most abundant congener was PCB 138, showing the highest concentration of 25.34 ng g^{-1}. The concentrations were all lower than the maximum levels of 75 ng g^{-1} required by the European Union (European Commission, 2011). Referring to the overview of the literature studies reported in Table 4, the concentration of PCBs in mussel samples were in according to those found by Herceg-Romanic´ et al. (2014), which found PCB 138 as one of the most abundant congeners, but with a lower maximum concentration compared to our results (6.34 ng g^{-1}).

PAHs were detected both in mussels and clams, with the highest prevalence in mussels. This could be because the discharges of maritime transport of petroleum

products (oil spills) are mainly composed of PAHs and are viscous fluid mixtures having a density lower than water, so PAHs tend to remain on the water surface (Gonzalez-Doncel, Gonzalez, Fernandez-Torija, Navas, & Tarazona, 2008; Fingas, 2016). The most frequent compound detected was Benzo(a)pyrene, with a maximum concentration of 7.05 ng g^{-1}. Also for these contaminants, all of the samples were compliant to Regulation No 1259/2011 (European Commission, 2011). As also reported by Pizzini et al. (2016), the PAHs concentration is higher in mussels than in clams, but in our study, the difference was greater, maybe due to the reasons described above. In fact, the level of PAHs in mussels was 13.95 ng g^{-1}, while this value was 4.35 ng g^{-1} in clams (approximately three times lower than in mussels).

Concerning OCPs, only DDT metabolites were found. In particular, 4,4'-DDE was detected only once in mussels, and was never detected in clam samples; 4,4'-DDD was found both in mussels and clams with a low prevalence of 10 and 8 %, respectively and a highest concentration of 16.34 ng g^{-1}. The concentration of DDTs found in our mussel samples was higher compared to the results of Herceg-Romanic´ et al. (2014), who found a highest concentration of 2.61 ng g^{-1}.

PBDEs were found only in four mussel samples and one clam sample at the LOQ. Despite low prevalence, the concentrations found are higher than reported by Hu et al. (2010), which detected PBDEs at concentrations ranging from 25.4 to 58.9 pg g^{-1}.

Regarding PFASs, up to 11 compounds (both acid and sulfonate forms) were detected in almost all clam samples, showing an evident higher contamination in terms of frequency and concentration than in mussels. The most contaminated clam pool was fished in the FAO area 37.2, confirming the pollution of this area, as reported by Vianello et al. (2013). The most abundant compound in clams was PFOA, with 97% of positivity and the highest concentration of 31.03 ng g^{-1}. Of the tested compounds, PFBA was present at the highest concentration (both for mussels and clams). This is because, as discussed by Water Research Foundation project #4322 (Fulmer, 2016), conventional treatment at wastewater treatment plants and most drinking water treatment plants are ineffective at removing this shorter chain PFAS. It should be emphasized that this analyte was always present even in the background contamination

of the extractive procedure at a maximum concentration of 4 ng g^{-1}, evaluated through the analysis of a batch of 10 procedural blanks during each analytical session. The evidence of a major contamination in clams (Fig. 2) is present also in the study of Nania et al. (2009). This could be explained by the fact that clams can absorb both from seawater and sediments, as reported by Berger et al. 2004 and Nakata et al., 2006. PFOA prevalence was found to be higher than PFOS, which is in line with the results of Nakata et al., 2006. In clams, the PFOA concentrations were also higher than those of PFOS, as reported in the last study about sea sediments. However, in mussels, this trend is reversed, even if the concentrations of PFOA and PFOS were quite similar.

3.11.3.3. Risk assessment

Considering the absence of maximum limits for PFASs, a risk assessment was carried out on the basis of our results referring to the established tolerable daily intake [TDI] for PFOA and PFOS (1.5 µg Kg^{-1} b.w. per day and 150 ng Kg^{-1} b.w. per day, respectively (EFSA, 2008)). Considering a person of 70 Kg, the threshold dose is 105 µg per day for PFOA and 10.5 µg per day for PFOS; on the basis of data reported by EUMOFA (European Commission, 2016), the annual per capita consumption is 1.27 Kg for mussels and 0.33 Kg for clams. Considering these tolerable intakes and, with a conservative approach, the highest concentration of PFOA and PFOS found in our samples were 0.55 and 3.64 ng g^{-1} (in mussel) and 31.03 and 7.20 ng g^{-1} (in calm) respectively. These concentrations could result in a daily intake of 1.91 ng of PFOA and 12.66 ng of PFOS in mussels and 27.93 ng of PFOA and 6.48 ng of PFOS in clams. These intake values are well below the suggested TDI. Thus, in this case, the consumption of mollusks does not represent a risk for consumers. This consideration could also be extent by taking into account the other contaminants, which have MRLs. In fact, all of the concentrations found were well below the limits provided by the legislations, confirming that all samples were compliant.

3.11.4. Conclusions

Due to anthropogenic activities, various contaminants could be present in the environment, increasing the pollution of marine ecosystems. Bivalve molluscs have been used as contamination indicators of the marine ecosystem. For this purpose, we used mussels and clams, belonging to diverse areas, to evaluate the occurrence of PCBs, PFASs, OCPs, PAHs and PBDEs, related to the different habitats of the two mollusc species. Mussel cultures are generally suspended to hard substrates placed at 2 to 5 m in the seawater, while clams usually live buried in the sand or the muddy seabed in brackish waters (Nania et al., 2009). Considering the different chemical-physical properties of the selected contaminants, two sensitive, specific and robust analytical methods, based on LC-HRMS and GC-MS/MS, were developed and validated for the analysis of mussel and clam samples. The results showed a greater contamination of PCBs, OCPs and PAHs in mussels than clams, whereas this trend was reversed for PFASs. These data could be accounted for by the different contamination sources, different chemical-physical properties of the selected classes, and different distribution in the marine layers.

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Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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Table 1. Retention time (tr), precursors, main products, polarity and collision energies of the compounds analysed by LC-HRMS and GC-MS/MS.

Compound LC-HRMS	Formula	t_r (min)	Precursor (m/z)	Main product (m/z)	Polarity
PFBA	$C_4HF_7O_2$	9.07	212.97920	168.98836	(-)
PFPeA	$C_5HF_9O_2$	11.68	262.97601	218.98560	(-)
PFBS	$C_4F_9HO_3S$	12.02	298.94299	98.95434	(-)
PFHxA	$C_6HF_{11}O_2$	13.22	312.97281	268.98288	(-)
PFHpA	$C_7HF_{13}O_2$	14.36	362.96962	318.97949	(-)
PFHxS	$C_6F_{13}HO_3S$	14.39	398.93660	98.95437	(-)
PFOA	$C_8HF_{15}O_2$	15.27	412.96643	368.97681	(-)
PFNA	$C_9HF_{17}O_2$	16.03	462.96323	418.97385	(-)
PFOS	$C_8F_{17}HO_3S$	16.00	498.93022	79.95598	(-)
PFDA	$C_{10}HF_{19}O_2$	17.96	512.96004	468.97064	(-)
PFUdA	$C_{11}HF_{21}O_2$	18.48	562.95684	518.96729	(-)
PFDS	$C_{10}F_{21}HO_3S$	17.35	598.92383	79.55599	(-)
PFDoA	$C_{12}HF_{23}O_2$	18.98	612.95365	568.96387	(-)
PFTrDA	$C_{13}HF_{25}O_2$	19.50	662.95046	618.96057	(-)
PFTeDA	$C_{14}HF_{27}O_2$	20.06	712.94726	668.95823	(-)
PFHxDA	$C_{16}HF_{31}O_2$	20.80	812.94088	768.95184	(-)
PFODA	$C_{18}HF_{35}O_2$	21.81	912.93449	868.94513	(-)
MPFNA	$[13]C_5C_4HF_{17}O_2$	16.03	467.98001	422.98703	(-)
MPFOS	$[13]C_4C_4F_{17}HO_3S$	16.00	502.94364	79.95592	(-)

Compound GC-MS/MS	t_r (min)	Precursor (m/z)	Product Ions (m/z)	Collision Energy (V)
PCBs				
PCB 28	18.76	256	**186** *	20
		258	186	25
PCB 52	20.25	292	**222***	25
		292	257	10

PCB 101	24.46	324	254	25
		326	**256***	25
		328	256	25
PCB 138	28.99	360	**290***	25
		360	325	10
PCB 153	30.25	360	**290***	20
		360	325	30
PCB 180	34.06	394	**324***	25
		394	359	10
		396	324	25
PBDEs				
PBDE 28	27.95	246	139	10
		248	**139***	10
		408	248	10
PBDE 33	28.05	246	139	30
		248	**139***	30
		406	246	10
PBDE 47	34.34	326	217	30
		328	219	30
		484	**326***	30
PBDE 99	38.17	410	297	30
		406	297	30
		564	**404***	20
PBDE 100	39.05	410	297	30
		406	297	30
		564	**404***	10
PBDE 153	40.88	484	377	25
		642	**482***	10
PBDE 154	41.76	484	324	30
		486	326	30
		644	**484***	20
OCPs				
α HCH	15.27	181	**145***	10
		181	146	10
		219	183	10

Hexachlorobenzene	15.45	284	**249***	20
		286	214	30
		286	251	20
β BHC	16.69	181	**145***	10
		183	148	10
		219	183	10
Lindane (γ HCH)	16.44	181	**145***	10
		183	145	10
		219	183	10
Heptachlor	19.27	272	**237***	10
		274	237	10
		274	239	10
Aldrin	20.84	261	**191***	30
		263	193	30
		265	193	30
Heptachlor epoxide	22.77	353	**263***	10
		353	282	10
		355	265	10
Trans chlordane	23.96	373	264	20
		373	**266***	20
		375	266	20
Endosulfan I	24.64	373	**266***	20
		375	266	20
		377	268	20
pp' DDE	25.96	246	**176***	30
		248	176	30
		328	248	20
Endrin	27.06	245	173	30
		263	**193***	30
		281	245	10
Endosulfan II	27.65	195	**159***	10
		241	206	10
pp DDD	28.18	235	**165***	20
		237	165	20

op DDT	28.27	235	**165***	20
		237	165	20
Endosulfan sulfate	29.88	272	**237***	10
		274	237	10
		274	239	10
PAHs				
Chrysene	37.37	228	202	20
		228	226	30
		226	**224***	30
Benz(a)anthracene	37.18	226	224	30
		228	202	20
		228	226	30
		226	**223***	30
Benzo(b)fluoranthe	41.61	250	248	30
		252	250	30
		250	**224***	30
Benzo(a)pyrene	42.57	252	250	30
		253	227	20
		253	251	30
		252	**226***	30

*= quantifier ion

Table 2. Validation parameters of the investigated POPs.

Compounds by GC-MS/MS	LOQ ($ng\ g^{-1}$)	CV %	Recovery %
PCB 28	0.5	11	85
PCB 52	0.5	9	87
PCB 101	0.5	9	83
PCB 138	0.5	12	97
PCB 153	0.5	12	85
PCB 180	0.5	10	88
PBDE 28	0.5	2	93
PBDE 33	0.5	3	79
PBDE 47	0.5	9	94
PBDE 99	0.5	7	81
PBDE 100	0.5	11	80
PBDE 153	0.5	7	70
PBDE 154	0.5	9	84
α HCH	5	18	119
β BHC	5	20	120
Hexachlorbenzene	5	16	100
Lindane	5	20	116
Heptachlor	5	20	120
Aldrin	5	12	89
Heptachlor epoxide	5	10	93
Trans chlordane	5	12	94
Endosulfan I	5	12	95
Endosulfan II	5	10	84
pp' DDE	5	16	90

Endosulfan Sulfate	5	14	75
Endrin	5	10	120
op DDT	5	20	120
pp DDD	5	3	102
Chrysene	0.5	3	82
Antracene	0.5	6	75
Benzofluoranthene	0.5	3	75
Benzopyrene	0.5	2	77

Compounds by HPLC-HRMS	**LOQ (pg g^{-1})**	**CV %**	**Recovery %**
PFBA	5	7	82
PFPeA	10	10	114
PFBS	10	11	102
PFHxA	20	6	110
PFHpA	5	5	112
PFHxS	15	9	103
PFOA	5	5	113
PFNA	5	10	95
PFOS	5	12	83
PFDA	20	13	84
PFUdA	20	6	85
PFDS	20	8	83
PFDoA	20	8	89
PFTrDA	15	8	87
PFTeDA	5	15	91
PFHxDA	5	18	85
PFODA	5	18	84

Table 3 Prevalence and concentration ranges of the selected contaminants.

Compounds	Prevalence (%)		Concentration range (ng g^{-1})	
	Mussels	**Clams**	**Mussels**	**Clams**
Σ PCBs	58	n.d.	n.d. - 49.02	n.d.
Σ PAHs	36	28	n.d. - 13.95	n.d. – 4.35
Σ DDTs	12	8	n.d. - 16.34	n.d. - 14.96
Σ PBDEs	8	2	n.d. - 0.5	n.d. - 0.5
Σ PFAs	70	100	n.d. - 91.80	n.d. - 120.75

n.d. = not detected

Table 4. Literature data on POPs distribution in mollusks.

Reference	Compounds investigated	Analytical technique	Concentration range in ng g^{-1} (average values)	
			Mussels	**Clams**
Choi et al. (2016)	18 PCBs	GC-ECD	70.6-159 [a]	69.3-109 [a]
	DDTs		38.6-102 [a]	40.3-49.3 [a]
	α- , β- , γ- and δ-HCH		9.00-13.5 [a]	6.25-17.8 [a]
Pizzini et al. (2016)	127 PCBs	GC-MS	< LOD - 4.57 [b]	< LOD - 3.68 [b]
	16 PAHs		< LOD - 7.03 [b]	2.32 - 5.67 [b]
Dodder et al. (2014)	2 PFASs	LC-MS/MS	< LOD - 29 [c]	not investigated
	11 PBDEs	GC-MS/MS	< LOD - 68 [c]	not investigated
Herceg-Romanic´ et al. (2014)	17 PCBs	GC-ECD	1.12 - 23.86 [b]	not investigated
	α- , β- and γ-HCH		0.40 - 1.61 [b]	not investigated
	Hexachlorobenzene (HCB)		0.01 - 0.12 [b]	not investigated
	DDTs		0.15 - 2.61 [b]	not investigated
Wille et al. (2011)	14 OCPs	LC-MS/MS	< LOD - 28 [c]	not investigated
	10 PFASs	LC-ToF	< LOD - 4 [c]	not investigated
Nania et al. (2009)	2 PFASs	LC-MS/MS	< 1.5 – 3 [b]	< 2 – 16 [b]

a = expressed as lipid weight

b = expressed as wet weight

c = expressed as dry weight

Fig. 1. Map of sample collection sites.

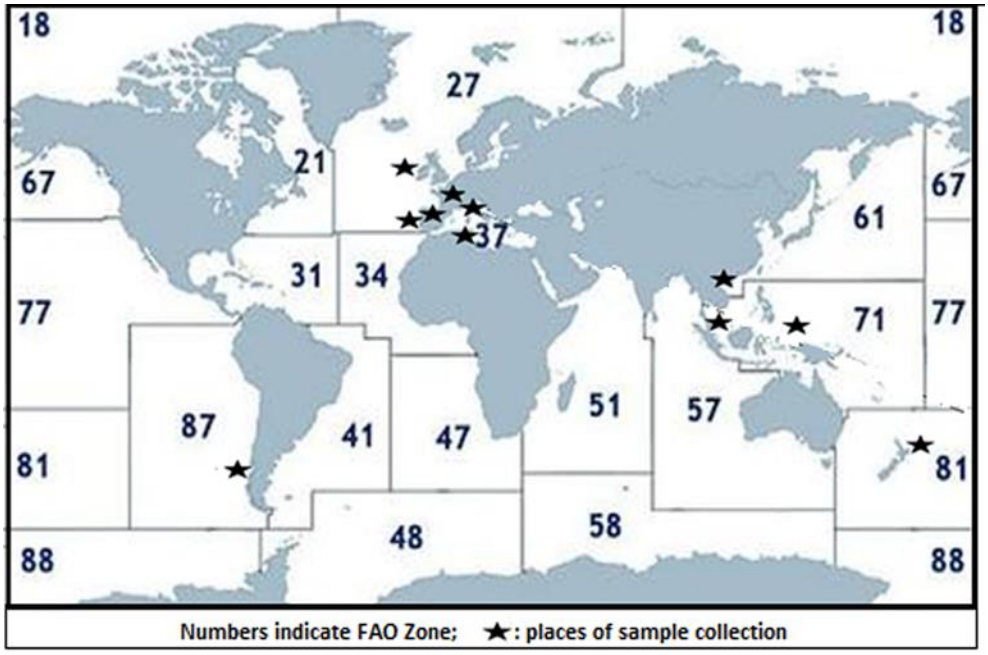
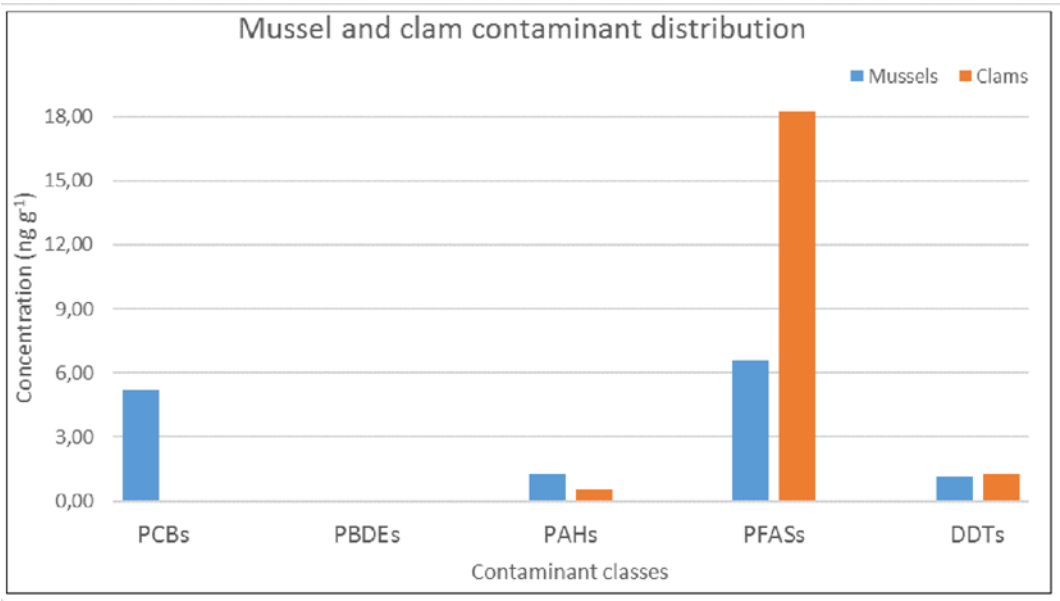


Fig. 2. Mean values of the Σ PCBs, Σ PBDEs, Σ PAHs, Σ PFASs and Σ DDTs in mussels and clams.



3.12. Unraveling estradiol metabolism and involvement in the reproductive cycle of non vertebrate animals: The sea urchin model

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In this study I contributed to the execution of preliminary trials and analyses, data processing and writing of the article concerning the LC-MS/MS analysis following a consultancy

Highlights

•Estradiol presence in sea urchin body fluids was confirmed by LC–MS.

•We administered physiological E2 doses to sea urchins for short and long-term periods.

•Despite increased E2 level no effect was observed on the reproductive parameters.

•Estrogens are not involved in the regulation of sea urchin reproductive cycle.

•Evidences of class-specific hormonal mechanisms warns against Phylum generalization.

Abstract

Estradiol (E2) is a well-known hormone in vertebrates whereas in invertebrates its unambiguous presence was verified only in some species. Weather this presence is also associated to similarly conserved roles in animal phylogeny is similarly uncertain. Due to their phylogenetic position, echinoderms represent ideal experimental models to provide evolutionary insights into estrogen appearance and function. Therefore, in this research, we investigated if E2 is truly present and has a role in the reproductive biology of the sea urchin Paracentrotus lividus. Presence of 17b estradiol in body fluids was confirmed by liquid chromatography–mass spectrometry. By immunological methods (RIA) we evaluated the physiological circulating E2 levels of adult specimens and, on the basis of these, we directly administered E2 to study its metabolism and its putative effects on gonad development at physiological doses. Although different E2 tested concentrations, a correspondent dose-dependent increase of hormone levels was not found in both body fluids and gonads, suggesting the presence of potent homeostatic/detoxification mechanisms. These latter do not involve enzymes such as aromatase-like, sulfotransferase-like and acyltransferaselike, whose activities were not affected by E2 administration. Despite the increase of endogenous E2, the treatment did not induce significant variations in none of the considered reproductive parameters.

Overall, this research (1) provides definitive evidence of E2 presence in sea urchin tissues and (2) demonstrate that, differently from vertebrates and starfish, E2 does not play a key role in sea urchins reproductive processes. Intra-phylum differences suggest

the existence of class-specific hormonal mechanisms and highlight the risk of Phylum generalization.

Keywords: Estradiol, Sea urchin, Reproductive cycle, Hormone metabolism

3.12.1. Introduction

Estrogens are involved in many physiological processes of vertebrates, having an essential role in their reproduction, metabolism, development and behavior [18]. As for all sex steroids, they were originally considered vertebrate-specific hormones but in the last decades this perspective partially changed. Indeed estrogen- like compounds have been found in almost all invertebrate groups [17]. Nevertheless, only in a limited number of cases the unambiguous presence of estrogens (mainly 17b estradiol, E2) was clearly demonstrated by direct methods (e.g. gas chromatography– mass spectrometry), including mollusks (for a review see [29]), tunicates [5] and echinoderms [47]. Besides their presence, it is still under debate also whether estrogens are endogenously synthesized and have a conserved physiological role in animal phylogeny [21,29,30]. As basal deuterostomes, echinoderms occupy a key-phylogenetic position [4,40], which can provide a relevant perspective on evolutionary insights related to estrogen appearance and function in metazoans [7]. In echinoderms, estrogen-like compounds has been detected in different tissues, including gonads, but mainly by means of indirect methods [1,2,13,19,48] and only in asteroids (i.e. starfish) their presence was confirmed by GS–MS analyzes [47]. The biosynthesis of estrogens in echinoderms has been – and is still-similarly under debate [12,21]. A P450 aromatase-like activity was measured in sea urchin digestive tube, suggesting this tissue might be the main putative biosynthesis site of estrogens [1,19]. Nevertheless, the molecular structure of this putative estrogen biosynthetic enzyme is likely to be different from the vertebrate aromatase (Cyp 19) since an homologous gene was not found in the completely sequenced sea urchin genome [33]. This is in agreement with Markov et al. [21] who proposed an independent evolution of steroidogenic enzymes in vertebrates and invertebrates, which may have led to a functional evolutionary convergence in structurally different proteins. Even so, echinoderm P450 aromatase-like activity was

affected by triphenyltin (TPT), a well-known inhibitor of vertebrate aromatase [19]. Echinoderm tissues can also efficiently metabolize exogenously administered estrogens. In sea urchins, estrogens were rapidly converted to estrogen-conjugated: aqueous-soluble, mainly estrogen-sulfates, and lipophilic compounds [6,12]. In the mussel Mytilus galloprovincialis estradiol is mainly transformed to esterified-estrogens [15], indeed esterification renders steroids to an apolar form, which is retained in the lipid matrices of the body and therefore may act as a long-term hormone storage. Accumulation of these compounds indicates that they are major estrogen metabolites also in sea urchins, although their biological significance has still to be elucidated. Besides their presence, synthesis and metabolism, the involvement of estrogen-like compounds in the regulation of echinoderm reproduction was suggested by several authors since their physiological levels varied according to the reproductive cycle and in a sex-specific manner [1,2,13,46,48,51]. Additionally, in the last decades a number of experiments of direct hormone administration have been performed both in vivo and in vitro on asteroid and echinoid species, in order to elucidate the physiological significance of estrogens in these invertebrates (see Table 1). In most studies, E2 treatment apparently resulted in appreciable physiological effects on different parameters, although a clear and positive influence on ovary/oocyte maturation and development could be inferred only for asteroids, similarly to what described for vertebrates and other invertebrates. Conversely, different and non-conclusive results were reported for sea urchins, moving from absence of effects [39,42] to positive regulation of ovarian growth [49]. The specific mechanism of action of E2 in echinoderms is also unknown as, similarly to the biosynthetic enzymes, no classic estrogen receptor (ER) gene was found in the sea urchin genome [33] and nothing is known for starfish. Despite these discrepancies within the same Phylum and the so-far presented ‘‘still open questions”, in the literature echinoderm reproduction is usually reported as estrogen-sensitive, a fact that may lead to dangerous generalizations. Additionally, the increasing use of echinoderms in research addressed to endocrine disruption assessment [37,38] necessarily require a better understanding of their baseline endocrinology to really understand their potential susceptibility to these environmental contaminants. On the basis of this, the aim of this work was to (1)

confirm the presence of estrogens (17b estradiol and estrone) in the sea urchins Paracentrotus lividus by direct methods (i.e. chromatography coupled to mass spectrometry) and provide a validation of the routinely used (and more practical) immunological analyses (Radioimmunoassays, RIA); (2) verify the putative involvement of E2 in sea urchin reproduction by assessing the effects of E2 administration at physiological doses. This was done by looking at (a) estradiol biosynthesis and metabolism and (b) gonad development; particular attention was paid to the experimental design (doses, reproductive cycle resetting, triggering environmental cues, feeding rates) in order to reduce the individual variability, possible source of misinterpreted results, and synchronously activate sea urchin gametogenesis.

3.12.2. Materials and methods

3.12.2.1. Animal sampling and maintenance

For GS–MS confirmation of estrogen presence, 15 adult specimens of P. lividus, were maintained in laboratory conditions (artificial sea water) and fed with an artificial diet for about one month. Body (coelomic) fluids were collected, immediately frozen in liquid nitrogen and stored at 40 °C until chromatographic analyzes. For evaluation of physiological circulating E2 levels by RIA, 102 adult specimens of P. lividus were monthly collected for a whole year in the Protected Marine Area ‘‘Isola di Bergeggi” (44°14’N; 8°26’E; Tyrrhenian Sea). After their arrival to the laboratory, animals were immediately sacrificed; body fluids (coelomic fluids) were collected with a syringe, frozen in liquid nitrogen and stored at 80 °C until RIA analyzes. One gonad was processed for sex and reproductive stage evaluation. For the experiment of direct E2 administration, 146 P. lividus adult specimens (diameter about 45 mm) were collected in the same location on July 2009, immediately transported to the laboratory and distributed in 50 L aquaria filled with artificial sea water (Instant Ocean; salinity about 37‰w/v) and provided with internal circulation system. Sea urchin health conditions as well as all physical and chemical water parameters were daily (temperature and salinity) or weekly (pH, KH, GH, NO_2, NO_3) monitored throughout the experimental period and promptly adjusted if necessary. Additionally, at the end of the experimental period (12

weeks), 10 more specimens were collected from the same field population. These animals represented the ‘‘environmental controls” (T_{2env}).

Table 1. Summary of the main experiments of E2 administration in echinoderms found in the literature.

Species	Exp. approach	E2 administr. type and freq.	Exp. period	Effects	References
Starfish					
Asterina pectinifera	*in vitro*	a; daily	3 d	↑ Oocyte diameter and ↑ % of oocyte in advanced reprod stage	[41]
Asterias rubens	*in vivo*	b; daily	16 d	↑ Oocyte diameter; ↑ GI ♀; ↑ MI ♀; ↑ E1 levels	[28]
Asterias rubens	*in vitro/in vivo*	a & b; 1st & 7th day	8 d	↑ Lipid content in pyloric caeca	[43]
Luidia clathrata	*in vivo*	b; every 2 days	16 d	↑ Activity of metabolic enzymes (G-6-PDH and 6-PGDH)	[50]
Sclerasterias mollis	*in vivo*	b; daily	16 d	↑ Oocyte area, ↑ ovarian protein, ↑ E1 level	[3]
Sea urchin					
Dendraster excentricus and *Strongylocentrotus purpuratus*	*in vitro*	a	4 h & 24 h	Synthesis of novel protein in non-gravid females	[10]
Pseudocentrotus depressus	*in vivo*	c; daily	1 m	No effects	[42]
Lytechinus variegatus	*in vivo*	c; daily	36 d	↑ GI ♀; ↑ protein percentage	[49]
Strongylocentrotus. purpuratus	*in vivo*	b; 1/week	8 w	↓Embryo sensitivity to E2; ↑ embryo sensitivity to TBT and DDD; ↑ SpSHR2 transcript in the eggs	[27]
S. nudus	*in vivo*	c	48 h	↑ Protein synthesis	[44]
S. intermedius	*in vivo*	c	48 h	↑ Ovarian protein synthesis; no effect before spawning	[45]
P. lividus	*in vivo*	b; 2/week	2 w & 12 w	No effect	[39]

a = culture medium; b = injection; c = diet; m = month; w = week; d = day; h = hours; ↓ = decrease; ↑ = increase; GI = Gonad Index; MI = Maturity Index; E1 = estrone; G-6-PDH = glucose-6-phosphate dehydrogenase; 6-PGDH = 6-phosphogluconate dehydrogenase; TBT and DDD = endocrine disrupting compounds, tributyltin and dichlorodiphenyldichloroethane, respectively; SpSHR2 = orphan steroid receptor.

3.12.2.2. Liquid chromatography–mass spectrometry

3.12.2.2.1. Sample extraction

40 ml of coelomic fluid (pool of 5 different individuals kept at −40 °C) were spiked with the internal standard estradiol-d2 to the final concentration of 10 ng ml^{-1} and centrifuged

before purification by Affinimip SPE catridges, previously equilibrated with 3 ml acetonitrile and 3 ml ultrapure water (flow rate 2 drops per second).

The sample was loaded by gravity and then washed with 3 ml ultrapure water and 3 ml of 60/40 ultrapure water/acetonitrile (flow rate 1 drop per second). Finally, the sample was eluted with 3 ml of methanol (flow rate 1 drop per second). The elution fraction was evaporated until dryness under nitrogen before derivatization with 100 µl of dansyl chloride (1 mg ml^{-1} in acetone) and 100 µl of 0.1 M sodium bicarbonate in water, heated at 60 °C for 3 min. The derivatized extract was reconstituted in 1 ml of methanol:water (70:30 v/v) and transferred in an auto-sampler vial. The injection volume was 10 µl.

3.12.2.2.2. LC–MS/MS analyzes

LC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation of the four estrogens (estradiol, estrone, estriol and 17α-ethinylestradiol) and the internal standard estradiol-d2 (Sigma-Aldrich, St. Louis, MO, USA) was achieved using a Synergi Hydro RP reverse-phase HPLC column (150 × 2.0 mm, 4 µm internal diameter), with a C18 (4 × 3.0 mm) guard column (Phenomenex, Torrance, CA, USA), which was kept at 30 °C. The mobile phase consisted of methanol (solvent A) and 0.1% aqueous formic acid (solvent B). The gradient program began at 70% A for 1 min, changing to 95% A in 9 min, which was then held for 2 min. Then, it returned to 70% A in 2 min and equilibrated for another 6 min. The flow rate was 300 µl min^{-1} and the overall run time was 20 min. The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher, San Jose, CA, USA) equipped with an electrospray interface (ESI) set in the positive (ESI+) mode.

3.12.2.3. E2 administration: experimental design

The experimental design of E2 direct administration experiment is summarized in Fig. 1, where further experimental details are reported. Estradiol administration was preceded by six weeks of starvation to reset and synchronize the reproductive cycle among the animals [35]. Feeding re-started 10 days before the first E2 administration to partially restore the animals after starvation stress. Animals were individually fed in excess with fresh carrots (3/week) or pellets of artificial diet (4/week; Wenger Manufacturing, Inc.,

patent n° 085115204). A mean “daily feeding rate” (DFR) was calculated at the end of the overall experimental period (12 weeks) for each tank: DFR = (n° pieces of eaten carrots or pellets/n° pieces of carrots or pellets provided in the aquarium) × 100. During starvation, temperature was set at 15 ± 1 °C and photoperiod at 15 h:9 h (dark:light; winter conditions). When feeding re-started the parameters were gradually increased to 20 °C and 10 h:14 h (dark:light; summer conditions), and remained fixed for the whole administration period. Indeed, changes in water temperature and daylight length (winter → summer) are fundamental cues to stimulate the onset of echinoid gametogenesis [31].

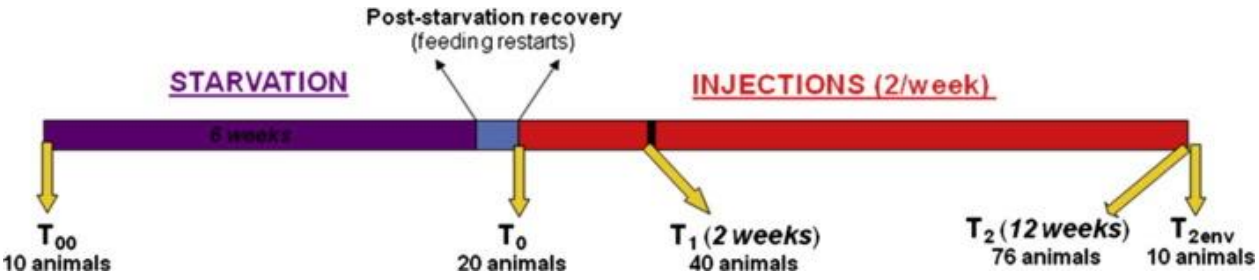

Fig. 1. Schematic representation of the experimental design, including the numbers of animals sacrificed at each time point.

Evaluation of circulating E2 level in field specimens allowed to calculate the E2 doses subsequently administered *via* injection in body fluids. A short-term (T_1: 2 weeks) and a long-term (T_2: 12 weeks) E2 treatment were planned and three different E2 concentrations plus one control solution were tested (see below). In the long-term experiment, each concentration was tested in duplicates (tank A and B) and a further control aquarium (CTL+: not injected sea urchins) was set up in order to check any long-term stress due to the experimental manipulation (injections). Ten animals were put in each aquarium/tank, except for the CTL+ aquarium (6 specimens). Animals were homogeneously distributed for size and weight among the aquaria. At each time point (T_0, T_1, T_2) a specific number of specimens was sacrificed (Fig. 1). Body fluids were collected with a syringe from each animal, frozen in liquid nitrogen and stored at −80 °C for the subsequent E2 analyzes. Sea urchins were dissected and the five gonads were removed and weighted for Gonad Index (GI) calculation [GI = (gonad fresh weight/total sea urchin fresh weight) × 100]. Then, one gonad was used for reproductive stage evaluation, whereas the remaining ones were collected, frozen in liquid nitrogen and

stored at −80 °C for E2 analyzes and lipid concentration measurement. Digestive tube was also collected, frozen and stored at −80 °C for enzymatic assays. Regardless of the time point (T_1 and T_2), animals were sacrificed 4 days after the last E2 injection. Additionally, T_{2env} specimens were processed for GI calculation and reproductive stage evaluation, as previously described.

3.12.2.3.1. Solution preparation

All chemicals were of reagent grade. A 17β-estradiol (Sigma, St. Louis, MO, USA) solution (2 mg ml^{-1}) in acetone (Merck, Darmstadt, Germany) was prepared and diluted (10^{-4}) in autoclaved and filtered ASW, in order to reach the highest tested concentration (200 ng ml^{-1}). The other concentrations were obtain by further dilutions (1:10) in ASW. Maximum acetone concentration was 0.01% v/v, therefore control solution consisted of 0.01% v/v acetone in autoclaved and filtered ASW. Fresh solutions were prepared each time.

3.12.2.3.2. Estradiol administration

Hormone administration occurred twice a week *via* intra-coelomic injection at the level of the peristomial membrane. Discontinuous injections were favoured to daily administration to reduce sources of mortality and stress for the animals. Three different E2 concentrations were tested: 2, 20, 200 ng ml^{-1}. 5 pg ml^{-1} was considered as "physiological" E2 level (see Section 3, Table 2) and selected as theoretical concentration to be reached in the fluids of the lowest dose treated group. The nominal concentration of the lowest dose was then calculated as follow: 5 pg ml^{-1} × 40 ml*/0.1 ml (individual injected volume) = 2 ng ml^{-1} (*mean fluid volume of the sea urchins). The medium and the highest tested dose were selected 10 and 100-fold more concentrated (20 and 200 ng ml^{-1}, respectively), in order to reach high but still rather close to the physiological E2 range.

Table 2. E2 levels measured by RIA in body fluids of field adult specimens (*P. lividus*) which had been monthly collected over a whole year.

	Total	Males	Females	Males		Females	
				NG	G	NG	G
N	99	46	53	19	27	23	30
Median	4.8	4.6	4.8	4.2	5.2	7.5	4.4*
Mean	22.3	9.1	33.7	7.4	10.2	60.0	13.6
25% percentile	3.5	3.3	3.8	3.1	3.5	3.9	3.6
75% percentile	11.3	7.4	19.9	8.2	7.2	140	5.7
Min	<2.0	<2.0	<2.0	<2.0	<2.0	3.0	<2.0
Max	292	131	292	35	131	292	151

Descriptive statistics (number of samples, mean, median, percentiles and min/max values) are reported separately for the sexes alone or the sexes and the reproductive condition i.e. NG = non gametogenic reproductive stages (*Spent, Recovery*), G = gametogenic reproductive stages (*Growing, Premature, Mature*). Three of the 102 collected animals were sex-undeterminate and therefore they were excluded from the analyzes. The overall median E2 level (males + females) was 4.8 pg ml^{-1}. G females display significantly lower (**P* = 0.015, Mann–Whitney test) E2 concentrations that NG females.

3.12.2.4. Reproductive stage evaluation

Reproductive stages were determined by histological analyzes (paraffin embedding), as described in Barbaglio et al. [1]. All solvents and reagents were of analytic grade (Merck, Darmstadt, Germany). Five stages were considered: non-gametogenic stages as *Spent* (immediately after spawning) and *Recovery* (phagocytosis and nutrient accumulation phase), progressive stages of gametogenesis as *Growing*, *Premature* and *Mature*.

3.12.2.5. Lipid content

The lipid fraction of gonads was analyzed in 38 T_2 animals (nine-ten specimens for each experimental group, excluded CTL+) as described in Sugni et al. [39]. Briefly, after lyophilisation, known amounts of dry samples were placed in cellulose thimbles (25 × 100 mm, Whatman, England) and then extracted using a Soxhlet apparatus (Falc Instruments, Lurano, Italy) for 12 h using 100 ml of n-hexane. Each extract was concentrated in a rotary evaporator to a volume of 1 ml, then transferred to a small vial, after which solvent evaporation was completed under gentle nitrogen stream until constant weight. The final weight was used to determine the dry-weight-lipid fraction (g lipid g^{-1} d.w.) expressed as percentage.

3.12.2.6. Hormone levels by RIA

Radioimmunoassay kits for 17β-estradiol were obtained from Beckman Coulter (Marseilles, France). Solvents and reagents were of analytic grade (Merck, Darmstadt, Germany). Analysis of E2 levels was performed on coelomic fluids of all the specimens (146) and on gonads of 46 T_2 individuals (10/experimental group plus 6 CTL+ sea urchins). Analyzes were performed as described in other works [2]. Efficiency of the extraction procedure was 80 ± 3%. Detection limits were of 30 pg g^{-1} w.w. in gonads and 2 pg ml^{-1} in coelomic fluids.

3.12.2.7. Enzymatic assays

Enzymes involved both in E2 synthesis and metabolism were investigated. Androstenedione and 17β-estradiol were obtained from Sigma (Steinheim, Germany); [1β-^{3}H]androstenedione (15–30 Ci/mmol) and [6,7-^{3}H]estradiol (49.7 Ci/mmol; >97% purity) were purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA, USA). PAPS (>99% purity) was purchased from Cal-Biochem, Darmstadt, Germany. All solvents and reagents were analytical grade (Merck, Darmstadt, Germany).

3.12.2.7.1. Subcellular fractioning

Digestive tubes of 4 T_1 and 6 T_2 individuals from each experimental group were homogenized in ice-cold 100 mmol l^{-1} potassium phosphate buffer pH 7.4 (150 mmol l^{-1} KCl, 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 1 mmol l^{-1} dithiothreitol (DTT) and 0.1 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF)). Homogenates were centrifuged at 500×*g* for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000×*g* for 20 min. The 12,000×*g* supernatant was further centrifuged at 100,000×*g* for 60 min to obtain 2 fractions: cytosolic supernatant and microsomal pellet. Microsomal pellets were then resuspended in a small volume of microsomal buffer (100 mmol l^{-1} potassium phosphate buffer pH 7.4 containing 150 mmol l^{-1} KCl, 20% w/v glycerol, 1 mM EDTA, 1 mM DTT and 0.1 mmol l^{-1} PMSF). Protein concentrations were determined by the method of (Lowry et al. [20], using bovine serum albumin as a standard. Microsomes and cytosols were stored at −80 °C until enzymatic analyzes were performed.

3.12.2.7.2. Aromatase activity

Briefly, microsomes (0.4 mg protein) were incubated at 25 °C for 3 h in a final volume of 1 ml of 100 mmol l^{-1} Tris–HCl pH 7.6, 10 µmol l^{-1} [^{3}H]androstenedione (1 µCi) and 0.2 mmol l^{-1} NADPH. Assays blanks containing 100 µl of buffer instead of microsomes were used for every run. The reaction was stopped by placing the tube on ice and organic metabolites and the excess of substrate were immediately eliminated from the aqueous phase by extraction with methylene chloride (3 × 3 ml). The possible remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in milli-Q water (4 ml). The solution was centrifuged (1500×*g*; 60 min) and two aliquots of the supernatant (1 ml) were counted for ^{3}H-radioactivity in a scintillation counter. The lowest aromatase-like activity detected by the method was 0.001 pmol h^{-1} mg^{-1} protein.

3.12.2.7.3. Palmitoyl-CoA: estradiol acyltransferase (ATAT) activity

Palmitoyl-CoA: estradiol acyltransferase activity was determined in digestive tube microsomes, as described in Janer et al. [15]. Microsomal proteins (200 µg) were incubated in 0.1 mol l^{-1} sodium acetate buffer pH 6.0 with 2 µmol l^{-1} [^{3}H]estradiol, 100 µmol l^{-1} palmitoyl-CoA and 5 mmol l^{-1} $MgCl_2$ in a final volume of 500 µl. The reaction was initiated by the addition of palmitoyl-CoA, and samples were incubated for 60 min at 30 °C. Reaction was stopped by adding 2 ml of ethyl acetate, and extracted twice. The ethyl acetate fraction was evaporated to dryness, the dry residue redissolved in methanol, and injected into the HPLC system. HPLC analyzes were performed on a PerkinElmer Binary 250 LC pump system equipped with a 250 mm × 4 mm LiChrospher 100 RP-18 (5 µm) reversed-phase column protected by a guard column LiChrospher 100 RP-18 (5 µm). Separation of estradiol and its palmitoyl-ester was performed at 1.2 ml min^{-1} with a mobile phase composed of (A) 56% water containing 0.1% acetic acid (pH 3), 13% acetonitrile, and 31% methanol, and (B) 60% acetonitrile and 40% methanol (all expressed as v/v). The run consisted of 9 min isocratic 100% A, 6 min of a linear gradient from 100% A to 100% B, and 25 min isocratic 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509, using Flo Scint 3 as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks. Metabolites were analyzed by gas

chromatography–mass spectrometry (EI+) as trimethylsilyl derivatives, and the chemical structures were identified by comparison of the retention times and the mass spectra with authentic standards. The detection limit of the method was 10 pmol h^{-1} mg^{-1} protein.

3.12.2.7.4. Sulfotransferase (SULT) activity

E2 sulfotransferase activity was determined in digestive tube cytosolic fractions, as described in Janer et al. [15]. All assays were carried out in duplicate, plus a control assay. Cytosolic protein (100 µg) were incubated in 50 mmol l^{-1} Tris–HCl buffer pH 7.4, containing 4 µmol l^{-1} $MgCl_2$, 2 mmol l^{-1} Na_2SO_3, with 100 nmol l^{-1} [^{3}H]estradiol in a final volume of 150 µl. The reaction was initiated by the addition of 10 µmol l^{-1} adenosine 3′-phosphate 5′-phosphosulfate (PAPS), and incubated for 60 min at 30 °C. The reaction was stopped with 3 ml methylene chloride, after addition of 200 µl of ice-cold Tris–HCl buffer (50 mmol l^{-1}, pH 8.7). The extraction of unconjugated estradiol was completed with 3 ml of methylene chloride, followed by 15 min centrifuge, and an aliquot of the aqueous phase, where sulphated estradiol remained, was quantified by liquid ^{3}H scintillation counting. The limit of detection of the method was 0.8 pmol h^{-1} mg^{-1} protein.

3.12.2.8. Statistical analysis

All statistical analyzes were performed using the software SPSS 18.0. Kolmogorov–Smirnov test was used to verify the normal distribution of the data. Whenever this was not obtained ($P < 0.05$) data were Log transformed (GI and E2 concentrations in fluids and in gonads) or analyzed by non parametric tests (Kruskall–Wallis or Mann–Whitney tests). No significant differences were observed between tank A and B for all the measured variables (*t*-Student test or Kruskall–Wallis test, $p > 0.05$), therefore data were pooled together. Correlation analysis (Pearson) was used to identify correlated variables. Generalised Linear Model (GLM) was used to analyze the effect of more factors (e.g. time, dose, sex, reproductive stage) on a dependent variable with Tukey's post-hoc test. A *p*-value of less than 0.05 was considered statistically significant. Data below the detection limits were considered as half of the limit value.

3.12.3. Results

3.12.3.1. LC–MS confirmation of estrogen presence

The reconstructed LC–MS/MS chromatograms of the standard solution (concentration 10 ng ml^{-1}) and of the body fluid sample are showed in Figs. 2 and 3. The chromatogram of the latter showed the compliance with the relative retention times for estradiol, estrone and obviously for the internal standard. Moreover, the transitions from the analyte molecular peak were monitored with a signal-to-noise ratio greater than 3. All ion ratios of sample were within the recommended tolerances as required by the Commission Decision 2002/657/CE when compared with standards. The concentration of derivatized estrogens, extrapolated from calibration curves built from 5 concentration points, were 2.4 ± 0.3 pg ml^{-1} and of estrone (1.2 ± 0.1 pg ml^{-1}) were.

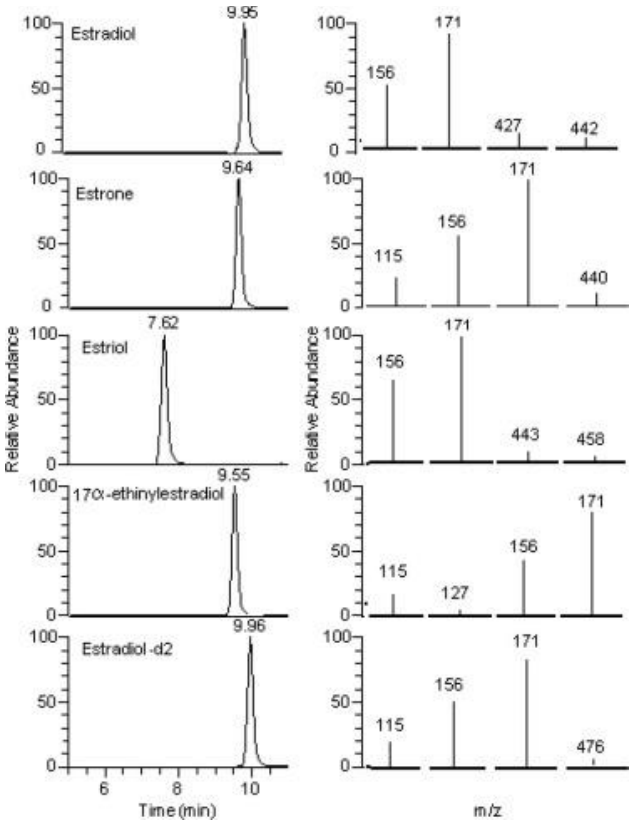

Fig. 2. LC–MS/MS chromatograms and related ion spectra of analytes and internal standard in the standard solution (concentration 10 ng ml^{-1}).

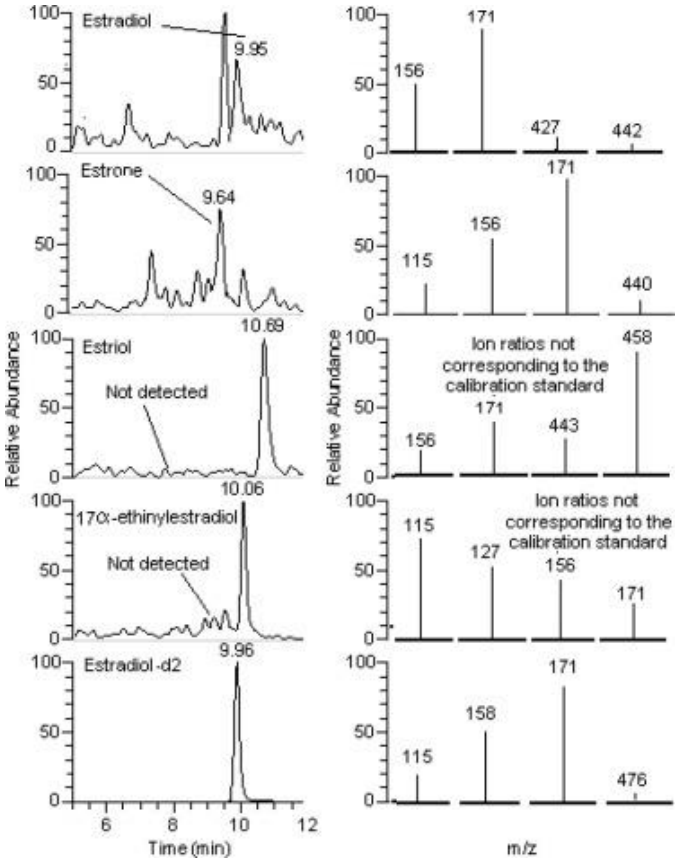

Fig. 3. Reconstructed LC–MS/MS chromatograms and related ion spectra of the analytes detected in sea urchin body fluids sample.

3.12.3.2. Circulating E2 levels in field specimens

Free E2 levels in body fluids were measured in 102 field specimens (FS) by RIA. Data were tested for their normality by Kolmogorov–Smirnov test, resulting in a strong non-Gaussian distribution. For this reason, medians rather than means were considered as more representative and 5 pg ml^{-1} was selected as reference physiological value for the following E2 administration experiment.

Sex did not significantly influenced E2 levels (Mann–Whitney test, $P = 0.13$), although it apparently affected their variability (Table 2). Additionally, differently from males, female specimens in active gametogenic stages (*Growing* + *Premature* + *Mature*) showed significantly lower E2 levels than those in non-gametogenic ones (*Spent* + R*ecovery*) (Table 2; Mann–Whitney test, $P = 0.015$).

3.12.3.3. Direct E2 administration experiments

3.12.3.3.1. Animal health conditions and feeding rate

No mortality was observed in the aquaria, except for the 200 ng ml^{-1} tank B, where all the animals suddenly died during the experimental period, possibly due to a bacterial infection. All the other specimens were healthy. No significant differences in feeding rates were observed between control and treated groups for both T_1 and T_2 sea urchins (Kruskal–Wallis test: $P > 0.05$). This allow to exclude that any possible difference in gonad development among experimental groups is due to a different food intake, as in sea urchins gonads are the main nutrient storage site and can be markedly influenced by the animal nutritional state [23].

3.12.3.3.2. Estradiol levels in body fluids and gonads

E2 levels in fluids were measured in 128 animals (Fig. 4). All these data in logarithm were analyzed by GLM, considering time, dose, sex and reproductive stage as variability factors. E2 was significantly affected by time and dose ($P < 0.001$ for both), whereas sex and reproductive stage did not influence this parameter ($P > 0.05$).

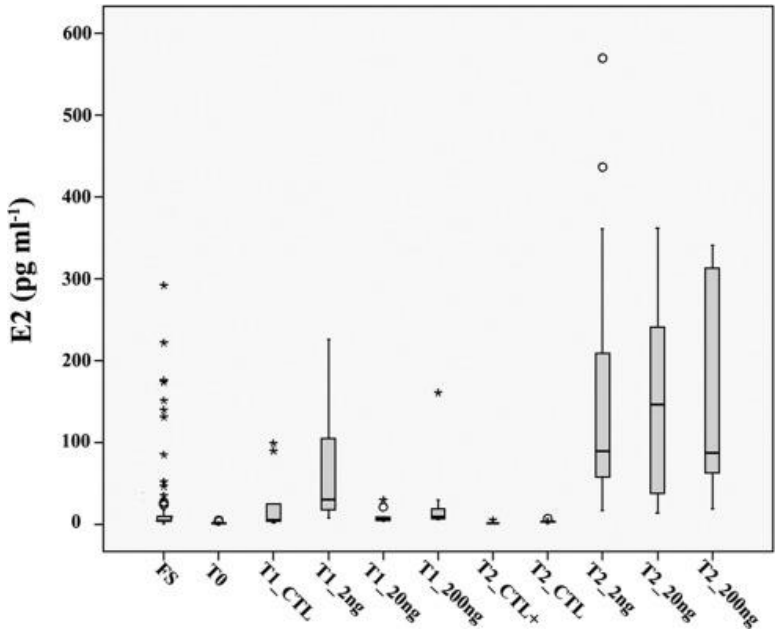

Fig. 4. Box-plot of free E2 levels in the fluids of the different experimental groups. Males and females pooled together. FS = field specimens monthly collected over a whole year before the administration experiments. T0 = time-zero specimens; T1_CTL, T1_2 ng, T1_20 ng, T1_200 ng = specimens analyzed after 2 weeks (T_1) of injections with control, 2 ng ml^{-1}, 20 ng ml^{-1}, 200 ng ml^{-1} E2 solutions respectively;

T2_CTL, T2_2 ng, T2_20 ng, T2_200 ng = specimens analyzed after 12 weeks (T_2) of injections with control, 2 ng ml^{-1}, 20 ng ml^{-1}, 200 ng ml^{-1} E2 solutions respectively; T2_CTL+ = specimens not injected. Circles and stars indicate values overcoming the III quartile of a distance higher than 1.5-fold and 3.0-fold the interquartile range, respectively. T1 2 ng vs T1 CTL and T1 20 ng = $P < 0.05$; T2 CTL vs T2 ng and T2 20 ng and T2 200 ng = $P < 0.001$ (Tukey post-hoc tests).

More in detail, all the control groups (T_0, T_1 CTL, T_2 CTL and T_2 CTL+) showed overall E2 levels (min < 2 pg ml^{-1}; max = 98 pg ml^{-1}) within the variability range of those previously found in field sea urchins (FS; Table 2, Fig. 4), but respect to these latter a much lower data dispersion was observed. In T_1 samples statistically significant differences were present considering treatment ($P = 0.019$, GLM with dose, sex, reproductive stage as variability factors), with a E2 peak at the 2 ng ml^{-1} group, which was higher than in control and 20 ng ml^{-1} groups (Tukey test, $P = 0.043$ and $P = 0.034$, respectively). Differently, in the long-term treated sea urchins (T_2), all the injected specimens displayed significantly higher (about 30-fold) levels of circulating hormone when compared to controls (Tukey test, $P < 0.001$), but no difference was observed among the three treated groups ($P > 0.99$). In CTL+ and CTL similar E2 levels were observed (Tukey-test, $P > 0.05$).

Gonad E2 levels were measured at the end of the experiment (T_2) in 44 animals (Fig. 5). Log E2 levels were significantly affected by dose and reproductive stage (GLM considering dose, sex and reproductive stage as variability factors, $P < 0.001$ and $P = 0.005$, respectively), but not by sex ($P = 0.44$). In detail, only the 2 ng ml^{-1} showed a significantly higher mean value compared to the control and the 20 ng ml^{-1} (Tukey test, $P < 0.001$ and $P = 0.013$, respectively) whereas CTL+ and CTL displayed similar hormone levels (Tukey test, $P = 0.32$). E2 levels were significantly lower in *Recovery* than in *Premature* stage (Tukey test, $P = 0.007$).

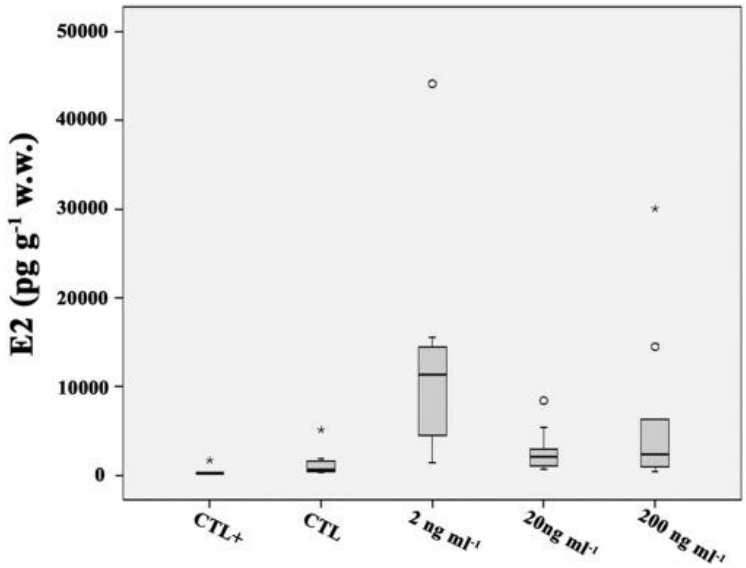

Fig. 5. Box-plot of the total (free + esterified) E2 levels in gonads measured at the end of experiment (T_2 = 12 weeks). Males and females pooled together. CTL = specimens injected with control solutions; CTL+ = specimens not injected. Circles and stars indicate values overcoming the III quartile of a distance higher than 1.5-fold and 3.0-fold the interquartile range, respectively. T1 2 ng vs T1 CTL = P < 0.001; T1 2 ng vs T1 20 ng = *P* < 0.05 (Tukey post-hoc tests).

E2 in gonads were positively correlated with E2 in body fluids (both log-transformed; Pearson test, *P* < 0.001).

3.12.3.3.3. Enzymatic activities in digestive tubes

All the enzymatic activities (aromatase, ATAT and SULT) were measured in the digestive tube at the end of the experiment (T_2) in 29 animals (Table 3). SULT activity was additionally determined in 16 T_1 specimens. For each enzymatic activity CTL+ and CTL samples displayed similar mean values (Tukey-test, *P* > 0.31).

Table 3. Activities of putative enzymes involved in E2 level homeostasis in control and E2-treated experimental groups and at different time-points (T_1 and T_2): aromatase-like (biosynthesis), sulfotransferase-like (SULT, metabolism), and palmitoyl-CoA: estradiol acyltransferase (ATAT, metabolism) measured in digestive tubes of control and treated sea urchins.

		Aromatase-like pmol h^{-1} mg^{-1} protein	SULT-like pmol h^{-1} mg^{-1} protein	ATAT-like pmol h^{-1} mg^{-1} protein
T_1	CTL	/	155.1 ± 13.4	/
	2 ng ml^{-1}	/	119.1 ± 49.3	/
	20 ng ml^{-1}	/	144.1 ± 24.7	/
	200 ng ml^{-1}	/	147.3 ± 12.8	/
T_2	CTL+	0.26 ± 0.20	79.8 ± 32.9	190.1 ± 56.4
	CTL	0.25 ± 0.37	85.4 ± 28.7	240.6 ± 44.8
	2 ng ml^{-1}	0.14 ± 0.13	90.2 ± 34.8	216.2 ± 34.2
	20 ng ml^{-1}	0.25 ± 0.24	115.8 ± 15.6	195.6 ± 32.6
	200 ng ml^{-1}	0.15 ± 0.19	104.9 ± 15.0	211.3 ± 18.4

Values are mean ± SD (n = 4–6). No statistically significant difference was found between controls and treated groups for any of the considered enzyme.

Considering aromatase, SULT and ATAT activity, GLM analysis did not show any significant effect of dose, sex and reproductive stage ($P > 0.46$), although SULT displayed significantly lower activity in T_2 samples than T_1 (GLM, $P = 0.001$).

3.12.3.3.4. Gonad lipid content

The lipid fraction of gonads was measured at the end of the experiment (T_2) in 38 animals.

Box plot analysis of all data revealed the presence of two outliers; excluding these data, GLM showed a significant effect of sex on the lipid fraction ($P = 0.012$), whereas dose and reproductive stage did not affect gonad lipid content ($P = 0.17$ and $P = 0.87$, respectively).

A significant regression was found between the lipid fraction and the Log E2 levels measured in gonads ($R^2 = 0.15$; $n = 33$; $P = 0.026$) (Fig. 6).

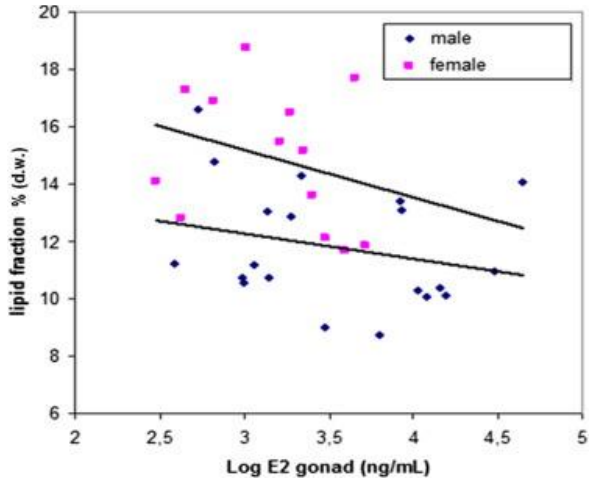

Fig. 6. Negative correlation between the lipid content and the Log E2 levels measured in gonads: a significant regression was found between the two parameters ($P = 0.026$). Data are reported for males (black) and females (gray).

3.12.3.3.5. Gonad Index

GI was measured in 137 experimental animals (T_0, T_1 and T_2) (Fig. 7). GI logarithms were analyzed by GLM: time and stage had a significant effect on GI ($P < 0.001$ and $P = 0.003$, respectively), whereas sex and dose did not ($P = 0.45$ and $P = 0.17$, respectively). A progressive increase of GI was observed during the experimental period, so much that T_2 samples showed significantly higher GI values (*T* test, $P = 0.004$) than T_{2env} animals.

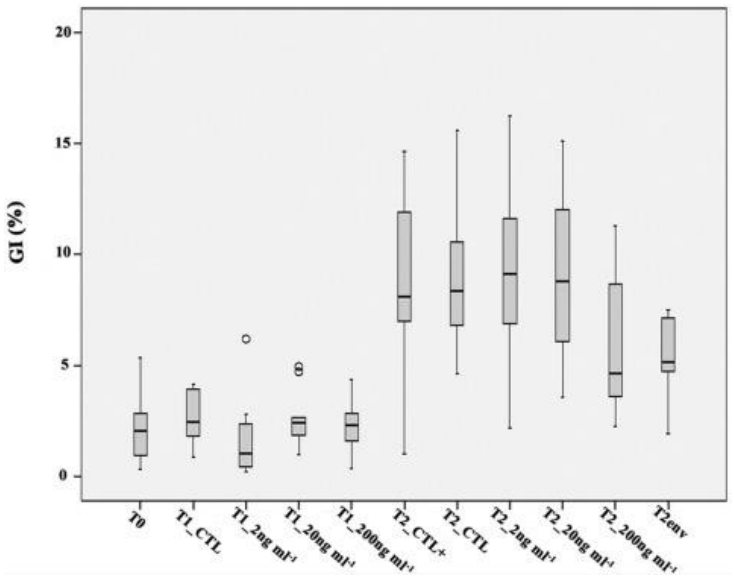

Fig. 7. Box-plot of the GI values in the different experimental groups. Circles indicate values overcoming the III quartile of a distance higher than 1.5-fold the interquartile range. GI progressively increase during

the experimental period (T1 samples are significantly lower than T2 samples, $P < 0.01$) and at the end of the experiment is significantly higher than the correspondent field samples (T_{2env}; $P < 0.01$).

In T_2 animals, considering mean values of each tank, GI was strictly correlated to DFR ($R^2 = 0.889$; $n = 7$; $P = 0.007$) excluding the value belonging to 20 ng ml^{-1} tank B group (GI = 8.9%; DFR = 77.5%) (Fig. 8).

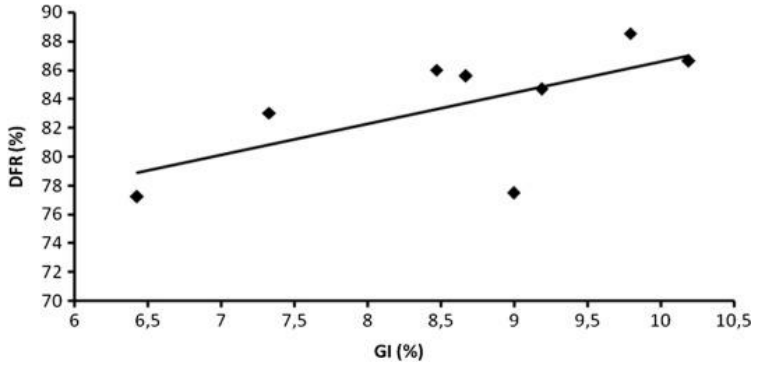

Fig. 8. Positive correlation between mean GI measured in each tank and their corresponding daily feeding rates (DFR) in T2 samples: the two parameter are significantly correlated (Pearson test, P = 0.007).

3.12.3.3.6. Reproductive stages

Reproductive stages were determined in 146 specimens (Fig. 9). After starvation (T0) almost all the specimens analyzed were in Recovery stage. Considering T1 control animals, some specimens in Growing stage were observed but the percentages of samples in Spent (30%) and Recovery (40%) stages were still high. At the end of the experiment, most of the control specimens (T2 CTL, CTL+ and T2env) were in active gametogenic stages (Growing, Premature and Mature) with only a small amount of samples in non-gametogenic stages (Spent and Recovery stages).

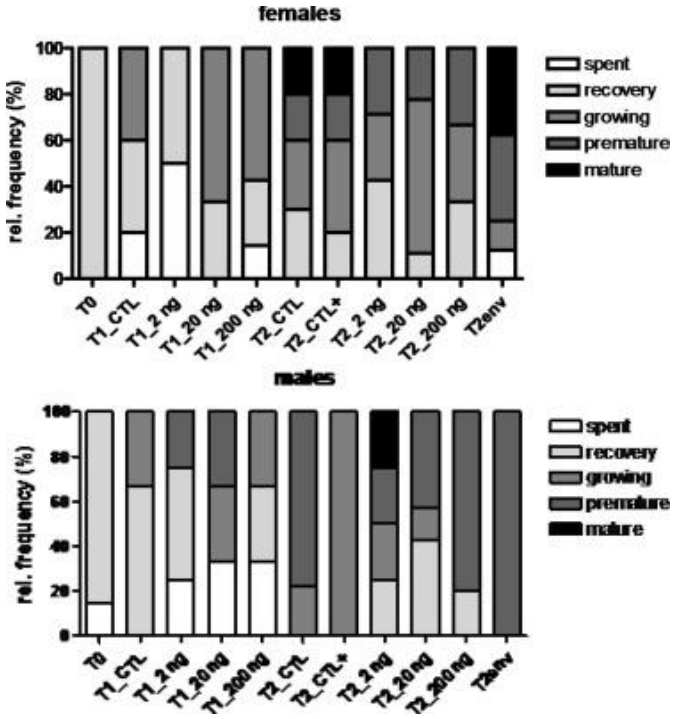

Fig. 9. Relative frequency of the reproductive stages (Non gametogenic = spent, recovery; gametogenic = growing, premature, mature) in the different experimental groups (males & females).

After 2 weeks of treatment (T1), no differences were observed between control and E2 treated groups in the relative frequency of the reproductive stages: in both males and females active gametogenic stages were found in almost all the experimental groups although non gametogenic stages were usually present in high percentages both in control and treated specimens. Similarly, after 12 weeks of treatment (T2), no marked differences in the relative frequency of reproductive stages were observed both among the experimental groups and between controls and T2env animals. Although non-gametogenic stages were still present, all the groups showed a higher percentage (>60%) of active gametogenic stages (Fig. 7).

As reported in paragraphs 2.5 and 2.2, reproductive stages significantly affected GI and gonad E2 levels (GLM analysis).

3.12.4. Discussion

In the present work, we explored echinoid endocrinology trying to clarify some of the debated aspects of estrogen physiology in these invertebrates. We focused on verifying

estrogen presence in sea urchin tissues and its putative physiological role in reproduction, specifically evaluating its metabolism and effects on gonad growth and gamete maturation after an exogenous administration of physiological doses of E2. Indeed, in echinoderms, and particularly in echinoids, estrogen physiology and function is still far to be elucidated, representing a profound gap of knowledge in the evolutionary pathway leading to the well-established endocrine system of vertebrates.

By means of direct methods i.e. LC–MS, we verified that estrogens, namely 17β estradiol and estrone, were present in sea urchin body fluids (coelomic fluids). Among the five echinoderm classes, this was done only for starfish [47], whereas for sea urchins, which belong to a different phylogenetic lineage, the Echinozoa, [25], this information is missing. Our LC–MS results provide evidence that in the fluids of the sea urchin P. lividus there are both 17β estradiol and estrone and their spectra are identical to the correspondent commercially available hormone. Furthermore, the E2 concentration calculated from chromatographic data are well in agreement with those measured by RIA and therefore provide a sort of validation for the immunological method, which comparing to chromatographic methods has many practical advantages (e.g. simultaneous processing of a higher number of samples).

In order to know the real physiological E2 levels we measured the concentrations of circulating hormone in field specimens of P. lividus collected during a whole year (Table 2). This guaranteed to include all the possible physiological and seasonal hormonal conditions experienced by the animals during their annual reproductive cycle. As expected, E2 displayed a wide range of concentrations (from <2 to 292 pg ml−1; Fig. 2) and this variability was particularly high in females (Table 2). For this reason, the median values of about 5 pg ml−1 are more representative of the overall physiological situation. Although slightly lower, the sea urchin ranges are quite similar to the reference values of E2 human plasma concentration, which range from <10 pg ml−1 (post-menopausal woman) to 350 pg ml−1 (pre-menopausal woman at ovulation), with a progressive increase during ovulatory follicle maturation [22]. Differently, in female sea urchins the hormone concentrations decreases in active gametogenic stages comparing to non-gametogenic ones (Table 2). Whether this result, together with the overall higher

mean levels observed in females, might suggest an E2 involvement in the control of oogenesis, had to be proven by a more “functional approach”. We therefore set up an experiment of direct E2 administration: if a high E2 level was the endogenous signal for maintaining gonads in resting condition we would have expected that, particularly in females, treated specimens remained in Spent–Recovery stage and did not start and accomplish gametogenesis. We administered three different E2 doses to obtain in body fluids final nominal hormone concentrations rather close to the physiological range.

RIA analyzes confirmed the actual increase of E2 in body fluids following the hormonal injections in a time-dependent manner. After 2 weeks of treatment, circulating E2 levels showed a high variability among the groups (Fig. 4) and only the 2 ng ml−1 group displayed significantly higher concentrations than control. Despite a 10 and 100-fold higher injected dose, the other experimental groups displayed lower E2 levels (Fig. 4). These data suggested that, differently from 2 ng ml−1 (the more “physiological” dose), higher E2 doses activated potent homeostatic or detoxification mechanisms to eliminate hormone excess. This would indicate the presence of a critical threshold of E2 level controlling the activation of such pathways. Long-term treated specimens showed a 30-fold increase of circulating E2 when compared to controls but no difference was observed between the E2 treatments. This further suggested the presence of protective mechanisms to maintain the E2 levels within a high but still “physiologically accepted” value (Fig. 4). However, after 12 weeks of treatment, mechanisms for E2 elimination were less effective than observed after the short-term treatment and even in the medium and high dose treated groups E2 concentrations considerably increased. The reduced metabolic efficiency might be due to long-term E2 addiction or to prolonged animal maintenance. A long-term reduced metabolism was also suggested by sulfotransferase analyzes reporting a significantly lower activity in T2 than T1 samples (Table 3).

We also measured the overall E2 concentrations in gonads of long-term treated animals. A positive correlation between E2 in body fluids and in gonads was found ($P < 0.001$), suggesting that the injected hormone was likely distributed via coelomic fluids to all body compartments, including gonads [1,19]. Here, the E2 levels displayed a

significant increase only in the 2 ng ml−1 group compared to control and 20 ng ml−1 groups, a “behavior” comparable to what observed in fluids of short-term treated animals (Fig. 4). Thus, homeostatic/detoxification mechanisms, responsive to high E2 doses, were apparently present also in gonads and they were similarly activated when E2 overcame a critical dose. These mechanisms could be important to protect the gonad from excessively high/toxic E2 concentrations [8] and in this organ they would efficiently work even after long-term hormone administrations. Nevertheless, in the lack of molecular data, we do not know a priori if these homeostatic mechanisms truly rely on endogenous enzymes specifically designed for E2 metabolism or are general xenobiotic detoxification enzymes which efficiently work on a wide range of substrates (including estrogens). Whatever the real situation, these homeostatic mechanisms apparently did not involve the enzymes considered in this study. In vertebrates, the overall E2 concentration is normally influenced by those pathways regulating its biosynthesis (e.g. aromatase) or its metabolism, among which sulfation and esterification might be relevant [14,36]. In the mussel M. galloprovincialis a dose-dependent increase of ATAT-like activity was actually observed following E2 exposure, thus indicating that the hormone excess was bound to fatty acids possibly to keep the endogenous level stable [15]. In the present study, no effect was observed on ATAT-like activity, suggesting that the esterification pathway is probably not a main homeostatic mechanism in P. lividus gonads and does not significantly contribute to restrain endogenous E2 levels. Similarly, also the aromatase-like and E2 sulfotransferase-like displayed comparable activity in all the experimental groups and were not influenced by the circulating E2 levels, which were probably controlled by other pathways. Possible alternative mechanisms include E2 conversion into estrone or other metabolites, the former being a well-documented process in echinoderms [11,12]. Indeed, in starfish an increase of estrone concentrations was reported after E2 treatment [3,28]. In the sea urchins Lytechinus variegates, studies on 17β-hydroxysteroid dehydrogenase-like activity indicated that the reaction equilibrium strongly favoured the production of E2 [12]. Nevertheless the presence of high concentrations of E2 – as we observed – might alter this equilibrium, leading to estradiol to estrone conversion. Alternatively, conjugation of E2 to glucuronide moieties could be another possible homeostatic

pathway, producing more polar and more readily excreted E2-conjugated compounds [24]. Previous research showed that in echinoderms estradiol was rapidly transformed mainly into aqueous soluble and secondarily in lipophilic metabolites [12].

Despite the actual increase of E2 levels in body fluids and, partially, in gonads, the hormonal treatment did not induced marked variations in the considered reproductive parameters.

The relative frequencies of both female and male reproductive stages (Fig. 9) were not significantly affected by E2, suggesting that the hormone was actually not involved in neither oogenesis nor spermatogenesis. The increased percentage of non-gametogenic stages (100%) in T1 females treated with the lowest dose (where a higher circulating E2 concentration was actually measured, Fig. 4) may give the impression of the “expected” inhibitory effect. Nevertheless, this is not supported by results obtained in animals treated for a longer period (T2) and having even higher E2 levels (Fig. 4), where both controls and E2 treated females displayed similar percentages of non-gametogenic (spent–recovery) and gametogenic stages (growing–premature–mature). Similarly, the lack of mature stages in all the T2 treated females is difficult to correlate to a hormone inhibitory effect, as indicated by the similar presence of premature stages in controls and treated animals. Since the classification in premature and mature stage only rely on a different proportion of histologically detected mature eggs and do not imply different physiological processes (as between growing and premature, with the onset of vitellogenesis), it would be difficult to explain why the hormonal treatment prevent the animals to reach the premature stage but not the mature one. So, overall, on the basis of our results, E2 seemed neither to prevent oocyte maturation, as we initially hypothesized, nor to enhance oocytes growth, as reported for asteroids [13,46,51]. This lack of E2 “reproductive responsiveness” in P. lividus is further supported by recent [39] and ongoing hormone administration experiments (Mercurio and Sugni, unpublished data), independently from the specific experimental conditions (administration type, food type, photoperiod/temperature manipulation). In other echinoids E2 treatment led to partially different and even contrasting results, including absence of effect in juveniles of Pseudocentrotus depressus[42] to inhibition of oocyte growth in Lytechinus

variegatus[49] and stimulation of gamete development in Strongylocentrotus intermedius[45]. These differences could be related to different species-specific mechanisms or to different experimental designs, which could have been affected by different feeding rates (not evaluated in some cases) or an original individual variability, due to a lack of reproductive cycle synchronization in animals at the beginning of the experimental period. Our experiment was successfully designed in order to synchronize the reproductive conditions of the animals to a basal stage (Recovery) and, then, artificially activate gametogenesis processes by environmental parameter manipulation and proper feeding. If these signals are lacking (as it can be in laboratory conditions) gametogenesis may not be activated at all, independently from any hormonal administration, as we observed in a previous comparable experiment [39]. This further indicate that factors other than estradiol strongly control gametogenic onset. From the synchronic basal reproductive stage the experimental animals gradually reached active gametogenic stages within the end of the 12 week-treatment (Fig. 6), although still displaying a certain individual variability. The latter might be related to slightly different food intake due to intra-specific food competition that, although we tried to reduce by individual feeding procedures, we may have not completely eliminated. However, even if a certain level of differences in individual food intake it must be recalled that no statistically significant different was found among the overall DFR of the different experimental groups.

The obtained results also allowed us to reject the hypothesis of a threshold oocyte size driving E2 effectiveness, as postulated for asteroids [28]: indeed no effects was observed on oocyte development despite all the gamete “sizes” were present in the control group within the end of the experiments (Fig. 7).

Gonad growth was not affected by E2 administration as well, since control and treated groups displayed similar GI values. Differently, in the echinoid L. variegatus, E2 dietary administration apparently enhanced ovarian growth [49], but lack of information on the initial conditions and the very short-acclimatization period (<1 w) do not exclude this observation was due to an originally unbalanced animal distribution. Rather, in the present work GI was apparently more influenced by food availability as demonstrated by

its clear correlation with the calculated DFR (Fig. 6). This result is in agreement with the well-documented critical influence of food quantity and quality on sea urchins reproductive cycle regulation [23,34].

Overall, our present research indicate that, although present in the body fluids (as confirmed by LC–MS), estrogens are not involved in the regulation of P. lividus reproductive cycle. Slight discrepancies with other research on sea urchins [49] might be related to species-specific hormonal mechanisms or, more possible, to different experimental designs. The lack of clear physiological effects can lead to two different "explanations":

(1) Estradiol is not an active endogenous hormone is sea urchins. This would fit with the apparent absence of both molecularly recognizable biosynthetic enzymes (CYP19) [21] and classical nuclear estrogen receptors (ER) in sea urchin genome [9]. Its presence in the tissues might be simply due to an uptake from the environment or from the diet, as hypothesized for mollusks [29,30]. If in principle this is possible, it makes difficult to explain why field and lab-maintained animals, which feed on highly different food (algae vs artificial pellets) and live in different sea water (natural vs artificial) display quite similar physiological E2 concentrations.

(2) Estradiol is an active endogenous hormone but its role is not the control of gametogenesis, as reported for vertebrates and asteroids. Other physiological process, which we have not considered in the present study, would be the target, as suggested also by other works [44,45]. This is not surprising as in vertebrates, besides reproduction, E2 is critically involved in many other physiological processes, as nervous system development, immunity, bone maintenance and lipid metabolism. In the present study, although no significant differences in gonad lipid content were found among the different experimental groups, a negative correlation between E2 measured in gonads and their lipid fraction was observed (Fig. 4). Whether this might be indicative of an E2-induced lipid consumption and therefore an E2 controlled lipid metabolism has still to be clarified by further more specific research.

Whatever the specific function, this second hypothesis would explain E2 presence in the tissues and the presence of an aromatase-like activity [19], that, however, independently evolved from the vertebrate enzyme in terms of structure but acquired a similar function (evolutionary convergence; [21]. Nevertheless, in this scenario the E2 mechanism of action would occur through a still unknown receptor. Interestingly, a novel estrogen receptor, GPR30 or GPER, which mediate different hormone effects was found [26] and underline the possible diversity of signal transducers even in the well-known vertebrates. Weather sea urchin possess GPER-like receptor is an intriguing issues we are currently investigating.

Differences with results obtained from starfish (Table 1) might be due to class-specific hormonal pathways, since the two taxa evolved separately quite early in echinoderm phylogeny [4,16,25]. In crinoids, the most basal echinoderms, estrogen-like compounds have been detected in whole body tissue homogenates and their levels are higher during vitellogenesis and drastically drop when complete maturation is achieved [2]. This is well in agreement with what happens in both vertebrates and starfish and may lead to the speculation that E2 physiology and sensitivity has been lost or independently evolved in the Echinozoa (echinoids and holoturoids) lineage, whereas the crinoids and the Asterozoa (asteroids and ophiuroids) would have maintained the vertebrate-like hormonal machinery. A support to this hypothesis is that crinoids and asteroids have a different gonad anatomy from sea urchins [32] and a slightly different oogenesis accomplishment.

Further studies are certainly needed to verify these fascinating hypotheses including to enlarge the analyzes toward a higher number of representative echinoderm models (including holoturoids and ophiuroids).

Overall, the present research contributes to a better knowledge of sea urchin endocrinology, and provide basic reliable information, which hopefully will help to shed light on the evolution of estrogen signaling system along the deuterostomian lineage. Furthermore, this work underline the risk of Phylum generalization (different echinoderm classes may present different situations) and necessarily induce a perspective revision

of the susceptibility of sea urchin reproduction to estrogen-mediated endocrine disruption: the toxicity of the so-called estrogen-mimicking environmental contaminant does not apparently occur via endocrine modulation.

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CHAPTER 4

Conclusions

3. CONCLUSIONS

Although the intense control of illicit growth promoter administration (e.g corticosteroids, anabolic steroids and antibiotics) within the European Union, the number of detected positives in the last few years seems very limited. The determination of residues of drugs in matrices of animal origin is often very complex due to the difficulties observed during the analysis, often related to the rapid or unknown metabolism of some drugs; recoveries for certain drugs could be much lower than for others within the same group, sensitivity for some compounds can be much lower than the other within the same group; the double origins (pseudoendogenous and/or exogenous) of some molecule (boldenone, prednisolone, nandrolone) is still under debate. There are also a ot of measures taken by the manufacturer, distributor and/or illicit user of some drugs as well as the low individual doses in cocktail of different products of the same or different group having additional or synergic effects; administration of synthetic analogues of known growth promoters or use of products which are not completely regulated from the member states. All these strategies are often adopted to circumvent the controls.

In addition, the problem of residues is related also to environmental contamination, both for residues of veterinary drugs and for new emergent contaminants, e.g. perfluoroalkyl substances (PFASs).

In this research study we proposed and investigated new unconventional matrices compared, where possible, to the conventional ones, analysed by multiresidual and multiclass methods by LC-MS/MS or LC/HRMS, to better clarify some issues mentioned above and improve the control framework. The analytical and instrumental strategies adopted dealt with the optimisation of instrumental performances as well as of all the steps of pretreatment of the samples, in order to achieve good levels of sensitivity, specificity and robustness of the validated confirmatory methods, culminating in considerations of qualitative, quantitative and statistical nature after their application to real cases often organised in groups for different gender, age or geographic areas, according to our different experimental plans.

Future perspectives of this study for analytical improvements could deal with the study of other veterinary drugs and new matrices taken from food chain animals or of animals purposely treated with known pharmacologically active principles, to identify residues

that are not found in the conventional matrices. In this regard, the potential of LC-HRMS could be exploited to investigate the fragmentation pathways of new molecules by focusing on the untarget analysis for the search of new markers useful for food surveillance at different stages of the food chain. Given the results achieved by the presented papers, some unconventional matrices could be considered for official controls giving a more complete view and allowing for adequate monitoring of residues in compliance with the withdrawal period, before the product comes to the consumer.

Certainly, the field of food safety is constantly changing, from both a regulatory and an experimental point of view, so this type of research will always play a major role in public health and further studies are strongly recommended.