Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXX

Settore Concorsuale: 07/H2

Settore Scientifico Disciplinare: VET/04

MYCOTOXIN DETERMINATION IN NON CONVENTIONAL MATRICES: DEVELOPMENT OF MASS SPECTROMETRY BASED ANALYTICAL METHODS

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Esame finale anno 2018

ABSTRACT

Mycotoxins are low-molecular-weight natural products produced, as secondary metabolites, by filamentous fungi. These molecules represent a wide chemical group with different toxicity effects in human and other animals.

These toxins can accidentally occur in food and feed, due to a direct or indirect contamination.

The aim of this work was to develop a method for the first time to quantitatively determine zearalenone and its metabolites (α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol, zearalanone) in bovine and human hair using LC-MS/MS.

Once the method was set-up for bovine hair, it was successfully validated according to Decision 657/2002/CE on three analytes, with satisfying performances. Moreover the applicability of the method was tested on human hair in a one-day validation with reasonable performances.

This method could be a useful tool to evaluate natural feed contamination or detect illegal use of α-zearalanol in bovines and to perform a first inventory of the occurrence of these molecules in bovine and human hair, as biomarkers for zearalenone exposure in future studies.

Another purpose of this work was to make a preliminary screening on mycotoxins contamination (aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, deoxynivalenol, zearalenone, fumonisin B1, fumonisin B2, ochratoxin, T-2 toxin and HT-2 toxin) in different pet food types for cats.

This research showed that mycotoxin occurrence in pet food for cats can represent an issue to put under control. Since pets are fed with the same type of pet food for long periods of their life, the necessity of evaluating mycotoxin presence in feed is evident.

Summary

1. GENERAL INTRODUCTION

Mycotoxins are low-molecular-weight natural products (250 to 720 Da) produced as secondary metabolites by filamentous fungi belonging to the phylum *Ascomicota* and mainly to the genera *Aspergillus*, *Penicillium* and *Fusarium* (Bennett et al. 2003; Brera et al. 2008; Botana & Sainz 2015; Alshannaq & Yu 2017). These molecules represent a chemically and toxigenically diverse category: they are brought together because the members are cabaple of causing disease and death in human beings and other vertebrates (Bennett et al. 2003).

Moreover they are extrinsic antinutrition factors that can accidentally contaminate feed and food. They are responsible for non-contagious and non-infectious pathologies known as mycotoxicosis (Nebbia 2009).

The word mycotoxin stems from the Greek word "*mycos*", meaning mould and the latin "*toxicum*" meaning poison: the term was coined in 1962 in the aftermath of an unusual veterinary crisis nearby London, called "Turkey X disease", that caused the death of 100.000 turkeys which had been fed a batch of peanut meal contaminated with *Aspergillus Flavus* spores (Bennett et al. 2003).

However the problem of mycotoxins does not represent a new issue and has probably influenced the history and health of peoples more than we thought (Brera et al. 2008).

Some researchers hypothesize that plagues described in the Old Testament could be attributable to this extent, which afflicted Egyptians between 1250 and 1300 B.C: reasonably some of them could be related with the ingestion of cereals contaminated by mycotoxins, resulting in lethal disease.

The Middle Ages and the following centuries were characterised by epidemics of ergotism, which hit various populations in Central and North Europe: at the time, it was considered a transmissible infectious disease. Subsequently it was ascertained that the pathogenesis of this disease was caused by ergotamine, a vasoconstrictor alkaloid produced by the *Claviceps purpurea* (rye ergot fungus) that contaminated cereal grains (Peraica et al. 1999; Piva et al. 2006).

Ergotism is also known as *ignis sacer* or St. Anthony's fire because, at the time, it was believed that a pilgrimage to the shrine of St Anthony would have provided relief from the severe burning sensation experienced. Moreover people affected by ergotism were exposed to lysergic acid diethylamide (LSD), a hallucinogen produced during the baking of bread made with flour contaminated by ergot (Peraica et al. 1999).

In conclusion, although it is plausible to debate that mycotoxins could have represented a relevant role in some of the animal and human diseases since the Roman era, the scientific

interest for their impact for animal and human health began only in the 1960s (Brera et al. 2008). A famous painting of the Flemish painter Pieter Bruegel the Elder (1525-1569), "The beggars" illustrates the devastating effects ergotism such as seizures, pains at limbs extremities, gangrene and deaths (Bennett et al. 2003; Piva et al. 2006).

Mycotoxins contamination is influenced by a broad range of climatic conditions. Furthermore it is estimated by the Food and Agriculture Organization (FAO) that the 25% of the world crops are affected by these contaminants: this phenomenon plays a significant economic impact not only for farmers and breeders, but also for the entire agri-food sector and national economies (Aidoo 2011).

Thus mycotoxins represent the hazard category with the highest number of border rejections reported by the Rapid Alert System for Food and Feed (RASFF) (Arroyo-Manzanares et al. 2014).

The fungi producing mycotoxins in food are divided into two groups: those which infest before harvest, commonly described as field fungi, and those which colonise the plant only after harvest, considered storage fungi. Thus there are three categories of toxicogenic field fungi: those which infest stressed or senescent plants, such as *Fusarium moniliforme* (fumonisin) and sometimes *Aspergillus flavus* (aflatoxin); plant pathogens such as *Fusarium graminearum* (nivalenol, deoxynivalenol); finally those that invade the plant before harvest and make them prone to mycotoxin contamination after harvest, such as *Aspergillus flavus* (aflatoxin) and *Penicillium verrucosum* (ochratoxin) (Ayalew 2010).

Animals fed vegetable matrices contaminated with toxic fungi are exposed to significant health risks; the same consequences may affect human health (Milani 2013).

Mycotoxins are molecules of fungal origin, while not all toxic compounds produced by fungi are considered mycotoxins. The concentration and the target of the metabolite are both crucial factors to take into account.

These contaminants are toxic to animal groups and vertebrates at low concentrations; while other low-molecular weight fungal metabolites, such as ethanol, are not regarded as mycotoxins because they exert toxicity only in high concentrations.

Moreover fungal products that are noxious to bacteria, such as penicillin, are usually described as antibiotics, while others that exert toxicity to plants are considered phytotoxins. Additionally, even if mushroom poisons are thought fungal metabolites that can provoke disease and death in humans and other vertebrates, they are kept out the field of mycotoxicology. The difference between a mushroom poison and a mycotoxin relies on the size of the producing fungus, but also on human intention. Mycotoxin contamination is almost always unintentional; on the

contrary, mushroom poisons can be served and consumed by people who had misidentified as a delectable species (Bennett et al. 2003).

1.1 MYCOTOXIN PRODUCTION

The production of secondary metabolites produced by fungi is closely influenced by many factors of different nature.

Essential factors influencing mycotoxin production involve both environmental influences (temperature, climate changes, activity water, humidity, mechanical damage of kernels caused by pest and insects attack), both plant stress (lack of a balanced nutrient absorption and the extreme soil dryness) (Brera et al. 2008).

These aspects can affect cereals cultivation, harvesting, drying and storage (Frisvad 1995; Wicklow 1995).

Mycotoxin formation in agricultural crops can happen at different stages in the food chain: at pre-harvest, harvest, drying, and storage. Inappropriate agricultural and harvesting actions, such as incorrect drying, handling, storage, packaging and transport conditions can stimulate fungal growth, enhancing the risk of mycotoxin production. Another key element to take into account relies on monitoring storing conditions, in order to prevent fungal contamination and mycotoxin production (Marin et al. 2013). Thus activity water is the key parameter to check: it represents the bioavailability of water in a food, although it would be better to use the percentage humidity value as an indicator to describe water content in a feed (Bryden 2012). However, when activity water of the stored products reaches levels that can allow fungal growth and mycotoxin production, toxins can also colonise processed products (Marin et al. 2013).

In the growing phase of plants such toxins are produced from saprophytic mushrooms, while during storage process they are produced by endophytic fungi (Hussein & Brasel 2001).

Moreover temperature and humidity are the most important factors for moulds and mycotoxins development. The critical levels for mushroom growth are 70-150 g / kg of moisture (depending on the raw materials) and a relative humidity of 80-85% (Bryden 2012).

Temperatures which promote mycotoxins production range from 0 ° C to 35 ° C, depending on the fungal species (Milićević et al. 2010).

Crops infection from fungi can occur in a range of optimal environmental conditions such as temperature, relative humidity and rain. These factors can also influence the colonization, toxin

production and survival. In addition climate change is expected to promote changes in fungal population and mycotoxin patterns leading to a superior mycotoxin contamination of crops (Miraglia et al. 2009; Paterson & Lima 2011; Marroquín-Cardona et al. 2014; Medina et al. 2014). However the complexity of mycoflora interaction to each crop and to the environment does not allow the formulation of any conclusions without specific studies.

1.2 CLASSIFICATION

Although more than 300 mycotoxins exist the scientific research has been converged only to approximately ten compounds, which present relevant toxicological impact on animal and human health (Brera et al. 2008).

In Table 1 are reported main important mycotoxins belonging to different genera, such as *Aspergillus, Fusarium, Penicillium.*

Aspergillus	• Aflatoxins B1, B2, G1,G2, M1 (AFB1, AFB2, AFG1, AFG2, AFM1).
Fusarium	• Trichothecenes: deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxin, diacetoxyscirpenol (DAS); • Fumonisins B1, B2, B3 (FB1, FB2, FB3); • Zearalenone (ZEA).
Penicillium	• Ochratoxin A, B, C (OTA, OTB, OTC); • Patulin.

Table 1. Main important mycotoxins from different genera.

1.2.1 Aflatoxins

Aflatoxins (AFs) are generated mainly by *Aspergillus parasiticus* and *Aspergillus flavus*, they can colonise different feed and food crops, together with *Aspergillus nomius* and *Aspergillus pseudotamarii* (Kurtzman et al. 1987; Ito et al. 2001). Hot and humid areas, such as tropical and subtropical ones, are considered the most susceptible ones to Aspergillus infection and aflatoxin contamination (Van der Fels-Klerx et al. 2016).

Temperatures above 25°C and humidity higher than 85% can promote the growth of aflatoxin-producing fungi during storage (Brera et al. 2008).

Aflatoxins are crystalline substances, soluble in moderately polar organic solvents (methanol, chloroform, dimethysulfoxide); on the contrary they scarcely soluble in water (10-30 mg/mL) and insoluble in non-polar organic solvents. Aflatoxins in a dry state show stability to heat up to their melting point.

Pure aflatoxins are destroyed by UV radiations, are unstable below pH 3 and above pH 10, and are susceptible to oxidizing components (Brera et al. 2008).

Aflatoxins M1 and M2, aflatoxin β-hydroxilated metabolites can occur in milk and derived products due to the quick biotransformation of aflatoxins B1 and B2 (AFB1, AFB2) in animals fed contaminated feeds (Brera et al. 2008).

Aflatoxins are molecules chemically similar to difuranocoumarin and can be categorised in two classes based on their chemical structure (table 2):

- the difurocoumarocyclopentenone group (AFB1 and AFB2);
- the difurocoumarolactone group aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxin M1 (AFM1).

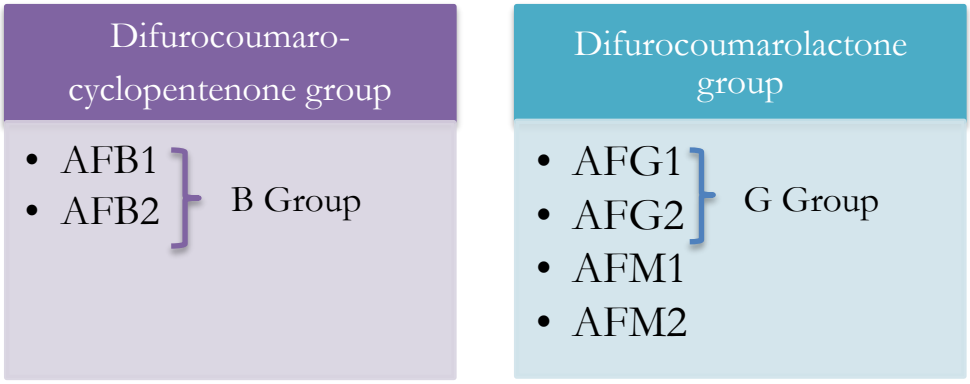


Table 2. Aflatoxin classes based on chemical structure.

The G group is characterised by a D-lactone ring, while the B group contains a cyclopentenone ring, which makes them the most toxic group (Figure 1).

Aflatoxins fluorescence is strong in UV light (ca. 365 nm): AFB1 and AFB2 are characterised by a blue fluorescence, while AFG1 and AFG2 by green fluorescence (Brera et al. 2008)

Maize, peanuts, nuts, pistachio nuts, and cotton seeds can be colonised by aflatoxins (Payne 1998)

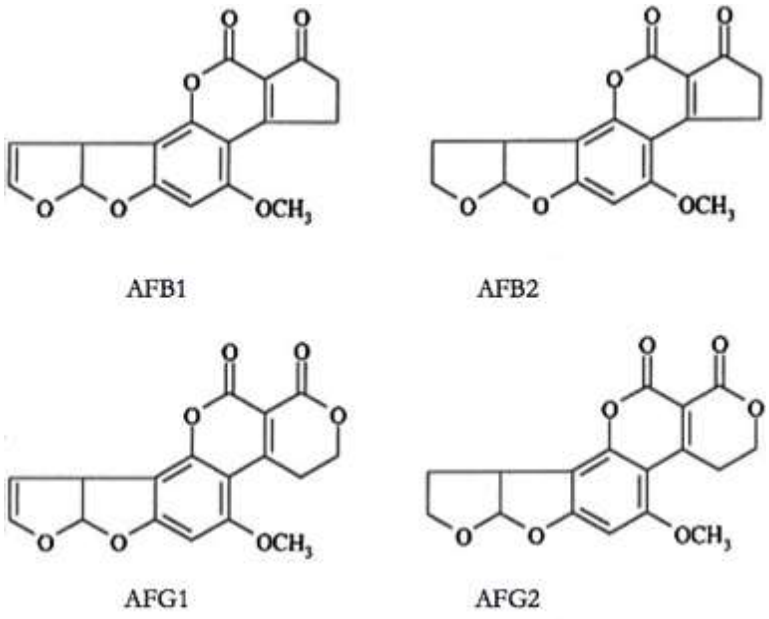


Figure 1. Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) structure.

1.2.2 Trichothecenes

Trichothecenes (Figure 2) are a broad class of about 150 mycotoxins generated by different species of various fungi of genera: *Fusarium*, such as *Fusarium poae*, *Fusarium langsethiae* and *Fusarium sporotrichioides*; *Stachybotrys*, *Myrothecium, Cephalosporium*, *Trichoderma*, *Trichothecium* and *Verticimonosporium* (Grove 1988; Smith et al. 1995; Thrane et al. 2004). These molecules present chemical similarities, infact they contain a basic group which consists of a common tetracyclic, sesquiterpenoid 12,13-epoxytrichothec-9-ene ring, the epoxide group that displays toxicity. Trichothecenes are categorised as group A and B, based on the presence of a side chain on C7 position: T-2 and HT-2 toxin and DAS belong to group A, while main trichothecenes of group B comprise deoxynivalenol (DON) (Figure 2), also known as vomitoxin, nivalenol (NIV), 3- and 15-acetoxy NIV and fusarenon X. They show chemical stability and can persist for long time once formed.

Type A trichothecenes (such as T-2 toxin, HT-2 toxin, DAS) show high solubility in ethyl acetate, acetone, dichloromethane, chloroform, and diethyl ether. Type B trichothecenes (such as DON, NIV, 3-acetylDON, 15- acetylDON) are relatively polar, highly hydroxylated and soluble in acetonitrile, methanol, and ethanol. DON shows one primary and two secondary hydroxyl groups: it contains a conjugated carbonyl system.

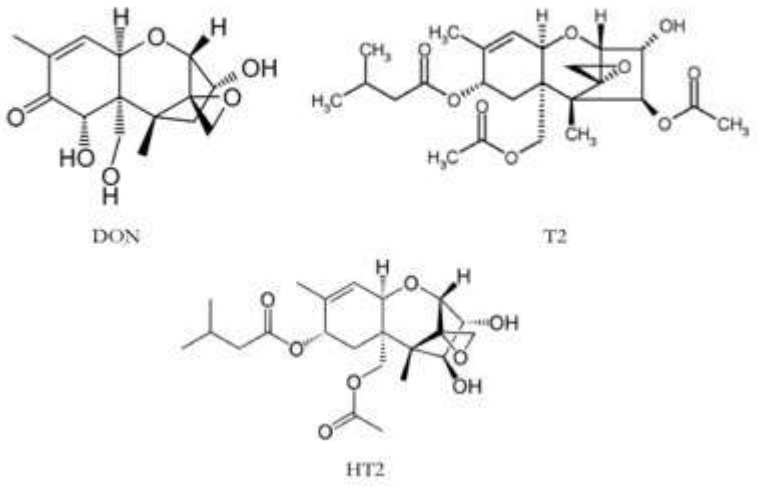
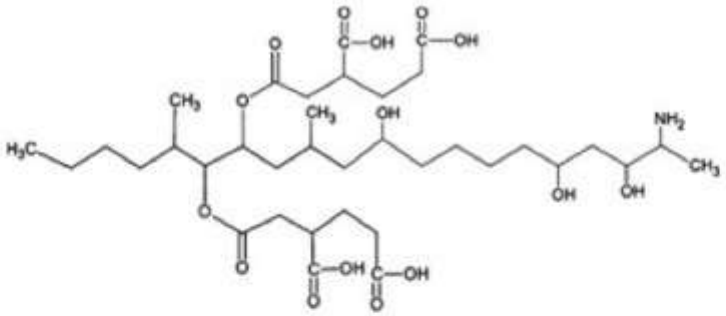


Figure 2. Deoxynivalenol (DON), T-2 toxin (T2), HT-2 toxin (HT2) structure.

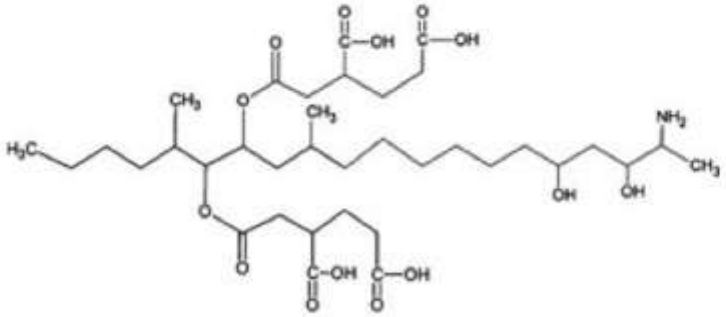
Moreover T-2 toxin and HT-2 toxin are produced by *F. poae*, *F. acuminatum* and *F. sporotrichioides*, which usually occur in different cereal crops (maize, wheat, oats, barley and rye) and processed grains (malt, bread and beer) (Figure 2).
T-2 and HT-2 toxin can colonise together infected cereals in almost all their growing areas in the world (Brera et al. 2008). DON represents the prevalent trichothecene of concern in Europe, whose ideal temperature for formation ranges from 26° to 30 °C (Paterson & Lima 2011).

1.2.3 Fumonisins

These molecules comprise a family of 28 members; they were characterised for the first time by Bezuidenhout in 1988 as a new class of mycotoxins that had been purified from *Fusarium moniliforme* coltures (Bezuidenhout et al. 1988). They are mainly generated by *Fusarium proliferatum*. *Fusarium verticillioides* (Sacc.) Nirenberg (former *F. moniliforme* Sheldon) (Geens et al. 2010).
Fumonisin B1 contains a diester of propane-1, 2, 3 - tricarboxylic acid and 2-amino-12, 16-dimethyl-3, 5, 10, 14, 15-pentahydroxyeicosane. Moreover, it is the most toxic and abundant one, accounting for 70–80% of the total FBs produced (Figure 3) (Friend et al. 1983; Gelderblom et al. 1988; Rheeder et al. 2002).
Fumonisin B2 (FB2) (Figure 3) is a deoxy-analogue of FB1, representing the 15-25% of total FBs.
Instead fumonisins B3 (FB3) stereochemistry is still unknown. However, the amino terminal of FB3 possesses the same configuration as that of FB1. MoreoverFB3 usually constitutes the 3-8% when cultured on rice, corn or in liquid medium.
Fumonisins pure substance is a white hydroscopic powder which displays solubility in water, acetonitrile-water or methanol. Fumonisins are soluble in polar solvents due to their four free carboxyl groups, the hydroxyl groups and the amino group. In addition they show insolubility in many organic solvents such as hexane and chloroform (Brera et al. 2008).



FB1



FB2

Figure 3. Fumonisin (FB1), Fumonisin (FB2) structure.

1.2.4 Zearalenone

ZEA (Figure 4) is a non-steroidal phenolic resorcyclic acid lactone estrogenic toxin generated mainly by *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium sacchari*. This mycotoxin with its main metabolites will be further described in the next chapter. This compound is a white crystalline one that displays solubility in water, slightly solubility in hexane and progressively higher solubility in acetonitrile, benzene, dichloromethane, ethanol, methanol and acetone.

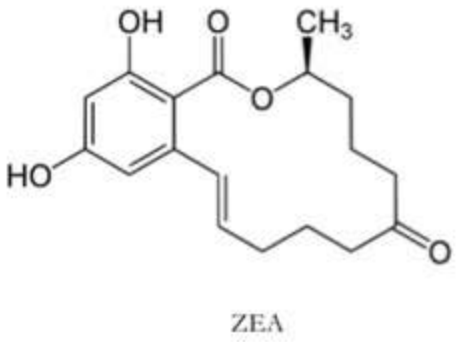


Figure 4. Zearalenone (ZEA) structure.

It also shows blue-green fluorescence when excited by long wavelength UV light (360 nm) and a more intense green one when excited with short wavelength UV light (260 nm) (Diekman & Green 1992; Brera et al. 2008).

Its presence is commonly related to DON production. The contamination of food and animal feeds occurs at a global scale, such as in maize, wheat, barley, soya, oat, beer, vegetables, dried fruits, sorghum, rice and beets (Zinedine et al. 2007; Van der Fels-Klerx et al. 2016).

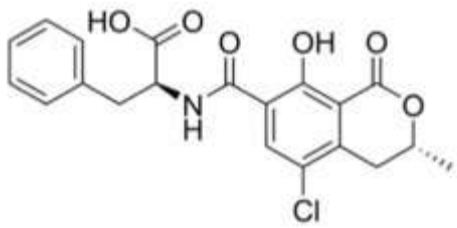
1.2.5 Ochratoxins

Ochratoxins A (OTA), B and C (Figure 5) are formed by a pentaketide derived from the dihydrocoumarins family coupled to β-phenylalanine.

OTA, the most harmful and relevant toxin, is characterised by a chlorine atom on the aromatic ring, which is responsible for its toxicity. It is a colourless crystalline substance which exhibitis blue fluorescence under UV light. In neutral and acidic pH OTA is soluble in polar organic solvents (alcohols, chloroform, ketones), slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons (el Khoury & Atoui 2010).

Aspergillus ochraceus is considered the main OTA producer, especially in cereals grown in warm climate. Moreover also other species of *Aspergillus section Circumdati*, *Aspergillus steynii* and *Aspergillus westerdijkiae* represent the most important fungi producers (Gil-Serna et al. 2015).

The optimal temperature for *Aspergillus ochraceus* which allows ochratoxins production ranges from 25 to 30 °C. Because of the broad range of fungi able to produce OTA, this molecule has been reported to colonise different areas of the world, in particular temperate climates, mainly in in various food products such as cereals, cocoa, coffee, wine (Sanchis & Magan 2004; Paterson & Lima 2011; Van der Fels-Klerx et al. 2016).



OTA

Figure 5. Ochratoxin A (OTA) structure.

1.3 EXPOSURE AND HEALTH EFFECTS

Food product contamination can occur as a consequence of a carry over from contaminated feed and can be present in food of animal origin such as eggs, milk and meat. Although some technological processes are able to lower mycotoxins levels of the raw commodity, processed food (beer and wine) can also shows contamination due to the use of contaminated raw material. Thus it is important to take into account that mycotoxins are resistant towards high temperatures and also cooking procedures, which are not able to destroy them (Brera et al. 2008).

Moreover humans and animals can also be exposed to these contaminants by inhalation of contaminated dusts. This phenomenon can occur both in certain working places such as in harbours, both in domestic environments due to the indoor contamination caused by wallpapers and mouldiness (Brera et al. 2008).

Mycotoxin ingestion can cause both acute and chronic toxicities: the former is characterised by a quick onset and toxic effects; the latter is resulted from low-dose exposure to these molecules over a long period of time, with adverse effects (hepatotoxicity, nephrotoxicity, neurotoxicity, immunosuppression, oestrogenicity, teratogenic, mutagenic and carcinogenic effects) (Malir et al. 2006; Wild & Gong 2009; Kensler et al. 2011).

Clinical symptoms usually regress when exposure from contaminated food or feed is ceased (Hussein & Brasel 2001).

In 1993 the International Agency for Cancer Research assessed the potential carcinogenic effect of the main mycotoxins (IARC 1993).

Aflatoxins can generate both acute toxicity and chronic carcinogenicity in human and animal populations. AFB1 is classified by (IARC) as a Group 1 carcinogen, inducing high risks for hepatocellular carcinoma (HCC) in people exposed to aflatoxins, while AFM1 is listed in Group 2B (possibly carcinogenic to humans) (Bennett et al. 2003).

In humans acute aflatoxicosis can cause vomitting, abdominal pain, coma, convulsions, and even death, while in animals can generate anemia, lowered milk and egg production, gastrointestinal dysfunction, reduced reproduction rate, lowered feed conversion and efficiency (Alshannaq & Yu 2017).

Trichothecenes were responsible causing alimentary toxic aleukia (ATA) toxicosis in the USSR in 1932. IARC placed DON in Group 3 Carcinogenesis: this molecule can be associated with nausea, vomiting, abdominal pain and diarrhea in humans. Moreover this class of contaminants can also cause also toxic effects in animals such as slow growth, intestinal hemorrhage, lowered milk production in cattle and drop in egg production in laying hens.

Fumonisin B1 has been classified as probably carcinogenic in 2B Group by IARC: fumonisins can cause severe toxicity on animals, and can exert toxic effects in kidney and liver.

Because of their hydrophilicity low amount of FB1 can accumulate in edible tissues (Richard 2007; Ostry et al. 2017).

Zearalenone is classified as a Group 3 carcinogen by IARC. Since ZEA is characterised by a strong estrogenic activity, due to the competitive binding to estrogen receptors, it can induce estrogenic effects both in human and animals. It can provoke significant alterations in laboratory and domestic animals such as infertility, hyperestrogenism, reduced milk production in cattle (Alshannaq & Yu 2017). This aspect would be further discussed in the next chapter.

Ochratoxin A is classified by IARC in Group 2B (possible human carcinogen) and it also interferes RNA and DNA synthesis. OTA is linked with acute nephrotoxicity and hepatotoxicity, and it can generate immunotoxicity, neurotoxicity, genotoxicity, teratogenicity, and embryotoxicity in both human and animals. It can also impact the productivity of food producing animals such as lowering feed conversion and body weight gain, decreasing egg production in laying hens. Since OTA is fat soluble it can accumulate into animal tissues, especially in pigs (Mantle 2002; Stoev et al. 2002; Heussner & Bingle 2015).

Animal and human health risks have been subject to a re-examination over the last few years: factors which affect the extent of toxicity include mechanisms of action and metabolism which are specific to each mycotoxin (De Liguoro 2006).

Metabolism and defense mechanisms are important aspects that help to better understand species-specific and individual toxicity. The role of species-specific mechanisms has been demonstrated by deeply examining the existing metabolic differences between ruminants and non-ruminants: the former seem to be more resistant to adverse effects of mycotoxins. *In vitro* studies showed the degradative capacity of ruminal flora towards these contaminants (Hussein & Brasel 2001).

These contaminants have attracted worldwide attention because the huge impact on human health and economic losses derived from contaminated foods (Ostry et al. 2017).

The presence of mycotoxins in zootechnical feeds, based on their concentration, can cause mycotoxicosis with subclinical manifestations relatively frequent but not easy to diagnose; while forms with clear clinical manifestations are rather rare and easier to diagnose, manifesting impairments of specific target organs (De Liguoro 2006). In the field of animal husbandry some toxic effects of mycotoxins are complex to detect, particularly the carcinogenic ones due to the short life span of livestock animals. There are other toxic effects occuring in animals fed contaminated feed at low concentrations of mycotoxins, such as hemorrhagic fever in poultry

meat, or liver degeneration in pigs caused by fumonisins which can alter the final food product (De Liguoro 2006).

It is important to point out that any animal species can be damaged not only by high exposure to mycotoxins but also at low levels of contamination. As previously described animals with highly active ruminal flora are less sensitive to mycotoxins because of the degradative action these microorganisms exert towards these contaminants. Instead pig species, rabbit, chicken show a high sensitivity to the main mycotoxins.

1.4 PREVENTION AND DECONTAMINATION STRATEGIES

In recent years many efforts have been made to prevent the onset of the main mycotoxins. European legislation highlighted the necessity to develop prevention systems and effective diagnostic tools in order to counter this issue.

In this regard, various studies have been carried out in the light of the Hazard Analysis Critical Control Point (HACCP) approach, which allows the identification of critical control points (CCPs) in any process relating food production (Magan 2006).

As previously described mycotoxins can colonise at pre-harvest, harvest and post-harvest phases. Therefore many key aspects can be performed in order to reduce mycotoxin contamination. Pre-harvest management is the optimal way for preventing mycotoxin contamination. The main effective actions include:

- a proper management of insect infestation (because damaged kernels can determine favourable conditions for growth of mould species);
- a correct management of crop rotation and residues;
- a suitable irrigation strategy to avoid drought or excessive moisture of soil;
- the use of naturally resistant plant varieties to fungal infection.

The genetic engineering research, pursued with the enhancement or addition of antifungal genes, is still in debate and a controversial issue, linked with ethic, economic, environmental aspects (Brera et al. 2008).

Procedures involving the avoidance of damaging kernels, effective drying processes should be taken into account during harvesting phase. These methods are essential to reduce mycotoxin production during storage. Some studies showed that crops left on the field for longer time may

generate higher levels of toxin contamination. Decontamination techniques for lowering or eliminating mycotoxins in food products have not yet been standardized worldwide due to the scarce results or high costs (Brera et al. 2008).
In this regard only few of decontamination techniques have been found effective such as the use of physical and chemical methods in post-harvest prevention systems.
Physical treatment includes:

- washing;
- cleaning;
- UV radiation;
- ultrasonic treatment;
- thermal treatment.

The efficiency of these techniques relies on the level of contamination and distribution of the raw material (Kolosova & Stroka 2012).
Moreover the use of chemical methods also requires sample preparation treatments, which make them laborious and expensive. In this sense numerous chemicals have been tested, such as oxidizing and reducing agents, acids, bases and salts for their ability to degrade mycotoxins; however only few of them allowed preservation of the nutritional properties.
Furthermore neither the use of chemical decontamination processes or batches blending (with the aim of reducing the level of contamination below the maximum tolerable level), are considered legal procedures in the European Union (Kolosova & Stroka 2012).
Recent studies allowed to develop strategies reducing mycotoxin contamination, so-called "mycotoxin binders". These molecules, if intentionally added to feed, are able to inhibit the level of such contaminants in *in vivo* animals. The use of adsorbent substances is authorized by the European Union, as they fall into the category of zootechnical additives (European Community 2003).
Some researchers, however, recommend the administration of these substances only occasionally, since these materials may also adsorb micronutrients and diminish the availability of essential nutrients, adsorpting the mycotoxins in the gastrointestinal tract and preventing distribution into target organs. The effectiveness of these binding substances seems to rely on involved actors: binders and mycotoxins. Therefore the physical structure of the adsorbent agent, the distribution of charges, the pore size and contact surface content are relevant aspects. However the intrinsic characteristics of mycotoxins also play a significant role, such as polarity,

solubility, shape, and charges distribution. Examples in this regard are provided by active carbons, polymers, sodium alumino-silicates, clay and sodium bentonite (Bennett et al. 2003)

1.5 LEGISLATION

The first food regulation in history was enacted approximately 3500 years ago by a king of the Hittites, in the area that nowadays corresponds to Turkey. This law already dealt with health protection and fraud prevention (Van Egmond HP 2013).

The awareness that mycotoxins can cause harmful effects both on humans and animals encouraged many countries to promote regulations on mycotoxins in feed and food in order to protect human health, as well as the businesses of producers and traders (FAO 2003).

In this regard there are specific aspects such as the availability of toxicological information, knowledge about mycotoxin distribution in foods, occurence in different commodities, availability of methods of analysis, political and economic aspects that should be taken into account (Van Egmond et al. 2007).

Establishing mycotoxin regulations represents a complex activity, which concerns many aspects and different stakeholders. Harmonisation of mycotoxin regulations in food and feed started from the 2000's in many areas such as the EU (European Union), COMESA (Common Market of Eastern and Southern Africa), Australia, New Zealand, MERCOSUR (Mercado Comun del Sur), GCC (Gulf Cooperation Council) and ASEAN (Association of South East Asian Nations) (Van Egmond 2013).

At least 99 countries in 2003 put specific limits for mycotoxins in both feedstuffs and foodstuffs, an increase of 30% compared to 1995 (Van Egmond et al. 2007).

Risk assessment is one of important process which leds to establish relevant mycotoxin regulations. Thus risk assessments have been carried out by many international and national public health and governmental authorities such as the European Food Safety Authority (EFSA), US Food and Drug Administration (FDA), JECFA (FAO/WHO Joint Expert Committee on Food Additives and Contaminants).

Risk assessment focuses on various aspects such as hazard identification, hazard characterization, exposure assessment and risk characterization.

While risk assessment is mainly carried out by scientific committees, risk management is conducted by regulators and policy makers. Finally risk communication is mediated by risk assessors and managers, and the stakeholders (FAO 2003; Van Egmond 2013; Alshannaq & Yu 2017).

Hazard identification, that represents the indication that a substance can provoke adverse effects on health, is usually part of the information showed to JECFA for evaluation.

In the same way hazard characterization, which relies on the quantitative and qualitative

evaluation of the adverse effects, is part of the data that are presented. Moreover the evaluation of toxicological data performed by JECFA can be needed estimating a Provisional Tolerable Weekly Intake (PTWI) or a Provisional Tolerable Daily Intake (PTDI) (FAO 2003).

The term "provisional" states the uncertained quality of the evaluation in view of the sparsity of reliable data about the consequences of human exposure at levels which JECFA is concerned.

As a matter of principle, the evaluation is based on the characterisation of a No-Observed-Adverse-Effect-Level (NOAEL) in toxicological studies and the application of an uncertainty factor. The latter one is obtained dividing the lowest NOAEL in animal studies by 100, 10 for extrapolation from animals to humans and by 10 for variation between individuals, in order to formulate a tolerable intake level.

In cases where the data are scarce, JECFA utilises a higher safety factor (FAO 2003). In table 3 are reported Tolerable Daily Intake (TDI) values for the main mycotoxins.

Mycotoxins	**Tolerable Daily Intakes**
Aflatoxin B1	ALARA
Ochratoxin A	TWI:120 ng/kg bw
Deoxynivalenol	TDI: 1 □ g/kg bw
Fumonisins	TDI: 2 □ g/kg bw
Zearalenone	TDI: 0.25 □ g/kg bw
T-2 and HT-2	TDI: 0.1□ g/kg bw

Table 3. Tolerable daily intakes of main mycotoxins. TWI: tolerable weekly intake; TDI: tolerable daily intake; ALARA: as low as reasonably achievable; bw: body weight.

European Commission (EC) set maximum allowed levels and recomedations for many mycotoxins in food (Commission Regulation 2006; Commission Recommendation 2013) and feed (Commission Directive 2003; Commission Recommendation 2006; Commission Recommendation 2013) among its Member States.

Guidance values and maximum levels for mycotoxins in animal feed have been established in Commission Directive 2003/100/EC and Commission Recommendation 2006/576/EC (Table 4 and 5) (Pinotti et al. 2016).

Methods of sampling and analysis have been set for the official control of the levels of mycotoxins in foodstuffs by Commission Regulation (EC) number 401/ 2006.

Regulations are also established by the US Food and Drug Administration.

In the late 1960s were set the first limits for aflatoxins, approximately ten years after the discovery of these contaminants: AFs have been closely controlled by the FDA since 1969. Among all mycotoxins, AFs are the only one provisioned by FDA; while others are regulated only by advisory levels (FAO 2003; Van Egmond 2013; Alshannaq & Yu 2017).

Table 4. Maximum levels for mycotoxins in cereals and cereal products for human consumption (Commission Recommendation No 2013/165/EU and Commission Regulation (EU) 1881/2006). Adapted and modified from Pinotti et al. 2016.

Mycotoxin	**Cereal and Cereal Products**	**Maximum Levels, µg/kg**
Aflatoxin B1	All cereals and all products derived from cereals	2.0
	Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuff	5.0
Aflatoxins, sum of B1, B2, G1 and G2	All cereals and all products derived from cereals	4.0
	Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	10.0
Deoxynivalenol	Unprocessed cereals other than durum wheat, oats and maize	1250
	Unprocessed durum wheat and oats	1750
	Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption	1750 750
Zearalenone	Unprocessed cereals other than maize	100
	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	350
	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption	75
	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100
Ochratoxin A	Unprocessed cereals	5.0
	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	3.0
Fumonisin B1 + B2	Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling Maize intended for direct human consumption, maize-based foods for direct human consumption	4000 1000
Sum T-2 and HT-2toxin(*)	Unprocessed cereals	-
	Barley and maize	200
	Oats	1000
	Wheat, rye and other cereals	100
	Cereals grains for direct human consumption	-
Sum T-2 and HT-2 toxin(*)	Oats	200
	Maize	100
	Other cereals	50

(*) indicates Recommendation.

Table 5. Maximum levels and guidance levels for mycotoxins in products intended for animal feed (Commission Directive 2003/100/EC, Commission Recommendation 2016/1318/EC, Commission Recommendation No 2013/165/EU). Adapted and modified from Pinotti et al. 2016.

Mycotoxin	**Cereal and Cereal Products**	**Maximum Levels, mg/kg**
Aflatoxin B1(*)	All feed materials	0.02
	Complete feedstuffs for cattle, sheep and goats with the exception of:	0.02
	Complete feedstuffs for dairy animals	0.005
	Complete feedstuffs for calves and lambs	0.01
	Complete feedstuffs for pigs and poultry (except young animals)	0.02
	Other complete feedstuffs	0.01
	Complementary feedstuffs for cattle, sheep and goats (except Complementary feedstuffs for dairy animals, calves and lambs)	0.02
	Complementary feedstuffs for pigs and poultry (except young animals)	
	Other complementary feedstuffs	0.02
	Complete feedstuffs for cattle, sheep and goats with the exception of:	0.005
Deoxynivalenol	Feed materials	
	Cereals and cereal products with the exception of maize byproducts	8
	Maize byproducts	12
	Complementary and complete feedstuffs with the exception of:	5
	Complementary and complete feedstuffs for pigs	0.9
	Complementary and complete feedstuffs for calves (<4 months), lambs and kids	2
Zearalenone	Feed materials	
	Cereals and cereal products with the exception of maize byproducts	2
	Maize byproducts	3
	Complementary and complete feedstuffs	-
	Complementary and complete feedstuffs for piglets and gilts (young sows)	0.1
	Complementary and complete feedstuffs for sows and fattening pigs	0.25
	Complementary and complete feedstuffs for calves, dairy cattle, sheep (including lambs) and goats (including kids)	0.5
	Compound feed for:	
	Adult dogs and cats other than for reproduction	0.2
Ochratoxin A	Feed materials(*)	
	Cereals and cereal products(**)	0.25
	Compound feed for:	
	pigs	0.05
	poultry	0.1
	cats and dogs	0.01

Mycotoxin	Cereal and Cereal Products	Maximum Levels, mg/kg
Fumonisin B1 + B_2	Feed materials	
	Maize and maize products	60
	Complementary and complete feedstuffs	-
	Pigs, horses (Equidae), rabbits and pet animals	5
	Fish	10
	Poultry, calves (<4 months), lambs and kids	20
	adult ruminants (>4 months) and mink	50
Sum T-2 and HT-2 toxin	Compound feed for cats	0.05

(*) Indicates Maximum levels.

In this regard EU set limits for ZEA and OTA in several foodstuffs (Commission Regulation 2006), while the US FDA did not put any regulatory guidelines for those molecules (Alshannaq & Yu 2017).
Moreover European Union put in place since 1979 the Rapid Alert System for Food and Feed (RASFF) which are harmful to human health. This system is based on a rapid information exchange among the authorities of the Member States, EFSA and European Commission.
In the case of problems in the food chain RASFF carries out the effective measures in order to safeguard the consumer safety (Van Egmond 2013).
From the 1st January 2017 to 1st December 2017 484 mycotoxin notifications were reported from food and 24 notifications in feed in the RASFF portal (RASFF 2017).

1.6 METHODS OF ANALYSIS

1.6.1 Sampling

The distribution of mycotoxin is a relevant factor to take into account in establishing regulatory sampling criteria. The distribution of contaminants can be heterogeneous. Mycotoxin concentration in an inspected batch can be wrongly assessed if appropriate procedures for representative sampling are not implemented (Barkai-Golan & Paster 2008).
Sample variation is a relavant error in estimating concentrations of mycotoxins in batches commodities. Mycotoxin concentrations display a distorted or uneven distribution in feed and food, particularly in whole kernels (or nuts). Therefore it is difficult to collect a sample that properly reflects the mean batch concentration: this one can be invalidated and it can bring to undesirable health and or economic consequences (Whitaker et al. 2010). Even though errors can be sometimes unpredictable, it should be considered that most errors are generated from the sampling stage rather than sample analysis. Thus 90% or more of errors are caused sampling variability as a consequence of the heterogeneity of the matrices (Zhang 2006).
Certainly the type of matrix to be analyzed can determine different approaches for heterogeneous samples (feed, granules) rather than for homogeneous samples (milk, cheese, flour); the homogeneity of the sample is a good assumption to obtain a significant sample, however not excluding the homogeneity and the representativeness of the batch. Also the

amount of sample to be analyzed may vary, so it will be necessary to collect batches in various parts and in larger part from heterogeneous samples, while collecting smaller quantities for homogeneous samples it will be required (Cast 2003).

The design of sampling procedures for multiclass mycotoxins and sample materials represented an international concern for several years (Krska et al. 2008).

International and national agencies, such as the European Union (EU), Codex Alimentarius and the United States Dept. Agriculture (USDA) have set sampling plans for a broad range of commodities.

Sampling plans defines the batch size (or a representative sub-batch in the case of very large lots), the size of the aggregate sample and the minimum number of incremental samples (Turner et al. 2015).

In the European panorama, as far as sampling (and analytical) methods are concerned for the official control of the levels of mycotoxins in foodstuffs, EC Regulation 401/2006 has precise indications for each type of matrix.

To obtain comparable data the Regulation 401/2006 (Commission Regulation 2006), directly applicable to all Member States, harmonizes in a single legal act the procedures for preparation, analytical methods, performance criteria for the official control of mycotoxins. Proper sampling it is, in fact, the basic prerequisite for a reliable analytical method: the quantity of sample must be as representative as possible of the original lot to be sampled, taking into account the distribution of the mycotoxins are very variable depending on the type of sample matrix (Tealdo 2006).

Moreover the whole analytical process, which includes sampling, sample preparation, clean-up and final determination, used by each laboratory for the enforcement and control of regulatory limits, needs to undergo to a validation procedure, in order to show that the method meets the prescribed criteria. Thus analytical methods available can be validated and accepted by official authorities, such as the European Committee for Standardization (CEN), the Association of Official Analytical Chemists (AOAC International) and the International Organisation for Standardization (ISO) (Krska et al. 2008).

Furthermore each laboratory can enforce quality assurance systems checking the precision and trueness of their protocols by analysing certified reference materials (CRM) and participating in proficiency tests (Krska et al. 2008).

The European Union Reference Laboratory (EURL) for mycotoxins, together with national reference laboratories, monitors and assesses the performance of analytical methods in order to ensure a reliable measurement capacity in Europe (De Saeger et al. 2016).

RIKILT (Wageningen University & Research) is the Dutch National Laboratory and the EU Reference Laboratory for analysis of biological residues produced by food-production animals for the presence of hormonal growth promoting compounds, sedatives and mycotoxins. As the others EURLs represents the interface between the European Commission (DG SANCO) and National Reference Laboratories in the Member States.

1.6.2 Analytical Techniques

The mycotoxins issue therefore demands robust analytical methods even though it is not always an easy task. The detection and identification of these molecules is often considered as a relevant paradigm for the analysis of environmental compounds. The complexity of the target, matrix, detection levels, time requirements and availability of suitable technology reprensent a challenge for the analytical process (Turner et al. 2015).

Screening methods are used to reveal the presence of a substance or a class of substances at a level of interest: these are methods of election adopted when it is necessary analysing a high number of samples in a short time and they are also economic methods with a good sensitivity (European Commission 2002).

Most used screening tests are enzyme immunoassays ELISA, Lateral Flow, polarization immunoassay fluorescence (FPIA), immunofiltration assays and, more recently, biosensor assays; however, the thin layer chromatography (TLC) is still widely used.

Lateral flow devices (LFD), also called "strips", are immunochromatographic analytical methods, whose reagents are incorporated directly into the device. This feature makes the method suitable for on field analysis, they can be used also by untrained personnel. The strips can be less precise than other screening methods, but they can be sufficiently sensitive and accurate.

Among quick methods based on immunochemical techniques that do not require clean-up or analyte enrichment steps, ELISAs became common tools for rapid monitoring of most mycotoxins, in particular for the screening of raw materials. Although ELISA tests can be affected by a high matrix dependence and possible overestimation of concentration levels, the benefits are speed, easy to use, good sensitivity and high sample throughput (Krska et al. 2008).

Furthermore biosensors are becoming more common in different fields (i.e healthcare, environmental, food sector): they rely on biological recognition such as enzyme-substrate, antibody-antigen or receptor-ligand) and they are connected to a transducer or detection system (i.e electrical, optical, acoustic or thermal) (Thakur & Ragavan 2013).

On the other hand confirmation methods provide complete information to identify a substance in a univocal way and, if necessary, quantify it at certain level of interest (European Commission 2002).

In general physico-chemical techniques, i.e separation by gas chromatography (GC), or high-performance liquid chromatography (HPLC) coupled with specific detectors are used to obtain highly reliable results. The detectors commonly used with these chromatograph techniques are flame ionization (FID), ultraviolet (UV), UV diode arrays, fluorescence (FL), electronic capture (ECD), and mass spectrometry (MS).

Currently liquid chromatography coupled with mass spectrometry (LC-MS) is probably the most used analytical method for mycotoxins analysis.

In the last few years technical progress has been made in this sector, allowing the simultaneous determination of different classes of mycotoxins (Meneely et al. 2011).

In comparison to other detection techniques, such as fluorescence or UV, mass spectrometry shows higher sensitivity and selectivity (even though fluorescence detection can be more sensitive for certain mycotoxins, such as aflatoxins), unequivocal confirmation of the analyte and allows the use of isotopically labelled substances as internal standards (Krska et al. 2008).

Furthermore relevant progress has been made in sequencing the entire genome of mycotoxins producing fungi: in which reference databases for genomic, transcriptomics, and proteomics analysis in the field of mycotoxin were created. Thanks to biomolecular methods (Real Time PCR, Microarray, 2-D DIGE) it is possible to identify the genes responsible for the biosynthesis of mycotoxins, their cluster organisation and their epigenetic regulation (Woloshuk & Shim 2013).

Because of the complexity of the matrices in which toxins are generally found, a pre-analysis step is required for most chromatographic methods, whereas for some immunological methods it may be not necessary. Although this step can involve extra cost and can be considered time-consuming, the improvements in sensitivity and robustness are relevant (Turner et al. 2015).

Different approaches for extraction and clean-up purification step have been proposed: the most popular methodology utilises solid-liquid extraction (SLE) followed by solid phase extraction (SPE), often carried out with immunoaffinity columns (IACs), which uses specific antibodies to link the analyte of interest (Arroyo-Manzanares et al. 2014).

SPE is an important support for sample preparation in mycotoxin analysis: there are many solid phase, including ion exchange, C_{18} materials, hollow microfibers and immunoadsorbent materials (Turner et al. 2015).

IACs are based on immobilised antibodies that selectively retain a certain mycotoxin or

mycotoxin class: they are characterised by high specificity, producing cleaner extracts and lowering interfering matrix components in order to produce excellent signal-to-noise ratios compared to less selective SPE sorbent materials. IACs have been developed for most mycotoxin classes such as aflatoxins, trichothecenes, ochratoxin A, zearalenone and their metabolites (Krska et al. 2008). On the other hand, IACs also represent a complex and expensive clean-up system which can be affected by low recoveries for some classes of mycotoxins.

As a consequence multiclass, simpler extraction systems are often used.

Among the different methods, the so-called QuEChERS (quick, easy, cheap, effective, rugged, and safe) and dispersive liquid-liquid microextraction (DLLME) are becoming the common treatments (Arroyo-Manzanares et al. 2014). QuEChERS is an inexpensive and fast method widely also used also for the extraction of pesticides: it presents some benefits such as its ease, minimum steps, and effectiveness for cleaning up complex matrices. It is based on two steps: the extraction and dispersive SPE (dSPE). The first step is based on partitioning via salting-out, implying the equilibrium between an organic and an aqueous layer; the second step allows further clean-up with combinations of $MgSO_4$ and different sorbents, such as C_{18} or primary and secondary amine (PSA). QuEChERS method is employed for the extraction of multiclass mycotoxins in bread, cereal products, eggs, spices.

DLLME relies on the specific solubility of the target analyte in a ternary component solvent system. It is considered the simplest method of pre-treatment available (Turner et al. 2015).

2. AIM OF THE THESIS

Mycotoxins are natural contaminants produced by several species of fungi as secondary metabolites. They can represent a health risk to humans and animals due to their toxicity and occurrence in food and feed. For this reason they have attracted worldwide attention because of the huge impact on health, the agri-food sector and national economies.
The available information regarding their occurrence in foodstuffs and feedstuffs, and harmful health effects have recently increased, highlighting the importance of further investigations.

The purpose of the research presented in this thesis was to:

1. develop a method for quantitative determination of mycotoxin zearalenone and its metabolites (α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone) in bovine and human hair using LC-MS/MS;

2. determine the occurrence of various mycotoxins (aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, deoxynivalenol, zearalenone, fumonisin B1, fumonisin B2, ochratoxin, T-2 toxin and HT-2 toxin) in different pet food for cats using LC-MS/MS.

   The first project was conducted at RIKILT, the Institute for Food and Feed Safety located in Wageningen (The Netherlands), during a traineeship held between April and August 2017.
   The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.
   Moreover RIKILT is the National Reference Laboratory (NRL) for monitoring residues and contaminants in food and feed, it is also the European Union Reference Laboratory (EU-RL) for mycotoxins and hormonal growth-promoting compounds.

   The second part was conducted at CABA-Lab (Laboratory of Analytical Bio-Agroalimentary Chemistry), set in the DIMEVET (Veterinary Medical Sciences Department) in Ozzano dell'Emilia, Bologna University. This laboratory carries out research activities in the field of residue and contaminant detection in food and feed and provides analytical support for veterinary-biomedical research.

3. ZEARALENONE AND METABOLITES

3.1 PROJECT INTRODUCTION

3.1.1 Hair composition and development

Hair is a keratinized tissue which is produced inside the hair follicle. Its growth comes from matrix cells which are located in the bulb region (Shimomura & Ito 2005).

Hair is a filament protein comprised of terminally differentiated dead keratinocytes, so called trichocytes, which form a fibre of massive strength, the hair shaft. Mammals are characterised by the presence of hair, which exerts a broad range of functions such as thermal isolation, physical protection, sweat and sebum dispersion, sensory and tactile functions and social interactions (Schneider et al. 2009).

Hair shafts originate from the hair follicle (Figure 6), a complex structure in the skin, which encompasses the pilosebaceous unit together with other structures such as the sebaceous gland, the apocrine gland and the arrector pili muscle (Schneider et al. 2009).

Bovine hair is made up of over 90% protein, with keratin as the main component (Reniers & Brebbia 2011).

Depending on its moisture content human hair is composed of 65–95% protein (specifically fibrous proteins, which are mostly alpha keratins), 15–35% water and 1–9% lipids, melanins, while mineral hair content ranges from 0.25 to 0.95%, based on dry weight (Boumba et al. 2006; Kempson & Lombi 2011).

The structural unit of alpha keratin is composed of three right-handed alpha helical polypeptides in a left-handed coil which is fortified by disulfide bonds. The thiol (–SH) group of cysteine is highly reactive and will bind another cysteine residue in order to create a disulfide bond (–S–S–), forming cystine. These bonds give rigidity, mechanical properties and tertiary structure of the protein (Kempson & Lombi 2011). Hair proteins contain amino acids glycine, aspartic and glutamic acid, threonine, cysteine and lysine (Boumba et al. 2006).

Lipid content is derived from secretions and sebum of apocrine glands.

This fraction is constituted by fatty acids, mono- di- and triglycerides, hydrocarbons, wax esters and alcohols. Three phases characterise the human hair lifetime: the anagen, catagen and telogen. The first represents the active growth phase of hair: this stage is characterised by rapid division of the cells in the bulb of the follicle.

Hair formation is based on the elongation of new cells which form a thin filament. Then hair cells advance into the follicular canal, differentiating into cuticle, cortex or medulla cells and starting the keratinization process. During this step, the hair growth is about 1 cm every 28 days, while scalp hair remains in this phase for 2-6 years (Boumba et al. 2006).

Hair usually grows 0.44 mm per day (ranging from 0.38 to 0.48 mm) for men and 0.45 mm per day (ranging from 0.40 to 0.55 mm) for women in the vertex region of the scalp.

Furthermore the hair growth rate is influenced by anatomical location, gender and age.

During this growing phase, the capillary blood which is located around the follicle supplies nutrients and may deliver also exogenous substances that might be in the blood stream such as drugs, trace metals (Boumba et al. 2006).

Moreover chemicals can be incorporated into hair in the keratinogenous zone thanks to surrounding tissues, intercellular or lymph fluids.

The catagen phase represents a rapid transitional stage that follows the anagen phase, which is characterised by interruption of cell division and the fully keratinization of hair shaft.

This stage lasts for about 2-3 weeks when the follicle becomes considerably shorter.

Finally telogen is a quiescent or resting phase, in which the growth of the hair shaft stops definitely. During this step the root allows hair anchorage into the follicle.

Then the germ cells below the root will generate the next anagen hair, while the old one will be forced out and lost. Scalp hair resting phase is mantained for 10 weeks, while in the rest body surface hair lasts for 2-6 years (Boumba et al. 2006).

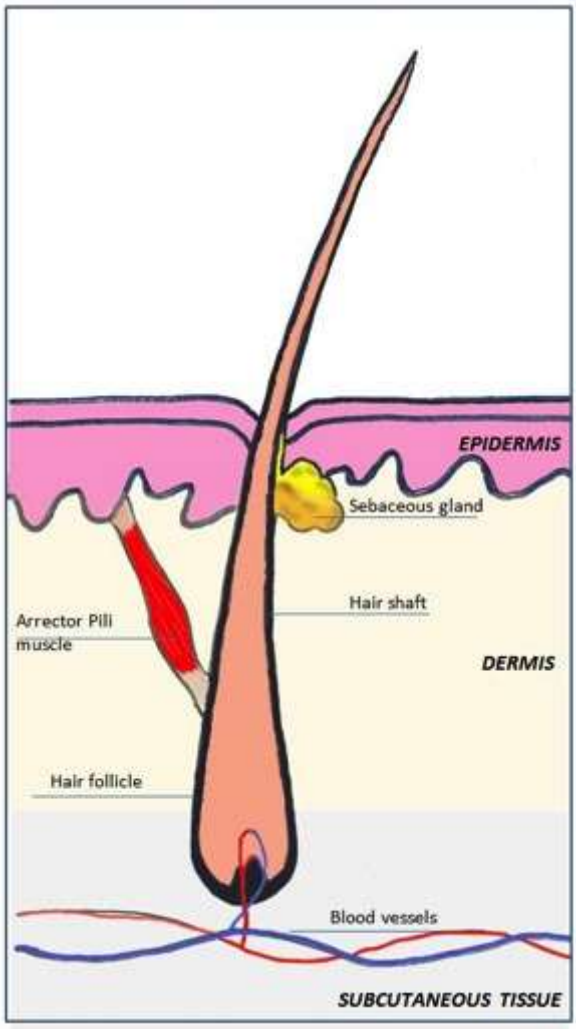


Figure 6. Hair longitudinal section, created by A. Repossi.

3.1.2 Mechanisms of xenobiotics incorporation into hair

The first case of poison identification in human hair was published into a 'Practical Guide to Legal Medicine' by Hoppe in 1858, where he described the arsenic determination in the hair in a body exhumed after 11 years. Approximately 100 years later, in 1954 Goldblum determined amphetamine content in the hair of a guinea-pig (Sachs 1997).

In general the uptake of environmental chemicals can happen by three ways: dermal absorption, inhalation and ingestion. This phenomenon is influenced by the physical and chemical properties

of the substance, together with the exposure time and the individual susceptibility.
Thus the body burden can be determined by absorption, distribution, metabolism and excretion (Esteban & Castaño 2009).
Chemical properties and individual variability can affect the magnitude of these phenomena and the final fate of the xenobiotic, which can be excreted in biological matrices like saliva, urine, breast milk or faeces, stored in bones or adipose tissue (Esteban & Castaño 2009).
Passive transfer represents the simplest model hypothesised for xenobiotic incorporation into hair. According to this scheme chemicals are transferred by passive diffusion from the bloodstream into hair cells located into the base of the follicle and then following the keratogenesis process they bind inside the hair shaft. The incorporation is influenced by the drug concentration in blood, which relies on the ingested drug dose. Segmental hair analysis represents the scientific base for determining the time course of the xenobiotic, since hair growth is assumed to be constant. According to this theory the position of the drugs along the hair shaft can be associated with the time the drugs were present in the bloodstream. Thus segmental analysis can represent a "calendar" of drug use in an organism (Boumba et al. 2006).
Many authors have found too many factors that give complexity to associate hair contamination to blood concentrations (Kempson & Lombi 2011).
Hence based on the current state-of-the-art literature, only a few publications focused on the incorporation mechanisms into hair were published; however the mechanism is not yet completely clear.
External hair contamination is closely related to this aspect. In fact any molecule that can be incorporated into the body by inhalation (vapour inhalation or smoking) needs to be considered as a possible source of contamination. This phenomenon can lead to positive results in hair analysis through passive transfer the hair shaft.
In the same way any other substance, solid or liquid, which is handled by an individual could be transferred into the hair. So a proper decontamination of hair is required in order to avoid false positives due to passive environmental exposure (Boumba et al. 2006).
A way to discriminate between external contamination and endogenous content relies on the assessment of metabolites: this aspect can represent a distinct advantage of drug analysis in hair, since a metabolite will only be present in case of consumption or ingestion (Kempson & Lombi 2011).

3.1.3 Hair preparation and decontamination

Some precautions need to be taken during the human hair sampling. Sample weight usually ranges from 100 to 200 mg of hair, moreover the cut must be taken from the occipital region of the head and as close to the scalp as possible in order to obtain the most suitable sample to identifying the selected analytes. Hair samples should be stored in a dry place, under dark conditions, at room temperature in aluminum foil, polyethylene bags or envelope (Boumba et al. 2006; Schramm 2008).

However some studies advise against storage in plastic bags because of possible contamination by softeners, such as plasticizers and because plastic can potentially take out lipophilic molecules from hair. Moreover only metal or quartz scissors should be used in order to not contaminate samples with the substance to be examined (Schramm 2008).

In this way most drugs or metabolites in hair are stable and can be detected after years of storage (Pragst & Balikova 2006).

Preparation for analysis is another step that follows hair collection.

As already discussed above, washing hair sample from external contamination is needed for two reasons:

1. to eliminate residue of hair care products such as shampoo, hair sprays, as well as sweat, sebum and dust that can lead to increased analytical background;

2. drugs could come from the environment of the individual, potentially leading to incorrect test results. Thus in order to exclude that a positive analytical result can originate from this type of external contamination, the first wash solution should be checked for subsequent analysis (Pragst & Balikova 2006).

Schramm et al. (1992) confirmed that washing step removes fat and sweat as well as exogenous substances from the surface of hair (Schramm 2008).

In general three types of solvents are used for washing external contaminants: solutions of surfactants in water such as 0.1-5% solution of sodium dodecyl sulphate, hydrophilic organic solvents and hydrophobic organic solvents. Moreover several types of cleaning solvents can be used, but also taking account that they should not damage the hair matrix (Pragst & Balikova 2006; Schramm 2008).

The importance of an effective method to differentiate between endogenous and external

contamination is obvious. The simplest way would be a cleansing protocol that would eliminate the contamination and leave the endogenously incorporated molecules (Kempson & Lombi 2011).

After the washing step hair is commonly dried in air or by a gentle flow of nitrogen.

Then size reduction is executed after the washing step and according to Zhang et al. the powder-like state of the sample will enhance the efficiency of subsequent extraction (Zhang et al. 2007).

Furthermore all compounds of interest should be extracted by solubilization (supercritical fluid extraction and liquid extraction), or with liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase micro-extraction (Schramm 2008).

3.1.4 Hair biomonitoring

Human hair is a durable matrix that shows various benefits for human biomonitoring, such as low cost, easy collection, non-invasive, easy storage and information about short and long-term exposure (Schramm 2008; Esteban & Castaño 2009).

Biomonitoring, which is the contraction of "biological monitoring" is considered as the assessment of human exposure to chemicals by directly measuring the molecules, their metabolites in biological matrices (Appenzeller & Tsatsakis 2012).

Hair is a unique matrix, differing from other human biological samples used for toxicological analysis, such as blood or urine because it possesses a longer detection window, from months to years, that enables retrospective investigation of past substances assumed.

Thus, because of its stability, hair analysis can be performed even centuries after growth (Pragst & Balikova 2006). Biomonitoring studies can provide lots of information, but also have some limitations: for example some molecules that are excreted quickly and therefore can be monitored for a short detection window after exposure.

However hair analysis cannot identify the sources of chemical contaminants that were detected. In fact hair analysis cannot discriminate between substances that have directly deposited onto hair from those coming from other route of exposure, such as ingestion of contaminated drinking water. In other words, hair analysis generally can not differentiate internal from external exposure (Pickle et al. 1995; Needham et al. 2002; Esteban & Castaño 2009).

Another factor to be considered is sensitivity that is particularly important in the case of hair analysis: the sample used is often limited to 50-200 mg compared to other matrices such as blood, serum, urine, in which the amount used for the determination of contaminants is usually 2-5 mL (Appenzeller & Tsatsakis 2012).

Even though hair has been used as a matrix since the 1950s to determine metals and since the 1970s to measure drugs, its later use for analysis to investigate environmental exposure was due to lack of reliable and sensitive analytical methods. The relevant analytical advances during the past years have allowed scientists to reach adequate sensitivity levels (Appenzeller 2015).

3.1.5 Bovine hair analysis

Farm animals can be treated with veterinary drugs in order to control and prevent a broad range of diseases. The illegal or improper use of these substances as growth promoters may results in

the presence of drug residues in food products derived from these animals.

The Council Regulation (EC) 2377/90 (today repealed) required that all veterinary drugs needed to be assessed: this regulation illustrated the procedure for the establishment, and in case of need, of the maximum residue limit (MRL) of a molecule in a certain matrix.

Currently MRLs are established according to the procedures laid down in Regulation (EC) 470/2009. Pharmacologically active substances and their classification regarding maximum residue limits are set out in Commission Regulation (EU) No 37/2010.

Furthermore the use of any drug as a growth promoter is banned within the European Union by the Council Directive 96/22/EC which was amended by Directive 2003/74/EC (Commission of the European Communities 1996a; Council Directive 2003). In order to establish a high level of consumer safety, European Union

Countries must implement residue monitoring plans to detect the illegal use or misuse of authorised veterinary medicines and the presence of environmental contaminants in food producing animals. The surveillance program, responsible to monitor the presence of residues of veterinary agents in food-producing animals and related products, is established by the Council Directive 96/23/EC and Commission Decision 2002/657/EC.

This document specifies the guidelines for sampling procedures on both farms and slaughterhouses, setting the procedures and criteria to validate analytical methods common criteria for the interpretation of test results (Commission of the European Communities 1996b; European Commission 2002). The proper application of these surveillance programs requires the development of sensitive and robust analytical methods in order to guarantee the control from authorities with effective tools (Gratacós-Cubarsí et al. 2006).

Veterinary drug residues are usually detected in urine and plasma matrices derived from living animals and also in tissue samples after slaughter such as muscle, kidney and liver.

Veterinary drugs are generally characterised by high clearance rates in these biological matrices, making the retroactive detection of banned substances really difficult. In the past years various publications investigated if hair analysis could be considered a suitable matrix to control this problem in food producing animals. As previously described hair has been considered as a reliable matrix for the detection of various organic drugs, since the publication by Goldblum in 1954 about amphetamine detection in hair of Guinea pigs (Gratacós-Cubarsí et al. 2006).

In recent years some authors described the possible applications of hair analysis in the field of fraudulent detection of beta-agonists and anabolics in animals (Sauer & Anderson 1994). However publications on veterinary hair analysis are still few and tend to focus only on a limited number of substances, although hair could represent a promising matrix.

The understanding of veterinary drug deposition is still not adequate and the influence of veterinary drugs uptake in the hair of livestock productions is not fully investigated (Gratacós-Cubarsí et al. 2006).

3.1.6 Zearalenone biotransformation and exposure

Zearalenone (ZEA) belongs to Fusarium mycotoxins and it is characterised by a ring system of a macrocyclic beta-resorcylic acid lactone (RAL). The given name of this compound comes from the its common occurrence in Zea mays, a mouldy maize, followed by the element RAL, and the alkene double bond ('en') and keto group ('one') of this substance (EFSA 2016).
Many species of the genus Fusarium frequently infest cereals, in particular maize and other plant products all over the world (Maragos 2010). This mechanism allows ZEA to enter into the food chain of both animals and humans, that can be affected by adverse health risks. In livestock animals such as pig, cattle, and sheep, ZEA can cause reproductive and infertility disorders.
In humans ZEA can cause hyperestrogenic siyndromes: this fact can be explained by the oestrogenic effect exerted by this compound. Thus zearalenone is an oestrogen analogue which is able to mimic the activity of naturally oestrogens (de Andrés et al. 2008).
Zearalenone presence in food and feed is regulated in various countries of the world. The European Food Safety Authority (EFSA) has established a TDI of 0.25 μg/kg body weight (bw), while the Joint Expert Committee on Food Additives (JECFA) set a provisional maximum tolerable daily intake (TDI) for ZEA of 0.5 μg /kg (bw) (JECFA, 2000; EFSA 2011).
Besides zearalenone and also its metabolites such as zeranol (ZAL) are considered oestrogen agonists in mammals, and they can occur in food such as cereal crops, fruits, vegetables.
The use of zeranol (ZAL) is permitted as a growth promoter in beef in the USA and Canada (Mukherjee et al. 2014).
These hormones can be produced naturally in the animal body, but they can also be artificially synthesized and added.
In United States about 30 growth promoters are currently used, such as estradiol, progesterone, testosterone, zeranol, diethylstilbestrol (DES), in order to increase feed-to-meat conversion in livestock production. Zeranol (ZAL) is also used as a growth-promoter in other countries, including Australia, New Zealand, Canada, Mexico, Chile, Japan, South Africa (Johnson 2015).
ZEA, ZAL and their metabolites were involved in precocious puberty cases in young girls of various countries, such as Italy in 1979, and Puerto Rico in the 1980s.

Other health effects associated with dietary exposure to ZAL residues in beef are not yet fully investigates (Mukherjee et al. 2014).

Zeranol together with DES have been banned as meat additives in the European Union (EU): this fact leads to a EU ban about the import of meat products from the US and Canada.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) set the Allowable Daily Intake (ADI) of ZAL in humans at 0.5 mg/kg body weight, which corresponds to a 35 mg allowable daily intake in a 70 kg adult. Furthermore the use of ZAL has been banned in sports by the International Olympic Committee and World Anti-Doping Agency, because of its anabolic effects (Haiyang et al. 2014; Mukherjee et al. 2014).

Alpha-zearalanol (α-ZAL) (Figure 5), known as zeranol, is a fungal metabolite produced from zearalenone (ZEA): α-ZAL can also occur naturally in some foods, thus its presence in animal products can be partly explained by its ingestion in feed: it can be produced endogenously in ruminants fed by contaminated lots with zearalenone and derivatives (Erasmuson et al. 1994; D'Mello et al. 1999; Zöllner et al. 2002; Zinedine et al. 2007).

Fusarium isolates from grazings in New Zealand produced zearalenone and also metabolites alpha-zearalenol (α-ZEL), beta-zearalenol (β-ZEL), alpha-zearalanol (α-ZAL), beta-zearalanol (β-ZAL) and possibly also zearalanone (ZAN). Also in *in vivo* studies it was not possible determine if the source of the alpha-zearalanol in the urine was extrinsic, due to its present in the fodder, or if it was generated by the animal, through the metabolism of ingested mycotoxins present in the pasture (Erasmuson et al. 1994).

Studies reveals zeranol can naturally be present in the bile of cattle following ingestion of *Fusarium* spp. toxins. Data showed that natural zeranol (α-ZAL) can derive in major part from a-zearalenol (α-ZEL), a molecule separated from zeranol by only one metabolic step (Kennedy et al. 1998).

Humans can be exposed to both ZEA and α-ZAL through consumption of contaminated grains although there were reported cases of inhalation exposure in farm workers.

However the most relevant exposure route for α-ZAL can comes from treated meat (Mukherjee et al. 2014).

The first study of ZEA *in vivo* metabolism was performed by Kiessling and Pettersson, who studied the livers of rats exposed to ZEA, indentifying two important steps: the reduction phase of zearalenone ketone group to zearalenol (ZEL), and the conjugation by glucuronic acid which leads to glucuronide form (Kiessling & Pettersson 1978; Mukherjee et al. 2014).

However the biotransformation of these molecules, especially the conversion rates to different

metabolites can vary widely across species. Furthermore the extents and rates of different metabolic reactions are essential because the metabolites of ZEA differ in their estrogenic potential. The oestrogenicity of these compounds follows this order: α-ZAL, α-ZEL, β-ZAL, ZEA, β-ZEL (Mukherjee et al. 2014).

Phase-I metabolism

The ketone group in ZEA or ZAN is reduced by aliphatic hydroxylation in order to form the corresponding alcohol. ZEA is converted to α-, β-zearalenol (α-ZEL, β-ZEL) (Figure 7) as catalysed by 3 α- and 3 β-hydroxy steroid dehydrogenase (HSD). Studies revealed a relevant variance of the reaction rates among species (Malekinejad et al. 2006; Pfeiffer et al. 2009).
Thus rats seem to produce more β-ZEL, while humans and pigs seem to show a preference towards α-ZEL. Estrogenic effects can vary in each species, due to interspecies differences in metabolism, which make pig the most sensitive animal (Mukherjee et al. 2014).

Phase-II metabolism

In this phase of metabolism occur the glucuronidation and sulfation of the parent compounds or the phase I metabolites occur. The glucuronic acid group is provided by uridine 5'-diphosphate glucuronic acid (UDPGA) and the conversion is performed by uridine 5'-diphosphate glucuronosyltransferase (UGT) (Pfeiffer et al. 2010; Mukherjee et al. 2014).

Excretion

In humans zeranol is mostly excreted by urine, whereas in other species, such as rats, fecal elimination represents the major route of elimination (Baldwin et al. 1983).
Studies reveal zearalanone (ZAN) is a major metabolite of zeranol in humans, while β-ZAL constitutes a minor one. Interestingly mammals dosed with α-ZAL showed no traces of ZEA or α- or β-ZEL: this fact indicates that α-ZAL and β-ZAL can hold a reversible relation with ZAN; but zearanols are not converted to their unsaturated forms in mammals.
In addition a peculiarity of ZAL human metabolism is that both ZAL and ZEA are metabolised to some extent in the intestine (Schaut et al. 2008; Mukherjee et al. 2014).
In cattle α-ZAL is metabolised into β-ZAL and ZAN and are excreted in urine; the presence of

α-ZAL and its metabolites in urine is used as a marker to detect the illegal use of ZAL (Haiyang et al. 2014).

Other studies were performed in pigs and heifers: α-ZAL and β-ZAL were detected in the urine of animals fed ZEA contaminated cereals. According to these facts it can be suggested that in mammals the reduction of α-ZEL, β-ZEL and of ZEA to ZALs and ZAN can negligibly occur, whereas there is no evidence that the opposite can happen (Kleinova et al. 2002; Zöllner et al. 2002).

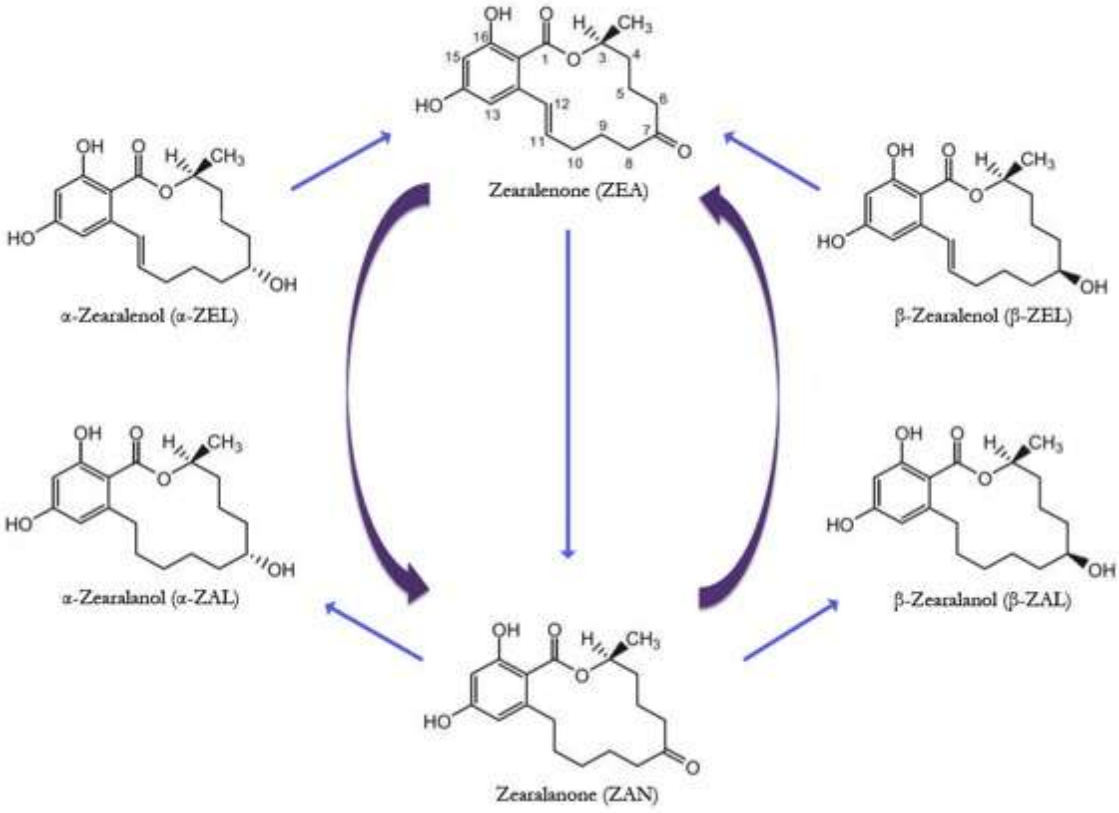


Figure 7. Compounds of the zeranol family of mycotoxins and their biotransformation pathways. Modified and adapted from Mukherjee et al. 2016.

3.1.7 Analytical challenges and current state-of-the-art literature

As previously described the European Union banned the use of many natural and synthetic hormones including α-ZAL and β-ZAL in livestock animals since 1985, therefore no residue of these compounds should be detected in samples of animal origin (Commission of the European Communities 1996a). On the other hand both these molecules can be naturally present in the urine from various animal species which were fed by contamined ZEA lots.

Highly sensitive and specific confirmatory methods capable of achieving very low detection levels are needed in order to identify the residues of resorcylic acid lactones (A4 group) in biological matrices of animal origin (Matraszek-Zuchowska et al. 2013).

However there are still many concerns about how to discriminate between ZEA environmental contamination and α-ZAL abuse: a possible method can rely on testing samples for both for α-ZAL, its metabolites β-ZAL and ZAN, as well as for the presence of ZEA and its metabolites α- and β-ZEL (Launay et al. 2004; Blokland et al. 2006).

Moreover α-ZAL and ZEA can give identical metabolites, which justifies why these metabolites, including α-ZAL itself, can also be detected in ovine urine and bovine bile after *Fusarium* spp. toxin biotransformation.

To discriminate forbidden treatment of zeranol from ingestion of contaminated feed with *Fusarium* spp. toxin, it was suggested that analysis for ZAL abuse would imply quantification of zeranol and its metabolites β-ZAL and of ZEA and its two major metabolites α-ZEL and β-ZEL (Blokland et al. 2006).

The model may help laboratories involved in a ZAL testing, in order to distinguish between illegal use of zeranol and natural contamination. The main principle is based on the comparison of the sum of the α-ZAL and β-ZAL concentrations with the sum for ZEA and its metabolites, α-ZEL and β-ZEL (Blokland et al. 2006).

For many years the detection of RALs was performed by thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), HPLC and fluoroimmunoassay (FIA). These analytical methods are not enough sensitive or specific.

A factor to take into account is that with ELISA and RIA methods it is not possible to detect α-ZAL metabolites and other molecules from RALs simultaneously in only one analysis.

Chromatographic methods play a relevant role in the residue analysis of anabolic substances both for screening and for confirmatory analysis (Stolker & Brinkman 2005). Most of the published confirmatory analysis for α-ZAL and its metabolites in biological matrices is based on

GC/MS: a complex and usually time-consuming method because of derivatisation step (Seo et al. 2005; Blokland et al. 2006). Recently LC coupled with MS or MS/MS also been used for the determination of α-ZAL in animal matrices displaying benefits in terms of sensitivity and specificity (Launay et al. 2004; Matraszek-Zuchowska et al. 2013).

According to EFSA, the results from the European National Residue Control Programmes 2005 - 2010 displayed a "high incidence" of bovine samples non-compliant for α-ZAL; specifically 221 non-compliant samples out of 73626 total samples tested for RALs. Data reveals that the source of the low levels of zeranol and its metabolites detected in these samples may derive from cattle exposure to zearalenone in their diet. However taking account the non-compliant results that have been detected in most years of National Residue Control Programmes testing, which have been ascribed largely to dietary exposure, these molecules can represent a potential concern. Thus it is desirable that monitoring measures for RALs might converge on determining illegal use of these substances in bovine production in the EU (EFSA BIOHAZ Panel 2013). According to Choi et al. blood is the most common matrix in human biomonitoring studies (67%), followed by urine (57%) and hair (35%) (Choi et al. 2015).

A review paper from Appenzeller and Tsatsakis (2012) analysed various research works that described the possibility of identyfing different contaminants in hair, reflecting environmental or occupational exposure (Appenzeller & Tsatsakis 2012). Many papers used hair to monitor and determine different organic pollutants, such as workplace exposure to pesticides, dioxins, polycyclic aromatic hydrocarbons (PAHs) have been determined in hair (Covaci et al. 2002; Toriba et al. 2003; Nakao et al. 2005; Chan et al. 2007). There are also studies on perfluoroalkyl compounds, brominated flame retardants, plastic additives such as bisphenol A and parabens (Martín et al. 2015; Wang et al. 2017; Qiao et al. 2018).

Studies about human biomarkers related to mycotoxins began in the early 1990's to offer an overview into about mechanisms of action of aflatoxin B1.

Moreover aflatoxin M1 was considered a biomarker of exposure, determined in milk and urine during exposure assessments (Gambacorta et al. 2013). Changes in the sphingosine/sphinganine ratio were also used for biomonitoring fumonisins studies in humans (Gilbert et al. 2001; Shephard et al. 2007).

In recent years a large number of mycotoxins and its metabolites have been detected in various biological samples, such as urine and serum; some studies determined these contaminants on less common biological fluids such as breast milk, bile, saliva, nasal secretions, amniotic fluids (Escrivá et al. 2017).

Also the type of animal species in biomonitoring studies in serum are 50% from human

provenance, followed by pig (17%), chicken (13%), rat (9%) and in minor proportion fish, hens, horse.

Studies focusing on mycotoxin urine analysis were performed on human (80%), to a lower extent from pig (9%), bovine (6%), cattle (3%) and rat (2%) (Escrivá et al. 2017).

Sewram et al. (2001) showed for the first time in 2001 and 2003 that FB1, FB2 and FB3 can accumulate respectively in monkey/rat and human hair, as a result of contaminated feed and maize consumption (Sewram et al. 2001; Sewram et al. 2003).

To date there are few papers investigating the presence of mycotoxin in hair: Carrasquillas et al. determined for the first time anabolic compounds such as alpha and beta zearalanol in bovine hair using GC-MS (Hernández-Carrasquilla 2001).

Bordin et al. (2015) identified FB1 levels in human hair from volunteers in Brazil and found low concentrations associated with FB1 daily intake values (Bordin et al. 2015).

More recently Souto et al. (2017) determined fumonisin B1 presence in plasma, urine, feces and hair from pigs using LC-MS/MS (Souto et al. 2017).

3.2 MATERIALS AND METHODS

The present work was conducted to develop a method for quantitative determination of mycotoxin zearalenone and its metabolites (α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone) in bovine and human hair using LC-MS/MS.

This work was funded by the Dutch Ministry of Agriculture (WOT-02-003-070).

3.2.1 List of abbreviations

ACN	Acetonitrile
HCOOH	Formic acid
H_2O	Water
IAC	Immunoaffinity column
ISS	Internal standard solution
MeOH	Methanol
MMRS	Matrix Matched Recovery Standards
MMS	Matrix Matched Standards
MSS	Mix solution standards
PBS	Phosphate buffered saline
RALs	Resorcylic acid lactones

3.2.2 Sampling

Twenty four samples from both bovine and calf hair from The Netherlands, were chosen to perform a three day validation plan (Table 4). Red, brown, black, white, red and white, brown and white, black and white hair samples were collected. Samples were stored in plastic trays in freezer under dark conditions.

Bovine hair	Identification Number
A	Blanco_2005M0718
B	Bovine 408651
C	Bovine 408652
D	Bovine 408653
E	Bovine 408658
F	Bovine 408659
G	Bovine 408661
H	Bovine 408697
I	Calf 3
J	Calf 7
K	Calf 8
L	Calf 9
M	Calf 11
N	Calf 53
O	Calf 76
P	Calf 77
Q	Calf 6
R	Calf 26
S	Calf 27
T	Calf 39
U	Calf 50
V	Calf 67
W	Calf 75
X	Calf 87

Table 4. Bovine and calf hair samples.

3.2.3 Glassware and consumables

- Several pipets;
- Polypropylene (PP) tube 12 mL with screw cap (Greiner 163275);
- 12 mL glass tube (Beldico, 8739007);
- 10 mL glass tube with screw cap (16 x 100 mm Kimble/Kimax USA 45066A-16100);
- Polypropylene tube 50 mL with screwcap (Greiner 210261);
- Glass 2 mL HPLC vial (Waters 186000327c);
- IAC easi-extract zearalenone (R-Biopharm Rhône LTD RP90);

3.2.4 Lab Equipment

- Analytical balance with trueness of 0.02 mg (Mettler AT 261, Marshall Scientific);
- Balance with trueness of 1 mg or better (Mettler PG503-S, Marshall Scientific);
- Laboratory centrifuge (MSE Falcon 6/300);
- Evaporation device (Turbo-Vap LV Zymark 44467);
- Vortex mixer (iKA Vibrofix VF1);
- Milli-Q system with a resistivity of at least 18.2 M $\Omega\,cm^{-1}$ (Merck Millipore);
- Ultrasonic bath (Elma, Elmasonic S100);
- MM400 ball mill (Retsch, Germany) equipped with:
    - ✓ 2 buckets coated from the inside with zirconium oxide,
    - ✓ 5 zirconium oxide balls (d=10 mm).
- SPE system (Alltech) with vacuum pump (KNF Lab Laboport UN 842.3FTP);
- LC-MS/MS system consisting of Waters Acquity pump and injection system. Analytical column: Acquity UPLC CSH 2.1 x 100 mm x 1.7 μm (Waters 186005297).
  Mass spectrometer Micromass Triple Quadrupole XEVO TQS with ESI interface (Waters).

3.2.5 Solvents and reagents

Standards

- α-zearalanol (NMI Australian Government P 1801);
- ß-zearalanol (NMI Australian Government P 1802);
- α-zearalenol (Sigma-Aldrich, St Louis MO, USA, Z0166);
- ß-zearalenol (Sigma-Aldrich, St Louis MO, USA, Z 2000);
- Zearalanone (LGC Standards B-MYC 0580-1);
- Zearalenone (Sigma-Aldrich, St Louis MO, USA, Z-2125);
- α/ß-zearalanol-d4 (RIKILT EU/CRL 006): this is an ampul containing 0.05 mg α-zearalanol-d4 and 0.05 mg ß-zearalanol-d4;
- α-zearalenol-d4 (RIKILT EU/CRL 048): this is an ampul containing 0.1 mg α-zearalenol-d4;
- ß-zearalenol-d4 (RIKILT EU/CRL 049): this is an ampul containing 0.1 mg ß-zearalenol-d4;
- Zearalanone-d6 (TRC Z270442) this is a vial containing 0.5 mg standard;
- Zearalenone-d6 (TRC Z270502) this is a vial containing 1.0 mg standard.

Chemicals

- Acetonitrile (Biosolve 01203502);
- Ammonium formate 97% (Sigma-Aldrich, St Louis MO, USA, 15.626-4);
- Methanol (Biosolve 13683502);
- PBS (Sigma-Aldrich, St Louis MO, USA, P-5368).

Standard solutions

- Stock standard solutions 1000 mg/L: each standard was prepared by dissolving accurately (between 1 and 5 mg ± 0.02 mg) in MeOH. Stock solutions were stored in a freezer for two years period of storage.

- MSS I 10 mg/L: 100 µL of each standard stock solution were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for six months period of storage.

- MSS II 0.1 mg/L: 100 µL of MMS I were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- MSS III 0.01 mg/L: 1000 µL of MMS II were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- Stock solution internal standard I 10 mg/L: 1 mL MeOH was added to each ampoule, placed subsequently in an ultrasonic bath for one minute. Thus it was mixed and transferred in a 5 mL and 10 mL flasks. The content was filled up with MeOH and mixed. The stock solutions were stored in a freezer for five years period of storage.

- Stock solutions internal standard II 1000 mg/L: 0.5 mL MeOH was pipetted in a vial containing zearalanone-d6 internal standard and 1.0 mL MeOH was pipetted in a vial containing zearalenone-d6 internal standard. These solutions were mixed, vortexed and stored in a freezer for five years period of storage.

- ISS I 10 mg/L: 100 µL of each internal standard stock solution 1000 mg/L was pipetted in a 10 mL volumetric flask and made up to volume with MeOH. This solution was stored in refrigerator for six months period of storage.

- ISS II 0.1 mg/L: 100 µL of each internal standard stock solution ISS 10 mg/L were pipetted and put into a volumetric flask of 10 mL, made up to volume with MeOH. This solution was stored in refrigerator for one month period of storage.

- Working standard solution 1.7 µg/L: 40 µL MMS III and 20 µL ISS II were pipetted in a glass tube.

Solvents

- All solvents used for mass spectrometry analysis were LC-MS grade.
- Solvents employed during sample preparation were all analytical grade.
- Milli-Q water, referred to as water from here on.

Prepared solutions

- MeOH/water 40/60 v/v: 400 mL MeOH were mixed in 600 mL of water. This solution was stored at room temperature for three months period of storage.

- Ammonium formate 1 M LC-MS grade: 6.3 gram of ammoniumformate were dissolved in 100 mL of water. This solution was stored at room temperature for three months period of storage.

- ACN/water 90/10 (v/v) LC-MS grade: 900 mL ACN were mixed with 100 mL of water. This solution was stored at room temperature for three months period of storage.

- PBS solution: the content of the packet was dissolved in 1 L of water. This solution (pH 7.4) contained 0.01 M phosphate buffer, 0.138 M NaCl and 0.0027 M KCl.

- Mobile phase A: Ammonium formate (1M)/HCOOH/water 2/0.5/1000 (v/v/v): 2 mL of ammoniumformate (1M) were mixed with 0.5 mL of HCOOH and 1000 mL of water LC-MS grade. This solution was stored at room temperature for one month period of storage.

- Mobile phase B: Ammonium formate (1M)/HCOOH/ACN/water 2/0.5/900/100 (v/v/v/v): 2 mL ammoniumformate (1M) were mixed with 0.5 mL HCOOH and 1000 mL ACN/water 90/10 LC-MS grade. This solution was stored at room temperature one month period of storage.

3.2.6 Sample preparation and sample series

- Sample pretreatment

  A bucket, equipped with 5 balls, was filled with about 500 mg hair. The content was pulverised for 4 minutes at 25 Hz, subsequently pulverised hair were transferred in a 12 mL polypropylene (PP) tube.

- Sample series composition for each validation day

  - ✓ 6 blank hair samples which were spiked before processing (MMS), according to table 5;
  - ✓ blank samples which were included in each series;
  - ✓ hair samples;
  - ✓ MMRS sample obtained from blank hair sample which was spiked with matrix standard solution only after processing, according to table 5;
  - ✓ working standard solution.

- MMS, MMRS preparation

  Calibration was performed using MMS samples, while recovery rate was obtained using MMRS sample. Hair samples were weighed and put in polypropylene tubes (12 mL); subsequently samples were spiked with standard solution (normal standard and internal one), according to table 5. Then spiked samples were mixed with vortex and were let stand for 15 minutes at room temperature.

Matrix Matched Standards	µL MSS III (0.01 mg/L)	µL MSS II (0.1 mg/L)	µL ISS I (0.1 mg/L)
MMS A (0 µg/kg)			10
MMS B (0.25 µg/kg)	5		10
MMS C (0.5 µg/kg)	10		10
MMS D (1.0 µg/kg)	20		10
MMS E (2.0 µg/kg)	40		10
MMS G (5.0 µg/kg)		10	10
MMRS A (1.0 µg/kg)	20		10

Table 5. Scheme preparation of matrix matched standards (MMS) and matrix matched recovery standard (MMRS) used for each validation day.

- Sample preparation

  200 mg of samples were weighed and put in polypropylene tubes (12 mL), subsequently 10 µL ISS I (0.1 mg/l) were added to each sample. Finally they were mixed with vortex and were let stand for 15 minutes at room temperature.

- Extraction

  2 mL MeOH were added to each hair samples and then they were vortexed.
  Samples tubes were placed in ultrasonic bath for one hour, subsequently they were centrifuged for 5 min at 3600 G in order to obtain the upper layer, which was transferred in a new 50 mL polypropylene tube. Finally 11 mL PBS solution were added and mixed.

- Sample clean-up IAC

  IAC columns were placed onto a SPE unit: these columns were first conditioned with the filling solution being present at each IAC and then with 3 mL PBS solution.

Hair samples were loaded on to the columns and drained through the columns under gravity, at a speed of one drop per second. Then the columns were washed with 10 mL PBS solution followed by 10 mL of water to remove any impurities. Subsequently a gentle vacuum was applied for a few seconds and finally the valve was closed. All the solutions, which passed through the column until this step, were discarded.

Elution of target analytes was started with the addition of 2 mL ACN, before being stopped for 15 min after collecting the first 4-5 drops. Sufficient contact time with ACN was necessary to promote the unbinding between analytes and antibodies. After 15 minutes the eluition was restarted collecting the remaining volume of ACN by gravity. The eluate was evaporated to dryness at 55 °C under a gentle stream of nitrogen.

Subsequently the residue was reconstituted in 120 µL MeOH/water 40/60 and vortexed for few seconds. The final extract was transferred into a 2 mL conical vial for injection into the LC-MS/MS system.

A scheme resuming the extraction/clean-up protocol is presented by figure 8.

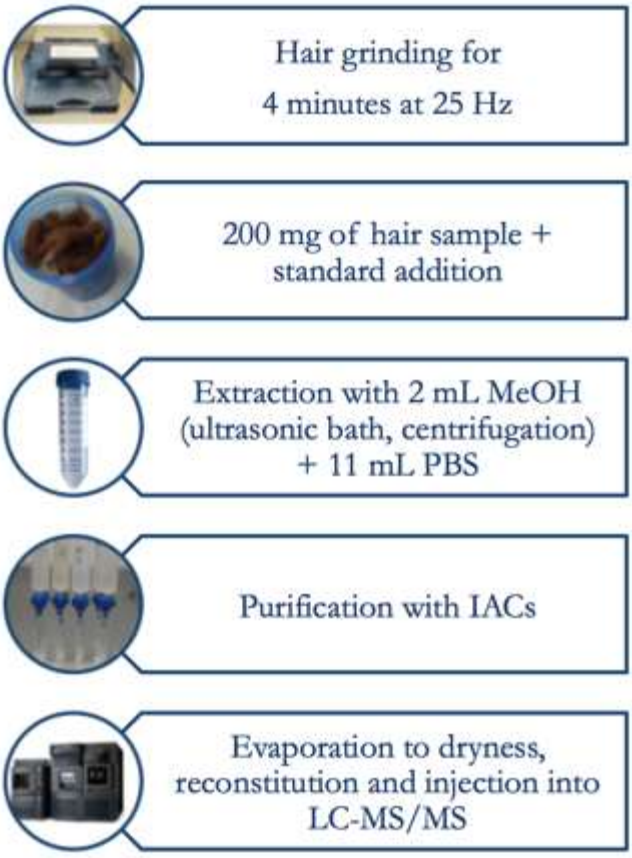


Figure 8. Sample extraction/clean-up scheme.

3.2.7 Analytical conditions

- LC conditions

Analytical column: Acquity UPLC CSH C_{18} 2.1 * 100 mm* 1.7 μm
Column temperature: 50°C
Vial tray temperature: 10°C
Flow: 0.5 mL/min
Injection volume: 10 μL
Gradient: see table 6
Run time: 10 minutes

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
2	50	50
6	30	70
7	0	100
8	0	100
9	80	20

Table 6. Gradient of chromatographic program.

- MS conditions

See the tables below (table 7 and 8).

Parameters	
Ionisation mode	ESI negative
Capillary voltage	2.5 kV
Cone	Component depends V
Source temperature	150°C
Desolvation temperature	600°C
Desolvation gas flow	800 L/hour
Cone gas flow	150 L/hour
Multiplier	750
Nebuliser gas flow (bar)	7
LM 1 resolution	3.0
HM 1 resolution	15.0
Ion energy 1	1.9
LM 2 resolution	3.0
HM 2 resolution	15.0
Ion energy 2	1.3
CID gas	Argon, p = 2.5 x 10^{-3} mbar purity > 99.998%
CID gas flow	0.22 mL/min

Table 7. Mass spectrometry parameters.

Compounds	Precursor	Product	Cone (V)	CE (eV)	IS
zearalenone	317.15	131.15	44	30	zearalenone-d6
		175.15	44	24	
zearalanone	319.15	275.15	30	21	zearalanon-d6
		205.15	30	22	
α/ß-zearalenol	319.15	160.15	30	30	α/ß-zearalenol-d4
		275.15	30	21	
α/ß-zearalanol	321.15	277.15	56	20	α/ß-zearalanol-d4
		259.15	56	26	
α/ß-zearalenol-d4	323.15	160.15	45	32	
α/ß-zearalanol-d4	325.15	281.15	50	20	
zearalanon-d6	325.15	281.15	50	20	
zearalenone-d6	323.15	175.15	50	23	

Table 8. Guidelines MS/MS fragmentation. Theoretical mono-isotopic masses of the precursor ions and product ions are shown in table. The deviation for every mass is ± 0.5 Da.

3.2.8 Method validation in bovine hair

- Bovine validation scheme

  In the table below (divided in two pages) (table 9) is reported the validation scheme.

Sample type	Number	Conc. µg/kg	Day 1 Validation		Day 2 Validation		Day 3 Validation *	
			Letter	ID Number	Letter	ID Number	Letter	ID Number
MMS	1	0	**A**	Blanco_2005M0718	**I**	Calve3	**Q**	Calve6
MMS	2	0.25	**A**	Blanco_2005M0718	**I**	Calve3	**Q**	Calve6
MMS	3	0.5	**A**	Blanco_2005M0718	**I**	Calve3	**Q**	Calve6
MMS	4	1	**A**	Blanco_2005M0718	**I**	Calve3	**Q**	Calve6
MMS	5	2	**A**	Blanco_2005M0718	**I**	Calve3	**Q**	Calve6
MMS	6	5	**A**	Blanco_2005M0718	**I**	Calve3	**Q**	Calve6
MMRS	7	1	**A**	Blanco_2005M0718	**I**	Calve3	**Q**	Calve6
MMRS	8	1	**I**	RIK_408668	**-**	-	**-**	-
MMRS	9	1	**Q**	RIK_408698	**-**	-	**-**	-
Sample	10	0	**B**	RIK_408651	**J**	Calve7	**R**	Calve26
Sample	11	0	**C**	RIK_408652	**K**	Calve8	**S**	Calve27
Sample	12	0	**D**	RIK_408653	**L**	Calve9	**T**	Calve39
Sample	13	0	**E**	RIK_408658	**M**	Calve11	**U**	Calve50
Sample	14	0	**F**	RIK_408659	**N**	Calve53	**V**	Calve67
Sample	15	0	**G**	RIK_408661	**O**	Calve76	**W**	Calve75
Sample	16	0	**H**	RIK_408697	**P**	Calve77	**X**	Calve87
Sample	17	0.5	**B**	RIK_408651	**J**	Calve7	**R**	Calve26
Sample	18	0.5	**C**	RIK_408652	**K**	Calve8	**S**	Calve27
Sample	19	0.5	**D**	RIK_408653	**L**	Calve9	**T**	Calve39
Sample	20	0.5	**E**	RIK_408658	**M**	Calve11	**U**	Calve50
Sample	21	0.5	**F**	RIK_408659	**N**	Calve53	**V**	Calve67
Sample	22	0.5	**G**	RIK_408661	**O**	Calve76	**W**	Calve75
Sample	23	0.5	**H**	RIK_408697	**P**	Calve77	**X**	Calve87

Sample type	Number	Conc µg/kg	Day 1 Validation		Day 2 Validation		Day 3 Validation *	
			Letter	ID Number	Letter	ID Number	Letter	Letter
Sample	24	1	**B**	RIK_408652	**J**	Calve7	**R**	Calve26
Sample	25	1	**C**	RIK_408652	**K**	Calve8	**S**	Calve27
Sample	26	1	**D**	RIK_408653	**L**	Calve9	**T**	Calve39
Sample	27	1	**E**	RIK_408658	**M**	Calve11	**U**	Calve50
Sample	28	1	**F**	RIK_408659	**N**	Calve53	**V**	Calve67
Sample	29	1	**G**	RIK_408661	**O**	Calve76	**W**	Calve75
Sample	30	1	**H**	RIK_408697	**P**	Calve77	**X**	Calve87
Sample	31	1.5	**B**	RIK_408651	**J**	Calve7	**R**	Calve26
Sample	32	1.5	**C**	RIK_408652	**K**	Calve8	**S**	Calve27
Sample	33	1.5	**D**	RIK_408653	**L**	Calve9	**T**	Calve39
Sample	34	1.5	**E**	RIK_408658	**M**	Calve11	**U**	Calve50
Sample	35	1.5	**F**	RIK_408659	**N**	Calve53	**V**	Calve67
Sample	36	1.5	**G**	RIK_408661	**O**	Calve76	**W**	Calve75
Sample	37	1.5	**H**	RIK_408697	**P**	Calve77	**X**	Calve87
Sample	38	1	**B**	RIK_408651	**J**	Calve7	**Q**	Calve6
Sample	39	1	**B**	RIK_408651	**J**	Calve7	**Q**	Calve6
Sample	40	1	**B**	RIK_408651	**J**	Calve7	**Q**	Calve6
Sample	41	1	**B**	RIK_408651	**J**	Calve7	**Q**	Calve6
Sample	42	1	**B**	RIK_408651	**J**	Calve7	**Q**	Calve6
Sample	43	1	**B**	RIK_408651	**J**	Calve7	**Q**	Calve6
Sample	44	1	**B**	RIK_408651	**J**	Calve7	**Q**	Calve6

Table 9. Validation scheme for three validation days.

* Six samples used for robustness were not reported.

- Criteria for individual samples

  For each sample were calculated:

  - ✓ the response factor;
  - ✓ the relative deviation of the relative retention time;
  - ✓ the relative deviation of the ion ratio.

  Calculations used to obtain these parameters are specified below.

- Calculations

  Equation I: ion ratio (R):

$$R = \left(\frac{A_{low}}{A_{high}} \right) \times 100\%$$

  With:

  R = ion ratio (%)

  A_{low} = area of the product ion with the lowest intensity

  A_{high} = area of the product ion with the highest intensity

Equation II: relative deviation of the ion ratio (D):

$$D = \left(\frac{R_{sample} - R_{aver}}{R_{aver}} \right) \times 100\%$$

With:

D = relative deviation of the ion ratio of the compound in the sample compared to the average ion ratio of the compound in MMS B to F before and after samples

R_{sample} = ion ratio of the compound in the sample

$R_{aver.}$ = average ion ratio of the compound in MMS B to F before and after samples

Equation III: response factor (RF)

$$RF = \frac{A_{analyt}}{A_{IS}}$$

With:

RF = response factor

A_{analyt} = sum of the peak areas of the product ions

A_{IS} = area of the product ion of the of the internal standard

Equation IV: concentration in sample (X):

$$X = \frac{RF - b}{a}$$

With:

X = concentration in sample (µg/kg)

RF = response factor, see equation III

b = intercept of the calibration line of the MMS before and after the samples (linear regression*)

a = slope of the calibration line of the MMS before and after the samples (linear regression*)

*They were applied least squares linear regression to the response factor and the concentration.

Equation V: relative retention time (RRT):

$$RRT = \frac{RT_{analyte}}{RT_{IS}}$$

With:

RRT = relative retention time

$RT_{analyte}$ = retention time of the compound

RT_{IS} = retention time of the internal standard

Equation VI: deviation of the relative retention time (ΔRRT)

$$\Delta RRT = \left(\frac{RRT_{sample} - RRT_{aver}}{RRT_{aver}} \right) \times 100\%$$

With:

ΔRRT = deviation of the relative retention time of the compound

RRT_{sample} = relative retention time of the compound, see equation V (%)

RRT_{ave} = average relative retention time of MMS B t/m F before and after samples see equation V (%)

Equation VII: Minimal response of the internal standard to secure the detection:

$$A_{min} = \frac{6 * A_{MMS\_RL}}{S/N}$$

With:

A_{min} = Minimal area internal standard for securing detection at 0.25 µg/kg

A_{MMS_RL} = Area of the least intense product ion of MMS 0.25 µg/kg before the samples

S/N = Signal/noise of the less intense product ion of MMS 0.25 µg/kg before the samples

- Validation criteria

Different samples were used each validation day (table 10):

- ✓ one for linearity (e.g. sample A for day 1);
- ✓ seven different ones for trueness, inter-day repeatability, within-lab reproducibility, selectivity, decision limit CCα, detection capability CCß (e.g. samlples B-H for day 1);
- ✓ one sample for intra-day repeatability (e.g. sample B for day 1).

Since no maximum residue limit (MRL) have been established for zearanols determination in hair, a guide value (GV) of 1 μg/kg has been set for all analytes.

Day	Linearity Recovery (MMS MMRS)	Trueness Within-lab reproducibility Inter-day repeatability CC_{α} $CC_{ß}$	Intra-day repeatability	Selectivity	Robustness	Stability MMRS	Total
1	7 (A)	7*3 (B-H)	7*(1xGV) B	7 (B-H)		1(I) 1(Q)	44
2	7 (I)	7*3 (J-P)	7*(1xGV) J	7 (J-P)			42
3	7 (Q)	7*3 (R-X)	7*(1xGV) R	7 (R-X)	6		48

Table 10. Different parameters tested during the validation process.

The following listed criteria were calculated based on the validation schemes during three validation days (table 10):

- ✓ **Linearity**

The linearity of the method was determined with the correlation of MMS calibration lines. The correlation of both lines should be ≥ 0.990.

As reported in table 10, linearity was calculated analysing the same sample type (MMS) at 0 - 0.25 - 0.5 - 1.0 - 2.0 - 5.0 x GV.

- ✓ **Recovery**

The recovery of the method was calculated analysing once the same sample used for linearity, spiking it after sample processing at 1 x GV.

- ✓ **Sensitivity**

The S/N of the less intense product ion of MMS B (Table 10) should be ≥ 6.

- ✓ **Trueness (T)**

Trueness was obtained analysing seven samples spiked at 0.5 – 1.0 - 1.5 x GV (Table 11).
According to 2002/657/EC this parameter at the selected validations levels should be between 50%-120% at 0.5 – 1.0 x GV, 70%-120% at 1.5 x GV.

- ✓ **Within-laboratory reproducibility / Inter-day repeatability/ Intra-day repeatability)**

The first two parameters were calculated analysing seven samples spiked at 0.5 – 1.0 - 1.5 x GV (Table 11), using single factor analysis of variance (ANOVA).
Intra-day repeatability was obtained analysing in seven-fold the same sample spiked at 0.5 – 1.0 - 1.5 x GV (Table 11).
The relative within-laboratory reproducibility (RSD_{RL}) and the relative repeatability (RSD_r) were calculated on the basis of Horwitz equation: these parameters have large values at low concentrations.
Thompson et al. prescribed that at concentrations below 120 μg/Kg, maximum RSD_{RL} can be used as 22%. This criterion results in a maximum RSDr of 14.7% (Horwitz 1980; Thompson 2000), which is applied to intra-day repeatability.

Compound	GV	Range (μg/Kg)	Criteria		
			T (%)	RSD_r (%)	RSD_{RL} (%)
All compounds	1	0.5	50-120	14.7	22.0
		1.0	50-120	14.7	22.0
		1.5	70-110	14.7	22.0

Table 11. Trueness (T), repeatability RSD_r (%) and within-laboratory reproducibility RSD_{RL} (%) criteria.

- ✓ **CCα / CCβ**

Seven different samples spiked at 0.5 – 1.0 - 1.5 x GV were used to calculate these parameters (e.g. B-H for validation day 1, see table 10).

The detection capability CCβ was set at a spiking concentration at which 95% of the sample identity of analyte was confirmed. The decision limit (CCα), per definition is below that value.

- ✓ **Selectivity**

Selectivity represents the discrimination between the analyte and closely related substances (e.g. isomers) or matrix interferents. Seven blank samples per each validation day (e.g. B-H for validation day 1, see Table 10) were tested: these samples were spiked with only internal standard and assessed for the presence or absence of a signal on the expected retention time.

- ✓ **Matrix effect**

Matrix effect was assessed by comparing the response of MMRS and standard prepared in solvent, which were spiked at the same level.

- ✓ **Stability**

In accordance with EC Decision 2002/657/EC the stability in solution and matrix must be determined.

Stability of extracts has been determined through MMRS samples. If the decrease of an analyte is <15% the analyte in the sample extract was considered stable. MMRS stability was assessed after one and two weeks of storage in the freezer respectively.vThe stability of zeranols in sample extracts was further verified by storing validation day 3 series in freezer. After one week the samples were re-analysed and the results were assessed based on validation criteria.

- ✓ **Robustness**

Robustness has been tested by applying three small changes in the method in duplicate. The robustness of the analysis was tested during method development and additionally during the validation. Three small deviations from the method were tested during the validation. This has not been carried out in accordance with the validation plan. Instead of three minor changes in duplicate, six minor changes were carried out and 0.5 µg/kg were tested instead of 1.0 µg/kg (table 12):

- ✓ 50 and 70 min ultrasonic bath instead of 60 minutes;
- ✓ Centrifugation at 3000G and 4000G instead of 3600G;
- ✓ Evaporation at 50 °C and 60°C instead of 55°C.

Level	Procedure	Change
1 * GV	60 min ultrasonic bath	50-70 minutes ultrasonic bath
1 * GV	60 min ultrasonic bath	50-70 minutes ultrasonic bath
1 * GV	Centrifugation at 3600 G	Centrifugation at 3000-4000 G
1 * GV	Centrifugation at 3600 G	Centrifugation at 3000-4000 G
1 * GV	Evaporation at 55^0 C	Evaporation 50°C-60^0 C
1 * GV	Evaporation 55^0 C	Evaporation 50°C-60^0 C

Table 12. Six minor changes for robustness test.

✓ **Change in response factor**

MMS samples were run before and after the samples.

✓ **Maximum deviation relative retention time**

The average relative retention time of the MMS was calculated before and after the sample analysis. The deviation in the relative retention time of each MMS sample was compared to the average; the final result should not exceed 2.5%.

✓ **Maximum deviation ion ratio**

Average ion ratio of the MMS was calculated before and after the samples analysis.
The deviation in the ion ratio of each MMS sample was compared to the average; final results should not exceed the 2002/657/EC criteria in table 13.

Average ion ratio MMS (R)	Permitted relative deviation (D)
R > 50%	≤20%
20% < R ≤ 50 %	≤25%
10% < R ≤ 20 %	≤30%
R ≤ 10%	≤50%

Table 13. Maximum permitted tolerances for relative ion intensities according to EU criteria.

✓ **Securing the detection**

To secure the reporting limit, every sample was checked by the signal of its internal standard. The minimal area of the internal standard in a sample was calculated where the reporting limit is secured using equation VII.

3.2.9 Method development in bovine hair

Many preliminary experiments were performed to set-up the final protocol (see 3.2.6 and 3.2.7 sections for the final protocol).

- This first part will focus on extraction-clean-up trials investigating the best extraction and clean-up method: Experiment A, B, C, D;
- The second part will focus on LC settings trials: Experiment E.

3.2.9.1 Extraction/Clean-up set-up

- **<u>Experiment A</u>**

Sampling

Same blank bovine hair sample was used.

Glassware and consumables

- Several pipets;
- Polypropylene (PP) tube 12 mL with screw cap (Greiner 163275);
- 12 mL glass tube (Beldico, 8739007);
- 10 mL glass tube with screw cap (16 x 100 mm Kimble/Kimax USA 45066A-16100);
- Polypropylene tube 50 mL with screwcap (Greiner 210261);
- Glass 2 mL HPLC vial (Waters 186000327c);
- SPE Bond Elut C_{18} (50 mg, 3 mL; Agilent, USA);
- Bond Elut-NH_2 (1g, 6 mL; Agilent, USA);
- Nitrile gloves.

Lab equipment

See 3.2.4 section.

Solvents and reagents

- Standards

  see 3.2.5 section.

- Chemicals

  - ✓ Acetonitrile (Biosolve 01203502);
  - ✓ MeOH (Biosolve 13683502);
  - ✓ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (≥98%) (Sigma-Aldrich, St Louis MO, USA);
  - ✓ Acetone (Biosolve BV, The Netherlands);
  - ✓ Methyl- tert-butyl ether (MTBE) (Sigma-Aldrich, St Louis MO, USA).

- Standard solutions

  - ✓ MSS I 0.01 mg/L: 1000 µL of mix standard solution were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

  - ✓ ISS I 0.1 mg/L: 100 µL of each internal standard solution were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- Solvents

  See 3.2.5 section.

- Prepared solutions

  - ✓ MeOH/water 40/60 v/v: 40 mL MeOH were mixed in 60 mL of water. This solution was stored at room temperaturefor three months period of storage;
  - ✓ MeOH/water 80/20 v/v: 80 mL MeOH were mixed in 20 mL of water. This

solution was stored at room temperaturefor three months period of storage;

- ✓ acetone/MeOH 80/20 v/v: 80 mL of acetone were mixed in 20 mL MeOH. This solution was stored at room temperaturefor three months period of storage;

Sample preparation

- Sample pretreatment, sample series composition

  See section 3.2.6.

- MMS preparation

  See section 3.2.6 and the scheme preparation of MMS/MMRS (table 14).

Sample	Type	Spiking level µg/kg	µL MSS I (0.01mg/L)	ISS I (0.1 mg/L)
Blank Hair	MMS	0	0	40
Blank Hair	MMS	0.25	5	40
Blank Hair	MMS	0.5	10	40
Blank Hair	MMS	1	20	40
Blank Hair	MMRS	1	20	40

Table 14. Spiking level of MMS and MMRS.

- Sample preparation

  See 3.2.6 section.

- Extraction

1. 200 mg of bovine hair were weighed accurately in 15 mL in polypropylene tube (PP), moreover MMS, MMRS, samples were spiked, as reported in table 12;
2. 2 mL Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (7.25 mg/ml water) were added and vortexed for few seconds;
3. PP tubes were put into head over head rotator (position 4) for one hour. Subsequently 4 ml MeOH HPLC grade were added;
4. PP tubes were centrifugated for 5 minutes at 1700 G and the upper (organic) layer was transferred into a new PP tube, with the addition of 5 ml distilled water.

- Sample clean-up

This clean-up method was made on the basis of a previous modified protocol, set-up for the zearanols determination in urine, which is summarised below.

5. SPE C_{18} cartridges were placed onto a SPE unit and conditioned with 5 mL MeOH followed by 5 mL of water;
6. samples were loaded onto C_{18} SPE cartridges and washed with 5 ml MeOH/water 40/60;
7. SPE cartridges were dried before the next step by applying slight vacuum;
8. eluition of target analytes were performed with 5 mL MeOH/water 80/20, then the eluate was collected in a glass tube and evaporated to dryness at 50°C under a gentle steam of nitrogen;
9. the dry residue was reconstituted in 5 mL acetone/MeOH 80/20;
10. meantime another SPE NH_2 cartridges were conditioned with 5 mL of acetone/MeOH 80/20;
11. samples, previously reconstituted in 5 mL acetone/MeOH 80/20; were loaded onto SPE NH_2 cartridge, then the eluate was collected into a glass tube and evaporated at 60°C under a gentle steam of nitrogen;
12. the final residue was redissolved in 120 μL of MeOH/water 40/60.

Analytical conditions

- LC conditions

  The mobile phases used were different from the final method: A1 water; B1 acetonitrile, using the same column and same gradient specified in the final set-up method (see 3.2.7 Analytical conditions).

- MS conditions

  See section 3.2.7.

Aim

It was decided to compare four different clean-up methods to investigate the best one:

- "Only C_{18}" cartridges: this protocol involved performing the above mentioned steps from 1 to 9, and then inject in the LC-MS/MS system.
- "C_{18} + NH_2" cartridges: this protocol involved the execution of the above mentioned steps from 1 to 12, finally injection in LC-MS/MS system was performed.
- "MTBE (methyl- tert-butyl ether) + C_{18} cartridges": this protocol involved MTBE addition (2mL) after step 2, and performing the protocol only until step 9.
- "MTBE + C_{18}+NH_2 cartridges": this protocol involved MTBE addition (2mL) after step 2, and then performing the protocol until step 12.

- **Experiment B**

Sampling

Three blank bovine hair samples were used: sample 646, 647, 647. This experiment was done in triplicate.

Glassware and consumables

- Several pipets;
- Polypropylene (PP) tube 12 mL with screw cap (Greiner 163275);
- 12 mL glass tube (Beldico, 8739007);
- 10 mL glass tube with screw cap (16 x 100 mm Kimble/Kimax USA 45066A-16100);
- Polypropylene tube 50 mL with screwcap (Greiner 210261);
- Glass 2 mL HPLC vial (Waters 186000327c);
- SPE Bond Elut C_{18} (50 mg, 3 mL; Agilent, USA);
- Bond Elut-NH_2 (1g, 6 mL; Agilent, USA);
- Nitrile gloves.

Lab equipment

See 3.2.4 section.

Solvents and reagents

- Standards

  see 3.2.5 section.

- Chemicals

  - ✓ Acetonitrile (Biosolve 01203502);
  - ✓ MeOH (Biosolve 13683502);
  - ✓ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (≥98%) (Sigma-Aldrich, St Louis MO, USA);
  - ✓ Acetone (Biosolve BV, The Netherlands);
  - ✓ Proteinase K from Tritirachium (>800 units/mL, Sigma Aldrich, St Louis MO, USA).
  - ✓ Tris-buffer (hydroxymethyl aminomethane) (Merck, Darrmstadt, Germany);
  - ✓ sodium hydroxide solution (Sigma Aldrich, St Louis MO, USA).

- Standard solutions

  - ✓ ISS I 0.1 mg/L: 100 µL of each internal standard solution were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- Solvents

  See 3.2.5 section.

- Prepared solutions

  - ✓ MeOH/water 40/60 v/v: 40 mL MeOH were mixed in 60 mL of water.
    This solution was stored at room temperaturefor three months period of storage;
  - ✓ MeOH/water 80/20 v/v: 80 mL MeOH were mixed in 20 mL of water.
    This solution was stored at room temperaturefor three months period of storage;

- ✓ acetone/MeOH 80/20 v/v: 80 mL of acetone were mixed in 20 mL MeOH. This solution was stored at room temperaturefor three months period of storage;
- ✓ Tris-Buffer solution 50 mM: 0.6057 g of Tris-Buffer were dissolved in 100 mL of water. This solution was stored at room temperature for three months period of storage.
- ✓ Proteinase K: 40 mg of this enzyme were dissolved in 20 mL Tris-Buffer solution 50 mM.
- ✓ Sodium hydroxide 2M: 2 mg NaOH were dissolved in 25 mL of water. This solution was stored at room temperature for three months period of storage.

Sample preparation

- Sample pretreatment, sample series composition

  See section 3.2.6. No MMS and MMRS were prepared for this experiment.

- MMS preparation

  No MMS and MMRS were prepared for this experiment.

- Sample preparation

  See 3.2.6 section.

- Extraction methods

  Three different extraction methods were tested:

  1. "TCEP" protocol: the same extraction method which was performed in "Experiment A", for the protocol see 3.2.9.1 section, except for the spiking levels: samples were spiked with only 30 μL ISS I (0.1 mg/L);

2. "Proteinase K" protocol:
    - ✓ 200 mg of bovine hair were weighed accurately in 15 mL in polypropylene tube (PP), subsequently they were spiked with 30 µL ISS I (0.1 mg/L);
    - ✓ 1850 µL Tris Buffer and 150 µL Proteinase K were added. The final pH was adjusted to 7.4 pH using few drops of 2M sodium hydroxide solution;
    - ✓ Samples were placed in shaker at 37°C overnight;
    - ✓ 4 mL MeOH were added, subsequently samples were vortexed and centrifuged for 5 min at 1700 G;
    - ✓ supernatant was collected in a new PP tube;
    - ✓ 5 mL of water were added and samples were vortexed for few seconds.

3. "ACN overnight" protocol:

    - ✓ 200 mg of bovine hair were weighed accurately in 15 mL in polypropylene tube (PP), subsequently they were spiked with 30 µL ISS I (0.1 mg/L);
    - ✓ 2 mL ACN were added to each sample;
    - ✓ samples were put in an ultrasonic bath at 40 °C overnight;
    - ✓ 5 mL of water were added, subsequently samples were vortexed and centrifuged for 5 min at 1700 G;
    - ✓ supernatant was collected in a new PP tube;
    - ✓ 5 mL of water were added and samples were vortexed for few seconds.

- <u>Sample clean-up</u>

  C_{18} +NH_2 cartridges were used for clean-up step (see Experiment A).

Analytical conditions

- LC conditions

  See Experiment A.

- MS conditions

  See section 3.2.7.

  Aim

  It was decided to compare three different extraction methods (in triplicate) to investigate which was the best one.

- **Experiment C**

Sampling

The same blank bovine hair sample was used. This experiment was done in triplicate.

Glassware and consumables

- Several pipets;
- Polypropylene (PP) tube 12 mL with screw cap (Greiner 163275);
- 12 mL glass tube (Beldico, 8739007);
- 10 mL glass tube with screw cap (16 x 100 mm Kimble/Kimax USA 45066A-16100);
- Polypropylene tube 50 mL with screwcap (Greiner 210261);
- Glass 2 mL HPLC vial (Waters 186000327c);
- IAC easi-extract zearalenone (R-Biopharm Rhône LTD RP90);
- Nitrile gloves.

Lab equipment

See 3.2.4 section.

Solvents and reagents

- Standards

  See 3.2.5 section.

- Chemicals

  - ✓ Acetonitrile (Biosolve 01203502);
  - ✓ MeOH (Biosolve 13683502);
  - ✓ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (≥98%) (Sigma-Aldrich, St Louis MO, USA);
  - ✓ Acetone (Biosolve BV, The Netherlands);

- Standard solutions

  - ✓ ISS I 0.1 mg/L: 100 µL of each internal standard solution were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- Solvents

  See 3.2.5 section.

- Prepared solutions

  None.

Sample preparation

- Sample pretreatment, sample series composition

  See section 3.2.6. No MMS and MMRS were prepared for this experiment.

- MMS preparation

  No MMS and MMRS were prepared for this experiment.

- Sample preparation

  See 3.2.6 section.

- Extraction methods

  Three different extraction methods were tested:

  1. "MeOH overnight" protocol:

     - ✓ 200 mg of bovine hair were weighed accurately in 15 mL in polypropylene tube (PP), subsequently they were spiked with 30 µL ISS I (0.1 mg/L);
     - ✓ 2mL MeOH were added, subsequently samples were vortexed and were put in an ultrasonic bath at 40 °C overnight;
     - ✓ 5 mL of water were added, then samples were vortexed for few seconds and subsequently centrifuged for 5 min at 1700 G;
     - ✓ supernatant was collected in a new PP tube;
     - ✓ 5 mL of water were added and samples were vortexed for few seconds.

  2. "ACN overnight" protocol: the method performed was previously described in Experiment B "ACN overnight";

  3. "TCEP" protocol: the method performed was previously described in Experiment A "TCEP"(Experiment A).

- Sample clean-up

  A different clean-up was utilised involving the immunoaffinity columns (IACs), for the IACs protocol followed see the "3.2.6 Sample preparation" section.

Analytical conditions

- LC conditions

  See section Experiment A.

- MS conditions

  See section 3.2.7.

Aim

Three different extraction protocols, which adopted a new clean-up methods (IACs), were compared in order to establish which one was the most suitable in terms of sensibility and peak shapes.

- **Experiment D**

Sampling

The same blank bovine hair sample was used. This experiment was done in triplicate.

Glassware and consumables

- Several pipets;
- Polypropylene (PP) tube 12 mL with screw cap (Greiner 163275);
- 12 mL glass tube (Beldico, 8739007);
- 10 mL glass tube with screw cap (16 x 100 mm Kimble/Kimax USA 45066A-16100);
- Polypropylene tube 50 mL with screwcap (Greiner 210261);
- Glass 2 mL HPLC vial (Waters 186000327c);
- IAC easi-extract zearalenone (R-Biopharm Rhône LTD RP90);
- SPE Bond Elut C_{18} (50 mg, 3 mL; Agilent, USA);
- Bond Elut-NH_2 (1g, 6 mL; Agilent, USA);
- Nitrile gloves.

Lab equipment

See 3.2.4 section.

Solvents and reagents

- Standards

  See 3.2.5 section.

- Chemicals

  - ✓ Acetonitrile (Biosolve 01203502);
  - ✓ MeOH (Biosolve 13683502);

- ✓ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (≥98%) (Sigma-Aldrich, St Louis MO, USA);
- ✓ Acetone (Biosolve BV, The Netherlands);

- Standard solutions

  - ✓ ISS I 0.1 mg/L: 100 µL of each internal standard solution were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- Solvents

  See 3.2.5 section.

- Prepared solutions

  - ✓ MeOH/water 40/60 v/v: 40 mL MeOH were mixed in 60 mL of water. This solution was stored at room temperaturefor three months period of storage;
  - ✓ MeOH/water 80/20 v/v: 80 mL MeOH were mixed in 20 mL of water. This solution was stored at room temperaturefor three months period of storage;
  - ✓ acetone/MeOH 80/20 v/v: 80 mL of acetone were mixed in 20 mL MeOH. This solution was stored at room temperaturefor three months period of storage;

Sample preparation

- Sample pretreatment, sample series composition

  See section 3.2.6. No MMS and MMRS were prepared for this experiment.

- MMS preparation

  No MMS and MMRS were prepared for this experiment.

- Sample preparation

  See 3.2.6 section.

- Extraction methods

  Three different extraction methods were tested:

  1. "IAC extraction" protocol: same of "MeOH overnight" (Experiment C);
  2. "C_{18}+NH_2 (MeOH)" protocol: same of "MeOH overnight" (Experiment C);
  3. "C_{18}+NH_2 (TCEP)" protocol: same of "TCEP" protocol (Experiment B);

- Sample clean-up

  1. "IAC extraction" protocol: it was performed IACs clean-up (see Experiment C);
  2. "C_{18}+NH_2 (MeOH)" protocol: it was performed C_{18}+NH_2 clean-up method (see Experiment A);
  3. C_{18}+NH_2 (TCEP): it was performed C_{18}+NH_2 clean-up method (see Experiment A).

Analytical conditions

- LC conditions

  See Experiment A.

- MS conditions

  See section 3.2.7.

Aim

Different extraction/clean-up methods were tested to investigate which was the best one.

3.2.9.2 LC conditions set-up

- **Experiment E**

Sampling

Samples from previous experiments (A-D) were used.

Glassware and consumables

None.

Lab equipment

See 3.2.4 section.

Solvents and reagents

- Standards

  None.

- Chemicals

  Acetonitrile (Biosolve 01203502);
  Ammonium formate 97% (Sigma-Aldrich, St Louis MO, USA, 15.626-4);

- Standard solutions

  None.

- Solvents

  See 3.2.5 section.

- Prepared solutions

  - ✓ MeOH/water 40/60 v/v: 400 mL MeOH were mixed in 600 mL of water. This solution was stored at room temperature for three months period of storage.

  - ✓ Ammonium formate 1 M LC-MS grade: 6.3 gram of ammoniumformate were dissolved in 100 mL of water. This solution was stored at room temperature for three months period of storage.

  - ✓ ACN/water 90/10 (v/v) LC-MS grade: 900 mL ACN were mixed with 100 mL of water. This solution was stored at room temperature for three months period of storage.

Sample preparation

None.

Analytical conditions

- LC conditions

  In this experiment were optimised:

  - ✓ mobile phases:

    samples from previous experiments were injected both with:

    - ❖ A: water,
      B: acetonitrile;
    - ❖ A: 2mM Ammonium Formiate, 0.05% Formic Acid, in H_2O,
      B: 2mM Ammonium Formiate, 0.05% Formic Acid, in ACN: H_2O (90:10).

- ✓ flow rate

  different flow rates were tested (0.4, 0.5, 0.6 mL/min).

3.2.10 Applicability of the method on human hair

The applicability of the method set-up on bovine hair was also tested on human hair to perform:

- one day validation;
- applicability of the method for the analysis of other human hair samples.

The protocol was adapted from the bovine hair method (See 3.2 Materials and Methods), so only small changes to the bovine method were applied.

- **List of abbreviations**

  See section 3.2.1.

- **Sampling**

  Human hair samples were collected from eight volunteers from the Gelderland area, during regular hair cuts (The Netherlands).

  ✓ <u>One day validation</u>

  Both blond, brown, white hair samples were collected between April-July 2017. Samples were stored in aluminium foils at room temperature, under dry and dark conditions (table 15).

Hair	Characteristics
A	Woman 21 years old
B	Woman 52 years old
C	Boy 6 years old
D	Man 21 years old
E	Man 75 years old
F	Woman 48 years old
G	Boy 17 years old
H	Man 63 years old

Table 15. Donors' human hair sample information.

✓ Applicability of the method for the analysis of other human hair samples

Both blond, brown, white hair samples were collected between April-July 2017. Samples were stored in aluminium foils at room temperature, under dry and dark conditions (Table 16).

Hair	Characteristics	Country
A	Woman 20 years old	The Netherlands
B	Woman 59 years old	The Netherlands
C	Boy 15 years old	The Netherlands
D	Man 63 years old	The Netherlands
E	Woman 50 years old	The Netherlands
F	Woman 52 years old	The Netherlands
G	Man 26 years old	The Netherlands
H	Woman 65 years old	The Netherlands
I	Man 65 years old	Italy
J	Man 73 years old	The Netherlands
K	Boy 11 years old	The Netherlands
I	Woman 71 years old	The Netherlands
L	Woman 67 years old	The Netherlands
M	Woman 30 years old	Italy
N	Man 23 years old	The Netherlands
O	Man 62 years old	The Netherlands
P	Man 27 years old	Germany

Table 16. Donors' human hair sample information.

- **Glassware and consumables**

- Several pipets;
- Polypropylene (PP) tube 12 mL with screw cap (Greiner 163275);
- Whatman 595 ½ folded filters (diameter 150 mm) (GE Healthcare Life Sciences);
- 12 mL glass tube (Beldico, 8739007);
- 10 mL glass tube with screw cap (16 x 100 mm) (Kimble/Kimax USA 45066A-16100);
- Polypropylene tube 50 mL with screwcap (Greiner 210261);
- Glass 2 mL HPLC vial (Waters 186000327c);
- IAC easi-extract zearalenone (R-Biopharm Rhône LTD RP90);

- **Lab equipment**

  See 3.2.4 section.

- **Solvents and reagents**

  - ✓ Standards

    See 3.2.5 section.

  - ✓ Chemicals

    Acetonitrile (Biosolve 01203502);
    Ammonium formate 97% (Sigma-Aldrich, St Louis MO, USA, 15.626-4);
    Dichloromethane 99.8% (ActuAll Chemicals, Randneer, The Netherlands);
    Methanol (Biosolve 13683502);
    PBS (Sigma-Aldrich, St Louis MO, USA, P-5368);
    Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (≥98%) (Sigma-Aldrich, St Louis MO, USA);

  - ✓ Standard solutions

    - Stock standard solutions 1000 mg/L: each standard was prepared by dissolving accurately (between 1 and 5 mg ± 0.02 mg) in MeOH. Stock solutions were stored in a freezer for two years period of storage.

    - MSS I 10 mg/L: 100 µL of each standard stock solution were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for six months period of storage.

    - MSS II 0.1 mg/L: 100 µL of MMS I were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

    - MSS III 0.01 mg/L: 100 µL of MMS II were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- MSS IV 0.001 mg/L: 100 µL of MMS III were pipetted into a volumetric flask of 10 mL and made up to volume with MeOH. This solution was stored in a refrigerator for one month period of storage.

- Stock solution internal standard I 10 mg/L: 1 mL MeOH was added to each ampoule, placed subsequently in an ultrasonic bath for one minute. Thus it was mixed and transferred in a 5 mL and 10 mL flasks. The content was filled up with MeOH and mixed. The stock solutions were stored in a freezer for five years period of storage.

- Stock solutions internal standard II 1000 mg/L: 0.5 mL MeOH was pipetted in a vial containing zearalanone-d6 internal standard and 1.0 mL MeOH was pipetted in a vial containing zearalenone-d6 internal standard. These solutions were mixed, vortexed and stored in a freezer for five years period of storage.

- ISS I 10 mg/L: 100 µL of each internal standard stock solution 1000 mg/L was pipetted in a 10 mL volumetric flask and made up to volume with MeOH. This solution was stored in refrigerator for six months period of storage.

- ISS II 0.1 mg/L: 100 µL of each internal standard stock solution ISS I 10 mg/L were pipetted and put into a volumetric flask of 10 mL, made up to volume with MeOH. This solution was stored in refrigerator for one month period of storage.

- ISS III 0.01 mg/L: 100 µL of each internal standard stock solution ISS II 10 mg/L were pipetted and put into a volumetric flask of 10 mL, made up to volume with MeOH. This solution was stored in refrigerator for one month period of storage.

- working standard solution: 80 µL MSS IV and 40 µL ISS III were pipetted in a glass tube. This solution is spiked at the same level as MMRS. This solution was stored in refrigerator for one month period of storage.

- ✓ Solvents

  See 3.2.5 section.

- ✓ Prepared solutions

  See 3.2.5 section.

- **Sample preparation and sample series**

  - ✓ Sample pretreatment

    Human hair were undergone to a decontamination step prior the extraction phase.

    - 1 g of hair was weighed and placed in a falcon tube;
    - 20 mL MilliQ water were first added to each sample, mixed for 5 minutes under head over head rotator;
    - hair samples were dried on folded filter paper for 2 hours;
    - 20 mL dichloromethane were first added to each sample, mixed for 5 minutes under head over head rotator;
    - hair samples were dried on folded filter paper for 30 minutes;
    - hair samples were pulverised through the use of ball mill and a bucket (equipped with 5 balls) which was filled with 500 mg hair. The content was pulverised for 4 minutes at 25 Hz and transferred in a 12 mL polypropylene (PP) tube.

  - ✓ Sample series composition for each validation day

    - 7 blank hair samples which were spiked before processing (MMS), according to table 17;
    - blank samples which were included in each series;
    - hair samples;
    - blank hair sample which was spiked with matrix standard solution only after processing (MMRS), according to table 17;
    - working standard solution.

  - ✓ MMS, MMRS preparation

    Calibration was performed using MMS samples, while recovery rate was obtained using MMRS sample. Hair samples were weighed and put in polypropylene tubes (12 mL); subsequently samples were spiked with standard solution (normal standard and internal one), according to table 17. Then spiked samples were

mixed with vortex and were let stand for 15 minutes at room temperature.

Matrix Matched Standards	µL MSS IV (0.001 mg/L)	µL MSS III (0.01 mg/L)	µL ISS III (0.01 mg/L)
MMS A (0 µg/kg)			20
MMS B (0.025 µg/kg)	10		20
MMS C (0.05 µg/kg)	20		20
MMS D (0.1 µg/kg)	50		20
MMS E (0.1 µg/kg)		10	20
MMS F (0.5 µg/kg)		20	20
MMS G (1 µg/kg)		40	20
MMRS (0.2 µg/kg)	40		20

Table 17. MMS and MMRS spiking levels.

✓ Sample preparation

See section 3.2.6.

✓ Extraction

See section 3.2.6.

✓ Sample clean-up IAC

See section 3.2.6

- **Analytical conditions**

  - ✓ LC conditions

    See section 3.2.7.

  - ✓ MS conditions

    See section 3.2.7.

3.2.11 Method validation on human hair

A one-day validation was carried out.

- Human validation scheme

  In the table below (divided in two parts) (table 18) is reported the validation scheme.

Sample type	Letter	Number	St mix µg/kg
MMS	A	1	0
MMS	A	2	0.05
MMS	A	3	0.1
MMS	A	4	0.25
MMS	A	5	0.5
MMS	A	6	1.0
MMS	A	7	2.0
MMRS	A	8	0.2
Sample	B	9	0
Sample	C	10	0
Sample	D	11	0
Sample	E	12	0
Sample	F	13	0
Sample	G	14	0
Sample	H	15	0
Sample	B	16	0.1
Sample	C	17	0.1
Sample	D	18	0.1
Sample	E	19	0.1
Sample	F	20	0.1
Sample	G	21	0.1
Sample	H	22	0.1

Sample type	Letter	Number	St mix µg/kg
Sample	B	23	0.2
Sample	C	24	0.2
Sample	D	25	0.2
Sample	E	26	0.2
Sample	F	27	0.2
Sample	G	28	0.2
Sample	H	29	0.2
Sample	B	30	0.5
Sample	C	31	0.5
Sample	D	32	0.5
Sample	E	33	0.5
Sample	F	34	0.5
Sample	G	35	0.5
Sample	H	36	0.5
Sample	B	37	0.2
Sample	B	38	0.2
Sample	B	39	0.2
Sample	B	40	0.2
Sample	B	41	0.2
Sample	B	42	0.2
Sample	B	43	0.2

Table 18. Validation scheme for one-day validation.

- Criteria for individual samples

  See section 3.2.8.

- Calculations

  See section 3.2.8.

- Validation criteria

  See section 3.2.8. (See Table 19).
  Since no MRLs have been established for zearanols determination in human hair a guide value (GV) has been established for all compounds: 0.2 μg/kg.

Day	Linearity Recovery (MMS MMRS)	Trueness Within-lab reproducibility CC_α $CC_ß$	Intra-day repeatability	Selectiviy
1	7 (A)	7*3 (B-H)	7*(1xGV) B	7 (B-H)

Table 19. Different parameters tested during one-day validation.

- ✓ **Linearity**: 7x the same sample at 0 - 0.05 - 0.1 – 0.25 – 0.5- 1.0- 2.0 μg/kg.
- ✓ **Recovery**: 1x same sample as used for linearity after reprocessing at 1 x GV.
- ✓ **Trueness**: 7 * 3 samples at 0.5 - 1 - 2.5 x GV.
- ✓ **Within-laboratory reproducibility**: 7 different samples at 0.5 - 1 - 2.5 x GV.
- ✓ **Intra-day repeatability**: 7 x the same sample at 1 x GV.
- ✓ **CCα / CCβ**: 7 * 3 samples at 0.5 - 1 - 2.5 x GV.
- ✓ **Selectivity**: 7 blank samples.

3.3 RESULTS AND DISCUSSION

Based on current state-of-the-art literature, this was the first work which identified zearalenone and its main metabolites in bovine and human hair using LC-MS/MS, moreover this research evaluated the suitability of this non-conventional matrix for biomonitoring studies.

As specified earlier, hair analysis is a promising alternative to improve the success of surveillance plans, offering multiple advantages compared to other biological matrices (liver, blood, kidney), because it a non-invasive, easy, low cost procedure which can give information about short and long time exposure (Schramm 2008; Esteban & Castaño 2009; Fernández et al. 2014).

Livestock biomonitoring could be important to better understand human exposure to mycotoxins, since this phenomenon may result from contamined plant-derived feed, and leading to carry-over into animal products, such as meat, eggs and milk.

Moreover the set-up method on bovine and human hair can provide useful information regarding zearalenone and its metabolites' occurrence in feedstuffs and foodstuffs, which can represent a huge impact on the agri-food sector and the economy.

The current research could represent a useful tool to evaluate natural feed contamination or detect not legal use of α-zearalanol in bovine, and to conduct an initial inventory of the occurrence of RALs as biomarkers for ZEA exposure in bovine and human hair in future studies.

3.3.1 Set-up and validation method in bovine hair

A multiresidual method was set-up to identify α-ZAL, β-ZAL, ZEA, ZAN, α-ZEL, β-ZEL in bovine hair, using LC-MS/MS technique.

The developed method was suitable to identify all six analytes, which showed satisfactory recoveries (≥ 79%), good linearity ($R^2 \geq 0.99$) and selectivity.

However the method was validated only for three analytes of interest (α -zearalanol, ß-zearalanol, zearalanone), according to European Union Decision 2002/657/EC (European Commission 2002).

For the results of the three validation days (see tables 20, 21 and 22) for the three different analytes (Validation day 1 to 3).

Spike level µg/kg	Day 1	Day 2	Day 3
0.5	0.56	0.50	0.52
0.5	0.55	0.51	0.52
0.5	0.58	0.48	0.46
0.5	0.57	0.48	0.48
0.5	0.55	0.48	0.44
0.5	0.56	0.47	0.49
0.5	0.60	0.52	0.50
1	1.01	1.04	1.04
1	1.10	1.04	1.03
1	1.08	1.10	1.09
1	0.99	1.08	1.05
1	1.06	1.05	1.09
1	1.12	1.04	1.07
1	1.10	1.04	1.03
1.5	1.65	1.53	1.65
1.5	1.60	1.58	1.60
1.5	1.58	1.53	1.52
1.5	1.54	1.52	1.64
1.5	1.67	1.52	1.61
1.5	1.58	1.52	1.57
1.5	1.61	1.48	1.57

Table 20. Validation results α-zearalanol in three different days.

Spike level µg/kg	Day 1	Day 2	Day 3
0.5	0.46	0.48	0.44
0.5	0.51	0.53	0.48
0.5	0.54	0.48	0.44
0.5	0.55	0.46	0.40
0.5	0.54	0.52	0.45
0.5	0.54	0.53	0.50
0.5	0.56	0.42	0.51
1	1.10	1.00	1.14
1	1.08	1.13	1.08
1	1.11	1.04	1.09
1	1.09	1.01	1.08
1	1.01	1.03	1.09
1	1.09	1.09	1.05
1	1.10	0.98	1.05
1.5	1.93	1.54	1.60
1.5	1.45	1.62	1.67
1.5	1.41	1.51	1.66
1.5	1.58	1.49	1.75
1.5	1.63	1.61	1.66
1.5	1.60	1.47	1.65
1.5	1.68	1.41	1.71

Table 21. Validation results for β-zearalanol in three different days.

Spike level µg/kg	Day 1	Day 2	Day 3
0.5	0.64	0.50	0.43
0.5	0.47	0.56	0.48
0.5	0.73	0.50	0.41
0.5	0.51	0.49	0.48
0.5	0.50	0.54	0.41
0.5	0.55	0.55	0.46
0.5	0.61	0.55	0.61
1	1.30	1.03	1.00
1	1.33	1.18	0.99
1	1.13	1.20	1.08
1	0.87	1.26	1.07
1	1.02	1.27	1.03
1	1.11	1.19	0.96
1	1.15	1.22	1.37
1.5	1.30	1.66	1.55
1.5	1.86	1.86	1.39
1.5	1.42	1.70	1.47
1.5	1.39	1.79	1.55
1.5	1.48	1.93	1.46
1.5	1.49	1.76	1.45
1.5	1.63	1.84	1.98

Table 22. Validation results zearalanone in three different days.

Compounds	Level	µg/kg	Trueness (%)	RSD_r (%)	RSD_{RL} (%)
Zearalanone	0.5*GV	0.5	104.6	13.1	15.7
	1.0*GV	1.0	113.0	11.4	11.9
	1.5*GV	1.5	107.8	10.2	13.4
α-Zearalanol	0.5*GV	0.5	103.1	4.3	9.5
	1.0*GV	1.0	105.9	3.3	3.5
	1.5*GV	1.5	105.0	2.6	3.6
ß-Zearalanol	0.5*GV	0.5	98.5	7.8	10.1
	1.0*GV	1.0	106.8	3.7	4.1
	1.5*GV	1.5	106.8	6.9	7.9

Table 23. Trueness, RSDr (Repeatability) and RSD_{RL} (Within-laboratory reproducibility) based on three validation days (seven different bovine hairs per each day).

Compounds	Level	µg/kg	RSD_r (%)
Zearalanone	1.0*GV	1.0	9.5
α-Zearalanol	1.0*GV	1.0	5.4
ß-Zearalanol	1.0*GV	1.0	6.4

Table 24. RSDr based on three validation days (ANOVA method, 7 x the same hair).

For zearalanone, trueness lied between 104.6 and 113%, for α-Zearalanol between 103.1 and 105.9%, for ß-Zearalanol between 98.5 and 106.8% (table 23).

For all compounds both the RSD_r and RSD_{RL} (%) complied with the criteria (see 3.2.8 section), moreover also RSD_r based on three validation days is satisfactory (table 24).

Zearalanone had an outlier according to Grubb's test. This value was not included in calculations.

CCα and CCβ

The calculation of CCα and CCβ was performed in accordance with the Decision 2002/657/EC. The calculated and stated CCα and CCß values are shown in table 25 below.

Compound	CCα µg/kg	CCß µg/kg
Zearalanone	0.48	0.95
α-Zearalanol	0.11	0.23
ß-Zearalanol	0.27	0.53

Table 25. Calculated CCα and CCß.

CCα and CCß were found to be equal or better than the lowest concentration level included in the validation, with the exception of zearalanone CCβ.

Selectivity

In almost all blanks samples zearalenone α-zearalenol and ß-zearalenol were found. These compounds probably derived from mouldy food that the cattle have eaten. Traces of zearalanone, α-zearalanol and ß-zearalanol were detected in three of the blanks used. These values have been corrected.

It was found also that:

- zearalanone and bromchlorbuterol have the same parent, however, bromchlorbuterol has others daughter ions and is measured in the positive mode;
- α-zearalanol, ß-zeralanol and chloramphenicol have the same parent, but they have different daughters.

Thus the method was selective to determine zearalanone, α-zearalanol and ß-zearalanol with a CCα as stated in the claim.

Robustness

In the robustness experiments none of the six minor changes influenced the reliability of the method.

Stability

Only zearalanon from MMRS 408668 did not meet the criteria after one week of storage in the freezer. The other components comply after one and two weeks in the freezer.
After the reinjections of samples run during the day 3 validation, the differences were tested against the repeatability obtained via Resval and difference in accordance with MMRS (<15%). Almost all differences of all re-injections were smaller than repeatability.
Despite of some deviations it can be concluded that extracts can still be analysed in the freezer after one week of storage.

Linearity

In the three series of the three validation days and in reinjections after one week linearity criteria were satisfied (table 26).

	Level	**Criterion R^2**
Day 1-3	0 – 0.25 – 0.5 – 1.0 – 2.0 – 5.0 * GV	≥ 0.990

Table 26. Linearity criteria.

Recovery

The recovery of the analytes was determined with a different sample on each validation day. The results are shown in table 27.

Compounds	**Day 1**	**Day 2**	**Day 3**	**Mean**
Zearalanone	72	81	85	79
α-Zearalanol	72	76	90	79
ß-Zearalanol	81	83	82	82

Table 27. Recovery per validation day and average recovery.

Zearalanone recovery values ranged from 72 to 85%, α-zearalanol between 72 and 90%, ß-zearalanol 81 and 82%. The latter one displayed better recoveries values.
However the results were satisfactory.

Matrix effect

The matrix effect of the analytes was determined by comparing a sample with spike after sample pre-treatment (MMRS) with a standard solution at a corresponding concentration level.
The matrix effect is determined for each batch on each validation day. At a value lower than 100 there is suppression. With a value higher than 100, there is enhancement. A summary of the results is shown in table 28.

Compounds	**Day 1**	**Day 2**	**Day 3**	**Mean**
Zearalanone	133	193	103	143
α-Zearalanol	110	163	92	122
ß-Zearalanol	107	127	87	107

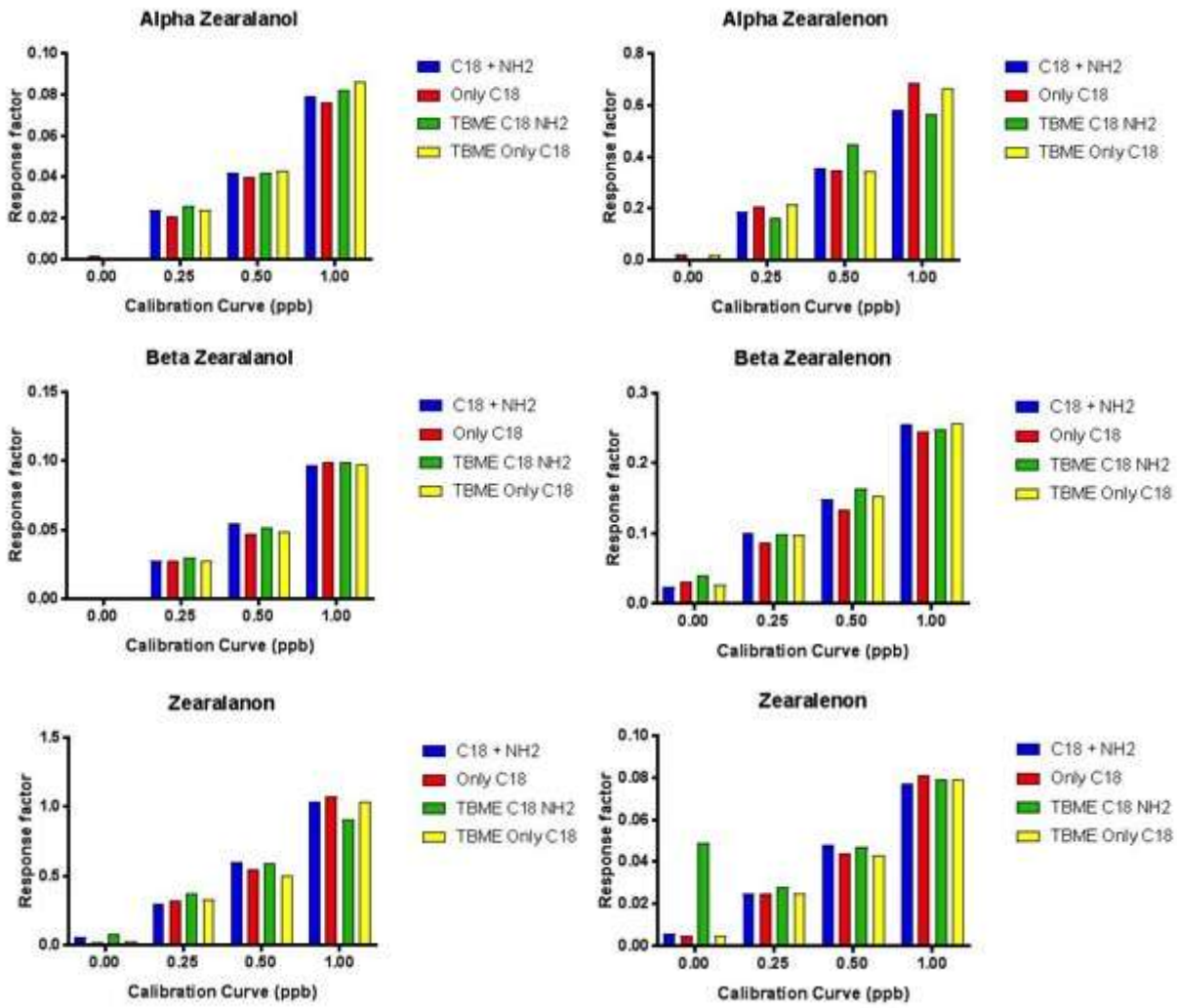
Table 28. Suppression and enhancement of zeranols.

For zearalanone in both three validation days has been displayed an enhancement of the signal, moreover α-Zearalanol and ß-Zearalanol showed an enhancement during the validation day 1, and 2. Day 3 validation was characterised by the lowest matrix effect values.

3.3.2 Method development in bovine hair

Experiment A

The additional MTBE step (graph 1) did not bring any significant enhancement to the protocol in all analytes, so it was decided to perform further future modifications to the extraction protocol, using the C_{18}+NH_2 clean-up step.



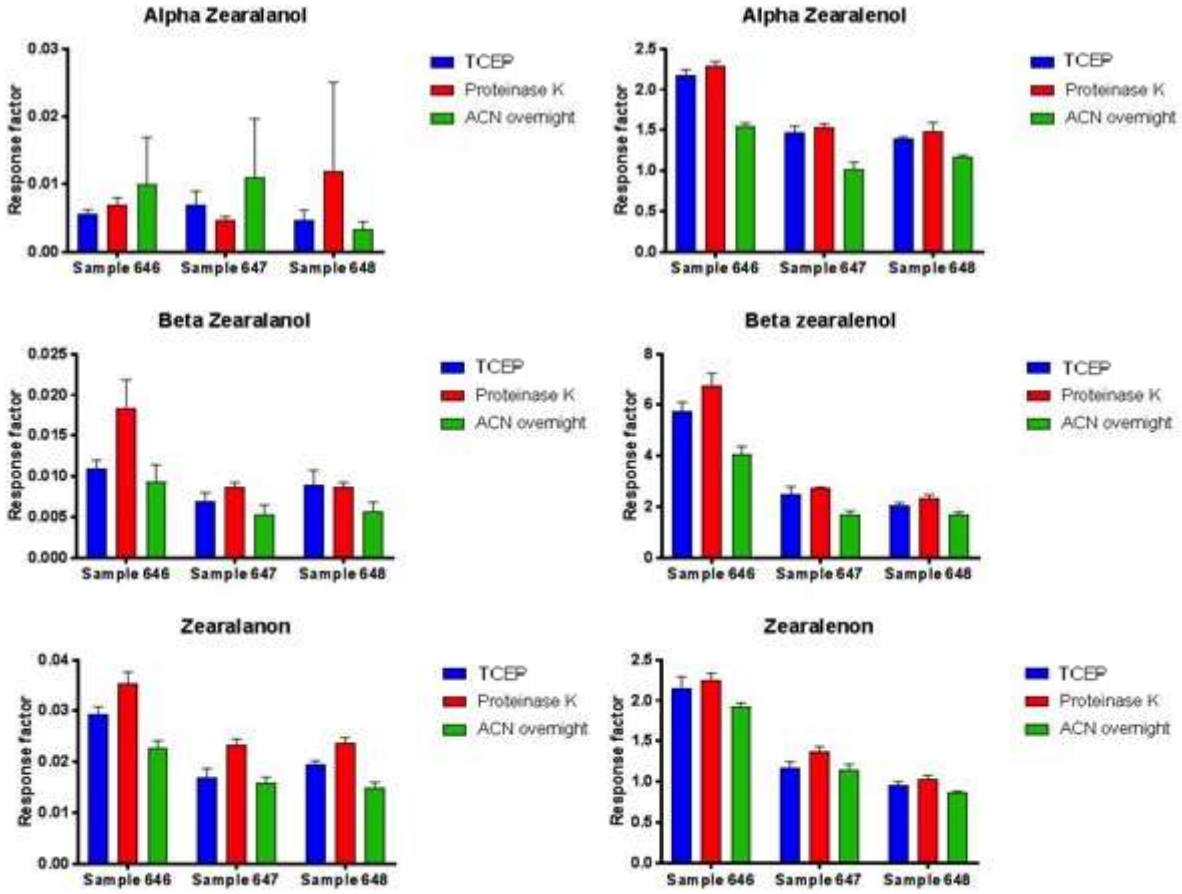
Graph 1. Response factor (Standard Area/Internal Standard Area) of different clean-up methods: C_{18}+NH_2, C_{18}, TBME C_{18}+NH2, TBME C_{18}.

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was employed in these protocols because it was reported it could reduce disulfide bridges of hair protein structure (Getz et al. 1999). Moreover it has been used in animal hair for drugs, steroid esters analysis and steroids analysis in human hair (Hooijerink et al. 2005; Nielen et al. 2006; Pozo et al. 2009).

Methyl-tert-butyl ether (MTBE) was also used in this experiment because many authors reported it improved extraction efficiency in bioanalysis for pharmaceutical compounds, moreover it has been used for drugs and growth promoters analysis in hair (Duffy et al. 2009; Regal et al. 2010; Jiang et al. 2012).

Experiment B

The performance of the three different extraction methods was compared (graph 2): there was not a huge difference among the three protocols, moreover the signal to noise ratio was also quite similar in all the methods tested. In conclusion, the method called "TCEP" remained the best option.



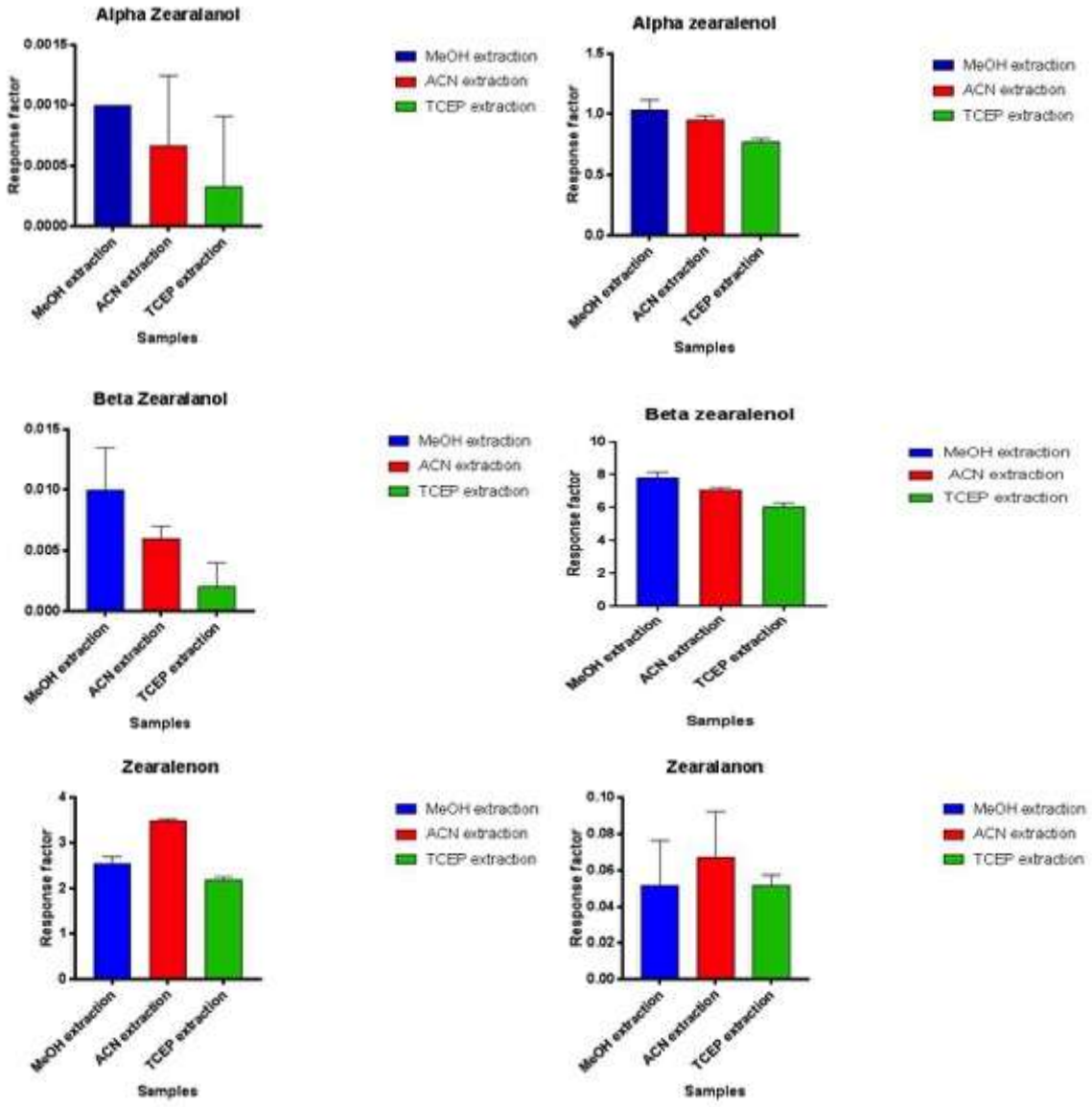
Graph 2. Response factor (Standard Area/Internal Standard Area) of different extraction methods: TCEP, Proteinase K and ACN overnight.

Proteinase K, a digestion enzyme with proven keratin hydrolyzing activity, was employed for the determination of drugs in human hair, and anabolic agents in bovine hair (Höld et al. 1998; Hernández-Carrasquilla 2001; Yamamura et al. 2002).

The Proteinase K protocol that was performed, was adapted from the optimised method set-up by De Kesel et al. used for caffeine and its metabolite paraxanthine analysis in hair (De Kesel et al. 2015).

Experiment C

Due to troubles generated by the PBS washing step, which did not allow a good sensitivity, many preliminary experiments were performed to set-up the IACs final protocol which involved.
PBS and water were tested in different volumes to wash IACs in order to minimize non-specific adsorbtion, which led to poor sensitivity and bad signal to noise ratio.



Graph 3. Response factor (Standard Area/Internal Standard Area) of different extraction methods (MeOH/ACN/TCEP) followed by IACs clean-up.

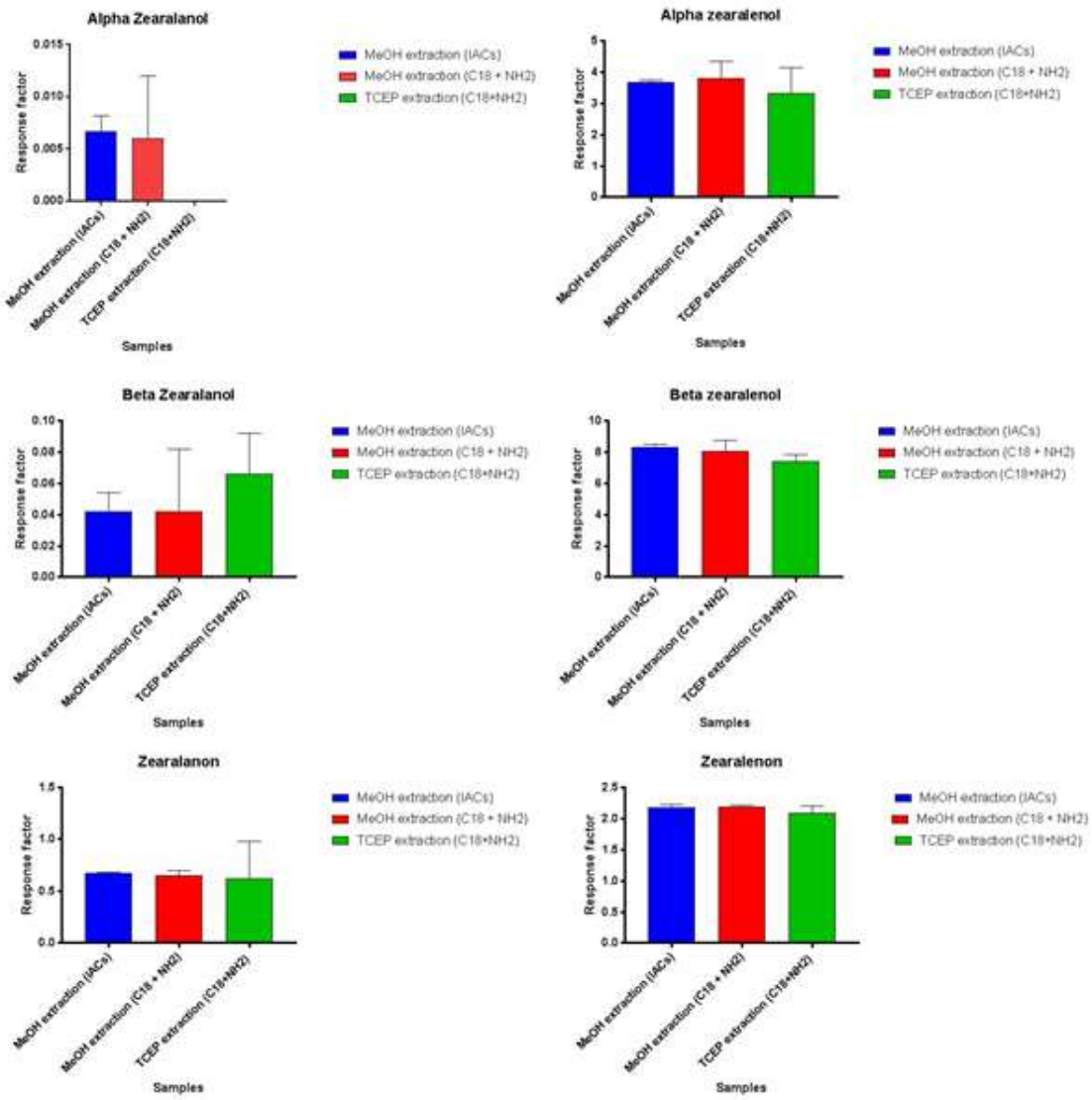
Both MeOH and ACN overnight extractions gave better responses, in comparison to the TCEP protocol; in addition immunoaffinity colums, although they represent an expensive clean-up method, provided good results in term of sensitivity and selectivity (graph 3).

IACs clean-up method was chosen because it had been performed previously to determine zearalenone and metabolites in many different biological matrices such as urine, plasma, faeces, which improved the selectivity of sample preparation (Songsermsakul et al. 2006).

Moreover IACs can show multiple advantages (Mateo et al. 2002):

- they can produce cleaner extracts due to the specificity of the antibodies towards a single or a group of mycotoxins;
- they are characterised by good accuracy and precision within a wide range of concentrations;
- they provide short analysis time.

Experiment D



Graph 4. Response factor (Standard Area/Internal Standard Area) of different extraction/clean-up methods: MeOH extraction (IACs), MeOH extraction (C_{18}+NH_2), TCEP extraction (C_{18}+NH_2).

MeOH extraction followed by IACs or the C_{18}+NH_2 method gave the better responses compared to the TCEP one (graph 4).

For the final extraction method it was decided to not use TCEP, since it did not improve the protocol in terms of sensitivity.

The IACs clean-up method was chosen as the definitive method for the high sensitivity achieved, high recovery and the reduced time of analysis, compared to that one of C_{18}+NH_2 method.

Experiment E

New mobiles phases were chosen:

- A: 2mM Ammonium Formiate, 0.05% Formic Acid, in H_2O
- B: 2mM Ammonium Formiate, 0.05% Formic Acid, in ACN: H_2O (90:10)

These ones gave better responses in terms of sensitivity and peak shapes, compared to A: water - B: acetonitrile.

Moreover, due to costant overpressure problem with the column, different flow rates were tested: it was finally decided to lower the flow from 0.6 mL/min to 0.5 mL/min.

3.3.3 Set-up and one day validation on human hair

The present work reported a sensitive method for the quantification of zearalenone, α -zearalanol, ß-zearalanol, α -zearalenol, ß-zearalenol, zearalanone in human hair (figure 9) based on an LC-MS/MS technique. The method was validated through a one validation day.
Based on the current state-of-the-art literature, this work identified for the first time zearalenone and its main metabolites in human hair using LC-MS/MS.

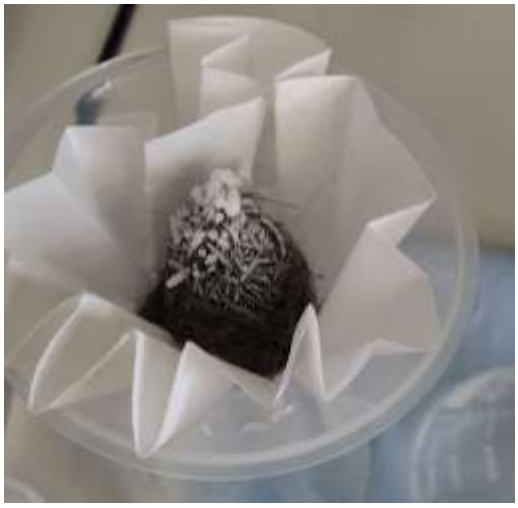


Figure 9. Human hair drying process after dichloromethane washing step: "white crystals" formation on hair was noticed, probably formed as a result of the heat loss during evaporation of the solvent.

Trueness, repeatability, whitin-lab reproducibility

The validation parameters of repeatability and inner-draft producibility were determined on the basis of 7 blank samples with additions prior to work-up of 0.5, 1 and 2.5 times the guide value and on the basis of one blank sample in sevenfold with addition before work-up of 1 time the guide value (GV) (tables 29 and 30).
For the calculations see "Validation criteria, 3.3.1 section".

Compounds	Level	µg/kg	Trueness (%)	RSD_r (%)	RSD_{RL} (%)
Zearalanone	0.5*GV	0.1	112	13.1	20.9
	1.0*GV	0.2	102	11.0	17.5
	2.5*GV	0.5	95	9.0	14.4
α-Zearalanol	0.5*GV	0.1	**123**	**20.8**	**33.2**
	1.0*GV	0.2	108	7.9	12.7
	2.5*GV	0.5	109	7.2	11.5
ß-Zearalanol	0.5*GV	0.1	117	14.7	***23.5***
	1.0*GV	0.2	104	5.8	9.2
	2.5*GV	0.5	99	7.7	12.2
Zearalenone	0.5*GV	0.1	105	12.7	20.4
	1.0*GV	0.2	95	3.5	5.6
	2.5*GV	0.5	100	6.3	10.0
α-Zearalenol	0.5*GV	0.1	**135**	**23.3**	**37.2**
	1.0*GV	0.2	108	14.5	***23.2***
	2.5*GV	0.5	97	7.9	12.7
ß-Zearalenol	0.5*GV	0.1	**136**	**23.5**	**37.6**
	1.0*GV	0.2	85	8.0	12.8
	2.5*GV	0.5	107	14.2	***22.7***

Table 29. Trueness, RSDr (repeatability) and RSD_{RL} (whitin-laboratory reproducibility) based on one test day (seven different human hairs).

All bold printed values did not meet the requirements set in the validation plan. Instead, the slightly too high values were accepted.

For all analytes trueness, RSD_r, RSD_{RL} displayed satisfying values at 1.0 and 2.5 GV, in accordance with the criteria specified (see Validation criteria, 3.3.1 section). While α-Zearalanol, α-Zearalenol, ß-Zearalenol did not show acceptable values at 0.5 GV, so the method still needs to be optimised to achieve better results.

Compounds	**Level**	**µg/kg**	**RSD_r (%)**
Zearalanone	1.0*GV	0.2	6.7
α-Zearalanol	1.0*GV	0.2	8.1
ß-Zearalanol	1.0*GV	0.2	6.1
Zearalenone	1.0*GV	0.2	9.1
α-Zearalenol	1.0*GV	0.2	14.4
ß-Zearalenol	1.0*GV	0.2	17.8

Table 30. RSDr based on one test day (Anova method, 7 x the same hair).

For all analytes RSD_r on the same hair displayed satisfactory results, in accordance with the Validation criteria, 3.3.1 section.

CCα and CCβ

The calculation of CCα and CCβ was performed in accordance with the Decision 2002/657/EC. The calculated and stated CCα and CCß values are shown in table 31 below.

Compounds	CCα µg/kg	CCß µg/kg
Zearalanon	0.09	0.17
α-Zearalanol	0.07	0.15
ß-Zearalanol	0.07	0.14
Zearalenon	0.06	0.11
α-Zearalenol	0.10	0.21
ß-Zearalenol	0.15	0.29

Table 31. Calculated CCα and CCß in human hair.

Selectivity

Selectivity represents the discrimination between the analyte and closely related substances (e.g.. isomers) or matrix interferents. In total 7 blank hair without the addition of the zeranols with only the internal standard were spiked and assessed for the presence or absence of a signal on the expected retention times. There were no disturbances on the retention times of the components. Thus the method is selective to determine all six compounds.

Linearity

For β -zearalenol and zearalanone, too low linearity was found in the MMS line for the samples ($R < 0.99$). For the other compounds the linearity was considered sufficient.

Recovery

The recovery of the analytes was determined with a different sample on each validation day. The results are shown in Table 32.

Compounds	Recovery %
Zearalanone	59
α-Zearalanol	66
ß-Zearalanol	74
Zearalenone	64
α-Zearalenol	51
ß-Zearalenol	82

Table 32. Recovery in human hair.

The recovery rates still need to be improved and optimized, specially for α-zearalenol, zearalanone, zearalenone, α-zearalanol.

3.3.4 Applicability of the method for the analysis of human hair samples

Zearalenone and other five metabolites were not detected in human hair from international donors.

4. MYCOTOXINS IN PET FOOD FOR CATS

4.1 PROJECT INTRODUCTION

4.1.1 Pet food for cats and mycotoxin risk

Pets play an important role in families, with purely emotional and other supporting functions in everyday life (Assalco 2017). In view of the intense human-animal companion bond and enhanced health awareness, as well as a greater consideration regarding pet welfare, the pet food industry is making so much efforts to pursue sustainability in food system, which implies also such as food quality and safety (Walsh 2009; Swanson et al. 2013).

In Western Europe cats are the most popular domestic animals: they are strictly carnivorous animals, moreover unlike other pets, cats need to have higher protein intake, which can affect both their food preferences and requirements. Thus their diet must contain specific nutrients, such as taurine, arginine, vitamin A, niacin, and arachidonic acid, without leaving out factors influencing food palatability (Zaghini & Biagi 2005).

Mycotoxin contamination in pet food represents a relevant health issue to domestic animals. Cereal grains and nuts are the main components in pet food formulations for companion animals such as cats, dogs, fish, birds and rodents (Aquino & Correa 2011).

There are types of commercial pet foods: dry, semi-moist, and moist or canned. The main difference relies on the water content of the food, for instance dry foods contain less than 11% water, semi-moist foods contain 25 to 35% water, finally moist or canned food contain 60 to 87% water (Zicker 2008; Aquino & Correa 2011).

Dry food particles are produced by the extrusion process, which uses the same technology utilised for breakfast cereals production. Other production methods include pelleting, baking, flaking and crumbling of raw materials to give them a dry form. Extruded food production involves a mixing and extrusion steps:. the ingredients are put into an extruder which utilises a combination of pressure (from 34 to 37 atm), steam and high temperature (between 100 to 200°C) in a short period of time to quickly cook foods (Aquino & Correa 2011).

In general food processes can reduce mycotoxin concentrations significantly, but do not eliminate them completely. However extrusion and roasting processing could lower mycotoxin concentrations, even though very high temperatures are required to lessen mycotoxin concentrations. According to Bullerman & Bianchini (2007) extrusion cooking at high temperatures (more than 150°C) can result in a proper zearalenone reduction, moderate one of aflatoxins, variable to low reduction of deoxynivalenol and a good reduction of fumonisins (Bullerman & Bianchini 2007).

However mycotoxin contamination represents a great concern in pet food, because the mycotoxins that can occur in cereals and other products cannot be completely degraded through food-processes and can still remain in the final product (Milani & Maleki 2014).

Aflatoxins can be degraded by temperatures ranging from 237 °C to 306 °C, moreover it was reported that temperatures above 150 °C can lead to the partial destruction of the molecules.

Deoxynivalenol is stable at 120 °C and moderately so at 180 °C; however it can be destroyed after 40 minutes at 210 °C (Samarajeewa et al. 1990; Rustom 1997; Milani & Maleki 2014).

Zearalenone is a chemically stable substance with a melting point of 164-165 °C, moreover it was reported that it can resist to 4 hours of heat treatments at 120 °C.

Fumonisins are quite heat stable and significant destruction occur during processes that are at temperatures greater than of 150 °C (Milani & Maleki 2014).

Ochratoxin A seems to be stable up to 180 °C (Raters & Matissek 2008). Few studies investigated the mycotoxin effects on cat health.

Newbern et al. reported that adult mixed-breed cats showed an aflatoxin B1 sensitivity similar to rabbit, dog and guinea pig, as a result of a single dose LD50 of 0.55 mg/kg. Deaths usually occurred after 48 – 72 hours. Furthermore gall bladder edema, abnormal growth and sometimes loose stools have also been found in cats (Newberne & Butler 1969).

Hughes et al. investigated the effects of dietary DON on dogs and cats: food was refused by cats when DON levels exceeded 7.7 mg/kg, while in dogs 4.5 mg/kg. These findings indicated a lower sensitivity in cats than dogs. Moreover food consumption diminished at DON levels of 7.5 μg/kg of cat food, and at 4.5 μg/kg of dog food (Hughes et al. 1999).

The discrepancy shown between cats and dogs could be explained by the tendency of felines to consume small amounts of food over time, instead of dogs which are more likely to feed with voracity. Based on the findings it has been suggested that deoxynivalenol levels in pet food should not exceed 0.5 mg/Kg. However deoxynivalenol is not the only trichotecene on which to focus in pet toxicology (Zain 2011).

Borison et al. intravenously administered T-2 toxin to cats at a concentration of 2 mg/kg, which

caused in hypovolemia and death, moreover, lower white blood cells content occurred at sub-lethal T-2 toxin dose (Borison et al. 1991).
Regarding ochratoxin toxicity kidney is the primary target organ in dogs and cats (Zain 2011).

4.1.2 Legislation

Directive 2002/32/EC and subsequent amendments of the European Union set specific maximum limits for aflatoxin B1 (AFB1) in complete and complementary feeding stuffs intended for animals: the established value is 5 – 20 μg/kg, which can be referred to products containing 12% moisture, with also several specifications for livestock animals (European Parliament and Council 2002). The feed category called "other complete feedingstuffs" probably consists of pet foods products, for which a limit of 10 μg/kg has been established.
In addition European legislation set recommendation regarding mycotoxin contamination in products intended from animal feed, on the basis of the different animal species considered.
UE Recommendation 2016/1319, which emended the Decision 2006/576/CE, established guidance values in pet food and/or cats/dogs for (see tables 4 and 5) (Commission Recommendation 2016):

- zearalenone
  - kittens, dogs and cats for reproduction: 100 μg/kg;
  - adult dogs and cats other than for reproduction: 200 μg/kg;

- ochratoxin A
  - cats and dogs: 10 μg/kg;

- fumonisins (FB1+FB2)
  - pet animals: 5000 μg/kg;

- T-2 + HT-2 toxin:
  - compound feed for cats: 50 μg/kg;

- deoxynivalenol:
  - compound feed for dogs: 2000 μg/kg;

4.2 MATERIALS AND METHODS

The present work was conducted to determine the presence and contamination level of most important mycotoxins in commercial cat dry food, using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

This work was performed in collaboration with the "Animal Productions and Food Safety Service" (SPASA) set in the DIMEVET (Veterinary Medical Sciences Department) in Ozzano dell¹Emilia, Bologna University.

The examined mycotoxins were aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), deoxynivalenol (DON), zearalenone (ZEA), fumonisin B1 (FB1), fumonisin (FB2), ochratoxin (OTA), T-2 toxin (T2) and HT-2 toxin (HT2).

In order to identify the risk mycotoxins can represent to the domestic cat, a survey study was conducted examining the levels of mycotoxins in sixty-four complete commercial extruded dry cat foods, within 9 category products, bought from Italian retailers.

4.2.1 List of abbreviations

AFB1: Aflatoxin B1
AFB2: Aflatoxin B2
AFG1: Aflatoxin G1
AFG2: Aflatoxin G2
DON: Deoxynivalenol
FB1: Fumonisin B1
FB2: Fumonisin B2
OTA: Ochratoxin A
ZEA: Zearalenone
LC: Liquid chromatography
QC: Quality control
DON-M: Labelled deoxynivalenol
FB1-M: Labelled fumonisin B1
ZEA-M: Labelled zearalenone
AFLAB1-M: Labelled aflatoxin B1
OTA-M: Labelled ochratoxin A

4.2.2 Sampling

Sixty-four complete commercial extruded dry cat foods were purchased from stores in the province of Bologna (Italy).
The products included 6 diet obesity, 7 diet renal, 6 diet gastrointestinal, 5 grain-free, 5 kitten-low, 5 kitten-premium, 5 senior-low, 5 senior-premium, 20 adult-low cat foods.
Samples were categorized also in two main groups, based on two price categories considered:

- premium pet food for cats: 6 diet obesity, 7 diet renal, 6 diet gastrointestinal, 5 grain-free, 5 kitten-premium, 5 senior-premium;
- standard pet food for cats: 5 kitten-low, 5 senior-low, 20 adult-low cat foods.

Samples (already pulverized in "Animal Productions and Food Safety Service" set in the DIMEVET) were stored in plastic containers in freezer under dark conditions.

4.2.3 Glassware and consumables

- Class A graduated glassware (cylinders, beakers, flasks, glass pipettes);
- Falcon tubes;
- Glass test tubes;
- Millipore filters Amicon Ultra 0.5 mL 3K;
- 2 mL glass vials with cap.

4.2.4 Lab equipment

- Analytical balance (trueness of ± 0.001 g);
- Vortex Scientific Velp;
- Hettich refrigerated centrifuge;
- Gilson P5000, P1000, P200 and P20 pipettes;
- LC-MS/MS system, consisting of a Waters ACQUITY UPLC pump equipped with a Waters ACQUITY BEH C_{18} column, coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford MA, USA).

4.2.5 Solvents and reagents

Standards

- DON: Deoxynivalenol, purity ≥98% (Sigma-Aldrich. St.Louis, USA);
- AFLAB1: Aflatoxin B1 from *Aspergillus flavus*, purity ≥98% (Sigma-Aldrich, St.Louis USA);
- AFLAB2: Aflatoxin B2 from *Aspergillus flavus*, purity ≥98% (Sigma-Aldrich, St.Louis USA);
- AFLAG1: Aflatoxin G1 from *Aspergillus flavus*, purity ≥98% (Sigma-Aldrich, St.Louis USA);
- AFLAG2: Aflatoxin G2 from *Aspergillus flavus*, purity ≥98% (Sigma-Aldrich, St.Louis USA);

- FB1: Fumonisin B1 from *Fusarium moniliforme*, purity ≥98% (Sigma-Aldrich, St.Louis USA);
- ZEA: Zearalenone, purity ≥99% (Sigma-Aldrich, St.Louis USA);
- OTA: Ochratoxin A from *Tetromyces albertensis*, purity ≥98% (Sigma-Aldrich, St.Louis USA).
- T2: T-2 toxin, (Sigma-Aldrich, St.Louis USA);
- HT2: HT-2 toxin (Orsell SRL, Modena Italy);
- Labeled DONC13: U-[$^{13}C_{15}$]-Deoxynivalenol, purity 99.5% (Biopure, Tulln Austria);
- Labeled AFLAB1: U-[$^{13}C_{17}$]-Aflatoxin B1, purity 99% (Biopure, Tulln Austria);
- Labeled FB1C13: U-[$^{13}C_{34}$]-Fumonisin B1, purity 97.8% (Biopure, Tulln Austria);
- Labeled ZEAC13: U-[$^{13}C_{15}$]-Zearalenone, purity 99.2% (Biopure, Tulln Austria);
- Labeled OTAC13: U-[$^{13}C_{20}$]-Ochratoxin, purity 98.7% (Biopure, Tulln Austria);

Chemicals

All the solvents used for mass spectrometric analysis were LC-MS grade, while those used for the extraction phase were HPLC grade.

- Methanol (Sigma-Aldrich, St Louis MO, USA);
- Acetonitrile (VWR);
- Ultrapure water (18.2 M Ω /cm) was obtained via a Human Power I lab water purification system (Human Corp., Seoul, South Korea);
- Acetic acid (Sigma-Aldrich, St Louis MO, USA);
- Formic acid (Fluka);
- Ammonium acetate (Fluka).

Standard solutions

The standard solutions used for the preparation of the QC and calibration samples were prepared according to the following procedure:

- Stock solutions: for each analyte a 20 ppm stock solution was prepared dissolving 1 mg of standard in 50 µL of solvent. Dilution is carried out in acetonitrile.
- Standard: 250 µL are taken from the "stock solution" solution at 20 ppm and is brought to volume in 5 mL of methanol in a volumetric flask, resulting in a final concentration of 1 ppm.
- Labeled standard: 250 µL is taken from the commercial solution of labeled standards at 25 ppm and is brought to volume in 1 mL of methanol in a volumetric flask, resulting in a final concentration of 5 ppm.

Reagents

- Solution for extraction (ACN/H_2O 50:50): 50 mL of acetonitrile, 50 mL of water are mixed stirred and stored in a refrigerator.

4.2.6 Sample preparation

The method was set-up and optimized from a protocol of Zhang et al. (Zhang et al. 2014).

Extraction

0.5 grams of feed sample was weighed in a beaker fortified with 100 µL of the internal standard solution and kept at rest for 15 minutes. Then 5 mL of the extraction solution (ACN/H_2O 50:50) were added to the sample and the whole is kept on a magnetic stirrer for 15 minutes (two phases are formed: the sediment and the surnatant one). Then 500 µL were taken from the supernatant and transferred to a Millipore filter and centrifuged for 30 minutes at 14000 rpm and at 20° C. The aliquots of the resulting filtrates were transferred into vials for LC-MS/MS analysis (figure 10).

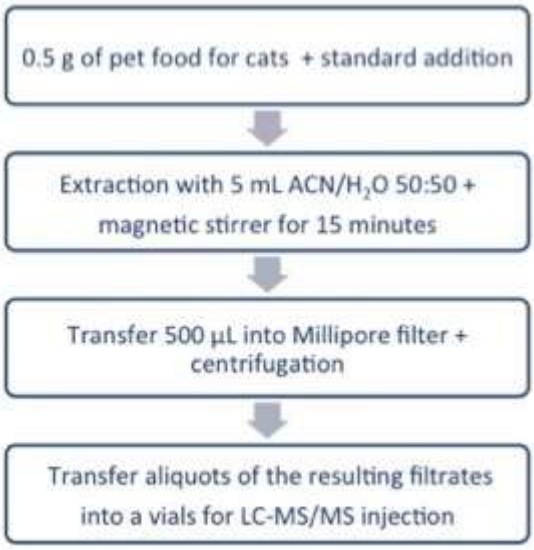


Figure 10. Sample extraction/clean-up scheme.

4.2.7 Analytical conditions

Chromatographic conditions

The method was set-up and optimized from a protocol of Gazzotti et al. (Gazzotti et al. 2015).

The chromatographic conditions for all analytes except for DON are described in table 33.

- Strong solution: water, acetonitrile and methanol in the proportions of 30:30:40, with 0.2% formic acid;
- Weak solution: water, methanol 95:5 with 0.1% formic acid.

Mobile Phase A	Water + 0.1% formic acid	
Mobile Phase B	Methanol + 0.1% formic acid	
Gradient	$\mathbf{T_0}$	95% A - 5% B
	$\mathbf{T_2}$	95% A - 5% B
	$\mathbf{T_4}$	90% A - 10% B
	$\mathbf{T_{12}}$	25% A - 75% B
	$\mathbf{T_{12,1}}$	1% A - 99% B
	$\mathbf{T_{14}}$	1% A - 99% B
	$\mathbf{T_{14,1}}$	95% A - 5% B
	$\mathbf{T_{16}}$	95% A - 5% B
Flow	0.42 mL/min	
Injection volume	10 µL	
Temperature	40 °C	

Table 33. Chromatographic conditions for all analytes, except for DON and labeled DON.

The chromatographic conditions for DON and labeled DON are described in table 34.

- Strong solution: water, acetonitrile and methanol in the proportions of 30:30:40;
- Weak solution: 5mM ammonium acetate in water and methanol in the proportions of 90:10.

Mobile phase A	Ammonium acetate 5 mM in Water	
Mobile phase B	Methanol	
Gradient	$\mathbf{T_0}$	99% A - 1% B
	$\mathbf{T_1}$	95% A - 5% B
	$\mathbf{T_2}$	25% A - 75% B
	$\mathbf{T_{2.10}}$	1% A - 99% B
	$\mathbf{T_4}$	99% A - 1% B
	$\mathbf{T_6}$	99% A - 1% B
Flow	0.3 mL/min	
Injection Volume	10 μL	
Temperature	40 °C	

Table 34. Chromatographic conditions for DON and labeled DON.

MS/MS Parameters

All the parameters are shown in Table 35 and 36.

Parameters	
Ionisation mode	ESI positive
Capillary voltage	3.0 kV
Cone	Component depends V
Source temperature	130°C
Desolvation temperature	450°C
Desolvation gas flow	446 L/hour
Cone gas flow	43 L/hour
Multiplier	690
LM 1 resolution	15.0
HM 1 resolution	15.0
Ion energy 1	0.2
LM 2 resolution	11.0
HM 2 resolution	11.0
Ion energy 2	1.0
CID gas	Argon p = 7.34 x 10^{-3} mbar purity > 99.998%

Table 35. Mass spectrometry parameters.

Compounds	Precursor	Product	Cone (V)	CE (eV)
Aflatoxin B1	313.2	241.3	45	36
		285.3	45	22
Aflatoxin B2	315.05	259.1	45	38
		287.1	45	33
Aflatoxin G1	329.1	243.3	45	26
		283.3	45	24
Aflatoxin G2	331.1	245.2	46	39
		313.25	46	33
Deoxynivalenol	297.25	231.25	19	12
		249.2	19	10
Fumonisin B1	722.2	334.5	52	45
		352.5	52	43
Fumonisin B2	706.3	318.5	50	40
		336.5	50	40
Zearalenone	319.3	185.2	20	30
		187.2	20	12
		283.2	20	12
Ochratoxin	404.15	221.2	25	25
		239.2	25	14
T-2 toxin	489.2	245.1	36	27
		387.0	36	22
HT-2 toxin	447.25	285.3	36	22
		345.3	36	20
Labelled Aflatoxin B1	330.3	255.4	45	38
		301.2	45	22
Labelled Deoxynivalenol	312.2	216.4	18	18
		263.4	18	13
		245.3	18	15
Labelled Fumonisin B1	756.3	356.6	52	45
		374.6	52	40
Labelled Zearalenone	337.3	199.4	17	18
		301.3	17	12

Labelled Ochratoxin	424.1	232.4	26	38
		250.4	26	25
Labelled T-2 toxin	513.3	260.3	40	28
		406.2	40	23

Table 36. Theoretical mono-isotopic masses of the precursor ions and product ions.

4.2.8 Statistical analysis

A statistical analysis of variance using Student's t-test was conducted on concentrations of the various mycotoxins determined in premium and standard dry food for cats: the aim was to identify any statistically significant differences between the two price categories evaluated.
Differences were considered statistically significant when $P<0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Measurement range

The following method shows the quantification limit (LOQ) and detection limit (LOD) for each molecule expressed in ppb (Table 37).

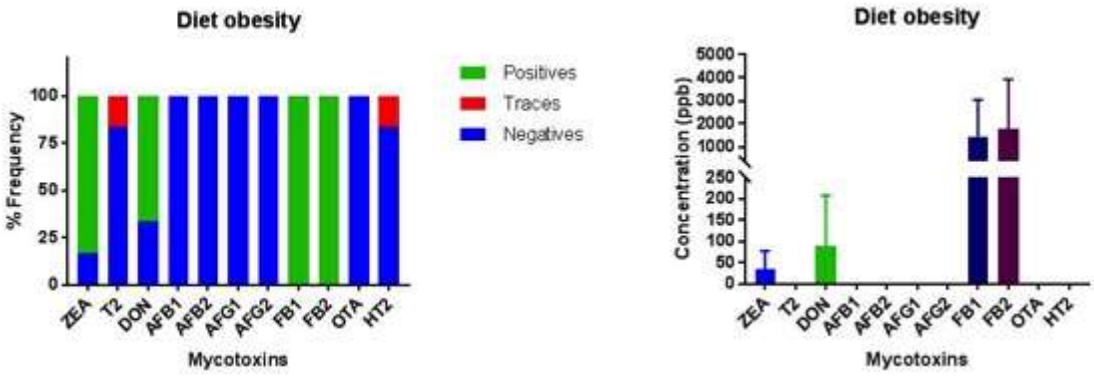
Compounds	LOQ	LOD
Aflatoxin B1	3	1
Aflatoxin B2	3	1
Aflatoxin G1	3	1
Aflatoxin G2	3	1
Deoxynivalenol	3	1
Fumonisin B1	3	1
Fumonisin B2	3	1
Zearalenone	5	2
Ochratoxin	5	2
T-2 toxin	10	5
HT-2 toxin	20	5

Table 37. Quantification limit (LOQ) and detection limit (LOD) for each molecule expressed in ppb.

4.3.2 Concentration of mycotoxins in commercial pet food for cats

Data relating to frequency of positive/negative/trace samples and different mycotoxin concentration in the nine pet food for cats categories were shown in the graphs below.

- **Premium diet obesity samples**

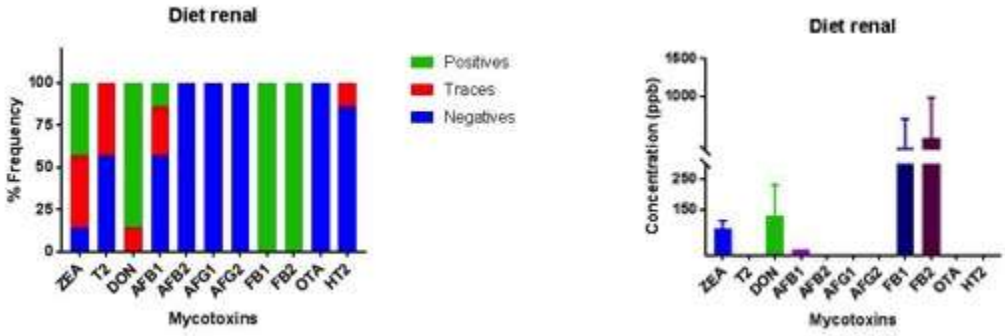


Graph 5. Diet obesity pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In premium diet obesity samples (n=6):

- ✓ AFB1, AFB2, AFG1, AFG2, OTA were not detected;
- ✓ with regards to FB1 and FB2 all samples were found positive; two samples exceeded the guidance values for FB1+FB2, showing 7494.4 ppb and 7932.6 ppb respectively;
- ✓ small amounts of ZEA and DON were found: in the 16.7% of the samples no ZEA was detected, while 83.3% of the samples were positive ranging from 5 to 112 μg/Kg; while in the 33.3% of the samples no DON was found and the remaining part was found positive (with a mean value of 88.1 ppb);
- ✓ T-2 and HT-2 toxins presented the 16.7% traces, while the other part was negative (graph 5).

- **Premium diet obesity samples**

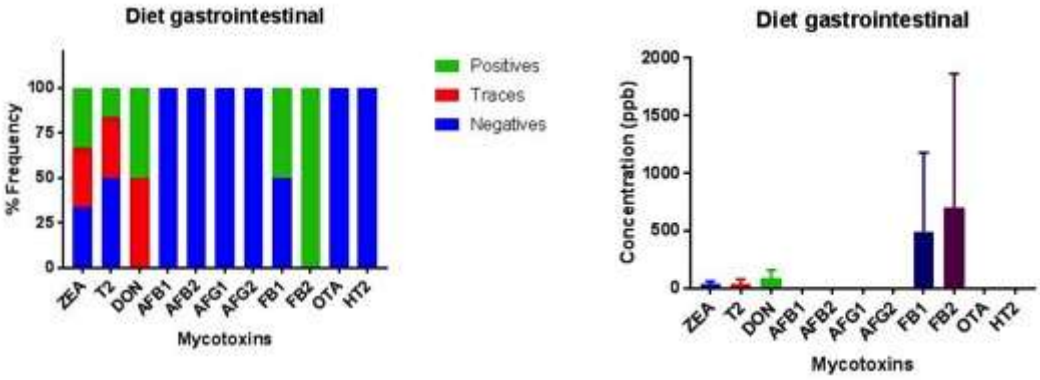


Graph 6. Diet renal pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In premium diet renal pet food for cats (n=7):

- ✓ AFB2, AFG1, AFG2, OTA were not present; AFB1 exceeded the set limit in one sample (17.4 ppb) and was present in traces in two samples;
- ✓ FB1 and FB2 were found in all samples, with mean values of 325.9 and 448.4 ppb respectively, not exceeding the guidance values specified in the above-mentioned recommendation;
- ✓ 14.9% of samples were negative for ZEA, while the 42.9% were at trace level and the other part was positive (mean value of 88.6 ppb);
- ✓ the major part of samples were positive to DON (85.7%, mean value of 129.2 ppb), while 14.3% contained traces;
- ✓ 57.1% of samples were negative for T-2 toxin, while the remaining part contained traces; the majority of samples were negative for HT-2 (85.7%), while 14.3% contained traces (graph 6).

- **Premium diet gastrointestinal samples**

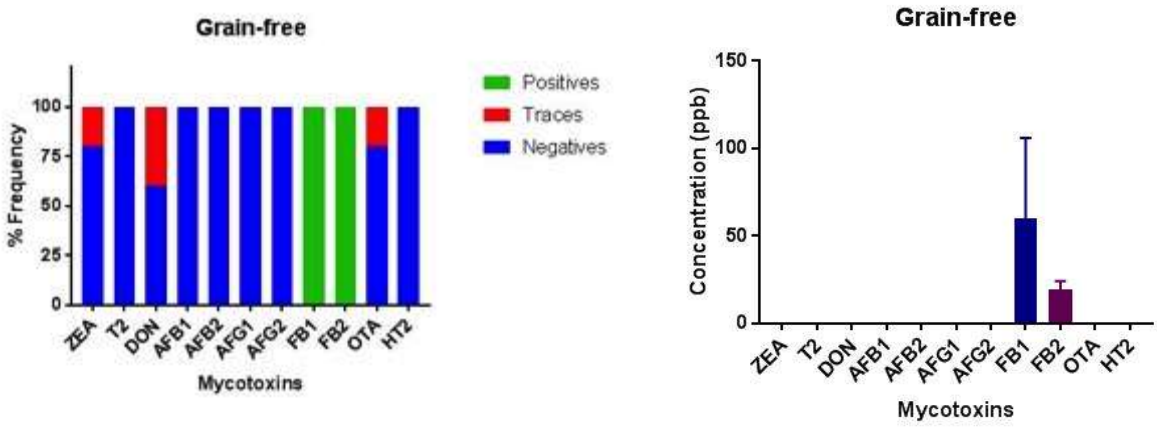


Graph 7. Diet gastrointestinal pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In premium diet gastrointestinal pet food for cats (n=6):

- ✓ AFB1, AFB2, AFG1, AFG2, OTA, HT-2 toxin were not present;
- ✓ FB2 was found in all samples, representing the most abundant mycotoxin; while FB1 was only present in the half of samples;
- ✓ ZEA and DON were present in small traces. Thus 33.3% of samples were equally positive, negative and presented trace levels for ZEA; while half of samples were positive to DON;
- ✓ More interestingly, although the half of samples were negative to T-2 toxin, and also with traces presented in the 33.3% of cases, two samples out of six were positive displaying respectively a content of 13.2 and 69.6 μg/Kg respectively (graph 7). The latter value did not conform to the UE Recommendation 2016/1319, which suggested a maximum level for T-2+HT-2 toxin at 50 μg/Kg.

- **Premium grain-free samples**



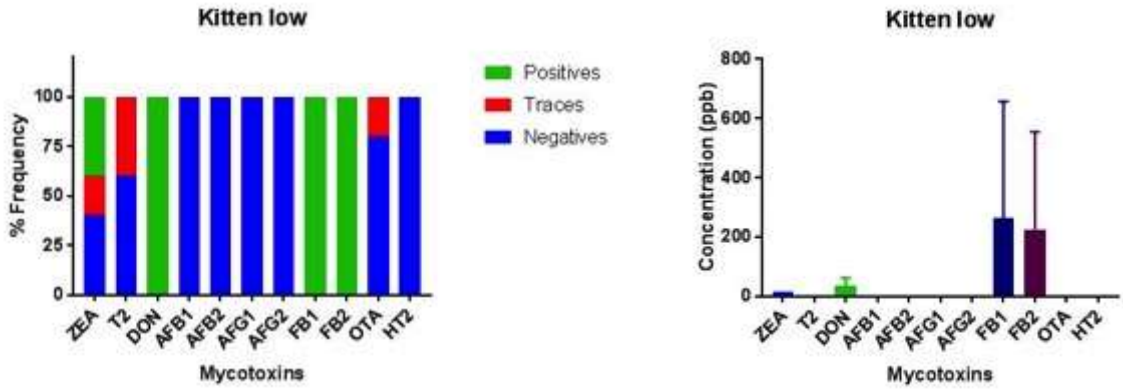
Graph 8. Grain-free pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In premium grain-free pet food for cats (n=5):

- ✓ AFB1, AFB2, AFG1, AFG2, HT-2, T-2 toxins were not present.
- ✓ ZEA and OTA were equally negative in 80% of the samples, while were present in trace quantities in 20% of them;
- ✓ DON traces were found in 40% of the samples, while the other part was negative (graph 8).

As expected the grain-free category showed the lowest mycotoxin contamination, displaying low levels of fumonisins (FB1+FB2).

- **Standard kitten low**

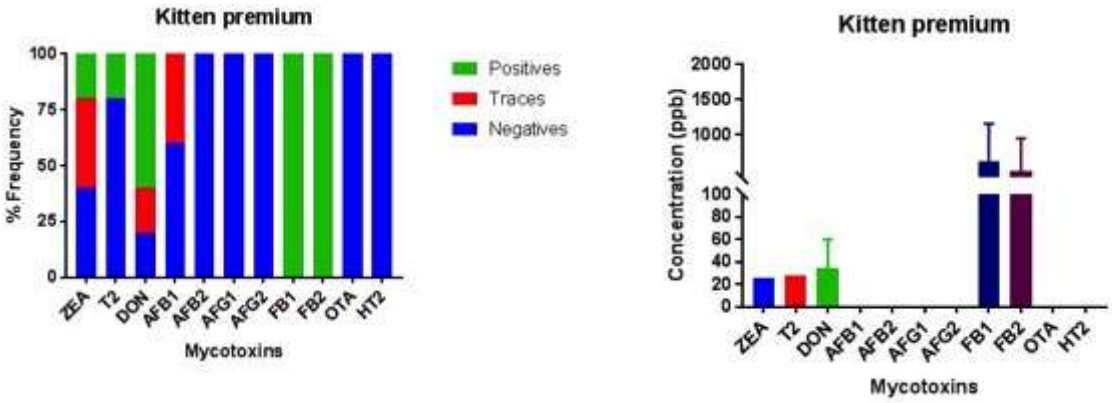


Graph 9. Kitten low pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In standard kitten low pet food for cats (n=5):

- ✓ no amount of AFB1, AFB2, AFG1, AFG2, HT-2 toxins was found;
- ✓ All samples were positive to DON ranging from 3 to 72.9 μg/Kg;
- ✓ ZEA was found in 40% of samples with a mean value of 11.3 μg/Kg;
- ✓ FB1 and FB2 were detected in all samples, with values in accordance to the EU Recommendation guidelines;
- ✓ 20% of samples contained OTA traces, while the remaining part was negative (graph 9).

- **Premium kitten samples**

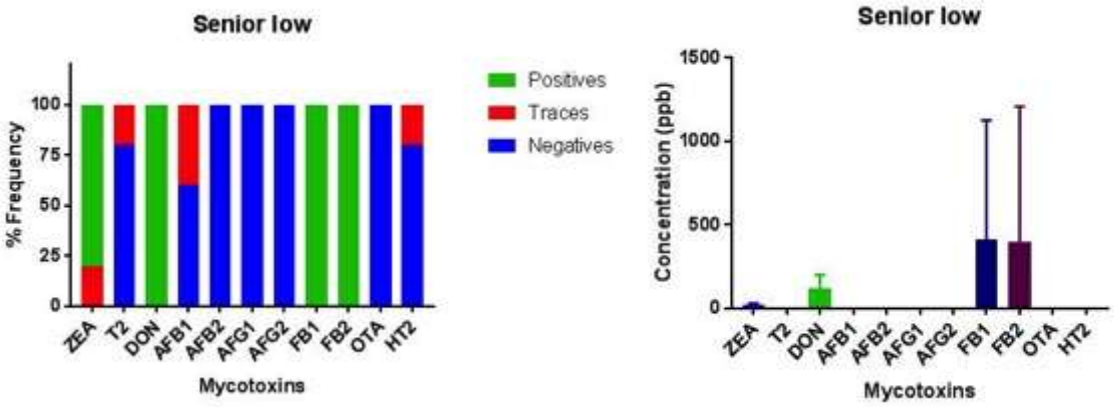


Graph 10. Kitten premium pet food for cats. Frequency of positive, negative, trace samples (on the left);mycotoxins concentration (on the right).

In premium kitten pet food for cats (n=5):

- ✓ no amount of AFB2, AFG1, AFG2, OTA, HT-2 toxins was found;
- ✓ FB1 and FB2 were present in all samples, displaying values in accordance to the EU Recommendation guidelines;
- ✓ 60% of the samples were positive for DON, ranging from 10.7 to 62.4 μg/Kg;
- ✓ Only one sample out of five was positive for ZEA, showing a content of 25.5 μg/Kg;
- ✓ Furthermore one sample was positive to T-2 toxin displaying a content of 27.9 μg/Kg, which represented more than the half value suggested by UE Recommendation (graph 10).

- **Standard senior low**

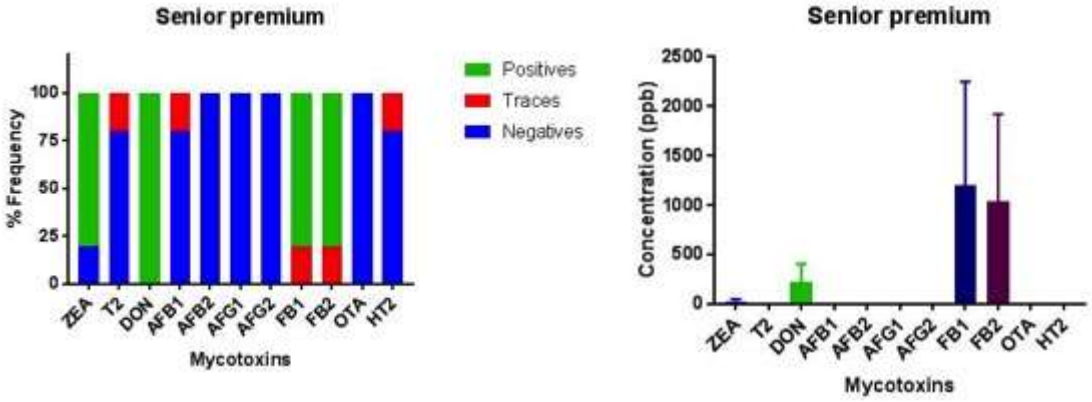


Graph 11. Senior low pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In standard senior low pet food for cats (n=5):

- ✓ AFB2, AFG1, AFG2, OTA were not detected;
- ✓ AFB1 was present in trace amount in the 40% of the samples, while the other part was negative.
- ✓ 80% of the samples were positive to ZEA, ranging from 11.5 to 31 μg/Kg;
- ✓ DON was found in all samples, with a mean value of 115 μg/Kg;
- ✓ T-2 and HT-2 toxins were equally negative in the 80% of samples, while the remaining part contained traces;
- ✓ FB1 and FB2 were present in all samples, with values in accordance to the EU Recommendation guidelines (graph 11).

- **Premium senior**

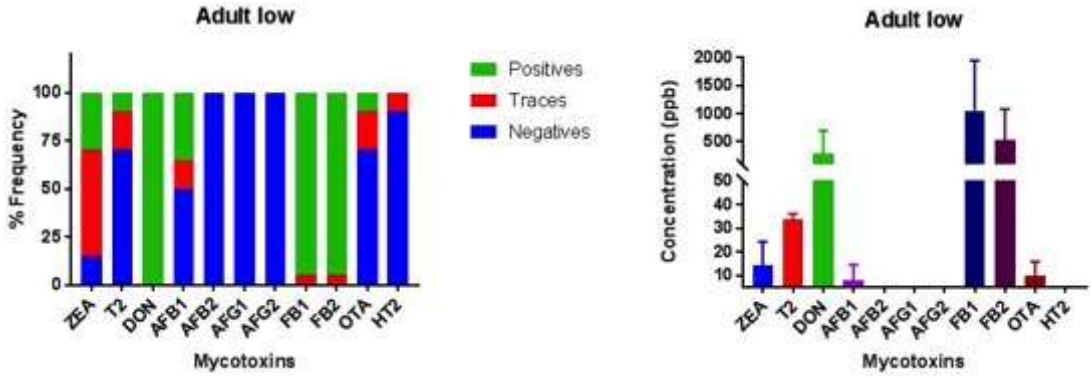


Graph 12. Senior premium pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In premium senior pet food for cats (n=5):

- ✓ AFB2, AFG1, AFG2, OTA were not detected;
- ✓ AFB1 trace was present only in one sample, the remaining samples were negative.
- ✓ 80% of the samples were positive to ZEA, ranging from 11.4 to 58.1 μg/Kg;
- ✓ DON was found in all samples, with a mean value of 224.6 μg/Kg;
- ✓ T-2 and HT-2 toxins were both negative in the 80% of the samples, the remaining part contained traces;
- ✓ FB1 and FB2 were found in 80% of the samples, these values were in accordance with the EU recommendation guidelines, a trace amount was detected in one sample (graph 12).

- **Standard adult low**



Graph 13. Adult low pet food for cats. Frequency of positive, negative, trace samples (on the left); mycotoxins concentration (on the right).

In standard adult low pet food for cats (n=20):

- ✓ AFB2, AFG1, AFG2 were not present; two samples exceeded the maximum value set for AFB1, with 18.4 μg/Kg and 16.5 μg/Kg respectively.
- ✓ 30% of samples were positive to ZEA, with a mean value of 13.9 μg/Kg;
- ✓ DON was found in all the samples, with a mean value of 273.9 μg/Kg;
- ✓ T-2 was present in 30% of the samples with a mean value of 33.2 μg/Kg, which represented more than the half of the guidance value set by EU Recommendation; HT-2 toxin was present in trace quantities in 10% of the samples, while the remaining part was negative;
- ✓ FB1 and FB2 were present in 95% of the samples, with values in accordance to the EU Recommendation guidelines;
- ✓ although 70% of samples were negative for OTA, 20% presented traces and two samples were positive with 14 μg/Kg and 5.1 μg/Kg (graph 13).
  The first one exceeded the guidance value set by the EU Recommendation.

The present study showed that four premium samples for adult cats and three standard samples for adult cats exceeded the guidance values for some mycotoxins.
Surprisingly two premium "diet obesity" samples, one premium "diet renal" sample and one premium "diet gastrointestinal" sample exceeded the guidance values/limit for FB1+FB2, AFB1 and T2 respectively.
The standard adult low category displayed that three samples exceeded the limit/guidance values for AFB1 (two samples) and OTA.
This fact could represent a critical point, since in this study some samples (designed with a specific formulation for cats diagnosed with pathological conditions or addressed to a selected age group) exceeded the guidelines/limit for some mycotoxins. Contaminations could also cause nutritional deficiencies despite a correct diet formulation (Di Cerbo et al. 2017).

This research also revealed that:

- one of the most common co-contamination was represented by ZEA, DON, FB1+FB2, since these mycotoxins were the most detected ones in the samples analysed;
- AFB2, AFG1, AFG2 were not present in any of these 9 categories;
- all samples contained quantified amounts of at least two types of mycotoxins, FB1 and FB2;
- most of the samples from the diet obesity-, kitten low-, senior low-, and senior premium-category displayed a contamination by four different types of mycotoxins;
- most of the samples from the diet renal-, diet gastrointestinal-, and kitten premium-category revealed a contamination by five different types of mycotoxins;
- as expected, premium grain-free samples, which did not contain starch from cereals but often from legumes and potatoes, represented the category with the lowest contamination level.

The co-occurrence of ZEA, DON, FB1+FB2 fairly agree with other studies conducted on pet food. A study from Oliveira et al. describing the occurrence of aflatoxins and fumonisins in corn intended for pet feed industry, revealed that fumonisins were detected in all samples evaluated (Oliviea et al. 2016).
Also a study from Błajet-Kosicka et al. 2014 described that DON and ZEA were identified in all dry pet food for cats and dogs analysed (Błajet-Kosicka et al. 2014).

Differently from a research monitoring mycotoxins in dry dog food (Gazzotti et al. 2015), this study showed an AFB1 contamination in 12.5% of samples evaluated. Moreover, as previously described, in the present work three samples exceeded the limit for AFB1 in pet food.

Regarding the statistical analysis ZEA was found with values significantly higher in premium products than in standard ones.
While DON was found with values significantly higher in standard products than premium ones.
So the present study pointed out that a high category price, such as for premium dry food for cats, does not automatically provide a mycotoxin free product.

5. CONCLUSIONS

In this research an LC-MS/MS method with IAC clean-up was developed to detect zearalenone, α-zearalanol, ß-zearalanol, α-zearalenol, ß-zearalenol and zearalanone simultaneously in bovine hair.

The method was also validated for three analytes (zearalanone, α-zearalanol, ß-zearalanol) in accordance with Decision 2002/657/EC, showing satisfactory recoveries and linearity, good precision, trueness and high sensitivity. Moreover the method was robust and stable, since the extracts could be stored in a freezer for one week before they were going to be analysed.

With this approach zearalenone and relative metabolites in bovine hair can be effectively monitored.

However the method also seemed capable to detect zearalanone, α-zearalanol, ß-zearalanol, zearalenone, α-zearalenol and ß-zearalanol in human hair in a lower range level.

For this a one-day validation was performed for all six analytes, showing reasonable results, although passable values in term of precision and trueness at 0.5 GV for α-zearalanol, α-zearalenol, ß-zearalenol and recoveries were obtained.

No previous study on zearalenone and related metabolites residues in bovine and human hair using LC-MS/MS has been published, thus the present work evaluated the suitability of this non conventional matrix for biomonitoring studies.

The exploring the potential of hair for biomonitoring studies of zearalenone and metabolites exposure is still in its initiation phase. Hair is a durable matrix that shows various benefits for biomonitoring, such as low cost, easy collection, non-invasive, easy storage and information about short and long-term exposure.

The set-up method on bovine and human hair can provide useful information regarding zearalenone and metabolites occurrence in feedstuffs and foodstuffs, which can represent a huge impact on agri-food sector and economy. Moreover using livestock for biomonitoring may represent an indirect method to better understand the contamination levels occurring in animal products, that can be a relevant part of human diet.

Furthermore this research could represent a useful tool both evaluating natural feed contamination or detect not legal use of α-zearalanol in bovine, both performing a first inventory of the occurrence of RALs in bovine and human hair as biomarkers for ZEA exposure in future studies.

The research study on pet food for cats showed that one of the most common co-contamination was represented by ZEA, DON, FB1+FB2.

Futhermore four premium samples exceeded the guidance values/limit for FB1+FB2, AFB1 and T2; while three samples from standard category exceeded the limit/guidance values for AFB1 (two samples) and OTA.

However these data highlighted the necessity to further investigate the potential synergistic effects that could occur.

Taking into account of the chronic exposure to which a cat is potentially exposed to when it is fed contaminated food for a long period of time, it is desirable to encourage pet food manufacturers to test for mycotoxins.

This aspect may also be achieved by improving the provisions of law, where necessary.

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