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**Food quality monitoring and analytical techniques
optimization of some aliments within plant-animal
correlation Contaminated aliments effects on the
detoxication enzymes**

Radu Corneliu Duca

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**Food quality monitoring and analytical techniques optimization of some
aliments within plant-animal correlation
Contaminated aliments effects on the detoxification enzymes**

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I dedicate my present work to my family, especially to my mother, who supported me unconditionally in all my demarches and goals, and to my dear wife who provided me a moral support and had sufficient patience to be close to me even from faraway.

Abstract

Zearalenone (Zen) is a secondary metabolite biosynthesised through a polyketide pathway by several *Fusarium* strains (*Fusarium graminearum*, *F. culmorum*, *F. equiseti*, and *F. crookwellense*). It is a non-steroidal estrogen or mycoestrogen and is frequently named phytoestrogen. Zen is a regular contaminant of cereal crops worldwide (Bennett and Klich, 2003). Zen resists to most common treatments occurred during food manufacturing and despite its non-steroidal structure, binds to estrogen receptors resulting in functional and morphological alterations in reproductive organs. It interacts not only with both types of estrogen receptors (Celius et al., 1999; Shier et al., 2001; Yu et al., 2004; Takemura et al., 2007), but also with the substrates for a number of hepatic enzymes. Zen is well-absorbed and is able to reach intracellular targets. The important disparity concerning the effects of Zen in animal species could in part result from the differences in their hepatic enzymes profile (Gaumy et al., 2001; Cavret and Lecoer, 2006). Zen metabolism is complex, dominated by conjugation reactions (considered as detoxification pathways) and reduction reactions which correspond to biological activation (Gaumy et al., 2001).

Our main objectives were the elucidation of the effects of contaminated aliments (especially with zearalenone) on the enzymes of detoxification (especially CYPs P450) and the understanding of Zearalenone effects on different species. In the available literature the effect of Zen on the expression and activity of detoxification enzymes is limited to *in vitro* experiments, to our knowledge *in vivo* researches has not been reported. In order to establish the zearalenone *in vivo* effects on the detoxification enzymes we performed several experiments: on rat, a classical animal model for the biological studies existing a large documentation within the specialized literature on the xenobiotics effects and in particularly on Zen effects, and on chicken, one of the species considered most resistant and “used” to Zearalenone presence. Complementary approaches have been used to determine zearalenone effects on the detoxification enzymes: a) development of analytical tools for the mycotoxins pharmacokinetics studies; b) determination of the *in vivo* effects of zearalenone on the detoxification enzymes expression and its becoming within animal organism c) *in vitro* zearalenone molecular mechanisms – the direct effects on hepatic detoxification enzymes d) species specificity and human risk assessment.

Important stimulatory effects on P-gp mRNA expression were observed, upon *in vivo* Zearalenone rat treatments, suggesting that P-glycoprotein might be implicated in the detoxification path of zearalenone. P-glycoprotein implication in Zen transport has been determined in Caco2 cell lines by Videmann et al., in 2008. The Zearalenone presence induces early and rapid metabolic responses, especially for CYP2C7, which could have an important role within Zen’s detoxification pathway in rats. An influence of Zearalenone treatments on the mRNA expression and enzyme activities of the P450 isoforms CYP2B2 and 3A was also stated. CYP2C7 and CYP3A1 are rat homologues of human CYP2C8 and CYP3A4, respectively (Nelson, 1999), cytochromes implication in the formation of a new 8-hydroxy Zen metabolite. The *in vivo* occurrence of this 8-hydroxy Zen metabolite was also determined. Zearalenone becoming within rat and poultry was assessed using new developed methods: HPLC-DAD and LC-MS (using enriched ¹³C Zen as internal standard). In rats, Zearalenone is rapidly eliminated in the urine within the first 6 hours after administration; about **60%** ($47.8 \pm 14.3 \mu\text{g}$) of the total amount of urinary eliminated Zen ($79.8 \pm 23.9 \mu\text{g}$), and the overall urinary eliminated Zen and metabolites are about 4% of the administrated Zen. In poultry muscle samples the levels of α -Zol ($13.42 \mu\text{g}/\text{kg}$) are higher than the JEFCA accepted level ($2 \mu\text{g}/\text{kg}$) and are not suitable for human consumption. A worldwide risk assessment of human zearalenone exposure, taking in to account the eating habits, was done resulting in an important and constant human health risk. Also the necessity of regulation changes concerning the acceptable maximum level of zearalenone in cereals, a $5 \mu\text{g}/\text{kg}$ we consider to be acceptable.

Résumé

Zéaralénone (Zen) est un métabolite secondaire de type polykétide biosynthétisé par différentes espèces de *Fusarium* (*Fusarium graminearum*, *F. culmorum*, *F. equiseti*, et *F. crookwellense*). Il s'agit d'un oestrogène non-stéroïdien (ou mycoestrogène) souvent nommé phytoestrogène. La Zen est un contaminant de cultures de céréales dans le monde entier (Bennett et Klich, 2003). Elle résiste à la plupart des traitements qui ont lieu au cours de la fabrication des denrées alimentaires. En dépit de sa structure non-stéroïdienne, le zen est capable de se lier aux récepteurs des oestrogènes et provoque des altérations morphologiques et fonctionnelles des organes de reproductions. Elle interagit non seulement avec les deux types de récepteurs aux œstrogènes (Celius et al., 1999; Shier et al., 2001; Yu et al., 2004; Takemura et al., 2007), mais aussi avec les substrats dans un certain nombre d'insuffisance des enzymes hépatiques. La Zen est bien absorbée et est capable d'atteindre des cibles intracellulaires. La disparité importante des effets de la Zen entre les différentes espèces animales pourrait en partie être due à la différence entre leur profil des enzymes hépatiques (Gaumy et al., 2001; Cavret et Lecoœur, 2006). Le métabolisme de la Zen est complexe, il est dominé par des réactions de conjugaison (considérées comme des voies de détoxification) et des réactions de réduction qui correspondent à l'activation biologique (Gaumy et al., 2001).

Nos principaux objectifs étaient l'élucidation des effets des aliments contaminés (en particulier avec le zéaralénone) sur les enzymes de détoxification (en particulier CYP P450) et la compréhension des effets de la zéaralénone sur les différentes espèces. Dans la littérature l'effet de la Zen sur l'expression et sur l'activité des enzymes de détoxification est limité à des expériences *in vitro*, à notre connaissance aucune recherche *in vivo* n'a encore été publiée. En vue d'établir les effets *in vivo* de la zéaralénone sur les enzymes de détoxification nous avons effectué plusieurs expériences: tout d'abord sur le rat, qui est un modèle animal classique pour les études biologiques et sur lequel on trouve une large documentation sur les effets des xénobiotiques et en particulier sur les effets de la Zen, et ensuite sur le poulet, l'une des espèces considérée comme la plus résistante et «habituee» à la présence de la Zéaralénone. Des approches complémentaires ont été utilisées pour déterminer les effets de la zéaralénone sur les enzymes de détoxification: a) Le développement d'outils d'analyses pour des études de la pharmacocinétique des mycotoxines; b) la détermination *in vivo* des effets de la zéaralénone sur l'expression des enzymes de détoxification et de son devenir dans l'organisme animal c) du mécanisme moléculaire *in vitro* de la zéaralénone - les effets directs sur les enzymes de détoxification hépatique d) la spécificité des espèces et l'évaluation des risques humains.

D'importants effets stimulants sur l'expression d'ARNm de la P-gp ont été observés *in vivo* chez le rat traité avec Zéaralénone, ce qui suggère que la P-glycoprotéine pourrait être impliquée dans la voie de détoxification de la zéaralénone. L'implication de la P-glycoprotéine dans le transport de la Zen a été déterminé dans des lignées cellulaires Caco2 par Videmann et al., en 2008. La présence de zéaralénone induit des réponses métaboliques précoces et rapides, en particulier pour le CYP2C7, il pourrait jouer un rôle important dans la voie de détoxification de la Zen chez le rat. Une influence des traitements par la Zéaralénone sur l'expression des RNAm et sur l'activité des isoformes de P450, CYP2B2 et 3A a également été réalisée. CYP2C7 et CYP3A1 sont respectivement les homologues chez le rat des formes CYP2C8 et CYP3A4 humaine (Nelson, 1999), ces cytochromes sont impliqués dans la formation d'un nouveau métabolite le 8-hydroxy Zen. *In vivo* l'apparition de ce métabolite (le 8-hydroxy Zen) a également été démontrée. Le devenir dans le rat et le poulet de la Zéaralénone a été évalué en utilisant les nouvelles méthodes développées: HPLC-DAD et LC-MS (en utilisant comme étalon interne Zen ¹³C enrichi). Chez le rat, la zéaralénone est rapidement éliminée. Elle est retrouvée dans les urines, pendant les 6 premières heures après l'administration : environ 60% (47,8 ± 14,3 mg) du montant total de la zéaralénone urinaire éliminé (79,8 ± 23,9 mg) et la Zen et ses métabolites éliminés par l'urine sont d'environ 4% de la zéaralénone administrés. Dans les échantillons de muscle de poulet les niveaux d' α -Zol (13,42 mg / kg) sont plus élevés que le niveau JEFCA accepté (2 mg / kg). Ces volailles sont donc impropres à la consommation. A l'échelle mondiale l'évaluation des risques de l'exposition de l'homme à la zéaralénone, en prenant en compte les habitudes alimentaires, a été réalisée et il en résulte un important et constant risque pour la santé humaine. Aussi la nécessité d'apporter des modifications au règlement concernant la teneur maximale acceptable de la zéaralénone dans les céréales a été surlignée, et nous considérons comme acceptable la valeur 5 µg/kg.

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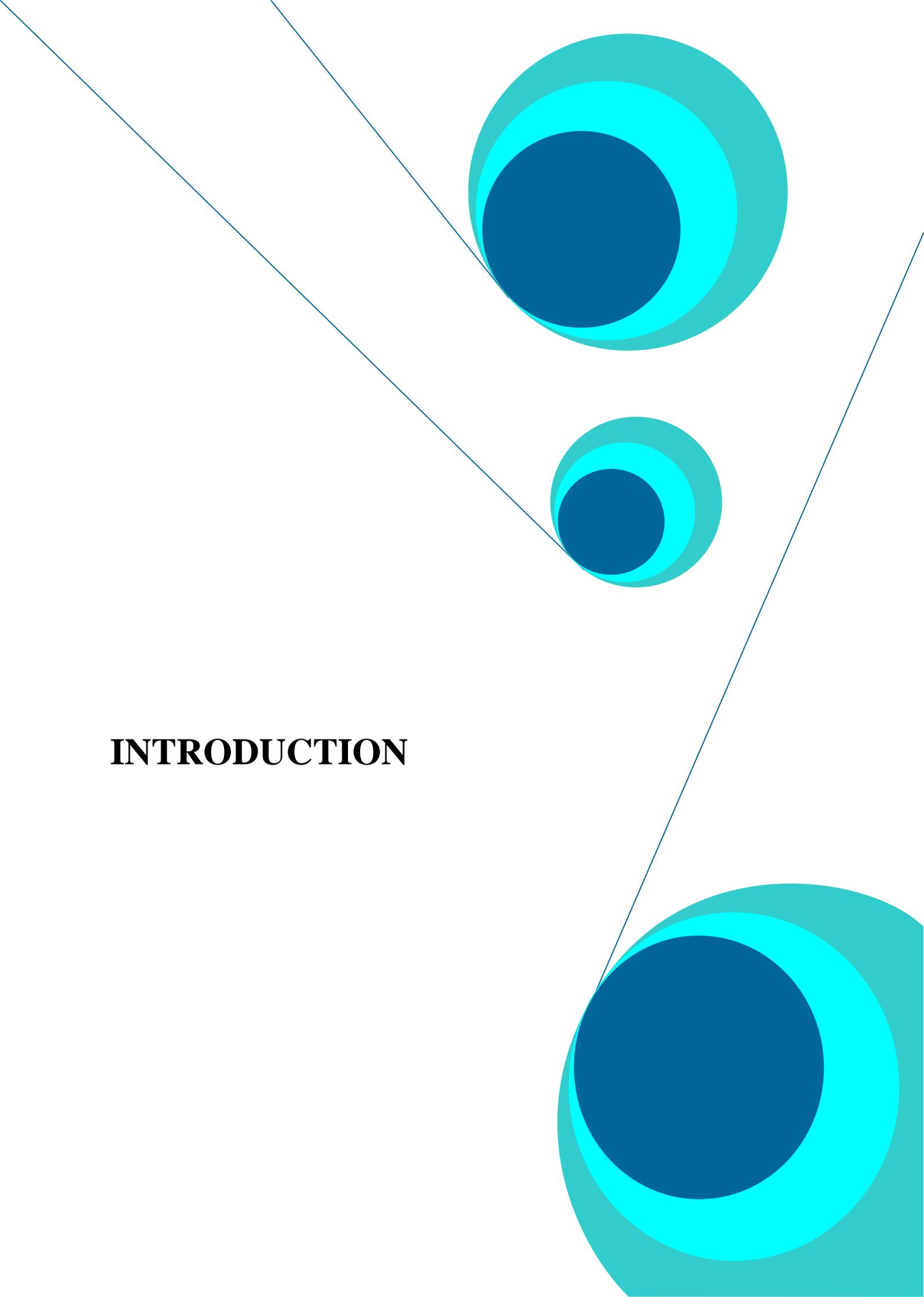
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List of abbreviations

ABC	ATP-Binding Cassette
ABCB1	P-gP: P-glycoprotein
ABCC, MRP	Multidrug resistance associated protein;
ABCG2, BCRP	Breast Cancer Resistance Protein
Ac Ac	Acid Acetic
ACN	Acetonitrile
AD	Added Dose
ADP	Adenosine Di Phosphate
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
ATP	Adenosine Tri Phosphate
b.w.	Body weight
CEA	Commissariat de l'Énergie Atomique
Clof	Clofibrate
COMT	Catechol-O-Methyl-Transferase
CYP	Cytochrome P450
CYP19	Aromatase
DM	Dexamethasone
DON	Deoxynivalenol
DSV	Direction des Sciences du Vivant
E ₂	Estradiol
ER	Estrogenic Receptor
FAO	Food and Agriculture Organization
FB 1,2,3	Fumonisin B1, B2, B3
FDA	Food & Drug Administration
G6P	Glucose-6-phosphate;
G6PDH	Glucose-6-Phosphate Dehydrogenase
GC	Gas Chromatography
GSH	Glutathione
HIF1B, ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
HPLC	High Performance Liquid Chromatography
HSD	Hydroxy Steroid Dehydrogenase
HSD17B	17-beta hydroxysteroid dehydrogenase
HSD3B	3-beta hydroxysteroid dehydrogenase
<i>i.p.</i> ,	IP: intra-peritonealy
IAC	Immunoaffinity Column
IARC	Internal Agency for Research on Cancer
ID ₅₀	Inhibition Dose for 50% of exposed individuals
INRA	Institut National de Recherche Agronomique
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD ₅₀	Lethal Dose for 50% of exposed individuals

LOAEL	Low Observed Adverse Effect Level
MRP	Multi Drug Resistance Associated Protein
MS	Mass Spectroscopy
NADP(H)	Nicotinamid Adenine Dinucleotide Phosphate (Hydrogenized or reduce form)
NCI	National Cancer Institute
NOAEL	No Observed Adverse Effect Level
NR1C1, PPARA	Peroxisome Proliferative Activated Receptor Alpha
NR1I2, PXR	Pregnane Xenobiotic Receptor
NR2B1, RXRA	Retinoid Xenobiotic Receptor Alpha
NR3A1, ESR1	Estrogen Receptor
OH-Zen	Hydroxy-Zearalenone
OMS	World Health Organisation
<i>p.o.</i> , PO	<i>per os</i>
PB	Phenobarbital
PBS	Phosphate Buffer Saline
qRT-PCR	quantitative real time PCR
SCF	Scientific Committee on Food
SG	NADPH Generator System
SPE	Solid Phase Extraction
TDI	Tolerated Daily Intake
TMS	Tri Methyl Silan
TST	Testosterone
UDP	Uridine Di Phosphate
UGT	UDP – glucuronosyl transferases
UV	Ultra Violet
Zan	Zearalanone
Zen	Zearalenone
α -Zal	α -Zearalanol
α -Zol	α -Zearalenol
β -NF	β -Naphtoflavone
β -Zal	β -Zearalanol
β -Zol	β -Zearalenol
FID	Flame Ionization Detector
MeOH	Methanol
DAD	Diode Array Detector
λ	Wave length
CV	Cyclic Voltammetry
DPV	Differential Pulse Voltammetry
GCE	Glassy Carbon Electrode



INTRODUCTION

Introduction

Understanding the influence of environmental factors¹ of synthetic or natural origin for health is a vast field of investigation. This implies to precisely quantify the acute or chronic exposure to such factors, to identify the role of each component in question and to quantify their contribution in the genesis or aggravation of many diseases, especially multifactorial ones and with unknown aetiologies. Knowledge of the interaction of these factors with other environmental determinants of health, whether behavioural, social or genetic, is a challenge for the scientific research. The challenge is to understand the means available to protect the health of population, and the conditions that must be met for an action to have the desired effect. National authorities shall ensure control over the commercialization, use, dissemination, and presence of certain products. For example: residues and degradation products in the environment or in food, chemicals, but also pesticides, additives, residues of medicinal products for human or veterinary use, or environmental contaminants (dioxins, polycyclic aromatic hydrocarbons, mycotoxins). The continuous human exposure to these compounds should be below levels likely to have adverse effects on consumer health (INRA, 2004)

Humans and animals have evolved different enzymatic systems to convert xenobiotics and drugs into hydrophilic metabolites, more easily eliminated via the bile and urine. These biotransformation reactions take place mainly in the liver, which expresses the most prominent class of biotransformation enzymes, but other organs including the lungs, kidneys and intestines may contribute to the overall xenobiotic biotransformation. Differences in biotransformation enzyme activities alter the systemic bioavailability and subsequently the efficacy of drugs; they may also provide protection against certain xenobiotics and environmental pollutants, but can increase the toxicity of others (Snawder and Lipscomb, 2000).

Among the food contaminants, some have hormone-mimetic properties. There may be estrogenic properties (similar to female hormones) or anti-androgenic (preventing the action of male hormones). These substances, called xenoestrogens, may act on the endocrine system of animals and humans (Afsset, 1996-2000). Among them, zearalenone, produced by fungi of the *Fusarium* family (filamentous fungi) is currently researched in

¹ In epidemiology, **environmental factors** are those determinants of disease that are not transmitted genetically. Stress, physical and mental abuse, diet, exposure to toxins, pathogens, radiation and chemicals found in almost all personal care products and household cleaners are common environmental factors that determine a large segment of non-hereditary disease.

animal feeds and meat products. This mycotoxin has been studied in this co-directed thesis; researches have been conducted in France at CEA Saclay in the Department of Life Sciences², under the supervision of Marcel Delaforge and in Romania in the Laboratory of Chemistry and Animal Physiology, INCDBNA³ and the Department of Analytical Chemistry⁴, University of Bucharest under the supervision of Luminita Vladescu. Our main objectives were the elucidation of the effects of contaminated aliments (mainly with zearalenone) on the enzymes of detoxification (especially CYPs P450) and the understanding of Zearalenone effects on different species.

Why Zearalenone?

The interest for this mycotoxin arises from the high human exposure to it and from its toxicological properties:

- ✓ It is a regular contaminant of cereal crops worldwide;
- ✓ It resists to most common treatments occurring during food manufacturing;
- ✓ It is well-absorbed and it is able to reach intracellular targets;
- ✓ It binds to estrogen receptors resulting in functional and morphological alterations in reproductive organs;
- ✓ Its biological and chemical properties may be feared, zearalenone presents a risk to human health by disrupting the hormonal balance;
- ✓ Its effects on the expression and activity of detoxification enzymes are limited to *in vitro* experiments.

To elucidate the effect of zearalenone on the detoxification enzymes (especially CYPs P450) and to understand Zen effect on different species, we have to answer several key questions:

- ✓ Can we develop new technological tools to track traces of zearalenone and its derivatives in biological matrices?
- ✓ Can we characterize zearalenone metabolisation and bio-transformation within the animal organism?

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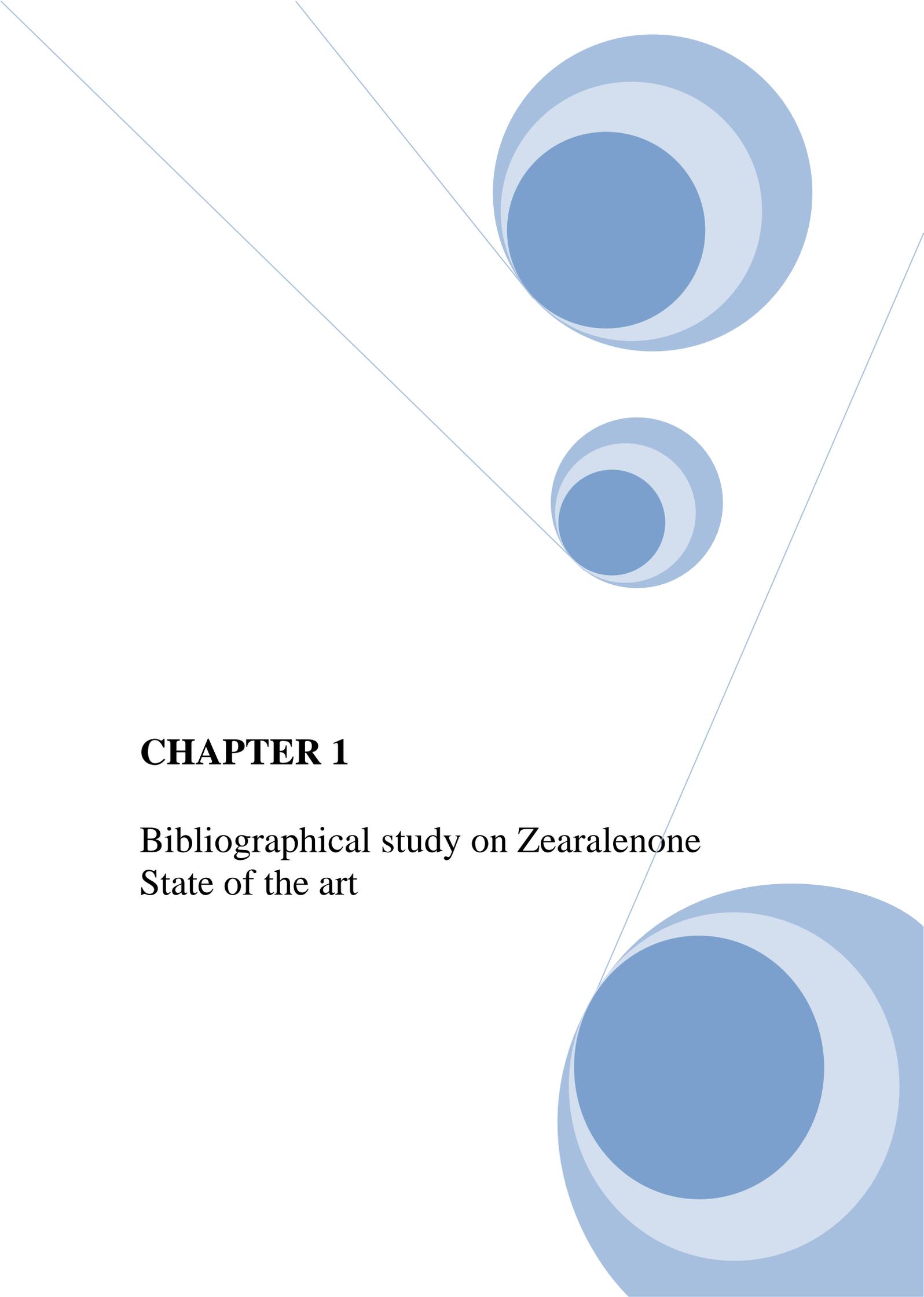
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Introduction

- ✓ Does zearalenone affect the hepatic detoxification enzymes? If yes, which are its *in vivo* effect and transformation, and its molecular mechanism?
- ✓ Are there species specificities linked to Zen metabolism? If yes, are humans undergoing a risk?

In order to answer to these questions, in the next sections we present:

1. The state of the art presentation of zearalenone concerning its occurrence and effects on animal / human organism.
2. The new analytical tools for zearalenone pharmacokinetics studies
3. The *in vivo* effect of zearalenone on the detoxification enzymes expression and its bio-transformation within the animal organism
4. The *in vitro* molecular mechanisms of zearalenone and the direct effect on the hepatic detoxification enzymes
5. The species specificity and human risk assessment

A decorative graphic on the right side of the page. It features three blue circles of varying sizes, each composed of concentric rings of different shades of blue. Two thin, light blue lines intersect at a point, forming a V-shape that frames the circles. The circles are positioned at the top, middle, and bottom of the right side.

CHAPTER 1

Bibliographical study on Zearalenone
State of the art

1.1. Mycotoxins

Among the secondary metabolites¹ of fungi, mycotoxins are toxic compounds that are difficult to define simply and comprehensively. Mycotoxins are natural compounds of low molecular weight, secondary metabolites of filamentous fungi belonging to the strains *Aspergillus*, *Penicillium* and *Fusarium* (Table 1.1).

Table 1.1. Mycotoxins and fungi found associated with producing feed and food (Risks assessment associated with the presence of mycotoxins in feed and food chain. AFSSA 2006)

	Mycotoxins	Major producing molds
Mycotoxins regulated or ongoing regularization	Aflatoxins B1, B2, G1, G2 Ochratoxin A Patulin Fumonisin B1, B2, B3 Trichothecenes (DON, NIV) Zearalenone	<i>Aspergillus flavus</i> , <i>A. parasiticus</i> , <i>A. nomius</i> <i>Penicillium verrucosum</i> , <i>Aspergillus ochraceus</i> , <i>Aspergillus carbonarius</i> <i>Penicillium expansum</i> , <i>Aspergillus clavatus</i> <i>Byssoschlamys nivea</i> <i>Fusarium verticillioides</i> , <i>F. proliferatum</i> <i>Fusarium graminearum</i> , <i>F. culmorum</i> <i>F. crookwellense</i> , <i>F. sporotrichioides</i> <i>F. poae</i> , <i>F. tricinctum</i> , <i>F. acuminatum</i> <i>Fusarium graminearum</i> , <i>F. culmorum</i> <i>F. crookwellense</i> .
Other mycotoxins	Ergot alkaloids (the ergot of rye) Citrinin Alternaria toxins (alternariol, alternariol methyl ether ...) Acid cyclopiazonique Sterigmatocystin Sporidesmines Stachybotryotoxines Toxins endophytes (ergovaline, lolitrem B) Phomopsines Toxins tremorgenes	<i>Claviceps purpurea</i> , <i>C. paspali</i> , <i>C. africana</i> <i>Aspergillus terreus</i> , <i>A. carneus</i> , <i>A. niveus</i> <i>Penicillium verrucosum</i> , <i>P. citrinum</i> , <i>P. expansum</i> <i>Alternaria alternata</i> , <i>Alternaria solani</i> <i>Aspergillus flavus</i> , <i>A. versicolor</i> , <i>A. tamaritii</i> <i>Penicillium</i> dont <i>P. camemberti</i> <i>Aspergillus nidulans</i> , <i>A. versicolor</i> , <i>A. flavus</i> <i>Pithomyces chartarum</i> <i>Strachybotrys chartarum</i> <i>Neotyphodium coenophialum</i> , <i>N. lolii</i> <i>Phomopsis leptostromiformis</i> <i>Penicillium roquefortii</i> , <i>P. crustosum</i> , <i>P. puberrelum</i> <i>Aspergillus clavatus</i> , <i>A. fumigatus</i>

If mycotoxins are of fungal origin, not all the toxic compounds produced by fungi are mycotoxins; the factors of importance being the target and the concentration of the

¹ **Secondary metabolites** are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms. Unlike primary metabolites, absence of secondary metabolites results not in immediate death, but in long-term impairment of the organism's survivability/fecundity or aesthetics, or perhaps in no significant change at all.

metabolite. Thus, fungi products that are toxic to bacteria (such as penicillin) are usually named antibiotics and ones that are toxic to plants are called phytotoxic (Bennett and Klich, 2003). There is a possible application of such products in agriculture: some herbicides may be selective (tentoxine), or total. Others may be peptide compounds, like α -amanitine produced by certain *Amanitas*, which is an inhibitor of eukaryotes RNA polymerase II (Labia and Michelot, 1988). Mycotoxins may develop on the plant, in the field or during storage, and are potentially toxic to human and animals.

More than 12 000 secondary metabolites have been identified from the AntiBase in 2008, but only thirty having toxic properties of a real concern. Toxins are natural contaminants of many food of plant origin: mainly cereals, but also fruits, nuts, almonds and feed compounds, manufactured for use as food and feed (Charmley, 1995; Underhill, 1996; Charmley, 2000).

It should be noted that certain aspects and effects of fungal secondary metabolites are of great interest to man and may be the subject of scientific research:

- As has been reported, the phytotoxicity was used in the agricultural industry;
- Certain toxic effects have been studied and found to have a real therapeutic value: this is the case for ergot. Medicine has benefited using its vasodilator effects. Today derived of ergot molecule are used in particular in the treatment of migraine crises or Alzheimer.

However, the balance toxicity / therapeutic properties is sometimes weak. Indeed, the reactivity of these compounds is closely related to their structure: the pharmacophore² and toxicophore³ layouts may be close. The reactive part of the molecule recognized by the pharmacophore (or another part of the molecule) may be unstable and the source of more reactive compounds (radicals or epoxy). Toxicity then covers the first therapeutic effects. This applies, for example, to cyclosporine, which, despite immunosuppressive properties, has proven to be toxic for the kidney at high concentrations.

Thus, it is important to keep in mind that 90% of medicines come from natural compounds and very few are entirely of synthetic nature. If mycotoxins, with their large number and low doses are still unclear, it remains true that alongside the pathogenic

² A **pharmacophore** is an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response.

³ A **toxicophore** is a feature or group within a chemical structure that is thought to be responsible for the toxic properties, either directly or via metabolic activation.

effects is now necessary to take into account that some have a certain therapeutic potential once adverse reactions identified and controlled. These can be limited for example by hemisyntheses in order to inactivate the toxicophore.

1.1.1. General overview

Mycotoxins are toxic secondary metabolites produced by fungi (molds). These fungal toxins are diverse on a chemical level - they belong to different chemical families - and their molecular weight varies from approximately 200 to 500. There are hundreds of known mycotoxins, but few have been researched and there are appropriate analysis methods only for a smaller number (Whitlow, 2001).

The fungi that produce them, can affect crops in the field, during handling or storage. From a practical point of view, a mycotoxin is a fungal metabolite that causes side effects in animals or humans who are exposed. Exposure usually occurs by consumption of contaminated foods, including foods for humans or livestock. The mycotoxicoses are diseases caused by exposure to foods contaminated by mycotoxins (Nelson et al., 1993). Mycotoxins produce various biological effects in animals, including liver toxicity and renal anomalies of central nervous system, estrogenic responses, respiratory diseases by inhalation (invasive aspergillosis) and other effects.

We distinguish among the mycotoxins, considered important from a food and health point of view (e.g. among the products tested and are subject to standards and recommendations): aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and trichothecenes (including deoxynivalenol and T-2 toxin) (Charmley, 1994). It should be noted that the toxicity may vary widely from one toxin to another and that the risk is not always the toxin itself, but also its metabolites. Historically, the oldest known mycotoxicosis in France is the ergotism. From the Middle Ages hallucinogenic effects produced by the ingestion of a parasite of rye, rye ergot or *Claviceps purpurea*, have been described. The symptoms took the form of delirium, prostration, severe pain, abscesses, and gangrene of the extremities, leading to severe and incurable disabilities. Epidemics have plagued the eighth and the sixteenth century due to the people miserable alimentation, especially the consumption of flour contaminated with sclerotia⁴ of the fungus. In France, the last episode occurred in 1951 in Pont Saint-Esprit in the Gard.

⁴ A **sclerotium** (plural *sclerotia*) is a compact mass of hardened mycelium stored with reserve food material that, in some higher fungi such as ergot, becomes detached and remains dormant until a favorable opportunity for growth occurs.

Today, we know that the "Fire of St. Anthony" is attributable to certain alkaloids produced by ergot. In addition, around 1900, Japanese researchers link specific clinical manifestations with the ingestion of moldy rice. In U.R.S.S. between 1942 and 1947 scores of people die (up to 10% in some communities) following consumption of wheat and millet contaminated with a microorganism of the *Fusarium* strains.

In the early 60s, the characterization of aflatoxin will be the starting point for systematic research on mycotoxins and their effects. The fact that aflatoxins have proved to be the most potent natural carcinogens, is not foreign to this sudden interest. Since then, the list of recognized molds capable of producing toxins continues to grow. In fact, toxigenic molds can grow in all climates, on all the media solid or liquid, if there are nutrients and humidity. This explains the great variety of foods and environmental substrates involved. Nevertheless, cereals have the greatest risk factor in view of their consumption and frequency of contamination. In the 80s, the Food and Agriculture Organization (FAO) estimated that at least 25% of the grains produced in the world were contaminated by mycotoxins. This phenomenon is even more important because it is becoming more important: with the warming climate, geographical areas that previously had no (or little) fungal contamination, become favourable to the development of mycotoxin-producing strains.

In addition to the health issues posed by the contamination of mycotoxins, inherent socio-economic aspects emerge recently. If, in order to export their grains, the developing countries are submitted to the sanitary regulations concerning food and feed of the developed countries, the local people remain largely exposed to a mycotoxic risk through uncontrolled local markets. This is the case of South Africa (oral presentation by Gordon Shephard at the XIIth International Congress of IUPAC on mycotoxins and phycotoxins, held from 21 to 25 May 2007 in Istanbul).

Chronic effects (repeated exposure to low or very low doses) are the most feared because of dietary habits and the power of persistence of these toxins. In addition, a food or other media can contain multiple mycotoxins simultaneously.

1.1.1.1. Occurrence

In the light of research conducted in North America, it is likely that the main types of fungi producing mycotoxins are *Aspergillus*, *Fusarium* and *Penicillium* (Whitlow, 2001). Several species of these fungi produce mycotoxins in food. Molds are fungi that grow into multicellular colonies, unlike yeasts that are unicellular fungi. Molds can grow and

produce mycotoxins before or after harvest or during storage, transport, processing or alimentation. The proliferation of molds and production of mycotoxins are associated with extreme weather, inadequate storage practices, poor quality food and bad alimentation conditions. In general, environmental conditions (heat, water, insect damages), stress and predispose plants to mycotoxins contamination in the field. Temperature, moisture content and insect activity are the main factors influencing the contamination of feed grains and feed by mycotoxins after harvest (Coulombe 1993).

Molds proliferates in temperatures ranging between 10 and 40°C, pH may be between 4 and 8, and moisture content higher than 0.7 a_w (water activity⁵). While yeasts require free water, molds can grow on a dry surface (Lacey, 1991). They can also grow on foods that contain more than 12 or 13% humidity. In the wet foods such as cheese, molds (e.g. *Penicillium roqueforti*) will develop in the presence of oxygen and a suitable pH. Since most molds are aerobic, the very wet media exclude adequate oxygen supply and can prevent mold growth. The most favourable conditions for their proliferation may not coincide with optimal conditions for the formation of mycotoxins in the laboratory. For example, it was observed that molds of the *Fusarium* strain proliferate between 25 and 30°C without producing important quantities of mycotoxins, while at temperatures close to freezing point, a large quantity of mycotoxins are produced when fungi present a minimal growth (Joffe, 1986). The fungicide treatments in the fields could reduce the proliferation of mold, and thus the production of mycotoxins, but stress or shock caused by the fungicide on the fungus could stimulate the production of mycotoxins (Boyacioglu et al. 1992; Ceynowa and Gareis, 1994). Compared to the *Fusarium* species, the *Aspergillus* strains are developing normally in environments where water activity has low values and temperatures are high. Therefore *Aspergillus flavus* and aflatoxins frequently infest maize grown under stress conditions caused by heat and dryness in the warm climates. Aflatoxin contamination is favoured by the damage inflicted by insects (before and after harvest) that creates openings for the fungi penetration. The species of the genus *Penicillium* are widespread and proliferate in the presence of a relatively low water and low temperature. Because *Aspergillus* and *Penicillium* requires only low water activity to grow, they are considered as storage fungi (Christensen et al., 1977).

⁵ **Water activity** is a dimensionless quantity used to represent the energy status of the water in a system. It is defined as the vapor pressure of water above a sample divided by that of pure water at the same temperature; therefore, pure distilled water has a water activity of exactly one. It is widely used in food science as a simple, straightforward measure of the dryness of food; foods typically have an optimum water activity at which they have the longest shelf life

1.1.1.2. Human and animal disorders

Most toxicity studies are related to the ingestion of contaminated food, but inhalation and dermal exposure are also caused signs of toxicity. Thus the toxic effects of several mycotoxins, including those that occur most commonly, are well documented in several animal species and humans (Trenholm, 1988; 1996-1997). The toxicity of these natural contaminants can be direct or indirect. Some mycotoxins have a very strong acute toxicity following exposure to a single high dose. In all cases, the chronic effects (repeated exposure to low or very low doses) are the most feared because of the persistence of these toxins often resistant to temperature and process technology used in the food industry (Sorensen and Elbaek, 2005). Toxicity is variable (Table 1.2), some mycotoxins are known or suspected to be carcinogens (aflatoxins, ochratoxin A, fumonisins). Some mycotoxins exert hepatotoxicity (aflatoxins), others are estrogenic (zearalenone), immunotoxic, hematotoxic (patulin, trichothecenes and fumonisins) or dermonecrotic (trichothecenes) (Bunger, 2004).

Table 1.2. Major mycotoxins identified or suspected effects and the cellular and molecular mechanisms of action identified experimentally (Assessment of risks associated with the mycotoxins presence in food and feed chain. AFSSA, 2006)

Toxin	Effects	Cellular mechanisms of action and molecular
Aflatoxin B1 + M1	Hepatotoxicity Genotoxicity Carcinogenicity Immunomodulation	Formation of DNA adducts Lipid peroxidation Bioactivation by cytochrome P450 Combination with GS-transferases
Ochratoxin A	Nephrotoxicity Genotoxicity Immunomodulation	Nephrotoxicity Impact on protein synthesis. Inhibition of ATP production Detoxification by peptidases
Patulin	Neurotoxicity In vitro mutagenesis	Indirect inhibition of enzymes
Trichothecenes (T-2 toxin, DON ...)	Haematotoxicity Immunomodulation Dermal toxicity	Induction of apoptosis in progenitor haematopoietic and immune cells Impact on protein synthesis Alteration of immunoglobulin
Zearalenone	Fertility and Reproduction	Binding to estrogen receptors Bioactivation by reductases Conjugation to glucuronyltransferases
Fumonisin B1	Lesion of the central nervous system Hepatotoxicity Genotoxicity Immunomodulation	Inhibition of the synthesis of ceramide Alteration of the report sphinganine / sphingosine Alteration of cell cycle

Table 1.3. Toxic effects of some mycotoxins on humans organs

Mycotoxin	Liver	Digestive tube	Nervous system	Rein	Endocrines glands	Skin	Blood
Aflatoxin B1	+	+	+	+	+		
Ochratoxin A	+		+	+			+
Vomitoxin	+	+	+			+	+
T2-toxin	+	+	+			+	+
Zearalenone				+	+		
Fumonisin B1							

Mycotoxins exert their effects through 3 main mechanisms:

(1) **Reducing the amount of nutrients available to the animal.** This effect is the result of a multi-factorial process. First, there may be an alteration of nutrient content of foods during the molding process. The proliferation of mold can reduce the nutrient such as vitamins, and the content of amino acids such as lysine (Kao and Robinson, 1972). Thus, the mold tends to reduce the energy value of feed. Secondly, some mycotoxins, because of qualitative considerations (taste, smell, etc.) reduce food consumption and therefore nutrient. Thirdly, irritation of the digestive system caused by mycotoxins can reduce the absorption of nutrients. Fourthly, some mycotoxins disrupt the normal metabolism of nutrients: like in the case of protein synthesis inhibited by T-2 toxin.

(2) **Effects on the endocrine and exocrine glands.** The effect of zearalenone on the reproductive performance, because of its estrogenic action, is an example. The estrogenic effects of zearalenone result from the mycotoxin and its derivatives affinity for the estrogen receptors of the animal (Kiang et al., 1978).

(3) **Immunosuppression.** The effects of mycotoxins on the immune system have been studied (Sharma, 1993). Trichothecenes, such as DON and T-2 toxin, reduce immunity by inhibition of protein synthesis and thus cell proliferation. Some mycotoxins have a cytotoxic action *in vitro* on lymphocytes. Corticosteroids produced in response to stress also reduce immune function.

Some of the symptoms observed in mycotoxicoses may be secondary in nature, e.g. they result from an opportunistic illness because of the immunosuppression. Thus, the growth and diversity of the symptoms are confusing and the diagnosis is difficult (Hesseltine, 1986; Schiefer, 1990). In fact, the diagnosis is also complicated by the lack of relevant

researches, the lack of feed analysis, by non-specific symptoms and interactions with other stress factors. Against this background, it should develop means of prevention strategies including agronomics (good agricultural practices including the selection of varieties, cultivation, processing plant ...), improving the conditions for harvesting and storage and improved monitoring throughout the food chain. The case of organic production method restricts the use of fungicide treatments, but focuses on techniques against contamination by mycotoxins such as crop rotation, tillage or low nitrogen inputs. The available data for contamination by mycotoxins of products from organic farming, even limited, show variable rates of infection but may not be relieved of significant differences with those of products of conventional agriculture (Bellon et al., 2000).

1.1.1.3. Risk assessment and legislative aspects

Results of contamination, generally recognized as of vegetal origin, mycotoxins are therefore a current problem concerning the quality and safety of feed and food stuffs. Risk assessment of mycotoxins remains difficult due to several reasons:

- ü this risk is depending of natural factors, which are independent of humans control (especially weather conditions),
- ü fungal contamination is hard to control,
- ü there may be multiple mycotoxins present due to the ability of the same mold to produce several mycotoxins (AFSSA, 2006).

Numerous studies have demonstrated that several mycotoxins may be found in the same feed (Hagler et al., 1984). For example, Abbas et al. have demonstrated that *Fusarium* species isolated from maize in Minnesota have produced multiple mycotoxins (Abbas et al., 1989). Given that animals are fed a mixture of feeds and molds, which produce a variety of mycotoxins, thus several mycotoxin interactions are possible.

Because of their partial degradation in the rumen, mycotoxins are less toxic to cattle than for most other animals. Nevertheless, mycotoxins are not completely degraded and some degradation products are toxic (Kiessling et al., 1984). Dietary factors known to interact with mycotoxins include nutrients such as fats, proteins, fibres, vitamins and minerals (Smith et al. 1971; Brucato et al. 1986; Coffey et al. 1989). The agglomerating agents used in feeds (clay) and other additives, such as glucomannan, bind some mycotoxins and thus reduce exposure of the animal (Diaz et al., 1999). Galvano et al. have recently studied the interaction of mycotoxins with dietary factors such as antioxidants, medicinal

herbs, plants extracts and minerals and biological agglomerating agents (Galvano et al., 2001). Some of these dietary factors modify the response of animals to mycotoxins, which has the effect of generating mixed reactions in the field, but also to provide data in order to modulate the toxicity to animals. Thus, the alimentary approaches appear promising in order to protect animals against the effects of mycotoxins and prevent the risk of mycotoxins that can contaminate food for human consumption.

Some of the factors that make diagnosis difficult also contribute to the difficulty of setting safety thresholds. These include the lack of research, sensitivity differences between animal species, the imprecision of the sampling and analysis, the large number of mycotoxins potential interactions with other mycotoxins and interactions with the stress generated by the environment and production (Hamilton, 1984; Schaeffer and Hamilton, 1991). The effects of mycotoxins are also modulated by factors such as gender, age, diet and duration of exposure. It is therefore impossible to provide specific guidance as to the concentrations of mycotoxins, which produce a mycotoxicosis on the ground. The recommendations provide hazardous concentrations of mycotoxins, rather than the lower concentrations of mycotoxins that have been associated with mycotoxicosis (Table 1.4). In this context, in Europe, from the 1st July 2006, a regulation fixing the fusariotoxin acceptable rates (deoxynivalenol, zearalenone and fumonisins) in cereal products used for human consumption (Regulation No. 856 / 2005 of 6 June 2005: Kyprianou, 2005) was introduced. These rates reflect the threshold levels of exposure estimated from the levels of contamination of cereals and toxicity studies conducted in animals (JECFA, 2000).

Table 1.4. European rules in force for the limitation of the levels of mycotoxins in foodstuffs for human (No. 466 of 8/03/2001, OJ L77, No. 472, 12/03/2002, OJ L75; No. 257, 12/02/2002, OJ L41)

Mycotoxins	Commodity	Maximum limit (µg/kg)
Aflatoxin B1	• Peanuts, nuts and dried, and derivatives	2
	• Peanuts subjected to physical treatment before consumption or ingredients	8
	• Nuts, dried and subjected to physical treatment before consumption or ingredients	5
	• Cereals and derivatives, direct or ingredients	2
	• Cereals subjected to physical treatment before consumption or ingredients	2
Aflatoxin B1	• Spices (pepper, pepper, paprika, nutmeg, ginger, saffron)	5
Aflatoxin M1	• Milk	0,05
Zearalenone	• Cereals and derived products, grains, raw	50
	• Vegetable oils	200
Ochratoxin A	• Cereals (including rice and buckwheat) and derived products, grains, raw	5
	• Derivatives and grain consumption	3
	• Raisins	10
Patulin	• Fruit juice (apple) and fruit nectar	50
	• spirit drinks, cider and fermented beverages	50
	• Products based on pieces of apple (compote and mashed)	25
	• Apple juice and products made from pieces of apple (compote and puree) for infants and young children	10

1.2. Zearalenone

Zearalenone is a macrocyclic lactone derived from resorcylic acid (Resorcylic Acid Lactone, hence the acronym RAL) of molecular formula $C_{18}H_{22}O_5$. Its formula is shown in Figure 1.1. Its scientific name is (-)-(3S, 11E) -3, 4, 5, 6, 9, 10 -hexahydro -14, 16 -dihydroxy - 3 - methyl - 1H - 2 -benzoxacyclotétradécin-1, 7 (8H)-dione (Merk Index, 1996).

Zearalenone is a natural form of the trans- isomer and an S configuration with its methyl group in position C3 (Kuo et al., 1967). It is thermostable and resistant to a temperature of 120°C for 4 hours (Trenholm, 1982).

It has natural metabolites: α - and β -zearalenol (α - and β -ZOL) and α - and β -zearalanol (α - and β -ZAL), which can be co-produced with the ZEN by fungi and is can end up with the ZEN in infected grains.

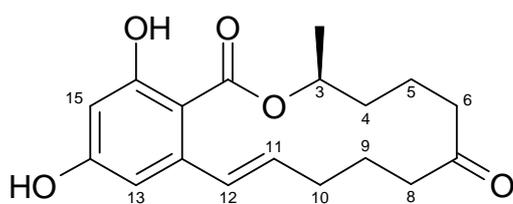


Figure 1.1. Zearalenone (ZEN)

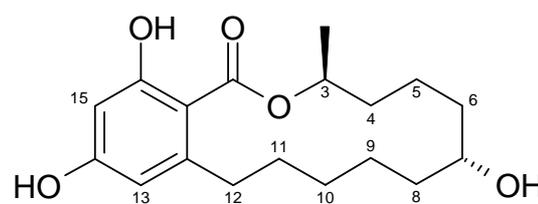


Figure 1.2. α -Zearalanol (α -ZAL)

Whether zearalenone is a source of toxicity in food, some of its metabolites such as α -zearalanol (Figure 1.2) present a significant hazard in terms of health. It is commonly used in the United States since 1969 as a growth promoter (Ralgro[®] ND - <http://www.ralgro.com/>) to improve the rate of fattening cattle. Use of this product has been banned within the European Union in 1989 but still permitted in North America and New Zealand (Gaumy et al., 2001). Extensive experiments on rat, dog and monkey have shown that α -zearalanol is an estrogeno-mimetic, whose main effects are changes in the mammary glands or organs of the reproductive system.

The study of physical and chemical properties of zearalenone suggests that this compound has ideal characteristics for a wide distribution within tissues. The major difference between species sensitivity to the zearalenone effects could partly result from metabolic differences.

1.2.1. Occurrence (cereals to feed and food)

Zearalenone is a mycotoxin produced by fungi of the genus *Fusarium*, particularly *F. graminearum*, *F. semitectum*, *F. equiseti*, *F. crookwellense* and *F. culmorum* but all strains are not producing zearalenone (Kuiper-Goodman et al., 1987). This mycotoxin is a natural contaminant of cereals, especially wheat and maize, but also some fruits and vegetables (bananas, beans, nuts ...) and can also be found in animal products such as milk, liver and eggs through contaminated feed. Contamination by zearalenone is a global phenomenon; the fungus producer develops easily in any type of weather but preferentially at low temperatures. An overview on the cereals and feedstuffs Zearalenone occurrence is summarized within the figure 1.3; as well as an estimation of human Zen daily intake depending on the different eating habits is presented in the figure 1.4. The Zen is produced by strains of *Fusarium* in Australia, Europe and North America (Vesonder et al., 1991), New Zealand (di Menna et al., 1997), the Philippines, Thailand and Indonesia (Yamashita et al., 1995). The Zen also has been detected in food in South America (Dalcero et al. 1997; Molto et al., 1997), Africa (Doko et al., 1996), Taiwan, China and Russia (Ueno et al., 1986).

From a quantitative point of view, the Zen and its metabolites were found in various cereals and derived products in Europe at rates ranging from a few micrograms per kg to 8000 µg/kg (Placinta et al., 1999). The estimated average intake for the French population is of 30 ng/kg bw⁶/day for adults aged of 15 years or more; and of 70 ng/kg bw/day in children aged from 3 to 14 years. The vector contributing the most to this exposure is constituted, in more than 60%, of cereals by-products and more particularly the breakfast cereals (between 12 and 23%)⁷.

EUROPE

Available data in Europe indicate that maize is the most prominent cereal at risk with high incidence and high levels of contamination with Zen, however wheat, oats, as well as soybean products have been found to be contaminated occasionally with Zen (EC, 2004). In 1988, Tanaka et al. published an overview on data about the occurrence of Zen from 19 countries including some European countries (Germany, Italy, Poland and UK).

⁶ bw: body weight

⁷ Study conducted within the Met@risk (Methods for Food Risk Analysis) unit of INRA for a period of 11 months on a French population (from southern, eastern and western areas of France) consisting in: 2492 individuals from which: 1474 adults of 15 years old or more, 1018 children of 3 to 14 years old, and a selection of 338 foodstuffs.

The paper reported the contamination of wheat, barley, maize, oat, sorghum, rye and rice by Zen. Placinta et al. (1999) reported the contamination of samples of wheat, barley, oat, rye and feeds from Bulgaria, Germany, Finland, Netherlands, Norway and Poland by Zen at levels from few $\mu\text{g}/\text{kg}$ to 8 mg/kg . Germany seems to be the European country where more data can be found about Zen in cereals are more available in Europe. Surveys of cereals and derivatives for several years confirmed their contamination with Zen (Muller et al., 1997a,b ; Schneewis et al., 2002; Schollenberger et al., 2005, 2006). In Yugoslavia, Zen was found at high levels (up to 10 mg/kg) in corn (Balzer et al., 1977) and in dairy cattle feeds (Skinjar et al., 1995). In Poland, the contamination of wheat by Zen was confirmed by Perkowski et al. (1990). Zakharova et al. (1995) reported a low contamination of cereal crop from Russia in 1993 and 1994 by Zen. The contamination of wheat by Zen was also found in Bulgaria (Vrabcheva et al., 1996). In Hungary, Fazekas et al. (1996) reported the contamination of mouldy and stored corn with ZEN that ranged between 0.01 and 11.8 mg/kg . Cereals from Finland (oats, barley) have been found to contain Zen together with deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON) (Hietaniemi and Kumpulainen, 1991). In the Netherlands, the occurrence of Zen was reported in wheat (Tanaka et al., 1990) and in feed ingredients (Veldman et al., 1992). In the United Kingdom, Zen was detected in corn and ingredients of animal feeding stuffs (maize and maize products) by Scudamore et al. (1998). In Scotland, according to Gross and Robb (1975), high contamination of barley stored (for 3 month to about one year) with Zen was detected and levels varied between 2.1 and 26.5 mg/kg . In Italy, the contamination of corn was found with Zen was reported from studies of Pietri et al. (2004) and Visconti and Pascale (1998). In Slovakia, Labuda et al. (2005) reported the contamination of poultry feed mixtures with Zen with the co-occurrence of DON, 3-ADON, 15-acetyldeoxynivalenol (15-ADON), T-2 and HT-2 toxins. Concerning recent data on human exposure in Europe to ZEN, the occurrence of the toxin was reported in 32% of mixed cereal samples (n = 4918) from nine European countries. The distribution showed that much of this contamination was in maize and wheat grains. A high incidence of Zen was found in samples of oat from Finland (47% of samples containing >0.2 mg/kg with a maximum level of 1.31 mg/kg being reported) and high incidence of Zen in wheat from France (16% of samples containing >0.2 mg/kg with a maximum level of 1.817 mg/kg being reported). Raw maize was the food commodity with the highest level of Zen, results reported the contamination of 14% of maize with a levels >0.2 mg/kg , the highest level (6.492 mg/kg) was reported in a sample of maize from Italy (SCOOP, 2003).

An overview on the cereals and feedstuffs Zearalenone occurrence in Europe is summarized within the table 1.5; as well as an estimation of human Zen daily intake depending on the different eating habits is presented in the table 1.6 (Zinedine et al., 2007; JECFA 2000, 2001; FAO, 2004)

Table 1.5. European occurrence of zearalenone within cereals and feedstuffs

EUROPE		Barley	Corn	Maize	Wheat	Oats	Feeds
ZEN	Mean	4,83	1.66	3.49	0.62	0.03	0.20
(mg/kg)	Max.	26.5	10	11.8	8.4	0.09	1.8

Table 1.6. European predicted human daily intake of zearalenone

EUROPE		
Food item	Food (g/day)	Zearalenone (µg/day)
Cereal derivatives		
Barley	20	< 0.16
Maize	0.0	0.000
Maize flour	8.8	< 0.11
Popcorn	0.2	< 0.002
Oats	2.0	< 0.010
Rice	12	< 0.087
Wheat bulgur	0.0	0.000
Wheat pasta	1.3	< 0.005
Wheat pastry	1.0	< 0.007
White bread	120	< 0.66
Wholemeal bread	59	< 0.33
Total grains	240	< 1.4
Legumes		
Soya beans	0.0	0.000
Other legumes ^a	12	< 0.12
Total legumes	12	< 0.12
Overall total	252	< 1.5
Total in µg/kg bw per day^b		< 0.025

^a The zearalenone value for tinned beans was used for other legumes

^b Based on 60 kg b.w.

NORTH AMERICA

In Canada, high concentrations of Zen (up to 141 mg/ kg) were reported in corn for animals (Funnell, 1979). Monitoring of Canadian foods (wheat, barley, soybeans, corn, corn-based foods and grain crops) by Stratton et al. (1993) and Scott (1997) reported also the presence of Zen at different levels. Recently, Zen was detected in infant cereal foods from the Canadian retail market (Lombaert et al., 2003). In the USA, an earlier investigation by Shotwell et al. (1977) showed the contamination of wheat with Zen. Corn from USA was found to be contaminated by Zen (Bennett et al., 1985; Bagneris et al., 1986; Hooshmand and Klopfenstein, 1995). High level values of Zen related to mouldy corn samples were reported by Abbas et al. (1988) and Park et al. (1996). The

contamination of sorghum and mouldy sugar beet root was also reported by Bagneris et al. (1986) and by Bosch and Mirocha (1992) respectively. On some occasions, phenomenally high concentrations of Zen have been reported, e.g. 2900 mg/ kg in a food sample from the USA (Pittet, 1998).

An overview on the cereals and feedstuffs Zearalenone occurrence in North America is summarized within the table 1.7; as well as an estimation of human Zen daily intake depending on the different eating habits is presented in the table 1.8 (Zinedine et al., 2007; JECFA 2000, 2001; FAO, 2004)

Table 1.7. North American occurrence of zearalenone within cereals and feedstuffs

NORTH AMERICA		Barley	Corn	Wheat	Sorghum	Feeds
ZEN	Mean	0.13	3.88	0.13	0.76	47
(mg/kg)	Max.	0.21	21.4	0.21	1.48	141

Table 1.8. North American predicted human daily intake of zearalenone

NORTH AMERICA		
Food item	Food (g/day)	Zearalenone (µg/day)
Cereal derivatives		
Barley	4.7	< 0.039
Maize	0.0	0.0
Maize flour	0.0	0.0
Popcorn	49	0.49
Oats	320	< 1.6
Rice	20	< 0.15
Wheat bulgur	64	<0.24
Wheat pasta	117	< 0.48
Wheat pastry	5.3	< 0.039
White bread	76	< 0.43
Wholemeal bread	38	<0.22
Total grains	315	< 1.6
Legumes		
Soya beans	0.0	0.0
Other legumes ^a	13	<0.53
Total legumes	13	< 0.53
Overall total	328	< 2.2
Total in µg/kg bw per day^b		< 0.037

^a The zearalenone value for tinned beans was used for other legumes

^b Based on 60 kg b.w.

SOUTH AMERICA

In Brazil, the occurrence of Zen was reported in corn by Sabino et al. (1989), Silva and Vargas (2001) and Vargas et al. (2001). The Contamination of wheat by Zen was also reported by Furlong et al. (1995). In Uruguay, a pilot study for monitoring mycotoxin contamination of foods and feeds showed the contamination of corn, barley, malt, dried fruits and dried vegetables with Zen (Pineiro et al., 1996a,b). In Argentina, Zen was found in grain (Lopez and Tapia, 1980), wheat (Quiroga et al., 1995), corn-based foods (Resnik et al., 1996) and poultry feeds (Dalcero et al., 1997, 1998). New data reported the contamination of cow feeding stuffs from Argentina with Zen at levels that ranged from 1.2 to 3.06 mg/kg (Cavaglieri et al., 2005).

An overview on the cereals and feedstuffs Zearalenone occurrence in South America is summarized within the table 1.9; as well as an estimation of human Zen daily intake depending on the different eating habits is presented in the table 1.10 (Zinedine et al., 2007; JECFA 2000, 2001; FAO, 2004)

Table 1.9. South American occurrence of zearalenone within cereals and feedstuffs

SOUTH AMERICA		Barley	Corn	Wheat	Feeds
ZEN	Mean	0.10	1.75	2.93	1.79
(mg/kg)	Max.	0.20	9.83	11.05	5.85

Table 1.10. South America predicted human daily intake of zearalenone

SOUTH AMERICA		
Food item	Food (g/day)	Zearalenone (µg/day)
Cereal derivatives		
Barley	6.5	< 0.054
Maize	1.5	< 0.061
Maize flour	40	< 0.51
Popcorn	0.2	< 0.002
Oats	0.8	< 0.004
Rice	86	< 0.64
Wheat bulgur	0.0	0.000
Wheat pasta	2.8	< 0.010
Wheat pastry	2.0	< 0.015
White bread	37	< 0.21
Wholemeal bread	75	< 0.42
Total grains	250	< 1.9
Legumes		
Soya beans	0.0	0.000
Other legumes ^a	23	< 0.23
Total legumes	23	< 0.23
Overall total	273	< 2.2
Total in µg/kg bw per day^b		< 0.036

^a The zearalenone value for tinned beans was used for other legumes

^b Based on 60 kg b.w.

AFRICA

Even though most African countries have a climate characterized by high humidity and high temperature which favour growth of moulds, little information is available on the occurrence of Fusarium toxins particularly Zen in foods and feeds. High contamination of the raw material is an ongoing problem. Regulatory issues are not available in the field of food exhibition and retailing, and mycotoxin problems have already been associated with some food contamination in some areas in Africa. An earlier investigation from Zambia reported the contamination of maize for beer brewing, beer and corn malt by Zen (Lovelace and Nyathi, 1977). In Nigeria, Gbodi et al. (1986a) reported that Zen was the most prevalent mycotoxin in mouldy acha⁸ (*Digitaria exilis* Stapf) samples. A high level of Zen (17.5 mg/kg) was also found in maize (*Zea mays*) from Nigeria (Gbodi et al., 1986b). Beer from Nigeria was also found contaminated with Zen (Okoye, 1987). In Egypt, several commodities were reported to contain Zen especially corn, wheat and rice (Abd Alla, 1997) and walnut (Abdel-hafez and Saber, 1993). Corn from Egypt was also found contaminated with high levels of Zen that ranged from 9.8 to 38.4 mg/kg (El-Maghraby et al., 1995). In South Africa, samples of cereals, animal feeds and brewed beers were found to be contaminated with Zen (Dutton and Kinsey, 1996; Odhav and Naicker, 2002). In North African countries, there is a lack of investigations on the occurrence of Fusarium toxins in foods and feeds. The first report from Morocco has reported the co-occurrence of Zen with fumonisin B1 and ochratoxin A (OTA) in corn (Zinedine et al., 2006).

An overview on the cereals and feedstuffs Zearalenone occurrence in Africa is summarized within the table 1.11; as well as an estimation of human Zen daily intake depending on the different eating habits is presented in the table 1.12 (Zinedine et al., 2007; JECFA 2000, 2001; FAO, 2004).

Table 1.11. African occurrence of zearalenone within cereals and feedstuffs

AFRICA		Corn	Maize	Feeds
ZEN	Mean	17.4	3.7	2.0
(mg/kg)	Max.	38.4	17.5	8.0

⁸ *Digitaria exilis* Stapf has the common names of acha, pene, fonio, petit mil, fundi, and hungry rice. It is one of the smallest cereals known. The grain is smooth and usually yellow. The grain weight varies from 0.4 to 0.5 mg. *D. exilis* is a tropical African plant grown in a region extending from Cape Verde to Lake Chad

Table 1.12. African predicted human daily intake of zearalenone

AFRICA		
Food item	Food (g/day)	Zearalenone ($\mu\text{g/day}$)
Cereal derivatives		
Barley	1.8	< 0.015
Maize	0.0	0.000
Maize flour	110	< 1.3
Popcorn	0.2	< 0.002
Oats	0.2	< 0.001
Rice	100	< 0.76
Wheat bulgur	0.0	0.000
Wheat pasta	0.0	0.000
Wheat pastry	0.0	0.000
White bread	19	< 0.11
Wholemeal bread	9.4	< 0.053
Total grains	330	< 2.3
Legumes		
Soya beans	0.5	< 0.005
Other legumes ^a	17	< 0.17
Total legumes	17.5	< 0.18
Overall total	347.5	< 2.5
Total in $\mu\text{g/kg}$ bw per day^b		< 0.041

^a The zearalenone value for tinned beans was used for other legumes

^b Based on 60 kg b.w.

ASIA

In Japan, the contamination of cereals (barley and wheat) with Zen was reported by Yoshizawa and Jin (1995) and Yoshizawa (1997). The 1990 barley and corn crops in Korea were reported to be heavily contaminated with ZEN because of the high rainfall and humidity (Park et al., 1992; Kim et al., 1993). Park et al. (2002) reported the contamination of barley, barley-based foods, corn and corn-based foods from Korea with Zen. In a recent investigation, rice collected from Korea was also found contaminated with Zen with the co-occurrence of OTA, aflatoxin B1 (AFB1), DON and nivalenol (NIV) (Park et al., 2005). Co-contamination of maize with Zen, NIV, fumonisins and aflatoxins is an emerging issue in Philippines and Thailand (Yamashita et al., 1995). In India, cereals including maize, wheat and rice, were reported to contain Zen (Phillips et al., 1996; Janardhana et al., 1999; Luo et al., 1990). Co-occurrence of Zen with AFB1, OTA, T-2 toxin, DON and citrinin was also reported in Indian maize (Janardhana et al., 1999). Foodstuffs available in Qatar including cereals and cereals products (rice, wheat, oats and cornflakes) were reported to contain Zen at low levels with the co-occurrence of

OTA, aflatoxins and DON in rice (Abdulkadar et al., 2004). The contamination of maize-based food and poultry feeds with Zen was reported in Indonesia (Nuryono et al., 2005). In Iran, Zen was found in pre-harvest maize (Hadiani et al., 2003), in corn flour and in a cheese snacks (Reza Oveisi et al., 2005).

An overview on the cereals and feedstuffs Zearalenone occurrence in Asia is summarized within the table 1.13 (Zinedine et al., 2007; JECFA 2000, 2001; FAO, 2004).

Table 1.13 Asian occurrence of zearalenone within cereals and feedstuffs

ASIA		Barley	Corn	Maize	Wheat	Rice	Feeds
ZEN	Mean	3.65	0.13	0.20	0.25	0.02	0.46
(mg/kg)	Max.	15.3	0.89	0.92	1.40	0.05	0.84

OCEANIA

In New Zealand, Zen was detected in maize at high levels (up to 16 mg/kg) (Hussein et al., 1989; Lauren et al., 1996), in corn germ, in fiber and in gluten (Lauren and Ringrose, 1997). In another survey, di Menna et al. (1997) reported also that Zen was found in leaf axils and blades (8–75 mg/kg) and in rachis fractions with high levels (up to 417 mg/kg). In Australia, a survey for mycotoxins and fungal damage in maize (*Zea mays* L.) reported that Zen contaminated four samples with concentrations that exceeded 1 mg/kg (Blaney et al., 1984). While in samples of wheat, Zen was detected with aflatoxins and 4-DON (Blaney et al., 1987).

An overview on the cereals and feedstuffs Zearalenone occurrence in Oceania is summarized within the table 1.14 (Zinedine et al., 2007; JECFA 2000, 2001; FAO, 2004).

Table 1.14. Oceania occurrence of zearalenone within cereals and feedstuffs

OCEANIA		Corn	Maize	Maize plants	Wheat	Feeds
ZEN	Mean	3.5	6.1	41.5	0.24	n.r.
(mg/kg)	Max.	4.8	16	75	0.43	n.r.

n.r.: not reported

An estimation of human Zen daily intake depending on the different eating habits is presented in the table 1.15 for Middle and Far East (Zinedine et al., 2007; JECFA 2000, 2001; FAO, 2004).

Table 1.15. Middle and Far East predicted human daily intake of zearalenone

Food item	MIDDLE EAST		FAR EAST	
	Food (g/day)	Zearalenone ($\mu\text{g/day}$)	Food (g/day)	Zearalenone ($\mu\text{g/day}$)
Cereal derivatives				
Barley	1.0	< 0.008	3.5	< 0.029
Maize	16	< 0.67	0.0	0.000
Maize flour	32	< 0.40	31	< 0.40
Popcorn	0.2	< 0.002	0.2	< 0.002
Oats	0.0	0.000	0.0	0.000
Rice	49	< 0.36	280	< 2.1
Wheat bulgur	0.3	< 0.002	0.0	0.000
Wheat pasta	1.0	< 0.004	0.3	< 0.001
Wheat pastry	3.0	< 0.022	0.5	< 0.004
White bread	220	< 1.2	76	< 0.43
Wholemeal bread	110	< 0.60	38	< 0.21
Total grains	430	< 3.3	450	< 3.1
Legumes				
Soya beans	4.5	< 0.046	2.0	< 0.020
Other legumes ^a	20	< 0.20	18	< 0.18
Total legumes	24.5	< 0.25	12	< 0.20
Overall total	454.5	< 3.5	462	< 3.3
Total in $\mu\text{g/kg}$ b.w. per day^b		< 0.059		< 0.056

^a The zearalenone value for tinned beans was used for other legumes

^b Based on 60 kg b.w.

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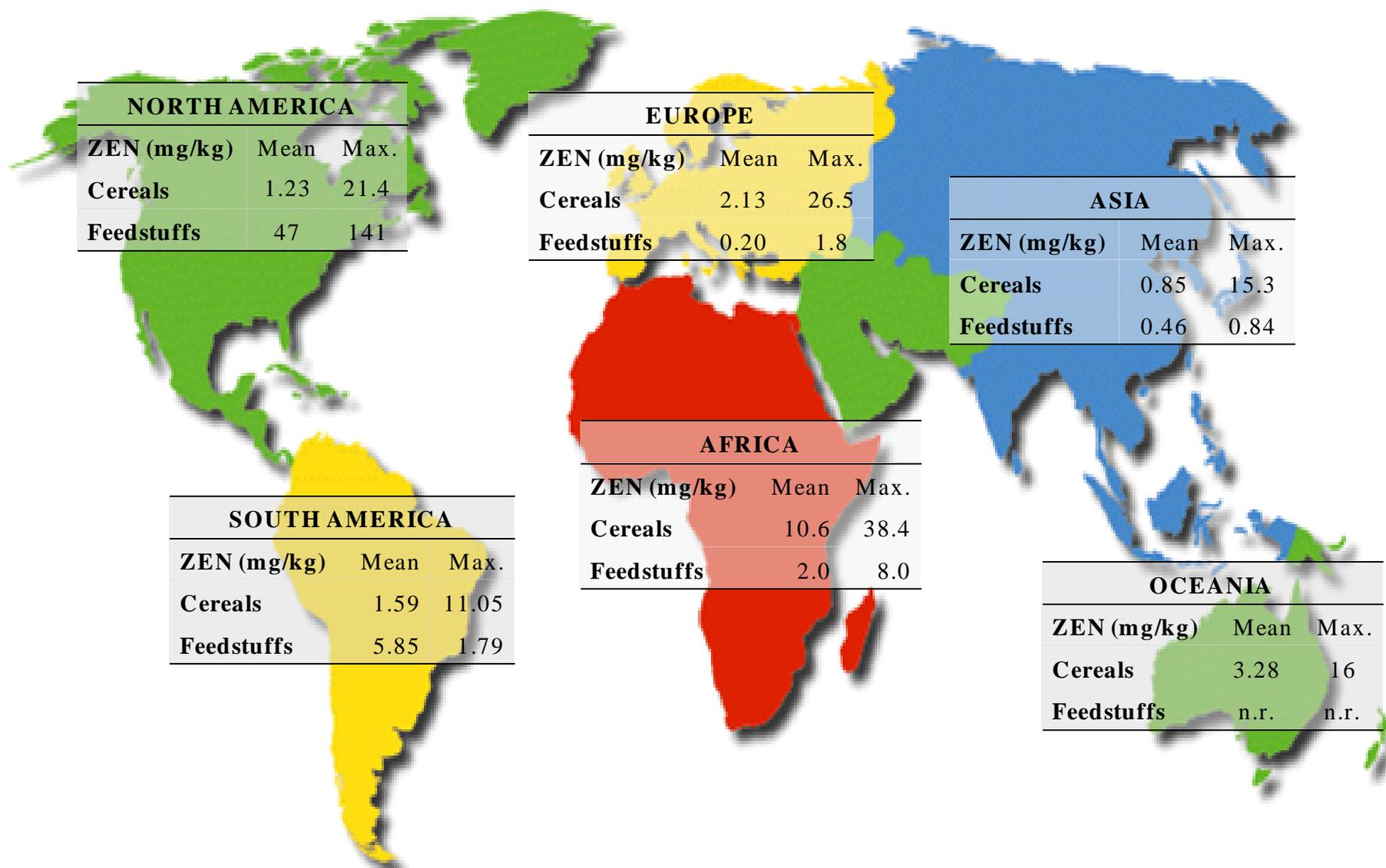


Fig. 1.3. Worldwide occurrence of zearalenone within cereals and feedstuffs
References: Zinedine et al., 2007; JECFA (2000,2001) ; FAO, 2004.

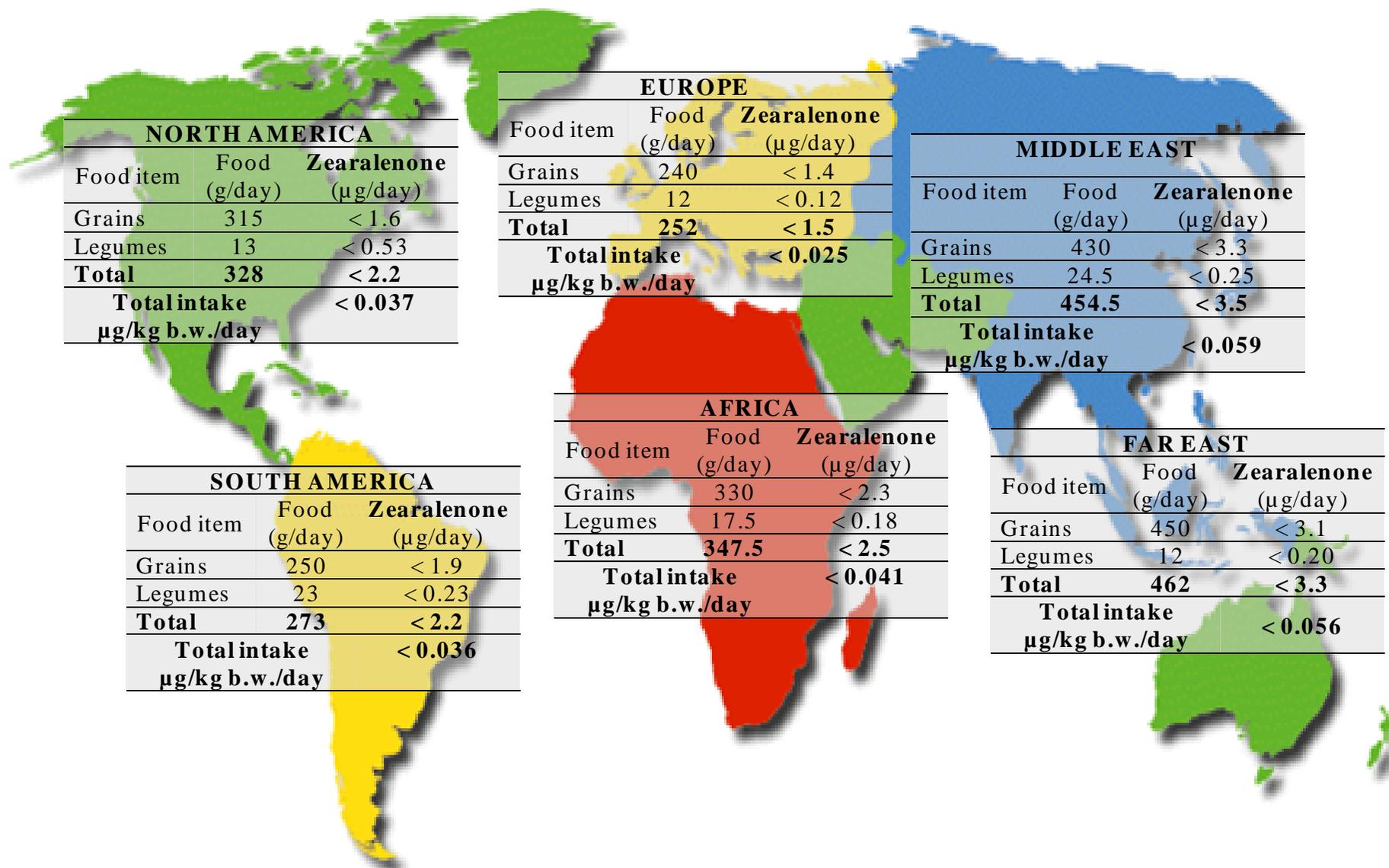


Fig. 1.4. Worldwide predicted human daily intake of zearalenone, in function of the eating habits
References: Zinedine et al., 2007; JECFA (2000, 2001); FAO, 2004.

1.2.2. Human and animal health disorders

Zearalenone is a molecule with estrogenic activity in animals, included in the category L (limited evidence of carcinogenicity) by the NTP in 1982 and in group 3 (not classifiable as to their carcinogenicity to humans) by IARC in 1999. According to the consumption of food contaminated by domestic animals, a high estrogen was observed in farm animals, particularly among sows (Liu et al., 1985) and cows (Diekman and Green, 1992). Experiments *in vivo* and *in vitro* indicate that Zen and its metabolites are substrates of different enzymes involved in the metabolism of steroids. Due to the interaction with estrogen receptors, Zen is associated with compounds known as endocrine disrupters. It is classified as non-steroidal estrogen, mycoestrogen or even as phytoestrogen. Despite its non-steroidal structure, Zen activates estrogen receptor involving functional and morphological changes of reproductive organs (see table 1.17) (Kuiper-Goodman et al. 1987, JECFA, 2000).

Zen structure is sufficiently similar to 17 β -estradiol in order to bind with high affinity estrogen receptors in target cells of mammals. Among farm animals, pigs are considered the most sensitive species. Clinical signs of exposure include, in females, ovarian atrophy, reduced fertility, increased foetal sorption, and decreased litter size, changes in weight of pituitary glands as well as changes in progesterone and estradiol serum levels. Fibrosis of the uterus, breast and vulvae swelling, and an increase of vesicular follicles in the ovaries were also mentioned (JECFA, 2000). Among males, Zen causes a decrease in reproductive capacity, testicular atrophy, inflammation of the prostate, a feminization of young males and a decrease in serum testosterone (Zinedine et al., 2007). Auto radiographic studies using [³H]-Zen in mice has shown that Zen localization depended on reproductive status: in pregnant mice, zearalenone and / or its metabolites were found only in foetuses in late pregnancy, mainly in kidney, bile and tissue (Appelgren et al., 1982).

It is important to note that zearalenone differs from its metabolites by its estrogenic potential. The first evidence of this potential difference was obtained in studies by Katzenellenbogen et al. in 1979 on hormone receptors. More recently, Shier and his team, 2001 have shown the relationship between structure and activity of zearalenone on cell experiments, using MCF7 cells from human breast adenocarcinoma, that require to proliferate the presence of agonists to estrogen receptors. By measuring the rate of proliferation of these cells, they showed that α -zearalenol has a relative estrogenic activity 92 times higher than zearalenone, while the relative estrogenic activity of β -zearalenol is only of 0.44 relative to that of the Zen (Table 1.16). Therefore the conversion of zearalenone in α -zearalenol must be seen as a

bioactivation reaction, and then the longer conversion into β -zearalenol can be considered as an inactivation of the parental compound.

Table 1.16. ZEN and its metabolites IC₅₀ (nM) of MCF7 cells proliferation (Shier et al., 2001) and affinity IC₅₀ for estrogen receptors ER- α and ER- β (Takemura et al., 2007).

	IC ₅₀ proliferation MCF7 (nM) (Shier et al., 2001)	IC ₅₀ link ER- α (nM) (Takemura et al., 2007)	IC ₅₀ link ER- β (nM) (Takemura et al., 2007)
E ₂		10,35	10,04
ZEN	1,1	240,4	165,7
α -ZOL	0,012	21,79	42,76
β -ZOL	2,5		
ZAN	0,44		
α -ZAL	0,060		
β -ZAL	0,31		

Since the biological effects of estrogen in target tissues depend on the presence of estrogen receptors, the characteristics of binding of Zen to such receptors have been studied: Zen binds and activates ER- α and ER- β within human cells transfected with ER- α and ER- β . For ER- α , Zen has proven to be a full agonist and for ER- β a partial agonist (Kuiper et al., 1998). Indeed, the lipophilicity of the Zen enables it to cross cell membranes and bind to cytosolic estrogen receptors. The complex formed is then translocated into the nucleus, where it binds to response elements of target genes (Riley, 1998). Both estrogen receptors sensitive to Zen, ER- α and ER- β are showing differences at the C-terminal domain, ligand binding site, and at the domain N-terminal, transactivation domain (Kuiper et al., 1998).

The lowest affinity to estrogen receptors in rodents was found for β -Zol (Celius et al. 1999, Shier et al., 2001). Affinity receptors on rat uterine cytoplasm for Zen and its derivatives are classified as follows: α -zearalanol > α -zearalenol > β -zearalanol > zearalenone > β -zearalenol (Kuiper-Goodman et al. 1987, Eriksen and Alexander, 1998). These reduction reactions compete in the intestine and liver conjugation reactions, especially after oral ingestion of the toxin through food.

Furthermore, Zen is also able to inhibit the synthesis of DNA and proteins. Moreover, in monkey kidney cells and cells from a human colorectal adenocarcinoma, Zen induced the

peroxidation of lipids in a dose-dependent way. The clastogenic⁹ effect of Zen by induction of lipid peroxidation may be related to its ability to disrupt the cell cycle and induce apoptosis (Essefi-Abid et al., 2004). However, no teratogenic¹⁰ effects of ZEN have been deferred (Kuiper-Goodman et al. 1987, JECFA, 2000).

1.2.2.1 Clinical signs associated with exposure to zearalenone contaminated feeds in farm animals

Reports on the adverse effects of zearalenone contamination of feedstuffs comprise case reports, field studies and controlled experiments, often conducted with crystalline zearalenone. Following oral exposure, zearalenone is rapidly absorbed from the gastrointestinal tract in monogastric species. In the liver, it is converted to two reduced stereoisomeric metabolites, α -zearalenol and β -zearalenol. The ratio of α -Zol/ β -Zol varies between animal species, and may count for differences of species susceptibility to zearalenone exposure, as α -zearalenol is more potent than the parent zearalenone (Fitzpatrick et al., 1989). The parent zearalenone, as well as the mentioned metabolites, can be glucuronized and excreted via urine and bile. Biliary excretion is followed by entero-hepatic recirculation that extends the duration of exposure (Biehl et al., 1993).

1.2.2.1.1. Pigs

Various studies have addressed the biological effects of Zearalenone exposure in pigs, considered to be the most sensitive animal species. Moreover, several studies have indicated that the female pre-pubertal pig (gilt) is particularly sensitive to the estrogenic effects of zearalenone. The first report on the oestrogenic effects of zearalenone describes a dose-dependent increase in the uterus weight of gilts following exposure to zearalenone (Hacking, A. 1983). Kordic et al. (1992) reported a dose-dependent decrease in the number of live foetuses following toxin exposure of gilts. Following dietary exposure corresponding to a dose of approximately 200 μ g/zearalenone per kg body weight (bw), the development and maturation of ovarian follicles was impaired (Zwierzchowski et al., 2005). Long-term exposure of 3 reproductive cycles to feed concentrations as low as 180 mg zearalenone/ Kg

⁹ **Clastogenic** describes the ability of an agent or process to act as a clastogen - an agent or process giving rise to or inducing disruption or breakages

¹⁰ **Teratogens** are substances or environmental agents which cause the development of abnormal cell masses during fetal growth, resulting in physical defects in the fetus.

feed resulted in prolonged return to oestrus, abortions and symptoms of hyperoestrogenism in the piglets, already in the first part of the study (Jadamus and Schneider, 2002).

Ovarian follicle atresia and apoptotic changes in granulosa cells, increased cell proliferation in the uterus and oviduct were observed at doses of 0.2 – 0.4 mg/Kg b.w. per day (Obremski et al., 2003).

In two comprehensive and well-conducted studies using naturally contaminated maize providing dietary concentrations of zearalenone from 0.01 to 0.42 mg/Kg and of DON from 0.2 to 3.9 mg/Kg feed clear estrogenic effects (increased swelling of cervix and increased mean relative uterus weight) was observed at the highest dose level of 0.42 mg Zen/Kg feed (Doll et al., 2003; 2004). A slight decrease in mean serum FSH concentration in the dosed groups was considered to be influenced by the co-occurrence of deoxynivalenol (DON) in the given feed. In other studies using crystalline zearalenone, estrogenic effects have been reported after exposure to feed concentrations ranging from 0.05 to 0.4 mg Zen/Kg feeding stuff (Bauer et al., 1987; Lusky et al., 1997; Coenen and Boyens, 2001; Obremski et al., 2003).

Adult multiparous and fattening pigs are less sensitive to Zen exposure. However, it is suggested that the developing foetus is exposed to zearalenone when pregnant sows are fed Fusarium-contaminated wheat during gestation (Danicke et al., 2007). The effect of a zearalenone-containing diet, fed to pregnant sows, on their full-term offspring was determined by Tiemann et al. in 2007. At necropsy, no macroscopic or microscopic lesions in spleen or liver were observed in the piglets, but effects on the reproductive tract were not determined.

These data indicated that the intensity of the effects of zearalenone in different age groups vary between pre pubertal, cycling and pregnant animals. Also is implied that not only the actual level of exposure, but also the duration of exposure determines the clinical significance of dietary zearalenone exposure. In sexually mature females, the adverse effects exerted by zearalenone include decreased fertility, ovarian atrophy, prolonged oestrus, oedematous swelling and reddening of the vulva and prolapsed uterus and rectum. Prenatal exposure of piglets may lead to stillbirth, reddening and swelling of vulva, nipples and the praeputium at the time of birth (Chang et al., 1979; Dacasto et al., 1995; Weaver et al., 1978; Yang et al., 1995).

Only a few studies have been devoted to the potential effect of zearalenone on male fertility. Boars seem to be quite resistant to the effects of zearalenone. Berger et al. (1981) investigated the effect of ZEN on the reproduction of boars by feeding those with 40 mg Zen/ kg diet from week 14 to 18 of age. Subsequently, reduced plasma testosterone concentrations and

decreased libido were observed. However, no adverse effects were detectable at week 36 of age. Several other studies showed also no effect of Zen exposure on libido, onset of puberty, semen quality or testicular weight of boars (Young and King, 1984; Stolla et al., 1987; Lusky et al., 1991).

1.2.2.1.2. Poultry

Poultry may access to zearalenone contaminated grains and maize with their diet. Some adverse effect attributable to zearalenone exposure has been described. In comparison to pigs, avian species are considered to be less sensitive to the hormonal effects of Zen. In 1989, it was postulated for the first time that mycotoxins might influence the egg-production of hens. In this study, the effect of corn *versus* grain on egg production was determined. The grain-fed hens showed a significant decrease in egg production, compared to the corn-fed hens. Analysis of the grain revealed the presence of mycotoxins zearalenone and deoxynivalenol and Branton et al. (1989) postulated that these mycotoxins may have decreased egg production. In a study by Garaleviciene et al. (2001), the effects of mould and toxin contaminated barley on laying hens performance was evaluated. Inclusion of mouldy barley in diets containing toxins, such as zearalenone and deoxynivalenol had an adverse effect on egg production and egg quality. However, the contaminated barley also reduced food intake. In addition, mycotoxin (both Zen and DON) contaminated feeds have been shown to significantly depress feed intake and daily egg production compared to the control group (Danicke et al., 2002).

Oviduct weight of growing broilers and growing turkeys remained unaffected following high Zen concentrations in diet (ranging from 50 to 800 mg Zen/kg diet) (Allen et al., 1981). Chi et al. (1980) administered crystalline zearalenone, either orally or intramuscularly, at doses of 50, 200, 400 and 800 mg/kg body weight/day for seven days to broilers. A dose-response related increase of the oviduct weights was found but the potency of zearalenone was estimated to be only 1.37 % of that of estradiol propionate. In another study, feeding of diets containing 50, 200, 400 and 800 mg of zearalenone/kg to broilers resulted in an increased incidence of ovarian cystic development, but increased oviduct weights were only seen in some birds at the highest zearalenone concentration (Chi et al., 1980b).

Growing male broilers exposed to a diet containing Zen and DON showed a linear decrease of feed intake and a slight decrease in weight gain (Danicke et al., 2003). The effect of zearalenone exposure on male turkeys was investigated by feeding turkeys a highly contaminated diet (800 mg Zen/kg) for a 2-week period (Olsen et al., 1986). Zen had no effect

on either feed consumption or body weight gain and hormone analysis revealed testosterone concentrations in blood plasma were the same in the control and the treated birds.

Growing broilers' testes weights were reduced following higher zearalenone concentrations in diet, whereas growing turkey testes weights remained unaffected (Allen et al., 1981). Dewlaps and combs showed an increased development and considerable strutting behaviour were observed at 400 and 800 mg Zen/kg diet. *Maaryamma et al* reported atrophy of testes in male chicks exposed to 10 mg Zen/kg bw/day for 20 days (Maryamma et al., 1992). Overall, these results show the effects of dietary zearalenone on male poultry are observed at high doses.

To determine the effect of Fusarium-contaminated wheat in Peking duck, growing ducks were fed a diet containing 0.05-0.06 mg Zen/kg and 6-7 mg DON/kg for 49 days (Danicke et al., 2004). It was concluded that these dietary concentrations did not adversely affect performance and health of growing Peking ducks. *Swamy et al* determined the effect of a 56 days diet of Fusarium mycotoxin contaminated wheat on the growth and immunology of broiler chickens and concluded that broiler growth decreased linearly with the contaminated wheat (Swamy et al., 2002). However, the wheat included contamination with Zen (concentrations ranging 0.4-0.7 mg/kg) as well as contamination with DON, fusaric acid and 15-acetyldeoxynivalenol; hence, the effect of Zen remains unclear. From the experiments with the poultry species tested so far, poultry can be regarded as quite tolerant to zearalenone because symptoms of hormonal effects were only observed at high zearalenone doses, which hardly occur under practical feeding conditions.

1.2.2.1.3. Ruminants

In ruminants, the fore-stomach flora effectively degrades significant amounts of Zen and only a fraction reaches the small intestines and is subsequently available for absorption.

Little is known on the hyperestrogenic effects of zearalenone on sheep. One study by Smith et al., in 1990, tested the reproductive performance of ewes after administration of zearalenone in several dosages. Results showed a dose-dependent decrease in ovulation rate, also cycle length decreased and oestrus duration increased with increasing Zen concentrations. There was no effect of zearalenone treatment after mating on pregnancy rate or embryonic loss. The authors concluded that a zearalenone intake of 3 mg/day or more before mating could depress ovulation rates and lower lambing percentages. Feeding of a diet containing 12 mg zearalenone/kg diet to rams influenced neither the volume of ejaculate or semen concentration, nor semen motility nor the percentage of semen abnormalities (Milano et al., 1991).

In 1990, 71 dairy cows and 25 heifers were accidentally given zearalenone and deoxynivalenol contaminated corn (Coppock et al., 1990). Concentrations zearalenone and deoxynivalenol were respectively 1.5 mg/kg feed and 1.0 mg/kg feed. Frequent episodes of behavioural oestrus of 2 to 5 days duration not synchronized with the ovarian cycle, were observed. Cows in the second and third trimester of pregnancy also had episodes of behavioural oestrus. Mammary development occurred in the prepubertal heifers, followed by sterility. However, the absence of a control group, unknown dosage per kg body weight and exposure to multiple toxins makes these results difficult to interpret.

Two experiments with heifers were reported by Weaver et al. who found the conception rate over 3 heat cycles to be decreased when 250 mg crystalline zearalenone (approximately 50 mg/kg diet) were fed per day (Weaver et al., 1986 a,b). No effects on the reproductive organs and no changes in the progesterone concentration in the blood could be detected when 500 mg crystalline zearalenone was fed per day. Moeser in 2001 fed contaminated oats with a dietary concentration of zearalenone at 1.25 mg/kg diet to heifers which gave effects that were found to be clinically insignificant. No deviations in the heat cycle and no pathologic or histological alterations of the reproductive organs were detected.

To determine the effect of zearalenone on veal calves, 17 to 21 day old calves were subcutaneously implanted with 0, 12, 24, 36 or 48 mg of α -zearalenol (Egan et al., 1993). Feed efficacy (gain/feed) improved in the 48 mg implanted group, while testicular weight decreased with increasing α -zearalenol concentrations.

Floyd et al determined the effect of α -zearalenol implantation on reproductive characteristics in bulls (Floyd et al., 1994). The implanted dosage was 36 mg of α -zearalenol on day 0, 36 mg on day 0 and 60 or no implant. After 40 days, scrotal circumference and testicular consistency were unaffected and no morphological changes in semen samples were observed. Another study concluded that feeding zearalenone-contaminated wheat (0.76 mg/kg feed) had no adverse effect on the growing performance of bulls (Danicke et al., 2002).

There are several field or case reports where the observed hyperestrogenic symptoms could not be related to the zearalenone concentrations found. This might reflect the variability in rumen microflora degradation of zearalenone. The zearalenone concentrations were reported to range between 0.1 and 75 mg/kg diet (Mirocha et al., 1968; Roine et al., 1971; Bloomquist et al., 1982; Drochner, 1990; Danicke et al., 2005).

Ruminants are regarded as quite resistant to zearalenone because of the detoxifying potential of rumen protozoa and microbes.

1.2.2.1.4. Horses

Only few reports are available regarding the toxicity of Zen in horses. With the increasing use of maize products in equine nutrition, the likelihood of exposure is increasing. Juhasz *et al.* investigated the effect of a daily oral administration of 7 mg purified Zen in cycling mares (Juhasz *et al.*, 2001). No effect on the length of inter-ovulatory intervals, luteal and follicular phases of uterus oedema were observed. The given dose represented a feed concentration of about 1 ppm (corresponding to 0.013 – 0.010 mg/kg bw given for 8 – 10 days). In experiments with cultures, in granulosa cells derived from the ovaries of mares, zearalenone was found to stimulate cell proliferation, but also increased the number of apoptotic cells (Minervini *et al.*, 2006). The authors concluded that these effects are attributable either to a direct oestrogen-receptor dependent effect or an inhibition of the aromatase / 17 β -HSD with a subsequent inhibition of the conversion of androstenedione to estradiol, as observed in human granulosa luteal cells (Lacey *et al.*, 2005).

Table 1.17. Reproductive and developmental effects observed after oral exposure of various species to zearalenone (after Kuiper-Goodman *et al.*, 1987; JECFA, 2000)

Species	Age / sex	NOAEL (mg/kg bw./day)	1 st effect dose (mg/kg bw/day)	Effect type
Rat	Adult (6-8 weeks)	0.1	0.85	Decreased fertility of males and females, disturbed spermatogenesis, disturbed cycling, decreased fertility of offspring
Guinea-pig	Adult, female	7	21	Reduced incidence of pregnancy, altered levels of progesterone, no effect on litter size, fetal size
Chicken/hen	Female	800	-	No lesion / No effect on reproductive function
Pig	Sow	< 0.28	0.28	Signs of hyperestrogenism, endometrial morphology
	Gilt	0.02	0.04	Swollen and inflamed vulvas
	Boar	> 2	-	No effect on copulatory behaviour or male reproduction
	Barrow	1.2	5	Precocious spermatogenesis, damage to germinal epithelium, interstitial-cell hyperplasia
Sheep	Ewe	0.03	0.06	Increased duration of oestrus, increased uterine weight
	Ram	> 0.48	-	No effect on volume of ejaculate or semen concentration, motility, or abnormalities
Cattle	Bull	<0.8	0.8	Degeneration of germinal epithelium, 75% incidence of sperm degeneration
Horse	Female	> 0.013	-	No effect on the length of inter-ovulatory intervals, luteal and follicular phases of uterus oedema

1.2.2.2 Human pathologies associated with exposure to zearalenone

Early reports suggest an association between dietary exposure to Zen (in maize products) and precocious puberty in girls (Ingle and Martin, 1986; Saenz de Rodriguez et al., 1985; Briones-Reyes et al., 2007). An increased incidence of early thelarche¹¹ was also reported to occur in Hungary, and these clinical observations were supported by increases serum levels of Zen associated with dietary exposures (Szuetz et al., 1997). Increased Zen tissue levels were found in female patients with endometrial adenomas, endometrial hyperplasia and proliferative endometrial disease (Tomaszewski et al., 1998). It remains to be elucidated whether or not dietary Zen exposure was the cause of these pathologies. The same uncertainties remain regarding the etiopathogenesis and prevalence of uterine carcinomas and blood levels of Zen as described by Gejacki et al., 2004. Zearalenone and its metabolites are able to stimulate the proliferation of cells that express one or both types of oestrogen-receptors. This has been demonstrated convincingly in *in vitro* experiments with human breast cancer cells (MCF-7 cells) as mentioned above. Based on these findings, it has been hypothesized that frequent dietary exposure to Zen might contribute to the development of human breast cancer (Yu et al., 2005).

Zearalenone was also found to suppress Wnt-7a expression in cultured endometrial adenocarcinoma cells. Genes of the Wnt family are known to be involved in signal transduction pathways important for transplacental carcinogenesis following intra-uterine exposure to, for example DES (Wagner and Lehmann, 2006).

Pillay et al. compared the plasma levels of Zen, α -Zol, and β -Zol in patients with breast cancer and cervical carcinomas with those of healthy volunteers, but found no significant differences (Pillay et al., 2002). These authors also stress the fact that the presented results do not exclude a causal relationship between long-term exposure to dietary Zen and oestrogen-dependent cancers.

¹¹ **Thelarche** is the first stage of secondary (postnatal) breast development, usually occurring at the beginning of puberty in girls. Thelarche is usually noticed as a firm, tender lump directly under the center of the nipple (papilla and areola).

1.2.2.3 Toxicological reference values

According to Van Egmond (1993), there are various factors that may influence the establishment of tolerances for certain mycotoxins, such as the availability of toxicological data, the availability of data on dietary exposure, the distribution of mycotoxins over commodities, legislation of other countries with which trade contacts exist, and the availability of methods of analysis.

The Scientific Committee on Food (2000) established in 2000, a temporary tolerable daily intake of 0.2 µg/Kg bw based on a short-term study in pigs (No Observed Adverse Effect Level - NOAEL de 40 µg/Kg bw/day and safety factor = 200). The committee of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established in 1999 a maximum daily dose of 0.5 mg/Kg body weight calculated from the hormonal effects observed in the most sensitive species, pig (NOAEL of 50 mg/Kg body weight per day). The “Conseil supérieur d'hygiène publique de France” (CSHPF) proposed in 1999 a tolerable daily intake (TDI) of 100 mg/Kg bw/day calculated upon the observed effects on reproduction of the monkey (INRA, 2004).

The joint expert committee of FAO and WHO on Food Additives (JECFA) estimated the maximum residue levels in the livers of 10 µg/Kg and in muscle of 2 µg/Kg. Recently the Food and Drug Administration (FDA) has established safety levels of concentration for total residues of α -zearalenol in edible tissues of cattle not cooked with values ranging up to 150 µg/Kg in muscle, 300 µg/Kg in liver, 450 µg/Kg in kidney and 600 µg/Kg in fat (Table 1.6). JECFA has established in 1999 a Provisional Tolerable Daily Intake (PTDI) equal to 0.5 mg/Kg bw/day based on a toxicological study performed on pigs for 15 days. The toxic effect relevant is the existence of estrogenic effect. The no-effect derived from this study is 40 µg/Kg bw/day. JECFA considered that a safety factor of 100 was sufficient in view of the Low Observed Adverse Effect Level (LOAEL) from this study (200 µg/Kg bw/day) and the TDI allocated to one of the metabolites zearalenone, the α -zearalanol used as a veterinary drug (banned in Europe). CWS has established in 2000, a PTDI of 0.2 mg/Kg bw/day based on the same study and the same effects. The safety factor was of 200 due to the small number of toxicological studies available (AFSSA, 2006).

Table 1.18. Maximum zearalenone concentration accepted in foodstuffs expressed in $\mu\text{g} / \text{kg}$ (Risks evaluation associated with the presence of mycotoxins in feed and food chain. AFSSA 2006)

Products	Maximum content $\mu\text{g}/\text{kg}$
Unprocessed cereals other than maize	100
Unprocessed maize	200
Cereals for direct human consumption, cereal flour, and his seed (excluding processed maize-based)	75
Maize intended for direct human consumption, maize flour, maize meal, maize grits and refined maize oil	200
Bread, pastries, cookies (excluding processed maize-based)	50
Corn snacks and breakfast cereals maize	50
Other snacks and cereal-based breakfast cereals	50
Processed maize-based foods for infants and young children	20
Other preparations based on cereals and baby foods for infants and young children	20

According to FAO (2004), Zen was regulated in 1996 by 6 countries, but by the year 2003 the toxin Zen was regulated in foods and animal feeds by 16 countries. Limits for Zen in maize and other cereals, currently vary from 50 to 1000 $\mu\text{g}/\text{kg}$. Current regulations of Zen in foods and feeds set by countries from Europe, Asia, Africa and America and reported by FAO (2004) are represented in Table 1.19.

Table 1.19 Maximum limits for Zen in foods and feeds in various countries (FAO, 2004)

Country	Maximum limit (µg/kg)	Commodity
Armenia	1000	All foods
Austria	60	Wheat, rye/durum wheat
	50	Feed for breeding-pigs
Belarus	1000	Barley, wheat, maize
Bulgaria	200	Cereals and processed products thereof intended for direct human consumption or as an ingredient in foodstuffs
Canada	3000	Feed for gilts and sows
Chile	200	All foods
Colombia	1000	Sorghum
Cyprus	2000	Feed materials
	1500	Complete feeding stuffs for piglets
	3000	Complete feeding stuffs for swine other than piglets
France	50	Cereals and cereal products
France	200	Vegetable oils
Estonia	1000	Wheat, barley, maize, cereal flours (wheat, barley, maize), cereal groats and flakes (wheat, barley, maize), pasta products, ordinary baker's wares, fine baker's wares, confectionery products, legume vegetables, fats, oils; isolates, concentrates and hydrolysates of cereals protein
	200	Complementary feedingstuffs for cattle, pigs and other farm animals
	100	Feeding stuffs of vegetable origin, complete feeding stuffs for cattle, pigs and other farm animals
	50	Complete feeding stuffs for young cattle, young pigs and other young farm animals
Hungary	100	Milled products, cereal-constituent of muesli
Indonesia	Not detectable	Maize
Iran	400	Barley
	200	Maize, wheat, rice
Italy	20	Baby foods
	100	Cereals and derived products
Japan	1000	Compound feeds
Latvia	1000	Cereals
	1000	Bread
Lithuania	300	Feed for piglet
	100	Feed for pig
Moldova	1000	Wheat and wheat flour, barley and barley flour, maize and maize flour
Morocco	200	Cereals, vegetable oils
Romania	20	Feeds
Russia	1000	Wheat, barley, maize, corn
Serbia and Montenegro	1000	Corn
	500	Feed for pigs (until 50 kg)
	1000	Feed for other type of swine
	3000	Feed for cow, sheep and goat
	5000	Feed for ox
	100,000	Feed for egg laying hen
Slovenia	1000	Feedstuffs for pigs
Ukraine	40	Grain-based babyfood products
	1000	Grains, beans; sunflower press; flour, bread; all nuts; all seeds to be used for immediate human consumption and for processing into the products for human consumption; vegetable oil; wheat middlings
	40	Combined feed for sows (pregnant, feeding), breeding boars, piglets younger than 2 months
	1000	Soya press for feed
	2000	Combined feed for pigs fed for pork lighter than 50 kg
	3000	Combined feed for pigs fed for pork over 50 kg of weight
Uruguay	200	Corn, barley

1.2.3. Fate in the body

1.2.3.1. Absorption

Although it is reported that zearalenone is relatively rapidly absorbed following an oral intake (Dailey et al., 1980; Olsen et al., 1985), limited quantitative data of this phenomenon seems to have been published. The kinetics of absorption has shown that Zen is rapidly absorbed at the intestinal level: between 80 and 85% in pigs (Biehl et al., 1993). In rats, the half-life of the intestinal Zen is 5 minutes and only 0.0095% of the dose initially administered as infusion into the intestine, were found in the intestine after an hour (Ramos et al., 1996). In pigs, using a model simulating the gastrointestinal tract was determined that the absorption of Zen is mainly in the jejunum (70% of the absorbed dose) and the ileum (30% of the absorbed dose) (Avantaggiato et al., 2003). These phenomena are preceded by a metabolic flora digestion and enterocytes, especially in ruminants (Kiessling and Pettersson, 1978, Kallela and Vasenius, 1982) and pigs (Olsen et al., 1987; Kollarczik et al., 1994)

1.2.3.2. Distribution

Zearalenone and zearalanol bind to globulins (which also bind the human sex hormones) (Martin et al., 1978). No mycotoxin is set at the α -fetoprotein rat (Sheehan et al., 1984). Blood concentrations zearalenone and α -zearalenol were followed in prepubertal gilts that received 192 g Zen/Kg bw/day for 4 days. Zearalenone and α -zearalenol were detected in the plasma in the half hours after the start of treatment until 5th days after treatment. During the administration, concentrations in plasma α -zearalenol are 3 to 4 times higher than those of zearalenone. The maximum concentration of zearalenone and its derivatives is found in the last day of treatment (it is about 10.4 ng/mL plasma). All of zearalenone and the α -zearalenol in plasma are conjugated with glucuronic acid (Olsen et al., 1985). Turkeys fed a diet containing Zearalenone 800 ppm for 2 weeks were presented at the end of the experiment plasma concentration zearalenone 66 ng/mL and 194 ng/mL in α -zearalenol. Almost all of zearalenone and α -zearalenol found in plasma are in the combined form (sulphate and glucuronoconjugate) (Olsen et al., 1986). In humans, the plasma half-life of α -zearalenol tritiated (oral) is approximately 22 hours. The concentration peak achieved is much higher among men than in other species (including monkeys) (Kuiper-Goodman et al., 1987).

Table 1.20. Toxicokinetics of zearalenone in farm animals (after Dänicke et al., 2008)

Species/category	Zen dose (mg/kg b.w.)	Toxin source	Route of administration	t _{max} (h)	t _{1/2 el} (h)
Pig					
• without bile removal	5	Standard	<i>i.v.</i>		86.6
• with bile removal	5	Standard	<i>i.v.</i>		3.34
Pig					
• undisrupted enterohepatic cycling	1	Standard	<i>i.v.</i>		2.63
• for 12 h disrupted enterohepatic cycling	1	Standard	<i>i.v.</i>		1.1
Broiler	5	Standard	<i>p.o.</i>	4-8	89
Laying hen	10	Standard	<i>p.o.</i>	2-4	

i.v. = intra venous, *p.o.* = orally

t_{max} = time at maximum plasma/serum concentration

t_{1/2 el} = plasma/serum elimination half life

1.2.3.3. Biotransformation

The intrinsic hepatic clearance of zearalenone in rats is approximately 100 g of zearalenone per gram of liver per hour. The main biotransformations of zearalenone are conjugation and its reduction. In rats, an unknown metabolite from 21 to 36% of the quantity metabolism would also be formed (Kiessling and Pettersson, 1978). Zearalenone can undergo reactions of glucuronoconjugation and sulfoconjugation. Pork is in deficit of sulfotransferases, only metabolites glucuronoconjugated are observed in the plasma. In rats, zearalenone is mainly glucuronoconjugated (Kiessling and Pettersson, 1978), only derivatives monoconjugated being synthesized, the combination being on the cycle. It is not known if the glucuronoconjugation is at the hydroxyl groups on C₂ and C₄ (Bories et al., 1991).

The reduction of zearalenone in zearalenol is catalyzed by the 3- α -hydroxysteroid dehydrogenase liver. According the incubation conditions, 25 to 50% of zearalenol and obtained can then be combined (Kiessling and Pettersson, 1978). The reduction of zearalenone constitutes a key step in its bioactivation. The 3- α -hydroxysteroid dehydrogenase liver are normally involved in the metabolism of steroids, androstenedione and zearalenone inhibit each other reduction (Kuiper-Goodman et al., 1987). Depending on the species, the reduction is done in the presence of NADH and/or NADPH. The cows have the greatest activity zearalenone NADH-dependent reductase, followed, in descending order by mice,

pigs, rats, rabbits and guinea pigs.

In rats, zearalenone is reduced by zearalenol S-9 fraction of liver homogenate in the presence of NADH or of NADPH (Ueno et al., 1983). The zearalenol reduction is multiplied by a factor of 3 during the addition of one or other of these two coenzymes (Kiessling and Pettersson, 1978). In this case, this reaction has 2 peaks of activity at pH 4.5 and 7.4. The hamster S-9 liver fraction shows optimal activity at pH 5.5 and 8 with NADH, and pH 6 and 8.5 with NADPH. This coenzyme is more effective than NADH only in this species.

These results suggest that there are 2 types of zearalenone reductase, NADH-dependent one, and other NADPH-dependent (Ueno et al., 1983). In rabbits, NADPH increases reduction activities in the cytosolic fraction, whereas the NADH enhances the reduction activity of the microsomal fraction. In the chicken NADPH enhances the activity of microsomal fractions reduced and cytosol of hepatocytes. These differences can be explained by the existence of 2 enzyme forms separate zearalenone reductase in fractions microsomal, whereas cytosolic reductases appear belong to the same enzyme form (Pompa et al., 1986). Thus, zearalenone undergoes *in vivo* a reduction of the group C₆ ketone, leading to the formation of α and/or β -zearalenol. Variability of inter specific are observed, they will lead to different proportions of these metabolites. These derivatives have a different toxicity. Differences in metabolism may explain differences in sensitivity to zearalenone (Pompa et al., 1986). The α -zearalenol is the major metabolite in the rat, mouse, pig, cow and rabbit liver at pH 4.5 with NADH or NADPH, and pH 7.4 with NADH. In the guinea pig, the α - and β -zearalenol are produced in quantities of essentially identical, irrespective of the pH and cofactor, while in hamsters, the β -zearalenol is the major metabolite (Ueno et al., 1983).

Compartmentalisation of intracellular activity reduction of zearalenone in the liver is described; it variation depending on the isomer formed. Among sows and goats, the α -zearalenol and β -zearalenol are mainly formed by the microsomal fraction. Cows and chickens metabolize almost entirely α -zearalenol with the microsomal fraction and β -zearalenol with the cytosolic fraction. At the sheep, the α -zearalenol is formed mainly by the fraction cytosolic (when NADPH is used as coenzyme) (Olsen and Kiessling, 1983). Chicken hepatocytes metabolize quickly and produce mainly β -zearalenol, whereas hepatocytes rabbit metabolize slowly and produce mainly of α -zearalenol (Pompa et al., 1986).

The major metabolite obtained rat is among the α -zearalenol. The NADPH is much more efficient NADH as the cofactor in this reaction (Chang and Devries, 1984). It is unclear whether these reactions occur in other species animal and whether the enzymes involved are identical to those present in hepatocytes.

Thus the reduction of zearalenone *in vivo* leads to the formation of α and β -zearalenol. It would seem that a reduction further, appearing on the vinyl group, C1 'and C2 ', takes place in sheep (Miles et al., 1996). The α - and β -zearalanol free and glucuronconjugated forms are detected in the urine. It appears that this biotransformation remains marginal compared to the reduction in zearalenol.

Table 1.21. *In vitro* conversion of ZEA into a-ZOL and b-ZOL by liver subcellular fractions of different animal species and their percentage of glucuroconjugation (after Malekinejad et al, 2006)

	Pig	Sheep	Cattle	Chicken	Rat
Zen					
<i>Post-mitochondrial fraction</i>					
% of glucuronidation*	90 ± 4.6	67 ± 2.8	75 ± 0.7	34 ± 3.0	66 ± 4.6
α-Zol					
<i>Microsomal fraction</i>					
Vmax (pmol/min/mg)	796 ± 123	364 ± 56	68 ± 1.6	326 ± 8.0	80 ± 4.3
<i>Post-mitochondrial fraction</i>					
Vmax (pmol/min/mg)	480 ± 62.3	587 ± 24	163 ± 7.1	192 ± 5.3	32 ± 3.6
% of glucuronidation*	88 ± 2	54 ± 32	39 ± 4.1	17 ± 5.7	61 ± 0.7
β-Zol					
<i>Microsomal fraction</i>					
Vmax (pmol/min/mg)	512 ± 99	693 ± 85	164 ± 3.6	1462 ± 30	495 ± 15
<i>Post-mitochondrial fraction</i>					
Vmax (pmol/min/mg)	208 ± 45	121 ± 8.5	78 ± 4.6	746 ± 25	72 ± 3.0
% of glucuronidation*	100	40 ± 1.1	34 ± 0.8	16 ± 2.3	72 ± 8.3

* Percentage of glucuronidation of Zen and its metabolites, at a concentration of 50 μ M, by the hepatic cytosolic fraction of the different animal species in the presence of UDPGA

1.2.3.4. Excretion

The excretion of zearalenone and its derivatives can be in bile, urine, but also milk. Urine and faeces are the main routes of elimination of Zen (JECFA, 2000). A summary of excretion of zearalenone and its metabolites in urine and in the faeces of cattle, pigs and rats is presented in Table 1.8. (Gaumy et al., 2001). Excretion rates measured show an inter-species variability. No studies on the transition from zearalenone and its derivatives in eggs have been made.

Table 1.22. Comparative Metabolism of Zen: Distribution of metabolites in urine and faeces (Mirocha et al., 1981).

Animal Species	Urine*			Faeces*		
	ZEN	α -ZOL	β -ZOL	ZEN	α -ZOL	β -ZOL
Bovine	29	20	51	25	12	58
Pig	63	32	5	91	9	ND
Rat	93	4	3	97	ND	3

* % of the excreted amount

Biliary excretion

The injection of tritiated zearalenone in mice revealed a rapid excretion of radioactivity in the bile as in urine (Appelgren et al., 1982). Sows have presented problems with reproduction; zearalenone and the α -zearalenol were detected in bile at concentrations of 40 ng/mL and 66.1 ng/mL (Meyer et al., 1997). This excretion is the cause of an entero-hepatic cycle. Thus, the intraduodenal administration of bile containing the marked zearalenone and its metabolites shows reabsorption of the mycotoxin. Approximately 65% of the original dose is excreted in bile and 21% in urine in less than 14h after single dose of 5 mg Zen/kg i.v. treatment. In gilts that received 5 mg/Kg of tritiated zearalenone intravenously or 10 mg/Kg orally, the plasma half-life of zearalenone and its metabolites (total radioactivity) is 86.6 hours in controls, against 3.3 hours in animals whose bile is removed (Biehl et al., 1993).

Thus, the high biliary excretion in pig, and the cycle enterohepatic important of zearalenone and its derivatives lead to a sharp increase in plasma half-life of these compounds in this species, which could explain their high sensitivity to the toxicity of this mycotoxin. In turkey that received a diet containing Zearalenone 800 ppm for 14 days, 182.2 μ g/g of zearalenone and 644 μ g/g α -zearalenol are eliminated in faeces. Only a few traces of β -zearalenol were found. Approximately 65% of the zearalenone and α -zearalenol is combined

in the form (Olsen et al., 1986). In chicken, the Zearalenone is excreted within 72 hours following its administration primarily as unchanged (93.1%) (Pompa et al., 1986). It is not known in poultry if these forms of excreta are related to biliary excretion, urinary or elimination of metabolites formed not absorbed by plants.

Table 1.23. Proportions of Zen and its metabolites, α -zearalenol (α -Zol) and β -zearalenol (β -Zol), in bile of various species determined after chronic oral Zen exposure (Dänicke et al., 2008)

Species/category	Dose (μ g/kg diet)	Duration (days)	b.w. (kg) ¹	Mean toxin proportion of the sum of Zen+ α -Zol+ β -Zol		
				Zol	α -Zol	β -Zol
Pigs						
Piglets	≤ 420	35	33	72	26	2
Fattening pigs	56	84	94	55	45	0
Gilts	≤ 358	35	120	42	56	2
Sows	358	35 ²	203	46	52	2
Piglets	358 ³	35	1.12	59	41	0
Sows	49	35 ⁴	192	57	43	0
Poultry						
Laying hens	1100	112	1.59	75	15	10
Pekin ducks	≤ 60	49	3.6	80	16	4
Turkeys	≤ 40	35	4.11	19	77	4
Growing bulls	100	152-160	460	24	8	68
Rabbits	≤ 297	21	2.68	17	37	45

¹Mean body weight at the end of the study

²Day 75 to 110 of gestation

³Diaplacental exposure

⁴Day 35 to 70 of gestation

Urinary excretion

In the gilt, nearly all of the urinary excretion of zearalenone and its metabolites is observed in the 48 hours after administration of the mycotoxin. Free zearalenone is majority in the urine, 31% of the total urinary zearalenone is in the glucuronoconjugated form. The zearalenol (α -zearalenol 90%), which appear mainly in the glucuronoconjugated form ($> 80\%$) constitute 37% of the total metabolites in urine. However, less than 7% of the zearalenone total administered was excreted in urine. None sulfoconjugated form has been detected (Mirocha et al., 1981). Another study confirmed that the products excreted in urine are zearalenone and α -zearalenol, but these times completely under glucuronoconjugated form (Olsen et al., 1985). In cow milk, zearalenone and zearalenol free form and in combination were detected in the urine. 84% of urinary metabolites of zearalenone are glucurono- and sulfo-conjugated. The ratio of concentrations total zearalenone/ α -zearalenol/ β -zearalenol is about 2/1/3; the β -

zearalenol is the metabolite whose concentration is highest in all tests (Mirocha et al., 1981). In rat, zearalenone and its glucuronoconjugated derivatives are the main metabolites excreted in the 96 hours after administration of the toxin (5 mg/rat). Zearalenol traces were detected. The fraction of the dose total excreted in urine is about 30% (Mirocha et al., 1981). These results seem to go hand in hand in accordance with another study showing that urine is a major route of excretion in rats (15-20% of the administered dose are excreted mainly free form). However, we can note that 55% of the initial dose of zearalenone is excreted in the materials stool (Fitzpatrick et al., 1988).

In rabbit, the zearalenone excretion is also mainly via urine (Kuiper-Goodman et al., 1987, Mirocha et al., 1981). Zearalenone, the α - and β -zearalenol are found more than 95% as glucuronoconjugates in urine, the excretion ratio is 2/1/1 respectively (urine collected 96 hours after administration of mycotoxin). In humans, detectable levels of radioactivity are observed until 4 days after a single oral marked mycotoxin treatment. The main forms are conjugated (Kuiper-Goodman et al., 1987). Metabolism appears to be similar to that of pigs, as regards the production of a large percentage of α -zearalenol glucuronoconjugated and metabolites, is similar to that of rabbits in relation to production of almost exclusive glucuronoconjugated forms. In both species for which the route of excretion is the major urinary tract, e.g. rabbits and humans disappearance in the blood is much slower than in other species.

Milk excretion

The observations of animal intoxication have shown that the vulva of gilts aged less than one week were inflated when the mother received a diet containing of zearalenone (Bristol and Djurikovic S, 1971; Dacasto M et al., 1995; McNutt et al., 1928; Pullar et al., 1937), which implies a transfer mycotoxin in milk. Palyusik has detected zearalenone in sow's milk, 42 - 44 hours after consuming feeds contaminated with 40 ppm zearalenone, and up to 5 days after withdrawal (Palyusik et al., 1980). The analysis of milk samples showed the presence from 82 to 86% of β -zearalenol, 13.4 to 15.7% of α -zearalenol and 0.5 to 1.3% of zearalenone. The maximum level of zearalenol found in sow's milk is of 0.575 to 0.790 ppm.

In lactating cows, passage of small amounts zearalenone in milk was measured (Hagler et al., 1980). An administration of 25 ppm zearalenone in food during 7 days is followed by an excretion of zearalenone and its derivatives (α -and β zearalenol) in milk (Mirocha et al., 1981). Compounds are excreted in milk as free and combined form (glucuronic and sulphate) in orders of magnitude equivalent. In a single administration of 250 ppm zearalenone, the

maximum milk excretion is observed the day after the administration. It is important to note that a single administration of a large amount of zearalenone (250 ppm in the food) are resulting fewer metabolites in milk (eight times less) than prolonged administration (7 days) of an amount ten times lesser of zearalenone (Mirocha et al., 1981; Prelusky et al., 1990).

Fate in the body of zearalenone is dominated by large interspecies differences. The conjugation of zearalenone is far outweighed by the reduction in most species studied. From a toxicological point of view the latter is the most important, because of the development of derivatives more toxic than the parent compound (bioactivation phenomenon).

1.3. Enzymes implicated within detoxification pathway

1.3.1. Metabolism, detoxification and transport of xenobiotics

The metabolism of a xenobiotic (drug, pesticide pollution, toxic, contaminating food, mycotoxins, etc.) corresponds to processing by an enzymatic reaction of the latter in one or more compounds, called metabolites, which may be pharmacologically inactive or active and sometimes toxic.

Metabolism is one of the phases of the elimination of an exogenous hydrophobic compound; the different stages of metabolism lead to the formation of more water-soluble substances, more easily removed by aqueous media such as urine, bile, saliva or sweat. It is important to note that this phenomenon can be utilized in a pharmacological approach. The metabolism of a drug does not necessarily represent its inactivation, e.g. the prodrugs which are pharmacologically inactive are rapidly metabolized into active metabolites.

Many tissues are the site of xenobiotic metabolism: liver, kidney, lung, intestine ... the main one being the liver hepatocytes, which are rich in enzymes involved in metabolism.

There are 3 main phases in the metabolism:

- The reactions of phase I
- The reactions of phase II
- Reactions known as phase III are similar to phenomena of efflux of parent compounds from the cell or its metabolites formed during the previous two phases (Figure 1.5).

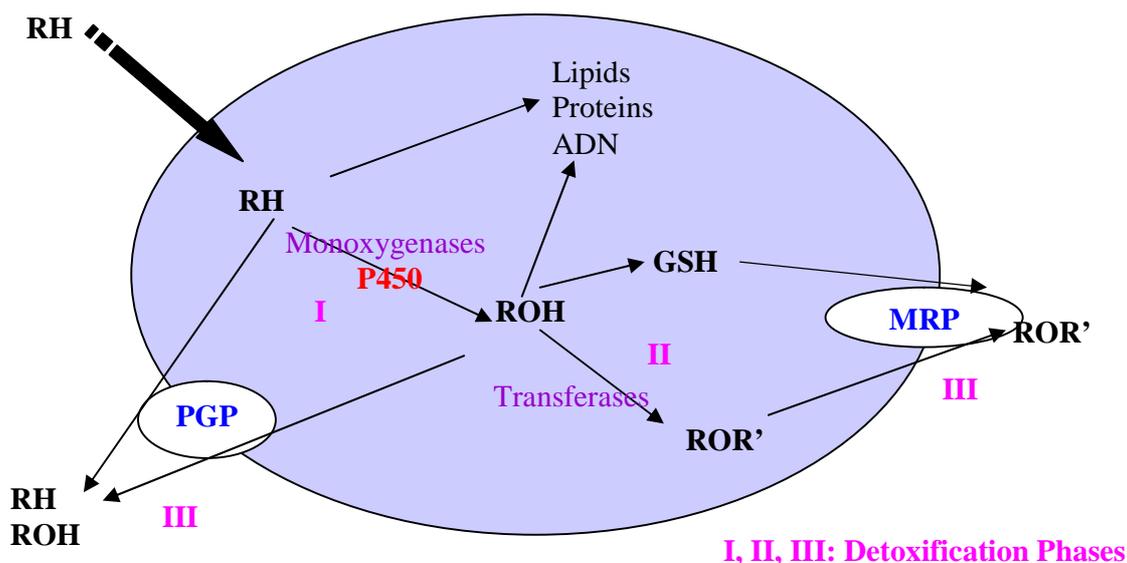


Figure 1.5: Diagram of a cell, the seat of the detoxification process: phases I, II and III. RH exogenous hydrophobic compound by a passive absorption is entering into the cell, then is undergone within phase I reactions via mono-oxygenases (e.g. P450) resulting in a compound of ROH type, which can be supported by transferases (UGT for example) during the detoxification phase II. The parent compound, as the successive metabolites, may be expelled from the cell by the transport proteins (Pgp and MRP) within the detoxification phase III.

1.3.1.1. Phase I

Mechanism of Action

Phase I is the step that leads to the formation of metabolites that can either be eliminated by taking over assets by the carriers, if they have reached a sufficient degree of water solubility, or continue the process of metabolism by Phase II. Phase I is not compulsory: some compounds can immediately undergo Phase II.

The reactions of phase I reactions are:

- Oxidation: involving mono-oxygenases such as cytochromes P450. They mainly occur in liver microsomes;
- Reduction, less frequent;
- Hydrolysis: taking place in the organs (kidney, liver, intestine, lung ...) but also in plasma. Esterase's involved in hydrolysis reactions are poorly specific.

The metabolites formed by the phase I reactions are the hydroxyl functional groups (OH), amine (NH₂) or carboxyl (COOH) which can then be conjugated by the phase II reactions (Figure 1.6).

The P450 cytochromes

Under the general term of cytochrome P450 is grouped a multigene superfamily of protein enzyme with redox properties, called "hemo-thiolate". Also known as the mono-oxygenases, cytochrome P450 has the ability to activate the oxygen molecules into highly reactive entities (ROS) and then insert molecular oxygen into a large and varied substrates of both level of a carbon atom, as well as nitrogen or sulphur. This activation of oxygen is made possible by the presence of an iron atom in heme. The cytochromes P450 (CYPs or P450s) are ubiquitous enzymes that are found in all living organisms with the exception of some primitive micro-organisms that have evolved, there are over 3.5 billion years. CYPs catalyze the oxidation reactions requiring oxygen and NADPH (Fig 1.6).

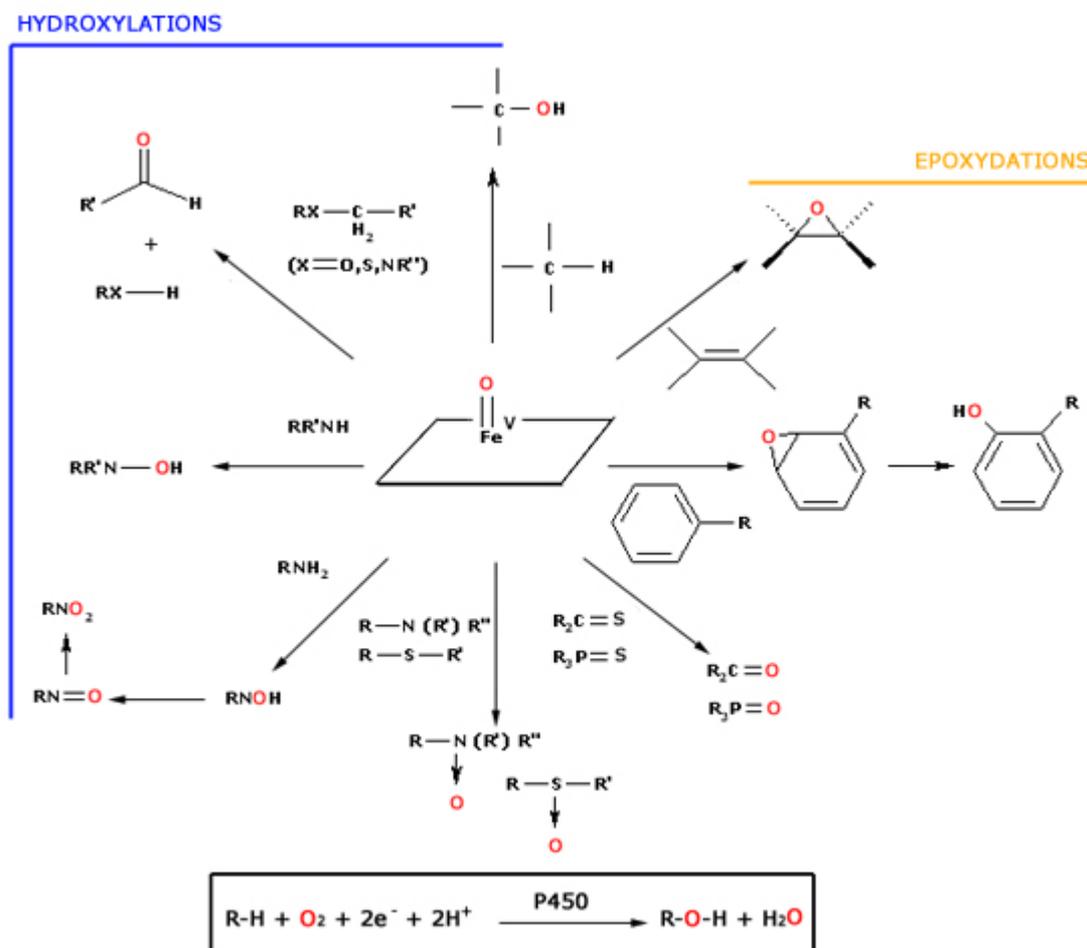


Figure 1.6: Various types of oxidation reactions usually catalyzed by P450s (According to Battioni and Mansuy, 1999).

The nature of substrates and the reactions generated by these enzymes are many and varied: the CYPs are responsible for the oxidative metabolism of diverse molecules, including both endogenous substances (steroid hormones, fatty acids, vitamins D, prostanoid, alkanoids, terpenes and phytoalexines other ...) and xenobiotics (drugs, pesticides, pollutants, toxins, carcinogenics ...). The reactions of biotransformation of xenobiotics catalyzed by CYPs are a process of detoxification avoiding the accumulation of potentially toxic substances in the body. These are in fact the same substrates that induce their own self detoxification reactions. The CYPs may catalyze the activation of certain chemical compounds (procarcinogenics ...) and produce toxic, mutagenic or carcinogenic metabolites (generation of ROS that damage DNA).

There are about fifty years; Klingenberg and Garfinkel have reported for the first time the appearance of a yellow-orange pigment in liver microsomal fractions of rat and pig in the presence of carbon monoxide (Garfinkel, 1958; Klingenberg, 1958). The pigment was then at first characterized as a hemoprotein, identified as an uncommon type b cytochrome, and later became what is known under the designation "P450". The designation "P450" was given because of its property of absorption maximum at 450 nm in the UV-visible spectrum when the heme iron, in the reduced state, is complexed to carbon monoxide (Omura and Sato, 1962). The 'P' of P450 refers to as the "pigment" resulting the term of P450 for Pigment at 450 nm. Since, this enzyme system has been the subject of much research and is still at the heart of many scientific projects today.

There are a large number of cytochrome P450 isoenzymes (57 isoenzymes identified in humans in 2004) (Figure 1.7). The cytochromes P450 are present in the liver microsomes in the liver but also in the intestine, kidney, lungs, skin, etc...

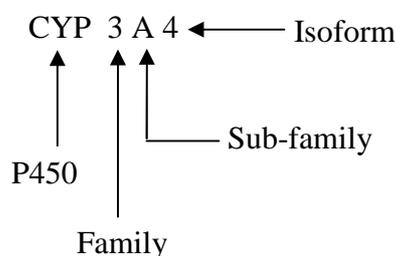


Figure 1.7: P450s nomenclature

Finally, all P450s identified to date means hemoproteins formed by a heme attached to the apoprotein by a cysteinyl group complexing the iron prosthetic group (Figure 1.8). Basically,

it is customary to represent CYPs like a large hemoproteins consisting of a single polypeptide chain, *apoprotein* (a molecular weight between 45 and 60 kDa) and a central prosthetic group, *heme* (a catalyst the enzymatic reaction). The latter is "non-covalent" linked to the apoprotein via a cysteine. The catalytic site of the enzyme is hydrophobic, enabling the reception of substrates.

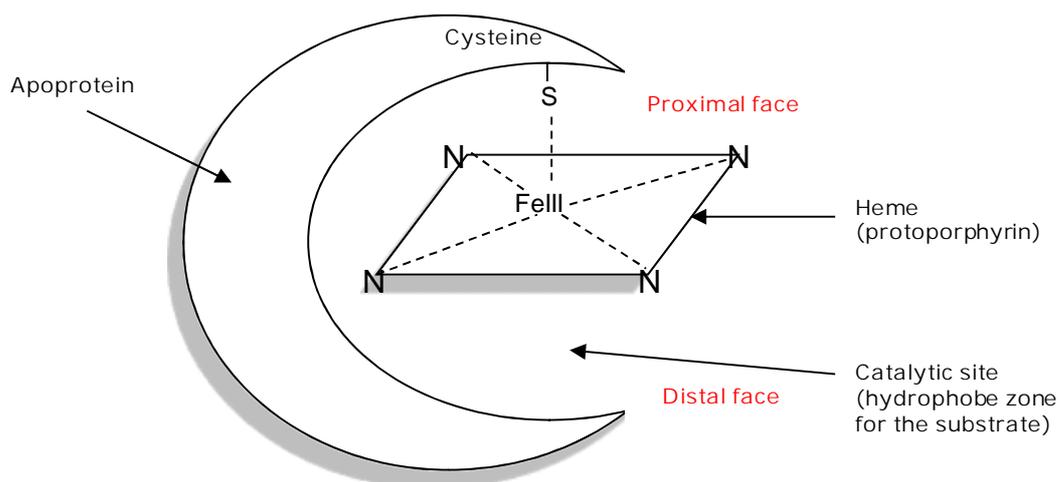


Figure 1.8: Schematic representation of a CYP (from the thesis of Thien-An Nguyen (Nguyen, 2007))

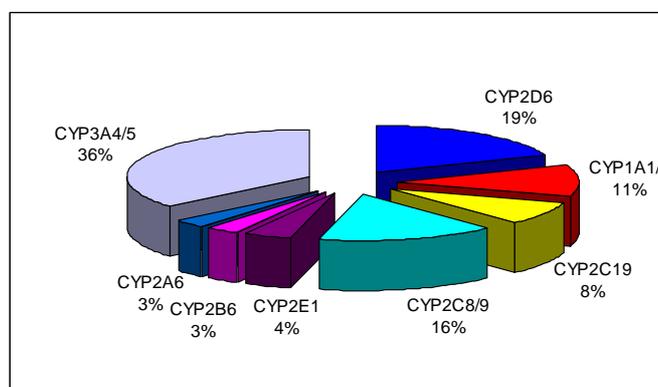


Figure 1.9: Distribution of drugs metabolized by different human CYP isoforms (Wrighton and Stevens, 1992).

The cytochrome P450 isoenzymes may be inhibited or induced by xenobiotics or foods that may cause drug interactions (fig 1.9).

Some substrates, enzyme inducers can increase the synthesis of cytochrome P450 and thus the enzyme activity. The enzymes induction effect is poorly specific. An inducer may increase the synthesis and enzymatic activity of one or more isoforms of CYPs, for example, phenobarbital induces the synthesis of CYP3A and CYP2B6, and rifampicin is an inducer of

CYP3A and 2C9.

The main enzyme inducers of CYPs are presented in Table 1.22, below. The most powerful enzyme inducers are some anti-epileptic drugs (phenobarbital, carbamazepine, and phenytoin) and rifampicin. The Millepertuis (St. John herb or St. John's worth, or *Hypericum perforatum*), a medicinal plant used in the treatment of mood disorders (anxiety, depression), is an enzyme inducer of CYPs. Millepertuis co-administered with low therapeutic drugs and metabolized by the CYPs (e.g. cyclosporine, theophylline, anti-vitamin K ...) causes a decrease in the therapeutic concentrations and thus their effects.

The inducing effect occurs:

- On the inductor own metabolism called self-induction, the metabolism of the inductor is increased, the dosage should be adjusted secondarily (e.g. phenobarbital);
- On other drugs when co-administration: the metabolism of other drugs metabolized by the induced CYP(s) increase, resulting in a decrease in the plasmatic concentration of the active product and of its half-life, resulting in a decrease of activity of the drug.

Inhibition of cytochrome P450 by certain foods or medicines, called enzyme inhibitors, is another variation of metabolism. Inhibition is the most often competitive and occurs rapidly with an increase in plasma concentration and half-life of the drug, whose metabolism was inhibited resulting in a risk of toxicity. Two drugs metabolized by the same CYP isoform without enzyme inducer or inhibitor, but only by being substrates of the same isoenzyme, will compete with a risk of reducing their metabolism. The enzyme inhibition is often the cause of drug interactions. The main used drugs enzymatic inhibitors (ketoconazole, macrolide antibiotics, ritonavir, grapefruit juice ...) are presented in Table 1.22.

Table 1.22: Principal substrates, inducers and inhibitors of CYPs (Booklet Vidal Drug Interactions ® 2003)

	Substrates	Inhibitors	Inductors
CYP1A2	Caffeine, imipramine, paracetamol, dimethylxanthine	Cimetidine, quinolones (ciprofloxacin, enoxacin, norfloxacin), fluvoxamine	Cigarettes smoke, omeprazole
CYP2C9	Non-steroidal anti-inflammatories, phenytoin, tolbutamide, S-warfarin		Barbiturates, rifampicin
CYP2C19	Citalopram, diazepam, imipramine, omeprazole, proguanil, propranolol		Barbiturates, rifampicin
CYP2D6	Antiarrhythmias: flecainide, propafenone Antidepressants: amitriptyline, clomipramine, desipramine, imipramine, nortriptyline, paroxetine Antitussives: codeine, dextromethorphan β -blockers: alprenolol, metoprolol, propranolol, timolol Neuroleptics: haloperidol, perphenazine, risperidone, thioridazine, zuclopenthixol	Cimetidine, fluoxetine, paroxetine, quinidine, ritonavir	
CYP3A4	Calcium antagonists: diltiazem, felodipine, nifedipine, verapamil Antiarrhythmics: amiodarone, disopyramide, lidocaine, quinidine, propafenone Benzodiazepines: alprazolam, diazepam, midazolam, triazolam. Antiinfectieux drugs: erythromycin, itraconazole, ketoconazole Statins: atorvastatin, simvastatin Other: carbamazepine, cyclosporine, dextromethorphan, digitoxin, ethinyl estradiol, ethosuximide, imipramine, terfenadine	Cimetidine, clarithromycin, erythromycin, itraconazole, ketoconazole, ritonavir	Carbamazepine, dexamethasone, phenobarbital, phenytoin, rifampicin

1.3.1.2. Phase II: Conjugation

Phase II is a phase conjugation that leads to the formation of conjugated substances, water-soluble and easily removed by the urine or bile. Metabolites or parent compounds undergo different reactions conjugation: glycu-ro-(or glucuronic or glucurono) conjugation; sulfo-conjugation, acetylation; alkyls (transfer of a methyl group); to give a conjugate that will be eliminated. Conjugation is the transfer of a functional group (OH, NH₂, and COOH) of a compound type sulfate, glucuronic acid, methyl (Figure 1.10)...

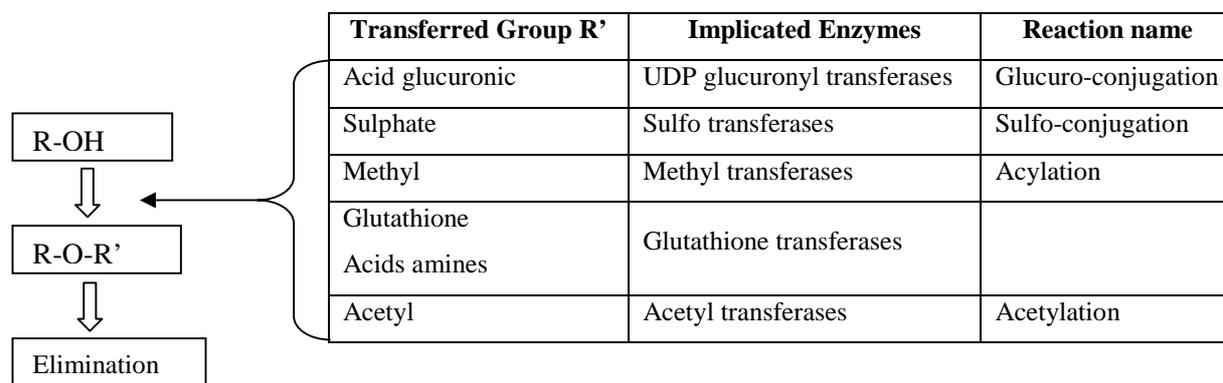


Figure 1.10: Main functional groups transferred to a compound of type R-OH / R-NH / R-CO₂H / R-SH (product of the phase I reaction of xenobiotic RH) during a phase II reaction, called conjugation by enzymes of the family of transferases.

1.3.1.3. Transport

To enter the body (oral, pulmonary, intra-venous, dermal etc.) xenobiotics will be confronted with barriers to cross cell to access to a target organ or target tissue. Several types of barriers exist: lung, gastric, intestinal, skin and so on. We will limit ourselves to the intestinal barrier, since the mycotoxin which we are interested is mostly absorbed in the small intestine: the Zen is absorbed more precisely in the jejunum, at the beginning of the small intestine (Avantaggiato et al., 2003).

The small intestine provides a first barrier against possible contaminated food that would have been ingested. This epithelial barrier is crucial to the passage through of mycotoxins ingested into the blood. It separates the light digestive (outdoor) environment from the inside of the body. It consists of a continuous layer of epithelial cells interconnected by tight junctions and modulates the membrane permeability via a paracellular transport (between the epithelial cells) or transcellular (passage through the epithelial cells). According to their

physicochemical properties and their affinity for membrane transport proteins present in the intestinal epithelium, the xenobiotic will be able to borrow these different transport channels. The transcellular transition of exogenous compounds may be altered by the intracellular metabolism

The different models for the study of the absorption mechanisms of xenobiotics and determination of their membrane permeability are models *in silico* (computer modeling), *in vitro* (pork gastrointestinal or pulmonary cell culture, etc.), *ex vivo* (inverted intestine) and *in vivo* (in rats or other animals).

The *in silico* approaches of xenobiotics absorption prediction are based in part on the physicochemical properties of molecules (size, lipophilicity, solubility, etc.) and are often used for the screening of new drugs (Smith et al. 2001). They are indeed: fast, inexpensive, reproducible and automatable. However, if they capture well the process of active transport, they do not incorporate possible multifactorial interactions (Deferme et al., 2008). However, methods using cell cultures or animal models used to study the mechanisms of passive and active absorption may involve membrane transporters. These are classified into two broad families of transporters: the family of active transport systems, ATP-binding cassette (ABC), which has 48 members and the family of carriers, solute carrier (SLC) that has over 300 members in mammals. At intestinal level, 8 carriers of the SLC superfamily and 7 of ABC have a known role in the intestinal transport of xenobiotics (Sai and Tsuji, 2004).

In order to determine how the trans-epithelial transfer of xenobiotics is done, different cell types of renal or intestinal origin are used for their ability to form a polarized epithelium in culture. Among them, the human Caco-2 cells line has a number of advantages including its spontaneous differentiation into enterocyte cell type, under certain culture conditions (Pinto et al., 1983). It is derived from human colorectal adenocarcinoma and was established for studying the mechanisms of carcinogenesis and cytostatic therapies (Fogh et al., 1977). Grown on filters, Caco-2 cells are widely used in the elucidation of the mechanisms involved in the absorption of xenobiotics (Artursson and Karlsson, 1991) and the screening of new drugs (Shah et al., 2006). The differentiation of human colon cancer cells in culture, defines their ability to self-organize morphologically as enterocyte cells and express the functional characteristics of normal intestinal epithelium.

1.4. Zearalenone biotransformation and effects on the hepatic enzymes

1.4.1. Hepatic biotransformations of zearalenone

The estrogenic mycotoxin zearalenone has been described to undergo both reductions into α - and β -zearalenols and further conjugations by using *in vitro* models such as rat liver homogenate (Galtier et al, 2008). Recently, it has been demonstrated that zearalenone is predominantly reduced by hepatic 3 α - and 3 β - hydroxysteroid dehydrogenases in mammals (Fig. 1.11) (Malekinejad et al., 2006).

Figure 1.11. Enzymatic analysis of Zearalenone biotransformation by porcine granulosa cells in the presence of different competitors (Malekinejad et al., 2006)

Products/kinetic values	ZEN	ZEN + 5 α -DHT	ZEN+ PGTN	ZEN + PGN
a-ZOL				
V _{max} (pmol/min/mg)	237.6 ± 21.7	179.0 ± 15.2*	118.5 ± 4.9*	209.8 ± 28.1
K _m (μM)	53.4 ± 10.2	45.1 ± 9.5	20.2 ± 2.9*	57.8 ± 16.1
r ₂	0.92	0.91	0.92	0.87
V _{max} /K _m	4.4	3.9	5.8	3.6
b-ZOL				
V _{max} (pmol/min/mg)	160.8 ± 15.9	151.8 ± 46.7	164.5 ± 70.4	127.9 ± 5.2*
K _m (μM)	76.5 ± 15.9	159.2 ± 56.5	174.1 ± 70.1	48.4 ± 9.4
r ₂	0.92	0.85	0.90	0.90
V _{max} /K _m	2.09	0.95	0.94	2.64

PGN, pregnenolone ; PGTN, progesterone ; 5 α -DHT, 5 α -Dihydrotestosterone.

*Differences considered significant for p < 0.05

Interspecies differences in the rate of absolute and relative metabolite productions in different subcellular fractions were identified. The highest amounts of α -zearalenol were produced by pig hepatic microsomes whereas chicken microsomes produced the highest amounts of β -zearalenol. Studies on the conjugation of zearalenone with glucuronic acid indicated significant interspecies differences in the rate of glucuronidation, suggesting differences in the affinity of the individual substrate or the presence of different isoforms of glucuronosyltransferase. In terms of toxicity, the estrogenic activity of α -zearalenol would be about 10 and 100 times greater than that of zearalenone and β -zearalenol. So, interspecies differences in α - reduction and conjugation of zearalenone could explain the large differences in estrogenic properties developed by this mycotoxin among animal species, particularly between pigs and poultry (Pompa et al., 1986).

Animal metabolism of zearalenone and the α -zearalenol was studied in several animal species and humans. The proportions depend largely on the species (Kleinova et al., 2002).

Biotransformation of zearalenone in rats is mainly in two ways:

- Reduction in α -zearalenol and β -zearalenol (Figure 1.11);
- A combination of zearalenone and its metabolites with glucuronic acid (Kiessling and Pettersson, 1978; Ueno and Tashiro, 1981).

The biotransformation of zearalenone in zearalenol is the reduction of the ketone function. This reaction shows similarities with the processes of drug metabolism that are catalyzed by hydroxysteroid dehydrogenases (HSDs) (Malekinejad et al., 2005). The HSDs belong to the super family of small chain dehydrogenases / reductases (SDRs) that catalyze the oxidation / reduction in the synthesis and inactivation of steroid hormones (Penning, 2003; Persson et al., 2003). The main phase I enzymes involved in the biotransformation of Zen are the 3 α - and 3 β -hydroxysteroid dehydrogenase (3 α - and 3 β -HSD). The organs that are the location of these transformations are the intestine, liver and target organs of Zen (e.g. gonads). Steroids are also handled by these enzymes, may modulate Zen, competition by the metabolism of steroids (Malekinejad et al., 2006).

The 3 α -HSD belongs to the superfamily of aldehyde ketone reductases, operating as oxidoreductases NAD(P)H-dependent reducing ketone alcohols. Among the endogenous substrates of this enzyme, are steroid hormones and prostaglandins. The 3 β -HSD belongs to her, the family of dehydrogenase / short-chain reductases that catalyze the conversion of hydroxy-3-ene steroids (e.g. conversion of pregnenolone to progesterone).

Malekinejad et al. showed great variability between species reports training α - and β -Zol on liver microsomes: the α -Zol is formed mainly in pigs and humans, whereas in poultry and ruminants, the β -Zol is predominant (Malekinejad et al., 2006).

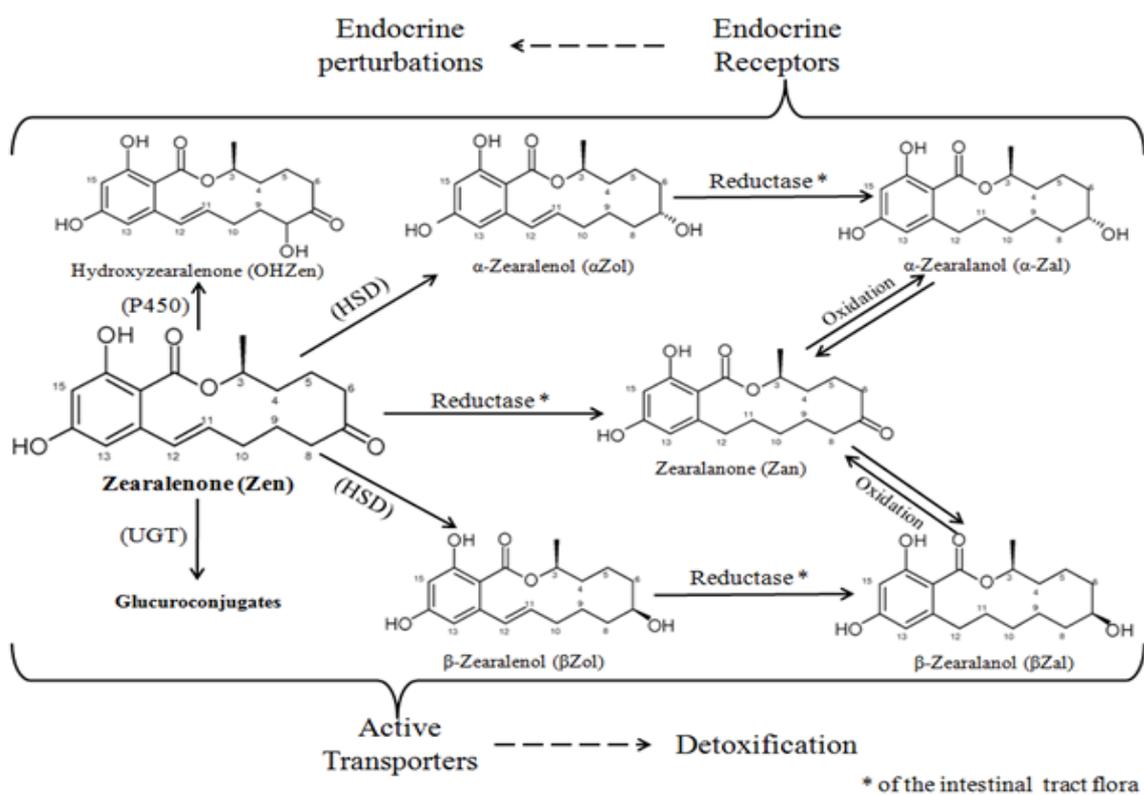


Figure 1.11: Diagram summarizing the different pathways of ZEN in the mammal.

It has also recently been highlighted (Malekinejad et al., 2005) that the formation of α -zearalenol increased in the presence of NADPH, whereas that of β -zearalenol increases only in the presence of NADH. The optimal pH for the production of α -zearalenol is 5.6 and the β -zearalenol is 7.4.

Finally, the ZEN and its derivatives can be conjugated by UDP-glucuronyl-transferases to be excreted in the urine and bile (Mirocha et al., 1981). Specifically, 27% of determined Zen was found in the form of glucuronic combined, 88% of determined α -Zol and 94% of determined β -Zol (Zollner et al., 2002).

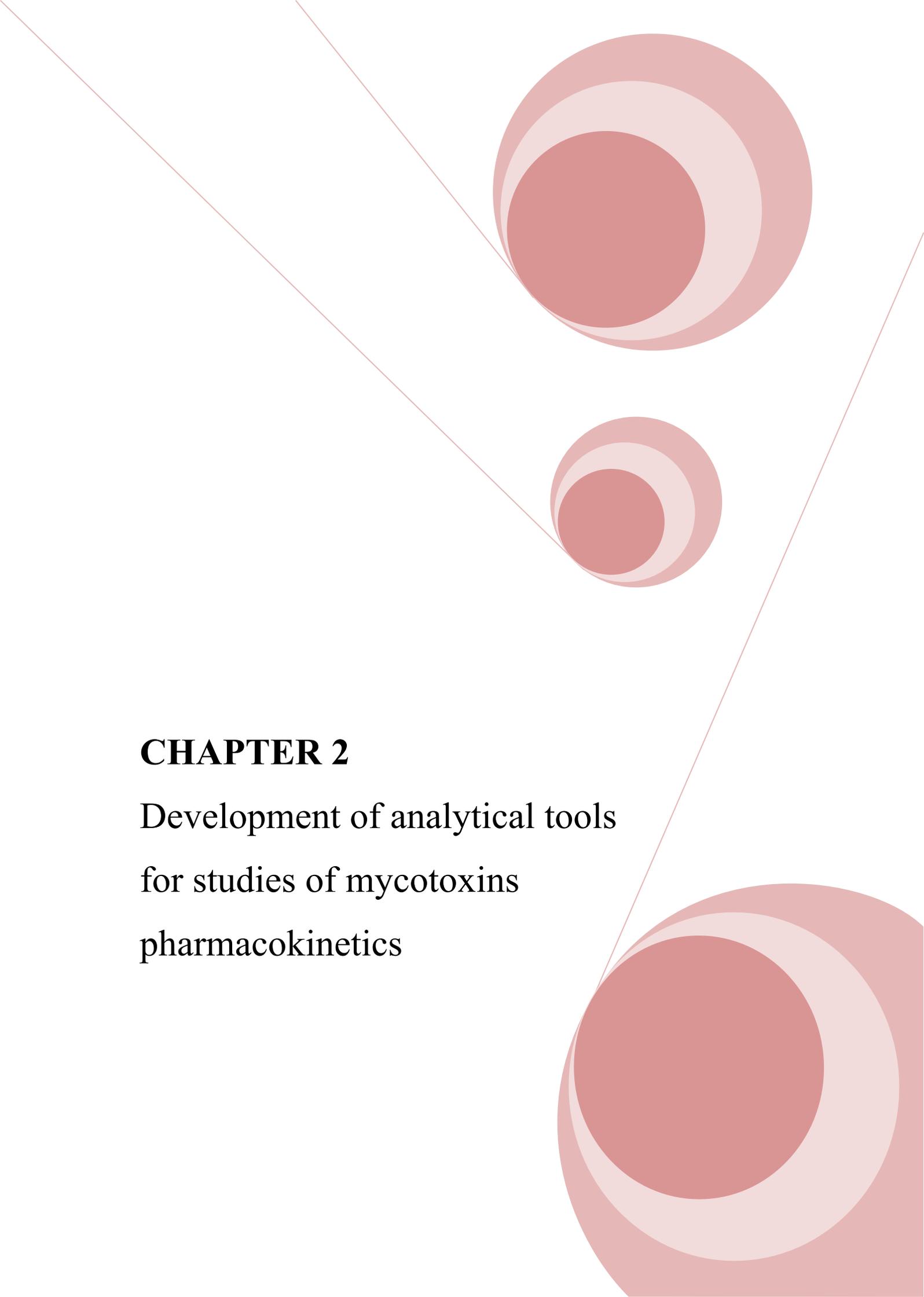
1.4.2. Effects of zearalenone on the activity and expression of liver drug metabolizing enzymes

In various *in vivo* experiments, an elevation of serum enzyme levels, indicative of liver cell damage, was observed. Significant increases in the activity of ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), γ GT(gamma-glutamyltransferase), and LD (lactatedehydrogenase) were observed in rabbits given Zen at doses between 10 and 100 μ g/kg bw (Conkova et al., 2001), which can induce dysfunction of blood coagulation (Maaroufi et al, 1996). Whether or not these effects are directly attributable to Zen or non-specified impurities in the given feeds remains to be elucidated. No clinical signs were associated with these alterations in serum enzyme activity. (Kullak-Ublick and Meier, 2000).

In a recent study, hemosiderosis in the liver of pig foetuses was observed as a typical alteration induced by Zen, although in these experiments, the maternal contaminated diet contained also measurable levels of deoxynivalenol (Tiemann et al., 2007). A typical feature of hepatocytes is the expression of drug-metabolizing enzymes of the cytochrome P450 superfamily. Zen was found to induce in human hepatocytes the expression of CYP3A4, which is controlled by the transcription factor PXR (xenobiotic receptor pregnane X receptor, PXR, NR1I2). PXR is a member of the nuclear receptor superfamily of ligand activated transcription factors (Blumberg et al., 1998). Analysis of the mechanisms involved in the CYP3A4 induction (and the corresponding reduction of the murine CYP3A11) revealed that Zen displaces the NR-corepressor protein from the human PXR, while recruiting at the same time the co-activating SRC-1 (steroid receptor coactivator-1), GRIP1 (Glucocorticoid Receptor-Interacting Protein 1) and PBP (PPAR-Binding protein) (Ding et al., 2006). These findings are of clinical significance as nutritional exposure to Zen may result in unexpected

alterations in drug metabolism and kinetics, as CYP3A4 is the major drug-metabolizing enzyme in the human liver.

Nevertheless, the effects of zearalenone on basal and TCDD (dioxin)-induced mRNA expression and enzyme activity of P450 1A1 and 1B1 were recently investigated in MCF-7 cells (Yu et al., 2004). 50 nM zearalenone significantly reduced both basal and TCDD-induced activity and mRNA expression in these cells. The oestrogen receptor antagonist 4-hydroxytamoxifen could attenuate these inhibition effects of zearalenone. Interestingly, zearalenone could promote basal and TCDD-induced CYP1B1 activity without any effect on CYP1B1 mRNA expression. These results suggest that the effect of zearalenone on both the TCDD-induced CYP1A1 activity and the gene expression, involved the oestrogen receptor pathway.

A decorative graphic on the right side of the page. It features three overlapping circles of varying sizes, each composed of concentric layers of different shades of red and pink. Two thin, light red lines extend from the top left towards the circles, and another line extends from the top right towards the bottom right circle.

CHAPTER 2

Development of analytical tools
for studies of mycotoxins
pharmacokinetics

2.1. HPLC assessment of Zen and metabolites in biological samples – Method development and validation

The literature describes several chromatographic methods for zearalenone and its metabolites' separation, identification and determination from biological samples. Most times, zearalenone is determined alone in cereal grain samples (Rosenberg et al., 1998; Pallaroni et al., 2002 a, b; Pallaroni and von Holst, 2003 a,b; Schneweis et al., 2002; Krska et al., 2003; Pallaroni and von Holst, 2004; Royer et al., 2004; Berthiller et al., 2005). Zearalenone metabolites α -Zal and β -Zal were determined by several methods from fat and kidney samples (Joos and Van Ryckeghem, 1999), from cow muscle and liver (Horie and Nakazawa, 2000) and from chicken liver (Fang et al., 2002). Other methods are dedicated to the separation and determination of Zen, α -Zol and β -Zol from beer (Zollner et al., 2002) and fish samples (Lagana et al., 2003). There are papers published which describe the separation and determination of Zen, α -Zol, β -Zol, α -Zal and β -Zal from river water (Lagana et al., 2001), pig urine and tissue (Jodlbauer et al. 2000), cattle urine (Launay et al., 2004), eggs (Sypecka et al., 2004) and milk samples (Sorensen and Elbaek, 2005). The latter methods used Zan as internal standard, but it has two major inconveniences: it is a natural metabolite of zearalenone and it has the same elution as Zen, if C18 columns are used (Sforza et al., 2006). Few are the liquid chromatography methods presented in the literature for zearalenone and all its metabolites separation from biological samples such as cattle urine (van Bennekom et al., 2002), pig liver, urine and muscle (Zollner et al, 2002; Kleinova et al., 2002; Zollner et al, 2003) and cattle liver, plasma and urine (Songsermsakul et al., 2006).

In the present chapter we present the optimisation and validation of a HPLC-DAD method for the separation of zearalenone and its metabolites, and its application on biological samples of chickens treated with 25 mg Zen/ Kg body weight.

2.1.1. Material and methods

2.1.1.1. Instrumentation

HPLC studies and analyses were performed on a HPLC Finigan Surveyor system (Thermo-Electron Corporation – Waltham, MA, USA); KjeltacTM 2300 and SoxtecTM 2055 (FOSS – Tecator – Hilleroed, Denmark) were used. A Gas-Chromatograph Clarus 500 system from Perkin-Elmer (Waltham, MA, USA) was also used. Chromabond[®] C18 polypropylene columns were obtained from Macherey-Nagel GmbH & Co. KG (Duren, Germania). HPLC system used in LC-MS coupling consisted of a LC 1100 series system,

(Agilent, CA, US) coupled with a Ion Trap Mass spectrometer (Esquire HCT) using an ESI interface operating in positive or negative mode (Bruker Daltonics, MA, USA). The immunoaffinity columns (IAC) ZearaStar were purchased from Romer Labs Diagnostic GMBH (Tulin, Austria). Water was purified in a Milli-Q[®] Ultrapure Water Purification Systems (Millipore – Billerica, MA, USA). Thermo SpeedVac[®] concentrator from Thermo-Electron Corporation (Waltham, MA, USA) was used. A GC-FID Perkin-Elmer with N₂ carrier gas with a capillary chromatographic column (BPX 70, 60m) was also used. Grindomix GM 200 knife mill from Retsch (Haan, Germany) was used for dried sample grinding. Centrifuge 2-16K Sigma (Deisenhofer, Germany) was used to centrifuge the biological samples; the GFL 3015 vibration shaking system (Burgwedel, Germany) was used for samples stirring; the SW23 Shaking Water Bath from Julabo Labortechnik GmbH (Sellback, Germany) was used to incubate the samples.

2.1.1.2. Chemicals and Solvents

Zearalenone, Zen, α -zearalenol, α -Zol, β -zearalenol, β -Zol, α -zearalanol, α -Zal, β -zearalenol, β -Zal, zearalanone, Zan, standards were purchased from Sigma (Deisenhofer, Germany); ammonium acetate, glacial acetic acid, sulphuric acids (density of 1.840 g/mL) disodium phosphate and monosodium phosphate as well as sodium hydroxide (all analytical reagent grade) were supplied by Sigma (Deisenhofer, Germany). Both buffer tampon solutions ammonium acetate - glacial acetic acid (50 mM; pH 4.8) and phosphate (50 mM, pH 7.4) were prepared in our laboratory. A standard mixture of fatty acids: PUFA No.2 (Animal Source) from Sigma (Deisenhofer, Germany) was used. Glucuronidase/ arylsulfatase from *Helix pomatia* (30/60 U/mL), HPLC grade methanol and HPLC grade acetonitrile were purchased from Merck (Darmstadt, Germany). The standards were individually dissolved in acetonitrile to give 10⁻³ M stock solutions which were stored at -20°C until use. Standard working solutions were prepared by diluting each stock solution with a mixture consisting of water : acetonitrile (50 : 50, v/v). Liquid nitrogen was purchased from Linde Gas (Saint Priest Cedex, France). Ultrapure Milli-Q water was used.

2.1.1.3. Sample collection and preparation

Poultry treatments

Leghorn broilers aged 2 weeks, housed and treated in agreement with the acting legislation in facilities authorised by the French Ministry of Agriculture within the French

Food Safety Agency (AFSSA), Ploufragan, France were used. The chickens were fed *ad libitum* with a standard diet. The birds (5 chickens per group) were treated intraperitoneally for 3 days as follows: group 1 – Control: NaCl 9‰ solution 1 mL/ day; group 2 – Zen 3d: 25 mg Zen/ kg bw every day; group 3 – PB-Zen: 80 mg phenobarbitone natrium salt/ kg bw every day, associated with a single treatment with 25 mg Zen/ kg bw the last day. The animals were slaughtered 24 h after the last treatment. The studies groups of broilers presented are part of a larger experiment (see chapter 3). The aims of the experiment is to detect the forms of cytochromes P450 induced by the presence of zearalenone in broilers (knowing that the broilers are the most “resistant” species to the presence of zearalenone) and to compare them with classical P450 inducers, like Phenobarbital.

Sample collection

The chicken muscles, blood and liver samples were collected at AFSSA Ploufragan.

The chicken muscle samples breast and whole left leg were collected. The chicken blood samples were collected in sterile tubes with heparin, left for 2 hours at room temperature and then centrifuged at 2500 rpm and the supernatants were collected. Liver samples, consisting of the whole liver, were also collected.

The chicken meat, blood and liver samples were frozen in liquid nitrogen and stored at -80°C.

Sample preparation

The samples were prepared for analysis according to Songsermsakul et al. (2006) as follows:

- Broiler muscle sample

1 g broiler meat sample, ground and dried at 65°C, was extracted thereafter in 50 mL water : methanol (50:50), while stirring at 150 rpm, for 60 minutes. The sample was centrifuged for 10 minute at 4000×rpm; 20 mL the supernatant was collected and mixed with 40 mL buffer solution acetic acid – ammonium acetate 50 mM, pH 4.8. This solution was incubated for 15 h at 37°C with 80 µL solution of glucuronidase/ arylsulfatase and then brought to pH 4.0 with glacial acetic acid. The solution was loaded into a Chromabond C18 extraction column preconditioned with 10 mL methanol and then rinsed with 10 mL ultrapure water. Thereafter, the columns loaded with the sample were rinsed with 5 mL ultrapure water and 5 mL methanol 30% and dried for 3 min under

vacuum. The analytes were eluted with 1.25 mL methanol. The eluate was mixed with 15 mL buffer phosphate solution 50 mM, pH 7.4. This mixture was introduced into a ZearaStar immunoaffinity column preconditioned by the manufacturer. The immunoaffinity column loaded with the sample was rinsed with 15 mL ultrapure water and dried for 3 minutes under vacuum. The analytes were eluted with 1.5 mL acetonitrile, the samples were dried using a Speed Vac concentration system and taken on 150 μ L mixture water: methanol (50:50, v/v). 20 μ L have been injected into the HPLC system for separation and determination.

- Plasma sample

1mL plasma was mixed with 6 mL buffer ammonium acetate solution 50 mM, pH 4.8. This solution was incubated for 15 h at 37°C with 25 μ L solution of glucuronidase/arylsulfatase before adding 6 mL phosphate buffer 0.1 M pH 7.4 and adjusted to pH 7.4 using NaOH 1 M. The sample was centrifuged at 4000×rpm; the supernatant was introduced into a ZearaStar immunoaffinity column preconditioned by the manufacturer. The immunoaffinity column loaded with the sample was rinsed with 15 mL ultrapure water and dried for 3 minutes under vacuum. The analytes were eluted with 1.5 mL acetonitrile, the samples were dried using a Speed Vac concentration system and taken on 150 μ L mixture water : methanol (50 : 50, v/v). 20 μ L have been injected into the HPLC system for separation and determination.

- Liver sample

The thawed liver samples were used to prepare microsomal extracts by successive centrifugation according to the method described by Peyronneau et al. (1992). We used 2.5 mg (10 mL S9 – subcellular fraction obtained after the first centrifugation) liver which was extracted in 40 mL water : methanol (50:50 v/v) mixture, while stirring at 150 rpm, for 60 minutes. The sample was prepared for analysis using the same methodology as for the broiler meat sample.

Chromatographic separation

Chromatographic separations were performed by reverse phase chromatography using a Hypersil Gold (C18), 150 x 4.5 mm column at 40°C using a water : acetonitrile, ACN : methanol, MeOH (45 : 8 : 47 v/v/v) mixture with a 50 mM ammonium acetate content as mobile phase, with a total flow rate of 1 mL/min.

2.1.2. Results and discussion

2.1.2.1. Method development

The determination of zearalenone and its metabolites content in biological samples is a current topic as supported by the wide contamination of the forages and implicitly of the animal products.

In developing a HPLC method for the simultaneous separation and determination of zearalenone and its metabolites in biological broiler samples, we started from the method described by Songsermsakul et al. (2006) for biological samples, horse urine, plasma and faeces. This method required the use of two serial chromatographic columns: a Polar-RP column with stationary phase containing phenyl-ether groups (150 mm×4.6 mm) and a Hydro-RP with stationary phase C18 (150 mm × 4.6 mm), and a mixture of water : ACN : MeOH (35:30:35, v/v/v) as mobile phase, with a flow rate of 1 mL/min. Working under these conditions, but using only a Hypersil Gold (C18) 150 x 4.6 mm column, the separation of a standard mixture containing 50 µM of Zen, Zan, α -Zol, β -Zol, α -Zal and β -Zal was performed. Three chromatographic peaks corresponding to the separation of mixtures Zen + Zan from α -Zol + α -Zal and from β -Zol + β -Zal were obtaining, but it was not possible to separate Zen from Zan, α -Zol from α -Zal and β -Zol from β -Zal.

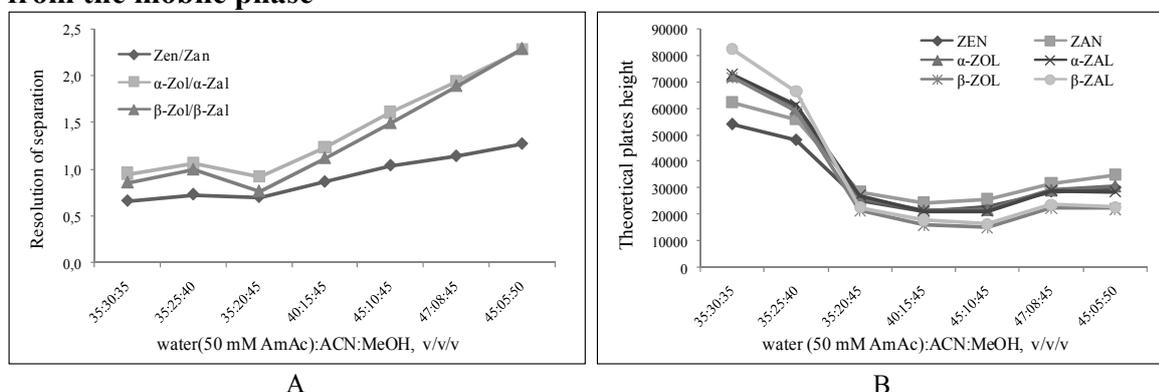
In order to optimise the method, the following parameters: mobile phase flow rate, ammonium acetate concentration in the solvent mixture, ratio of water : acetonitrile : methanol in the mobile phase and the chromatographic column temperature were varied successively.

By modifying the mobile phase flow rate only, an improved separation of Zen from Zan at a flow rate of 0.4 mL/min. was obtained, but not the separation of α -Zol from α -Zal and β -Zol from β -Zal. Knowing the beneficial effect of using the ammonium acetate, AmAc (Zollner et al., 1999, 2000, 2002, 2003; Jodlbauer et al., 2000; Kleinova et al., 2002; Lagana et al., 2001, 2003; Krska et al., 2003; Pallaroni and von Holst, 2004; Royer et al., 2004) we studied the influence of the ammonium acetate content between 10mM and 70mM in the mobile phase; we have thus improved the separation of the entire mixture at a content ≥ 50 mM ammonium acetate.

For an optimal separation of the pairs of substances which lead to neighbouring peaks: Zen from Zan, α -Zol from α -Zal and β -Zol from β -Zal, the influence of the proportion of solvents in the mobile phase water (containing 50 mM AcAm) : ACN : MeOH, starting from a 35:30:35 (v/v/v) ratio to a 45:5:50 (v/v/v) ratio, was studied.

The separation resolution for each of the three pairs of substances displayed minimal values when a mobile phase with a water (50 mM AcAm) : ACN : MeOH ratio of 35:20:45 was used, after which it increased continuously with the increase of the content of water solution and the decrease of the acetonitrile content in the solvent mixture (Fig. 2.1. a). Evaluating the separation resolution values as function of the solvent mixture composition and the time of analysis, the solvent mixture water (50 mM AcAm) : ACN : MeOH with 45:8:47 (v/v/v) ratio was chosen as optimal mobile phase composition. The correctness of this decision is also supported by the values of the equivalent theoretical plate height, H (Fig. 2.1 b).

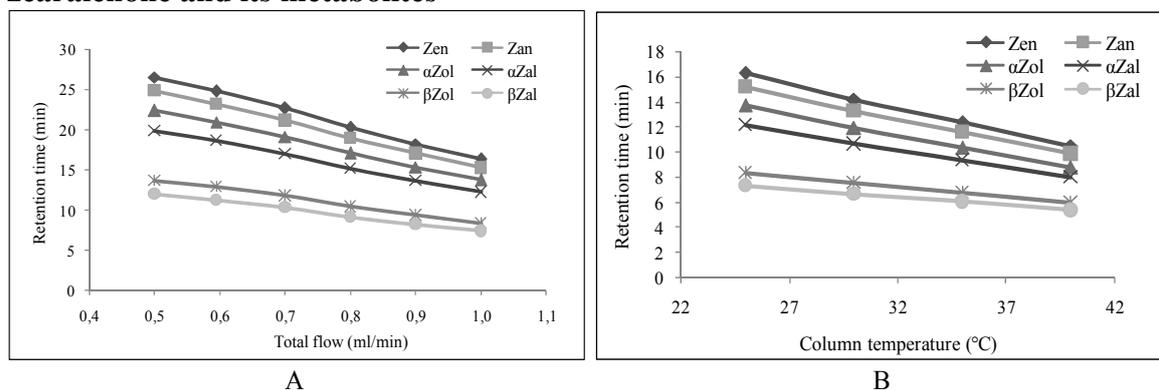
Fig.2.1 Variation of resolution for 3 pairs of neighbouring peaks (A) and of the height of the equivalent theoretical plates (B) function of the ratio of the components from the mobile phase



Hypersil Gold (C18) column, 150 x 4.6 mm; elution isocratic gradient – mixture of water (containing 50mM ammonium acetate) : acetonitrile : methanol; total flow rate of the mobile phase 0.4 mL/min; temperature of the chromatographic column 25°C; DAD detector, $\lambda = 280\text{nm}$.

In order to optimise the total analysis time, the influence of the mobile phase flow rate and of the chromatographic column temperature on the retention time values were studied. As shown in Fig. 2.2, varying the flow rate from 0.5 mL/min to 1 mL/min, the total analysis time decreased from 30 minutes to 20 minutes. Subsequently, by increasing the column temperature from 25°C to 40°C, the total analysis time decreased from 20 minutes to just 12 minutes. Because the resolution of β -Zol from β -Zal separation decreases with the increase of the flow rate, we did not exceed the flow value of 1 mL/min.

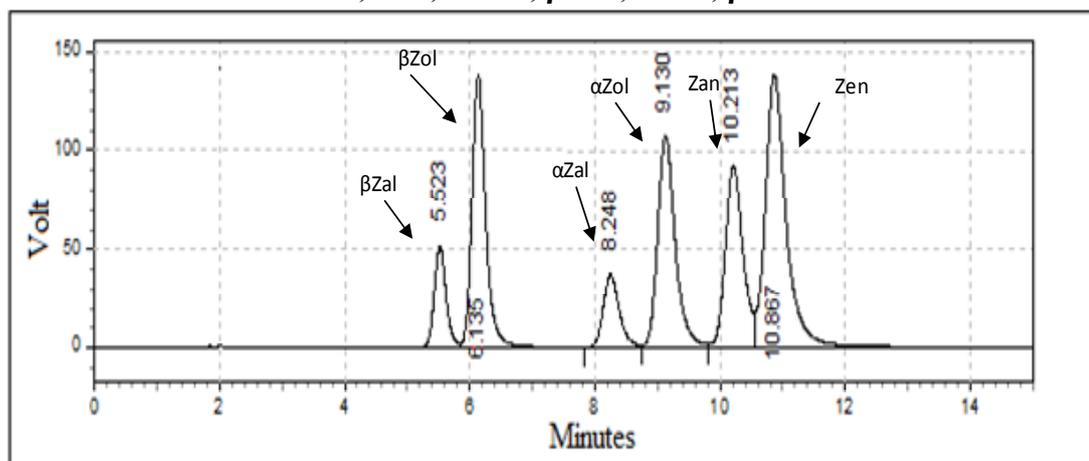
Fig. 2.2. Variation of the retention time according to the total flow rate of the mobile phase (A) and to the temperature of the chromatographic column (B) for zearalenone and its metabolites



Hypersil Gold (C18) column, 150 x 4.6 mm; elution isocratic gradient – mixture of water (containing 50mM ammonium acetate) : acetonitrile : methanol (45:8:47); DAD detector, $\lambda = 280\text{nm}$; temperature of the chromatographic column 25°C – A; total flow rate of the mobile phase 1 mL/min min – B

In conclusion, Zen and its metabolites can be separated with a good resolution within 12 minutes, using a Hypersil Gold C18 column (150 mm × 4.6 mm), the mobile phase water (50 mM AcAm) : ACN : MeOH, 45 : 8 : 47 (v/v/v) with a flow rate of 1 mL/min and a chromatographic column temperature of 40°C. Fig. 2.3 shows a chromatogram obtained in these working conditions.

Fig 2.3. Example of chromatographic separation of a standard mixture of 50 μM Zen, Zan, α -Zol, β -Zol, α -Zal, β -Zal



Hypersil Gold (C18) column, 150 x 4.6 mm; elution isocratic gradient – mixture of water (containing 50mM ammonium acetate) : acetonitrile : methanol (45:8:47); total flow rate of the mobile phase 1 mL/min; temperature of the chromatographic column 40°C; DAD detector, $\lambda = 280\text{nm}$

2.1.2.2. Method validation

The HPLC method was validated according to the international rules (ICH Topic Q2 A and B, 1995): the selectivity, linearity, limits of determination and quantification, precision, accuracy, robustness and stability of the solutions were determined.

Selectivity was studied on a standard mixture of Zen, Zan, α -Zol, α -Zal, β -Zol and β -Zal containing 50 μ M of each compound and was evaluated during the development of the assay method.

Linearity was checked for zearalenone and its metabolites using standard solutions having concentrations between 0.32 μ g/mL (1 μ M) and 31.8 μ g/mL (100 μ M).

The precision of the proposed method, expressed by **repeatability** (intra-day assays) was determined by performing 6 repeated determinations on 3 samples containing 12.7 μ g/mL (40 μ M), 15.9 μ g/mL (50 μ M) and 19.1 μ g/mL (60 μ M) standard mixture of Zen, Zan, α -Zol, α -Zal, β -Zol and β -Zal, respectively.

In order to determine the precision expressed by **reproducibility** (inter-day assays) 15.9 μ g/mL standard samples was analyzed within 5 consecutive days by 2 different analysts.

Precision was reported as percentage of relative standard deviation (Causon, 1997):

$$\text{RSD} = [(\text{standard deviation}/\text{mean})] \times 100$$

The **accuracy** was determined using the results obtained for the precision determination. Accuracy was reported as percentage of bias (Causon, 1997):

$$\text{Bias} = [(\text{measured concentration} - \text{true concentration})/\text{true concentration}] \times 100$$

The **lower limit of detection** (LLOD) and the **lower limit of quantification** (LLOQ) were determined on 6 consecutive determinations of a standard sample of 0.03 μ g/mL. The LLOD and LLOQ were calculated as a sum of 3 times standard deviations and 10 times standard deviations, respectively, and the average concentration determined for the used standard.

The **recovery** was determined by performing 10 repeated determinations on a standard sample of 15.9 μ g/mL and other 10 determinations on the same standard sample with spiked analyte up to a final concentration of 19.1 μ g/mL.

Stability of the solution was studied during two weeks, the standard solution being stored at -20°C. Robustness was explored during the development of the assay method.

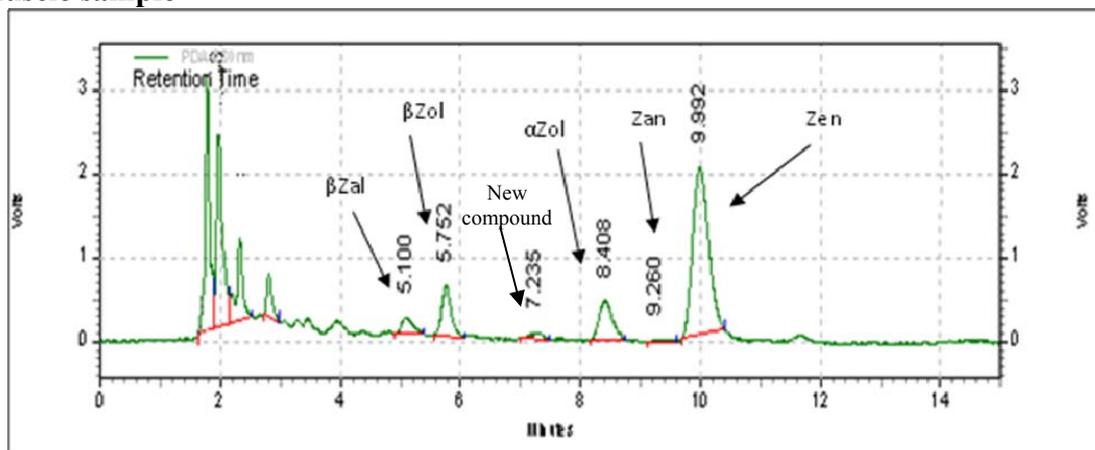
Table 2.1. Results obtained upon validation of the HPLC method for zearalenone and its metabolites determination

Compounds Parameters	ZEN	ZAN	α -ZOL	α -ZAL	β -ZOL	β -ZAL
ACCURACY						
Bias	0.76	0.78	1.31	1.35	0.65	0.35
calculated t	1.65	2.09	2.12	2.16	1.92	1.12
PRECISION						
Intra- day – RSD (%)	1.36	0.61	2.20	0.48	0.50	0.27
Inter- day – RSD (%)	2.22	2.21	2.00	2.18	2.00	1.86
LINEARITY						
Domain ($\mu\text{g/ml}$)	0.32-31.8	0.32-31.8	0.32-31.8	0.32-31.8	0.32-31.8	0.32-31.8
R ² value	0.9996	0.9990	0.9990	0.9969	0.9984	0.9980
LOWER LIMITS OF DETECTION AND QUANTIFICATION						
LLoD ($\mu\text{g/ml}$)	0.03	0.02	0.03	0.14	0.04	0.04
LLoQ ($\mu\text{g/ml}$)	0.04	0.03	0.04	0.17	0.05	0.07
RECOVERY						
Value (%)	104.93	102.28	98.86	103.64	119.87	107.13

The performance criteria required are: accuracy within the bias interval of (-2) – 2 %; maximal RSD value is set according to the analyte concentration, according to Horwitz equation ($\text{RSD}=2(1-0.5\log C)$); $\text{RSD} = 8.88\%$ for a concentration of $15.9 \mu\text{g/mL}$; $\text{LLOQ} > \text{LLOD}$ and the recovery values were in the interval 80 – 120%. The obtained results were evaluated using the Student T-test: the value given in the table is $t = 2.26$ for $p \leq 0.05$ and 9 degree of freedom and it is in all cases greater than the calculated ones.

2.1.2.3. Zearalenone determination within chicken biological samples

Using the optimised and validated HPLC method the concentrations of zearalenone and its metabolites in broiler meat (figure 2.4), plasma and liver samples were determined.

Fig. 2.4. Example of a chromatographic separation of Zen 3 days treated chicken muscle sample


The samples have been prepared as described in the sample preparation part. The chromatographic separation has been done on a reverse-phase Hypersil Gold (C18), $150 \times 4.6 \text{ mm}$, column; using an isocratic separation with water:acetonitrile:methanol (45:8:47, v/v/v) with a content of 50 mM ammonium acetate. The total flow was of 1 mL/min , column temperature of 40°C and the chromatographic separation total time of 11 minutes. The DAD detector lambda was set at 280 nm.

The samples were prepared as described in the material and method part. The obtained results are presented in detail in chapter 3. Based on the results obtained using the proposed method for samples analysis of muscle (35.72 ± 6.12 ng Zen/g dry matter (DM), 1.72 ± 0.68 ng Zan/g DM, 13.42 ± 2.50 ng α -Zol/g DM, 11.47 ± 3.95 ng β -Zol/g DM, 5.61 ± 0.94 ng β -Zal/g DM), of plasma (6.62 ± 1.04 ng Zen/mL) and of liver (3.01 ± 0.32 μ g Zen/g, 26.46 ± 2.22 μ g α -Zol/g, 23.69 ± 2.05 μ g β -Zol/g) collected from broilers treated with zearalenone, one can conclude that the method responds to the requirements to determine zearalenone and its metabolites at ng/g level in broiler biological samples

2.1.3. Conclusion

The method responds to the requirements to determine zearalenone and its metabolites at ng/g level in broiler biological samples, allowing the quality control of the samples; it could also be a useful instrument in the studies of zearalenone metabolism.

Zen and its metabolites can be separated with a proper resolution in 12 minutes using a HypersilTM Gold C18 column (150 mm \times 4.6 mm), using the mobile phase 50 mM AcAm : ACN : MeOH, with 45 : 8 : 47 (v/v/v) ratio, at mobile phase flow rate of 1 mL/min and chromatographic column temperature of 40°C

2.2. Study on the oxidation - reduction equilibriums involving Zearalenone

Zearalenone, Zen is a secondary metabolite of several *Fusarium* species (*Fusarium graminearum*, *F. culmorum*, *F. equiseti* and *F. crookwellense*.), which colonize the cereals particularly (corn, oats, barley, wheat and sorghum) under conditions of cold, wet weather, in the temperate and warm regions. The studies on the physical and chemical properties of zearalenone revealed traits ideal for an easy diffusion into the tissues. Zearalenone is low toxic and there is no evidence of its carcinogenetic potential, all studies being conducted on animals.

A critical research of the literature reveals the high stability of zearalenone. Until now there are no references related to the oxidation of Zen by means of common oxidative reagents. For this reason the oxidation-reduction equilibriums involving Zen and Ce (IV) were studied both, in aqueous and in mixed solvents water – acetonitrile solutions containing 1M H₂SO₄ and the voltametric behaviour of Zen was established, too.

2.2.1. Study on the oxidation - reduction equilibriums involving Zen and Ce (IV)

Material and methods

Chemicals

Zearalenone, Zen standard and acetonitrile HPLC grade were purchased from Sigma (Deisenhofer, Germany). Both, sulphuric acid (density of 1.840 g/mL) – supplied by Sigma (Deisenhofer, Germany) – and 0.1M aqueous solution of cerium sulphate in 1 M H₂SO₄ – purchased from Merck (Darmstadt, Germany) – were analytical reagent grade. 0.01 M aqueous solution of cerium sulphate containing 1M H₂SO₄ was prepared in a calibrated flask with exactly measured appropriate volumes of 0.1 M aqueous solution of cerium sulphate containing 1M H₂SO₄ and H₂SO₄ (density of 1.840 g/mL); the flask was filled up to the mark with ultra-pure water. Dichloromethane (analytical grade) was purchased from Merck. The stock solutions of Zen were prepared in 10 mL calibrated flasks where exactly weighted ($\pm 2 \cdot 10^{-4}$ g) quantities of Zen were dissolved in acetonitrile.

Instrumentation

The water was purified using a ULTRA CLEAR system. HPLC studies were performed on a HPLC Finnigan Surveyor system (Thermo-Electron Corporation – Waltham, MA, USA) having the patented LightPipe™ technology that provides up to five times the sensitivity of a conventional photodiode array detector and a integrated column oven that offers a good chromatographic stability. An UV-VIS spectrometer JASCO V530

provided with SpectraManager software was used to record the UV-VIS spectra. A pH/mV-meter Consort P501 provided with a platinum electrode and saturated calomel reference electrode (SCE) was used to record the potential of the solution.

Procedure

Oxidation - reduction equilibriums involving Zen and Ce (IV) were studied both, in aqueous and in mixed solvents water – acetonitrile solutions containing 1 M H₂SO₄. The solutions to be studied were prepared in Berzelius glass beakers having a volume of 50 mL, where exact quantities of Zen were weighted ($\pm 2 \cdot 10^{-4}$ g) and the appropriate reagents were added as follows:

- appropriate volumes of both 0.01 M or 0.1 M aqueous solution of cerium (IV) sulphate in H₂SO₄ 1 M and solution of sulphuric acid 1 M respectively, to a total sample volume of 20 mL – for the experiments in aqueous systems;
- 2 mL acetonitrile added for Zen dissolution and appropriate volumes of 0.01 M or 0.1 M aqueous solution of cerium (IV) sulphate in H₂SO₄ 1 M and sulphuric acid 1 M solution respectively, to a total sample volume of 20 mL – for the experiments conducted in mixed solvents systems.

After the given time of contact between the reagents, the precipitate was filtered by means of a quantitative paper filter, washed with ultra-pure water and both the filtrate and the washing solution were collected in a 25 mL calibrated flask filled up to the mark with ultra-pure water. A volume of 15 mL of this solution was quantitatively passed in a separation funnel where a volume of 10 mL of CH₂Cl₂ was added; the separation funnel was shaken for 5 minutes, after which the funnel needed to be left for 15 minutes while the layers separate out and settle down. The organic phase was completely evaporated using a rotary evaporator. The residuum was dissolved quantitatively in a 10 mL volumetric flask using acetonitrile as solvent. This solution is further cited as working solution. The solution obtained was analysed by HPLC.

The oxidation - reduction potential for each studied mixture (solutions containing Zen and Ce (IV) in different ratios) was measured at equilibrium using an mV-meter Consort P501 provided with a platinum electrode and saturated calomel reference electrode (SCE). The UV absorption spectrum of a 10⁻⁴ M Zen solution was registered using a JASCO V 530 spectrometer having 1 cm quartz cells.

HPLC method

Chromatographic studies were performed by reverse phase chromatography using a Hypersil™ Gold column having dimensions 250 x 4.6 mm, 5 µm particle size, purchased from ThermoElectron Corporation, USA. During the separation, the temperature of the column was kept at room temperature. The mobile phase was a mixture of acetonitrile - water. The elution was in gradient and the profile of them is presented in Table 2.2.

Table 2.2. Composition of the gradient

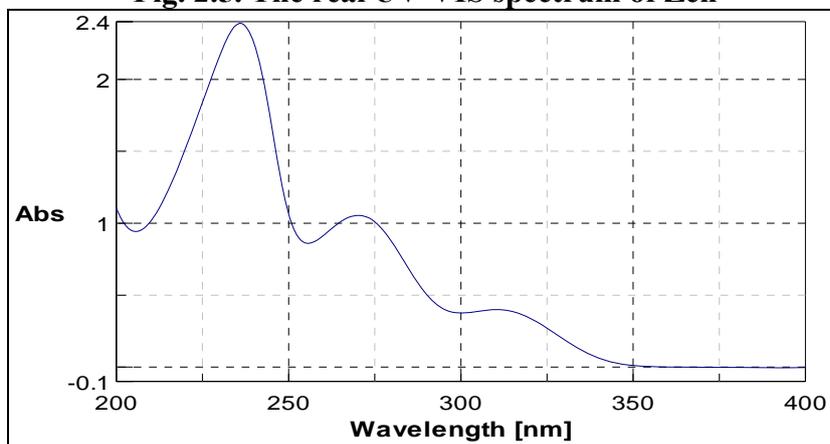
Time (min)	Water %	Acetonitrile %
3	90	10
40	10	90
55	90	10

The flow rate was 1 mL/min. The detection was made at λ : 236, 254 and 274 nm, respectively. The volume of injection was 10 µL. The data were acquired and processed by the ChromQuest Chromatography Data System.

2.2.1.1. UV-VIS spectra of Zen

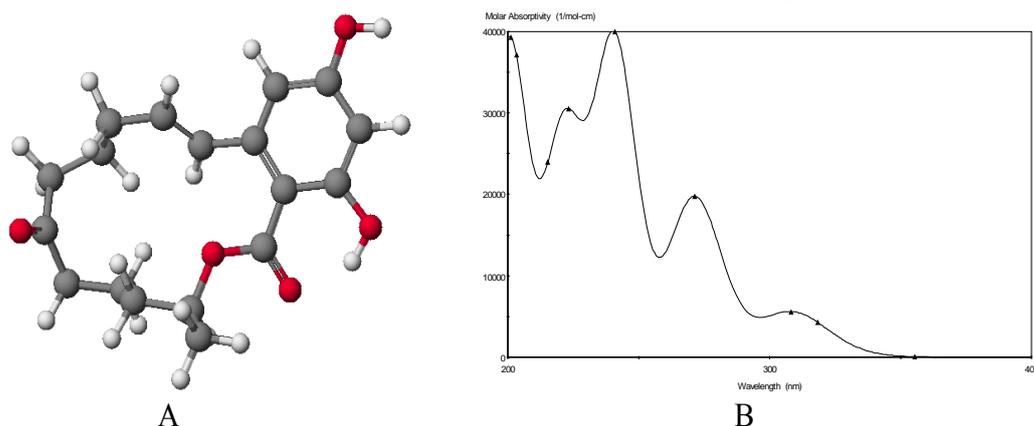
As the chromatograph is provided with a diode array detector that ensures the record of UV-VIS spectrum of each analyte during the separation, both real (Fig. 2.5) and theoretical (Fig. 2.7 B) spectra of Zen were obtained.

Fig. 2.5. The real UV-VIS spectrum of Zen



An UV-VIS spectrometer JASCO V530 provided with SpectraManager software was used to record the spectra. The real spectrum was recorded for a solution of Zen of 10^{-4} M in 5% ethanol-75% water against blank solution 5% ethanol-75% water.

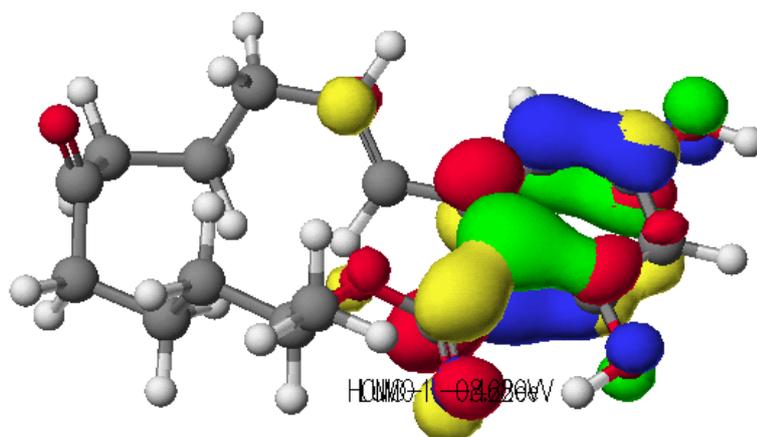
One can observe the existence of three maxima located at λ : 236, 270 and 310 nm, respectively.

Fig. 2.6. Optimised geometry (A) and theoretical UV-VIS spectrum (B) of Zen

The computed spectrum of Zen was obtained using a CAChe WorkSystem Pro version 6.01. The optimize geometry calculation was done with Augmented MM3 and MOPAC PM3. The UV-visible electronic transitions are calculated with ZINDO using INDO/1 parameters after optimizing geometry.

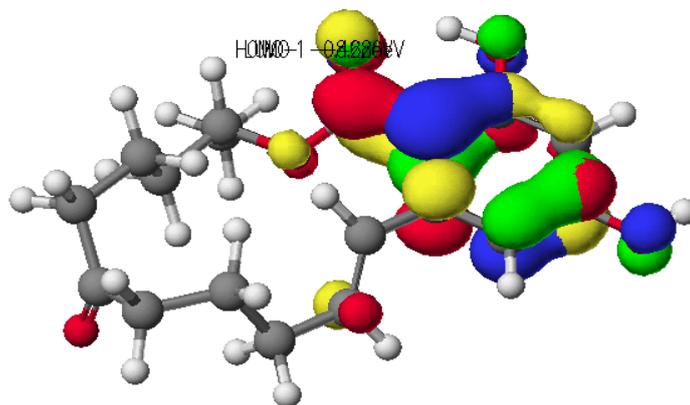
Based on the spectra presented in figures 2.5 and 2.6B one can conclude that there is a good agreement between both theoretical and real spectra of Zen. The extra peak observed at 240 nm in the theoretical spectra was observed as a shoulder in the real spectrum.

The attribution of absorption bands of theoretical spectrum to a structural fragment of the molecules was done as follows: the absorption band with maximum at 308 nm is assigned to the aromatic structural fragment (fig. 2.7); the absorption band with maximum at 272 nm is assigned to the aromatic and carbonyl structural fragment (fig. 2.8); and the absorption band with maximum at $\lambda = 240$ nm is assigned to the aromatic, double bond and carbonyl structural fragments (fig. 2.9).

Fig. 2.7. Structure of Zen with molecular orbitals generating the maximum located at $\lambda = 308$ nm.

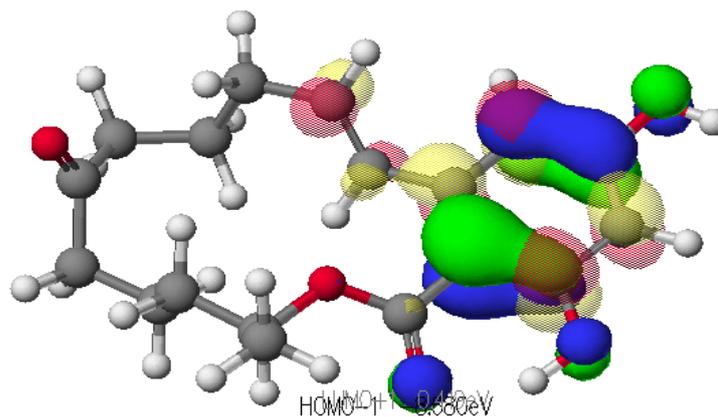
The absorption band having the maximum at $\lambda = 308$ nm was assigned using CAChe software to the aromatic structural fragment; the molecular orbitals were presented as described in the current figure.

Fig.2.8. Structure of Zen with molecular orbitals generating the maximum located at $\lambda = 272$ nm.



The absorption band having the maximum at $\lambda = 272$ nm was assigned using CAChe software to the aromatic and carbonyl structural fragment; the molecular orbitals were presented as described in the current figure.

Fig.2.9. Structure of Zen with molecular orbital's, generating the maximum located at $\lambda = 240$ nm.



The absorption band having the maximum at $\lambda = 240$ nm was assigned using CAChe software to the aromatic, double bond and carbonyl structural fragments; the molecular orbitals were presented as described in the current figure.

2.2.1.2. Studies of oxidation - reduction equilibriums involving Zen and Ce (IV) by HPLC

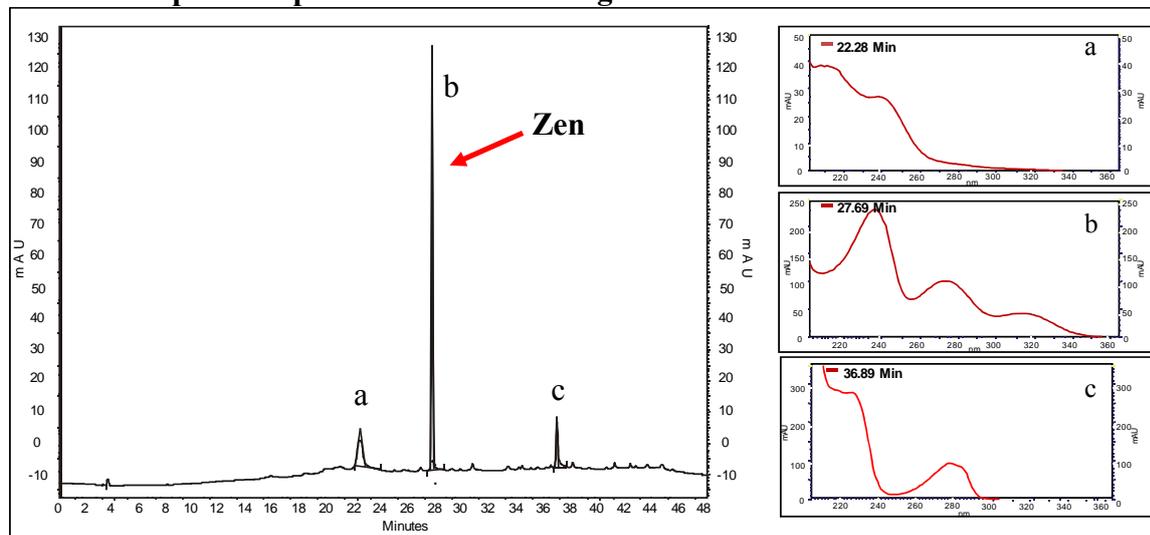
a. Studies performed in mixed solvents acetonitrile - water

In order to get information about the oxidative degradation of Zen, solutions having various ratios between Zen and Ce (IV) were prepared in acetonitrile - water using the general procedure as described in experimental part and then analysed by HPLC.

Fig. 2.10 shows a typical chromatogram of etalon solution of Zen (0.26 mg/mL, 0.8 mM) in acetonitrile, without Ce(IV). One can observe that the chromatogram contains not only Zen (retention time at 27.61 min), but also two compounds characterized by retention

times: 22.03 min and 36.71 min, respectively. The spectra recorded during the elution, for the three compounds are presented in medallions in Fig. 2.10.

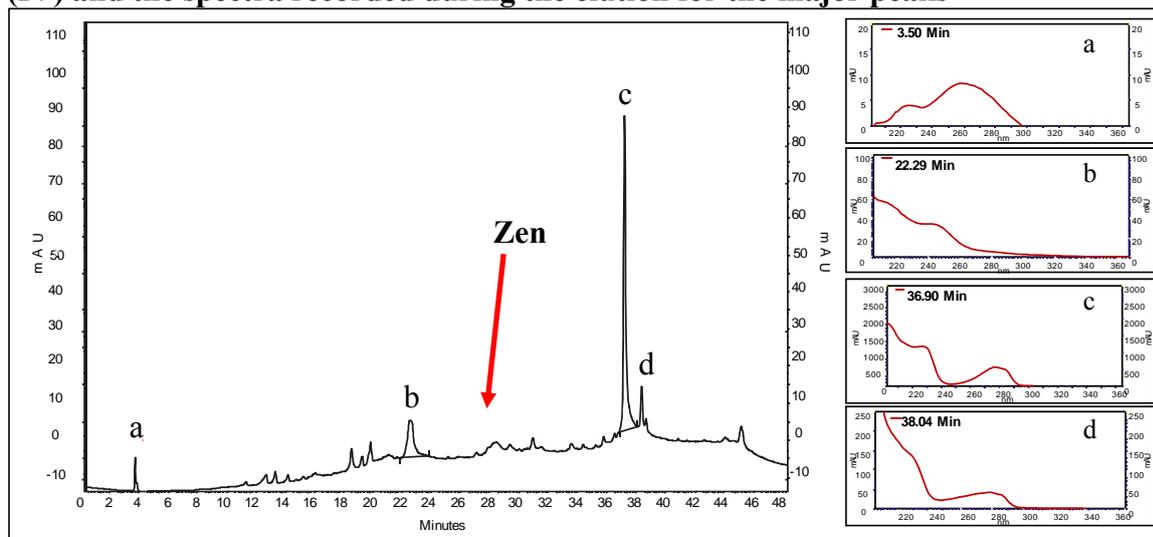
Fig. 2.10. Typical chromatogram of Zen (0.26 mg/mL) solution in acetonitrile - water and the compounds spectra recorded during the elution



The chromatographic study was performed by reverse phase chromatography using the method presented in the material and method part. The observed compounds spectra are: a) a first impurity spectrum; b) zearalenone spectrum; c) second impurity spectrum.

Solutions having a 1 : 3, 1 : 6 and 1 : 10 ratio between Zen and Ce (IV) were studied. The typical chromatograms obtained for a ratio Zen : Ce (IV) = 1 : 10 are presented in Fig. 2.11. This figure presents the spectra recorded during the elution for the major peaks that appear in chromatogram.

Fig. 2.11. Typical chromatogram of a sample prepared with a 1 : 10 ratio Zen: Ce (IV) and the spectra recorded during the elution for the major peaks



The chromatographic study was performed by reverse phase chromatography using the method presented in the material and method part. The observed compounds spectra are: a) new compound spectrum; b) first impurity spectrum – very stable; c) oxidized second impurity spectrum; d) second impurity spectrum – oxidizable.

The compound located at 22.0 min is an impurity of Zen that is very stable to the oxidation. The area of the peak corresponding to this product is the same, no matter what the ratio between Zen and Ce (IV) was. The area of the peak corresponding to the product located at 36.7 min increases when the quantity of Ce (IV) increases, meaning that this impurity is an oxidisable ones. A new compound is formed during the oxidation of Zen, located at 3.5 min.

The results computed based on area of Zen peaks in chromatogram of etalon solution and those obtained for working solutions are presented in Table 2.3.

Table 2.3. Percent of Zen oxidized in aqueous solutions having various ratio Zen : Ce (IV)

Sample No.	Ratio ZEN : Ce (IV)	Oxidized Zen* (%)
1	1:3	100
2	1:6	100
3	1:10	100

*Note: The chromatograms obtained for 1:3, 1:6 and 1:10 ratio ZEN:Ce (IV) did not contain the peak corresponding to ZEN; for this reason one can conclude that the oxidation occurs 100%.

b. Studies performed in aqueous solutions

In order to get information about the oxidative degradation of Zen, solutions having various ratios between Zen and Ce (IV) were prepared in water using the general procedure as described in experimental part and then analysed by HPLC.

In Fig. 2.12 a typical chromatogram of standard solution of Zen (0.024 mg/mL) in water is presented. One can observe that the chromatogram contains not only Zen (retention time at 27.61 min), but also two compounds characterized by retention times: 22.33 min and 36.84 min, respectively. The spectra recorded during the elution, for the three compounds are presented in medallions in Fig. 2.12.

Solutions having a 1 : 3, 1 : 6 and 1 : 10 ratio between Zen and Ce (IV) were studied. The typical chromatograms obtained for a ratio Zen : Ce (IV) = 1 : 10 is presented in Fig. 2.13. This figure presents in inserts the spectra recorded during the elution for the major peaks that appear in chromatogram.

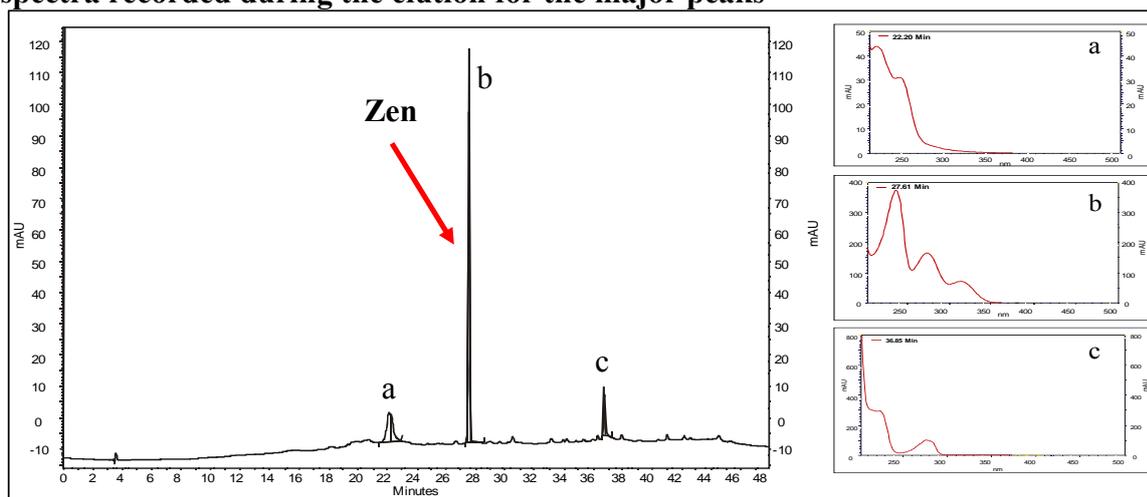
The results computed based on area of Zen peak in chromatogram of etalon solution and those obtained for working solutions are presented in table 2.4.

Table 2.4. Percent of Zen oxidized in aqueous solutions having various ratio Zen:Ce (IV)

Sample No.	Ratio ZEN : Ce	Oxidized ZEN (%)
1	1:1	37.80
2	1:3	94.78
3	1:6	96.34
4	1:10	100.0

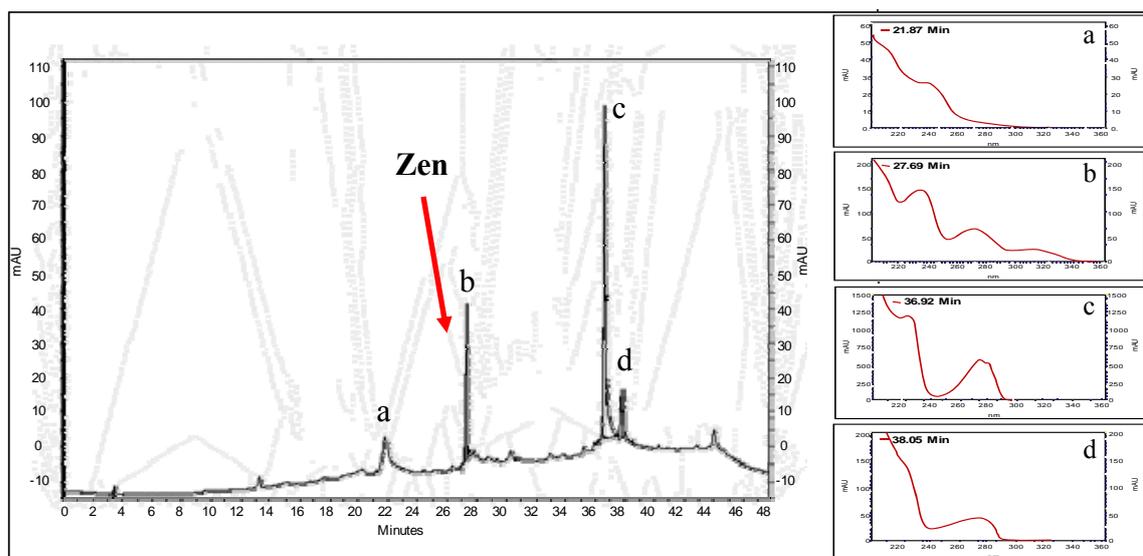
One can conclude that the oxidation of Zen depends on the ratio between the reagents Zen and Ce (IV) in the reaction system. The increase of cerium quantity determines a high degree of ZEN oxidation.

Fig. 2.12. Typical chromatogram of Zen (0.024 mg/mL) aqueous solution and the spectra recorded during the elution for the major peaks



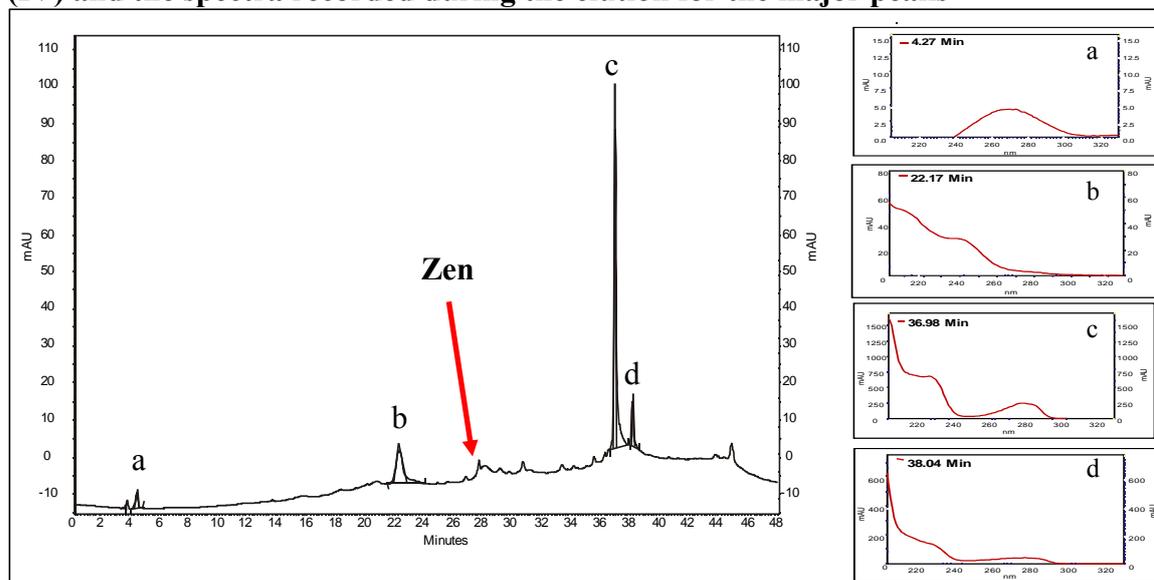
The chromatographic study was performed by reverse phase chromatography using the method presented in the material and method part. The observed compounds spectra are: a) a first impurity spectrum (RT=22.20 min); b) zearalenone spectrum (RT=27.81 min); c) second impurity spectrum (RT=36.86 min).

Fig. 2.13. Typical chromatogram of a sample prepared with a 1 : 6 ratio Zen : Ce (IV) and the spectra recorded during the elution for the major peaks



The chromatographic study was performed by reverse phase chromatography using the method presented in the material and method part. The observed compounds spectra are: a) first impurity spectrum – very stable (RT=21.87 min); b) zearalenone spectrum (RT=27.69 min); c) oxidized second impurity spectrum (RT=36.92 min); d) second impurity spectrum – oxidable (RT=38.06 min).

Fig. 2.14. Typical chromatogram of a sample prepared with a 1 : 10 ratio Zen : Ce (IV) and the spectra recorded during the elution for the major peaks



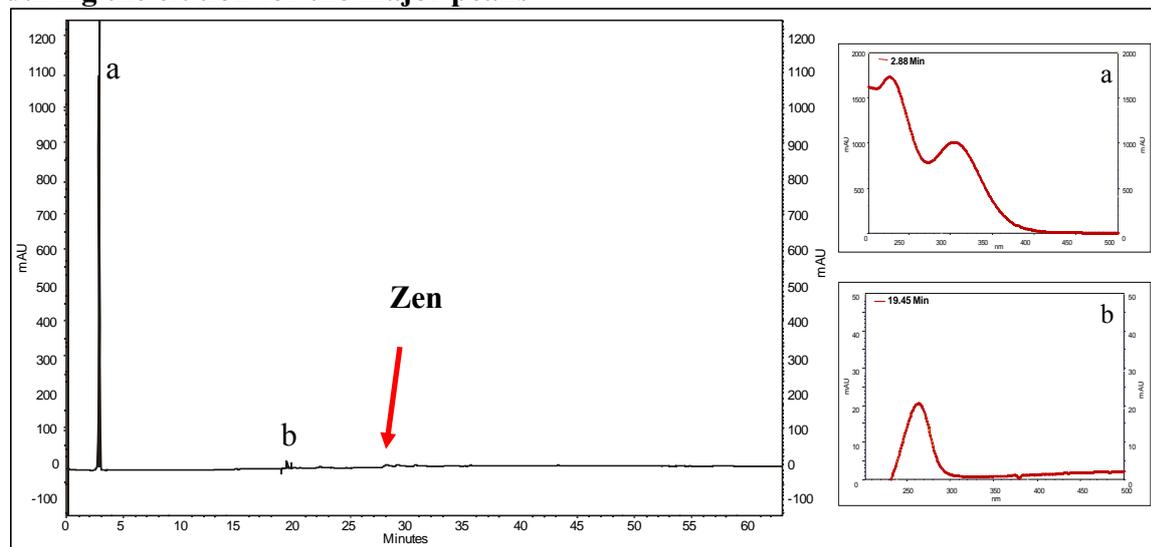
The chromatographic study was performed by reverse phase chromatography using the method presented in the material and method part. The observed compounds spectra are: a) new compound spectrum (RT=4.27 min); b) first impurity spectrum – very stable (RT=22.17 min); c) oxidised second impurity spectrum (RT=36.96 min); d) second impurity spectrum – oxidable (RT=38.04 min).

The UV-VIS spectra of all the oxidised compounds formed in solution containing Zen and Ce(IV) did not contain the absorption band located at $\lambda = 310$ nm in Zen spectrum. Based on the theoretical assignment of absorptive bands in Zen UV-VIS spectrum (Fig. 2.7-2.9) one can conclude that the resorcinol moiety of Zen molecules is not oxidised.

c. Studies performed in time, in mixed solvents acetonitrile - water

A solution of Zen and Ce (IV) prepared in mixed solvents acetonitrile – water containing the reagents in the molar ratio Zen : Ce (IV) = 1: 4 was kept at room temperature for 10 days. The sample was treated as described in the general procedure. The chromatogram recorded for this working solution is presented in Fig. 2.15. It can be observed that there are no peaks as those observed in chromatograms presented in figures 2.11 and 2.13. One can conclude that Zen, both impurities existing in etalon and all the products obtained during oxidation with various concentrations of cerium (IV) are oxidised in time and in the presence of an excess of Ce (IV).

Fig. 2.15. The chromatogram recorded for the working solution of Zen oxidized by Ce (IV) in time (10 days time contact of the reagents) and the spectra recorded during the elution for the major peaks



The chromatographic study was performed by reverse phase chromatography using the method presented in the material and method part. The observed compounds spectra are: a) new compound spectrum (RT=2.88 min); b) first impurity spectrum – very stable (RT=19.45 min).

Conclusions

The oxidation-reduction equilibriums involving Zen and Ce (IV) were studied both, in aqueous and in mixed solvents water – acetonitrile solutions containing 1 M H₂SO₄. Real and theoretical UV spectra of Zen were obtained. The chromatograms of the etalon solutions of Zen prepared in water and in mixed solvents water – acetonitrile contain not only Zen (retention time at 27.61 min but also two compounds characterized by retention times: 22.33 min and 36.84 min, respectively).

In order to get information about the oxidative degradation of Zen, solutions having various ratios between Zen and Ce (IV) were prepared in acetonitrile – water, as well as in water and the products of reactions were analysed by HPLC. Solutions having a 1:3, 1:6 and 1:10 ratio between Zen and Ce (IV) were studied.

The chromatograms obtained for the samples containing Zen and Ce (IV) in a ratio greater than 1:3 show 3 compounds located at: 22.03 min, 36.71 min and 3.50 min, respectively. The compound located at 22.03 min is an impurity of Zen that is very stable to oxidation. The area of the peak corresponding to this product is the same, no matter what the ratio between Zen and Ce (IV) was. The area of the peak corresponding to the product located at 36.71 min increased when the quantity of Ce (IV) increased, meaning that this impurity is an oxidisable one. A new compound is formed during the oxidation of Zen, located at 3.50 min.

One can conclude that the oxidation of Zen depends on the ratio between the reagents Zen and Ce (IV) in the reaction system. The increase of cerium quantity determines a high degree of Zen oxidation.

The UV-VIS spectra of all the oxidised compounds formed in solution containing Zen and Ce (IV) did not contain the absorption band located at $\lambda = 310$ nm in Zen spectrum. Based on the theoretical assignment of the absorptive bands in Zen UV-VIS spectrum, we can conclude that the resorcinol moiety of Zen molecules is not oxidised.

Zen, both impurities existing in the etalon and all the products obtained during oxidation with various concentrations of cerium (IV) are oxidised in time and in the presence of an excess of Ce (IV).

In order to confirm these results a MS determination of the observed oxidation compound, as well as of the observed impurities and their oxidation derivatives, will be done in the future in order to complete the presented primary researches.

2.2.2. Voltammetric behaviour of zearalenone

The nature of the electrochemical behaviour of Zen was investigated by cyclic voltammetry (CV). Its electrochemical behaviour was used to develop a rapid and simple method for Zen determination by differential pulse voltammetry (DPV).

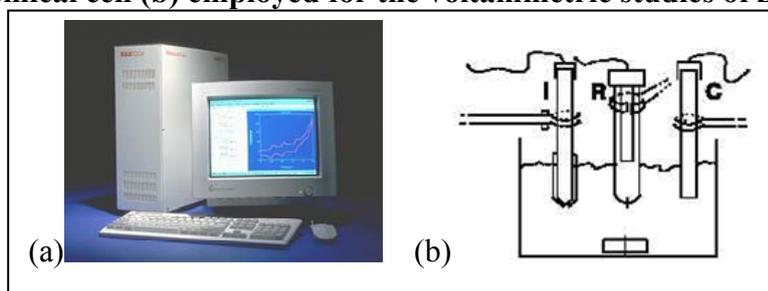
Material and Methods

Instrumentation

All voltammetric measurements were carried out on a BAS CV-50 W electrochemical analyser with a classical three-electrode cells comprising a glassy carbon electrode (3 mm diameter) (GCE) as working electrode (I), a Ag/AgCl (3 M KCl) as reference electrode (R) and a Pt wire as auxiliary electrode (C) (Fig. 2.16).

In order to provide a reproducible active surface and to improve the sensitivity and resolution of the voltammetric peaks, the glassy carbon electrode was polished to a mirror finish with 0.3 micron alumina on a smooth polishing cloth and then rinsed with double distilled water prior to each electrochemical measurement.

Fig.2.16. The BAS electrochemical system (a) and the schematic representation of the electrochemical cell (b) employed for the voltammetric studies of zearalenone.



Reagents and materials

A stock solution of $1 \cdot 10^{-2} \text{ mol} \cdot \text{L}^{-1}$ zearalenone (Zen) was prepared by dissolving the required amount of zearalonone in dimethylsulfoxide (DMSO) (Merck) containing $0.1 \text{ mol} \cdot \text{L}^{-1}$ tetrabutylammoniu perchlorate (TBAP) (Fluka) as supporting electrolyte. Dilute working standard solutions of $1 \cdot 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ and $1 \cdot 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ Zen were then prepared by appropriate successive dilutions of the stock solution with DMSO containing $0.1 \text{ mol} \cdot \text{L}^{-1}$ TBAP. All chemicals were of analytical grade and were used without further purification.

Procedure

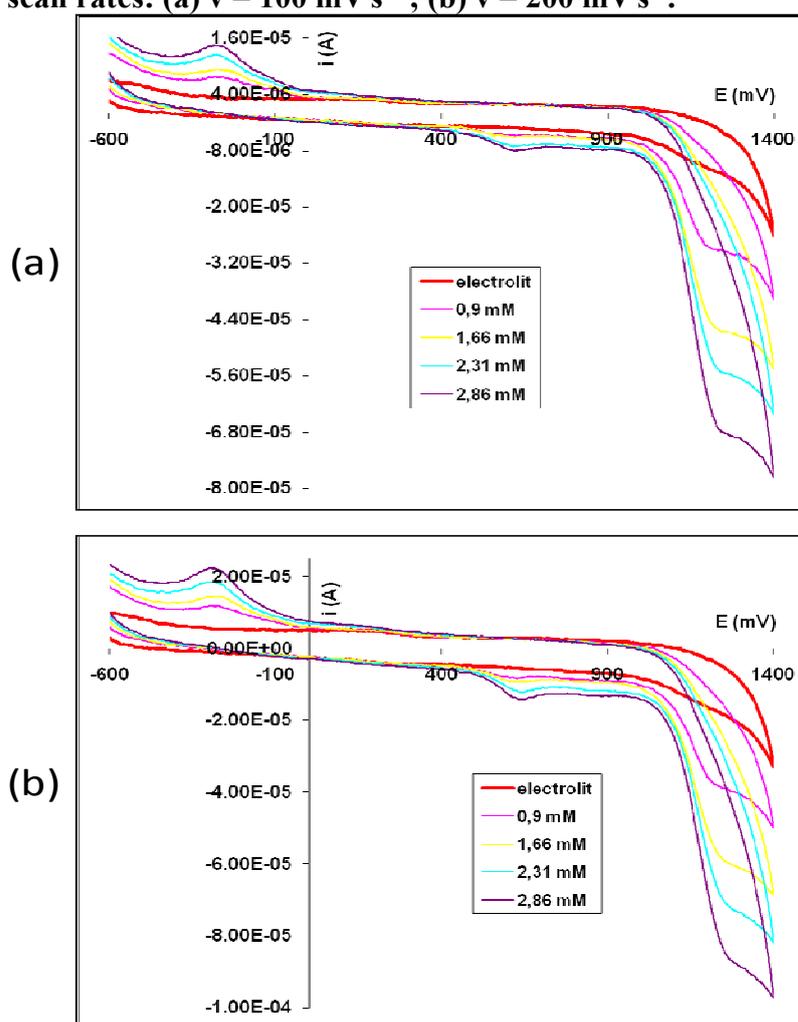
Cyclic (CV) and differential pulse voltammograms (DPV) were recorded for solutions with different concentrations (in the range $9 \cdot 10^{-4} - 2.9 \cdot 10^{-3} \text{ mol} \cdot \text{L}^{-1}$) of Zen in DMSO containing $0.1 \text{ mol} \cdot \text{L}^{-1}$ TBAP. These solutions were obtained by adding in the

voltammetric cell, of appropriate, exactly measured volumes of the working standard ($1 \cdot 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ or $1 \cdot 10^{-4} \text{ mol}\cdot\text{L}^{-1}$ Zen) or stock solution ($1 \cdot 10^{-2} \text{ mol}\cdot\text{L}^{-1}$ Zen) to 10 mL supporting electrolyte solution ($0.1 \text{ mol}\cdot\text{L}^{-1}$ TBAP in DMSO). The volume correction was made every time. Cyclic voltammograms at different scan rates (from 5 to $500 \text{ mV}\cdot\text{s}^{-1}$) were obtained by scanning the potential in the range -600 to 1400 mV. Differential pulse voltammograms were recorded from 850 to 1200 mV. Optimum DPV conditions used were: pulse amplitude, 150 mV; pulse width, 21 ms and scan rate, $25 \text{ mV}\cdot\text{s}^{-1}$.

2.2.2.1. Voltammetric behaviour of Zen

In order to obtain qualitative information about the electrochemical behaviour of Zen cyclic voltammograms were recorded on a GCE, in the potential range -600 to -1400 mV (vs the Ag/AgCl 3 M reference electrode).

Fig. 2.17. Variation of the maximum peak currents in cyclic voltammograms recorded at GCE for different Zen concentrations in DMSO containing $0.1 \text{ mol}\cdot\text{L}^{-1}$ PTBA, at two scan rates: (a) $v = 100 \text{ mV}\cdot\text{s}^{-1}$; (b) $v = 200 \text{ mV}\cdot\text{s}^{-1}$.



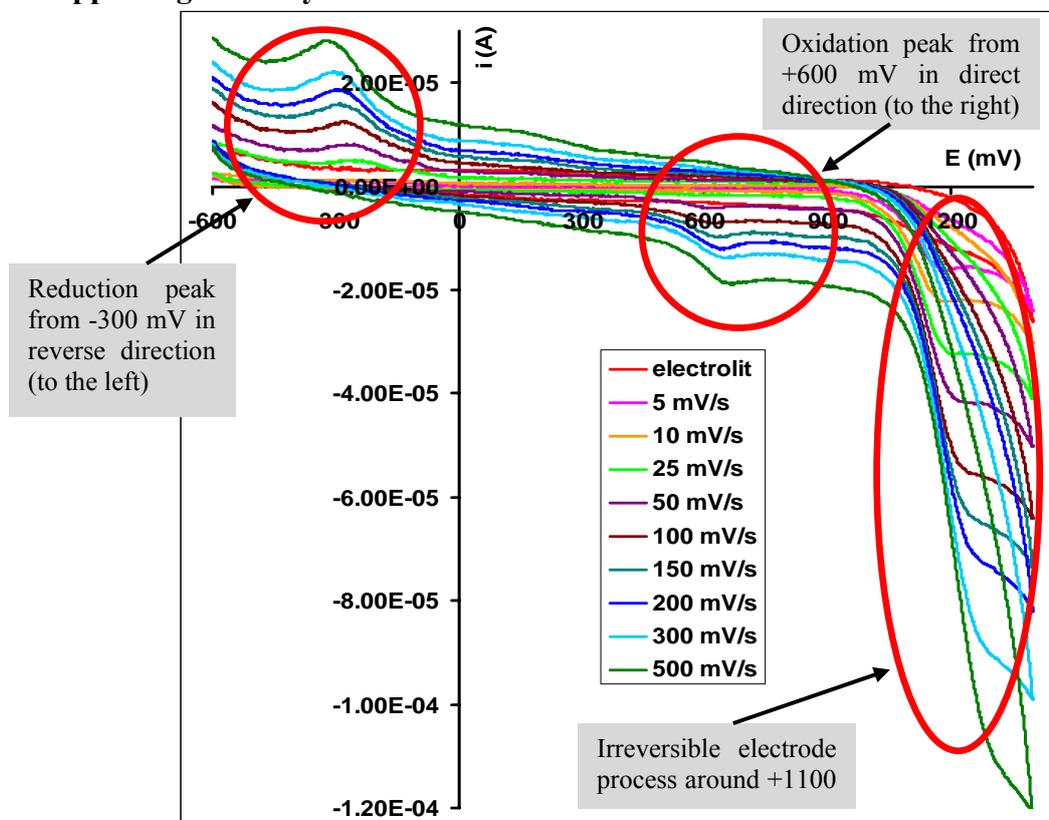
Cyclic voltammograms of zearalenone recorded on GCE in DMSO using $0.1 \text{ mol}\cdot\text{L}^{-1}$ TBAP as supporting electrolyte showed two oxidation peaks and a reduction one.

The height of the three peaks observed in Zen cyclic voltammograms (Fig. 2.17) increases with each addition of analyte solution indicating that these signals are due to the investigated compound.

Scan rate influence on the cyclic voltammetric behaviour of Zen

In order to establish the nature of the processes occurring at the electrode surface, the influence of the scan rate on the electrochemical behaviour of Zen was investigated by cyclic voltammetry (figure 2.18). The linear dependence of the maximum peak current on the square root of the scan rate, for both the reduction peak situated at about -300 mV and for the oxidation one with peak potentials around 600 mV , indicates the existence of Faradaic diffusion controlled processes.

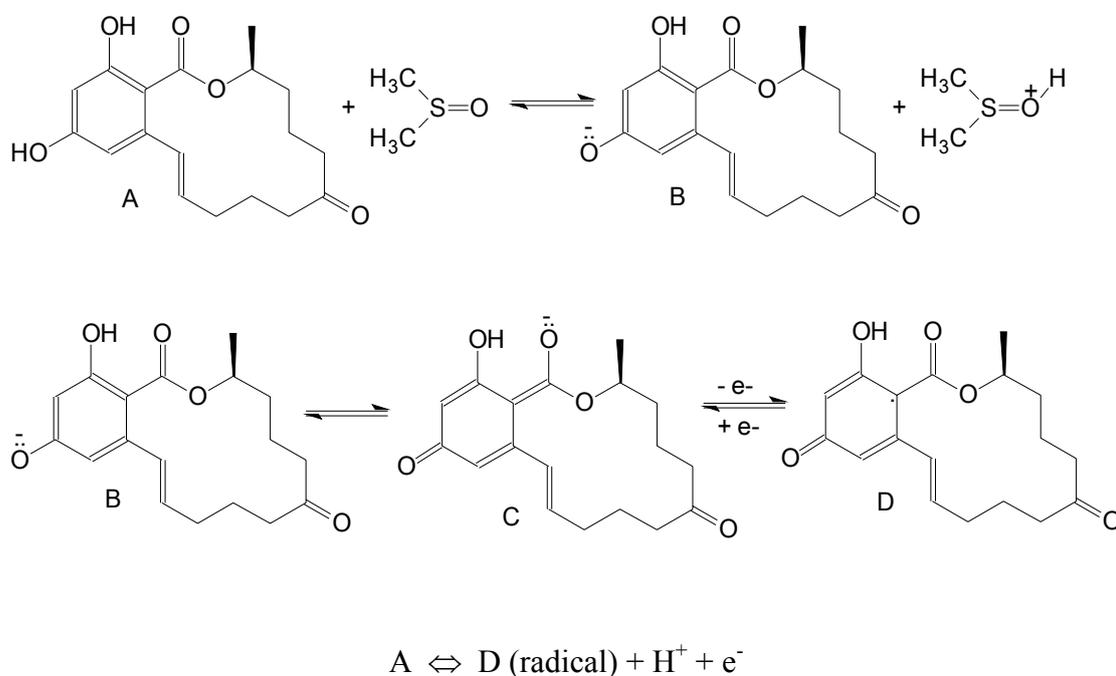
Fig. 2.18. Influence of the scan rate on the cyclic voltammograms recorded at GCE for a solution of $2.31\cdot 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ Zen in DMSO containing $0.1 \text{ mol}\cdot\text{L}^{-1}$ TBAP as supporting electrolyte



Determination of resorcinol on glassy carbon electrode modified by carbon nanotube and its application in wastewater analysis

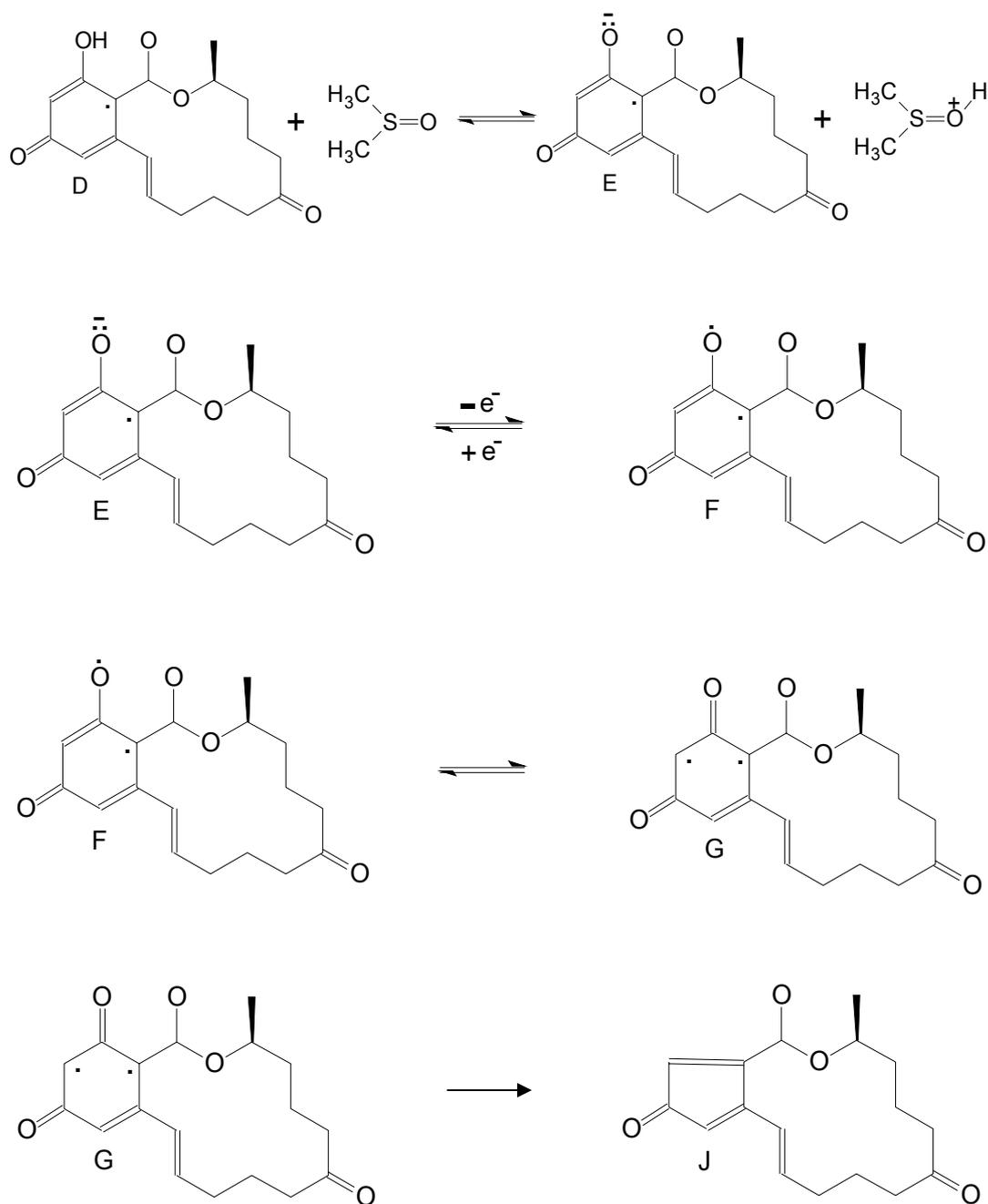
Correlating with literature data (Shuxuan et al., 2009) the oxidation peak situated at about 600 mV can be attributed to the oxidation of the resorcinol structural unit from the investigated mycotoxin molecule. The large separation between the potential of this peak and that of the reduction peak from -300 mV suggests the existence of a quasi-irreversible electrode process (figure 2.19).

Fig. 2.19. Proposed mechanism for the existence of the oxidation peak from +600 mV in the direct direction (to the right) and of the reduction one from -300 mV in reverse direction (to the left) (in correlation with Shuxuan et al., 2009)



The peak situated in the CV at very positive potentials (around +1100 mV) becomes less defined with increasing scan rates indicating slow electrode processes whereas the absence of the corresponding reduction peak is the proof of an irreversible electrode process which could be attributed to an oxidative cleavage with the irreversible lost of a small molecule (CO) according to figure 2.20.

Fig. 2.20. Proposed reaction mechanism explaining the appearance of the oxidation peak from + 1100 mV



2.2.2.2. Possibilities of voltammetric quantitative determination of Zen

The literature has few data regarding the quantitative determination of Zen using voltammetric methods. Andres et al. (2008) employed the electrochemical detection at +850 mV at a carbon nanotube glassy carbon modified electrode after a previous clean-up and pre-concentration on C₁₈ adsorption cartridges of Zen and its metabolites from urinary samples followed by liquid chromatographic separation. Ramirez et al. (2005) reported the quantification of this mycotoxin from maize samples by the square wave voltammetric (SVW) determination of Zen adsorbed on a glassy carbon electrode.

The present research describes a rapid and simple method for Zen determination by differential pulse voltammetry (DPV).

From the three peaks observed in the CV of Zen, the signal from +1100 mV was selected for the quantitative determination by DPV because it was the most sensitive peak (its height had the highest variation with Zen concentration).

In order to perform the most sensitive DPV determinations of Zen, the operational parameters of the method were optimised and were as follows: scan rate: $v = 25 \text{ mV s}^{-1}$, pulse amplitude: $\Delta E = 150 \text{ mV}$, pulse period: $PP = 200 \text{ msec}$, pulse width: $PW = 21 \text{ msec}$, sample width: $SW = 18 \text{ msec}$.

These optimised conditions were further used to investigate the influence of Zen concentration on the DPV maximum peak current. As it can be seen from figures 2.21 and 2.22, the peak height varies linearly with the analyte concentration, presenting different slopes in two concentration ranges.

Fig. 2.21. The influence of Zen concentration ($9.2 \cdot 10^{-5} - 5.1 \cdot 10^{-4} \text{ mol} \cdot \text{L}^{-1}$) on the DPV maximum peak current recorded at a GCE in DMSO containing $0.1 \text{ mol} \cdot \text{L}^{-1}$ TBAP ($v = 25 \text{ mV s}^{-1}$, $\Delta E = 150 \text{ mV}$, $PP = 200 \text{ msec}$, $PW = 21 \text{ msec}$, $SW = 18 \text{ msec}$).

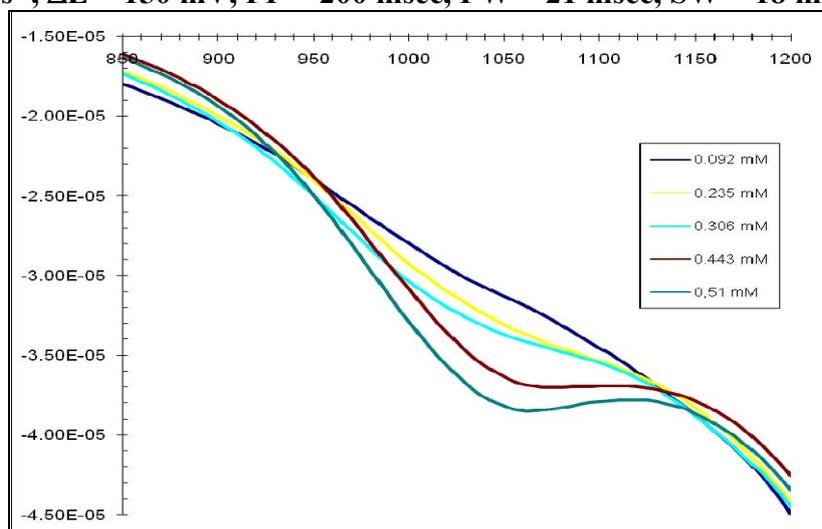
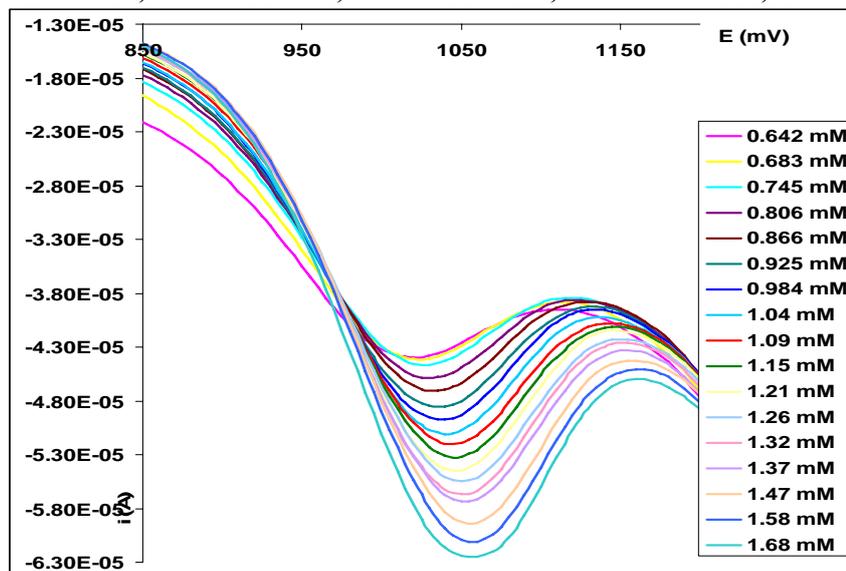


Fig. 2.22. The influence of Zen concentration ($5.77 \cdot 10^{-4} - 1.68 \cdot 10^{-3} \text{ mol L}^{-1}$) on the DPV maximum peak current recorded at a GCE in DMSO containing $0.1 \text{ mol}\cdot\text{L}^{-1}$ TBAP ($v = 25 \text{ mV s}^{-1}$, $\Delta E = 150 \text{ mV}$, $PP = 200 \text{ msec}$, $PW = 21 \text{ msec}$, $SW = 18 \text{ msec}$).



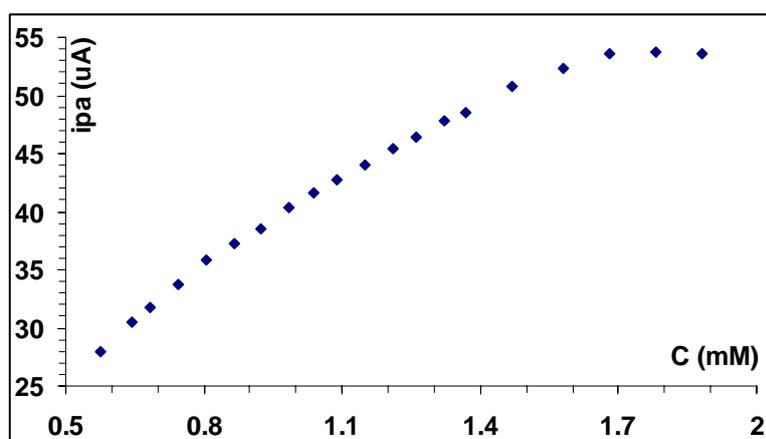
The equation of the calibration curve established from the experimental data for the concentration range $9.2 \cdot 10^{-5} - 5.1 \cdot 10^{-4} \text{ mol}\cdot\text{L}^{-1}$ is:

$$I_{pa} = 71.179C - 4.322 ; \quad r = 0.9986,$$

where I_{pa} is the maximum anodic peak current and r is the correlation coefficient.

In the concentration range $5.77 \cdot 10^{-4}$ and $1.68 \cdot 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ the maximum peak intensity varies linearly with concentration up to $1.48 \cdot 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ there upon the dependence is flattening (Fig. 2.23).

Fig. 2.23. DPV maximum peak currents variation with Zen concentration in the range $5.8 \cdot 10^{-4} - 1.9 \cdot 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ (experimental conditions as in Fig. 2.22)



The regression curve for the concentration range $5.77 \cdot 10^{-4}$ - $1.48 \cdot 10^{-3}$ mol·L⁻¹ Zen is:

$$I_{pa} = 25.4C + 14.455 \quad r = 0.9926$$

Applications

The developed method was applied to the Zen determination in corn, barley and oat samples using both the calibration curve and the standard addition method. The corn, barley and maize samples were prepared as described by Silva and Vargas, in 2001.

No Zen was detected in the maize and corn samples whereas the following concentrations were obtained for barley:

- by the calibration curve method: $6.78 \cdot 10^{-4}$ mol·L⁻¹,
 $7.05 \cdot 10^{-4}$ mol·L⁻¹;
- by the standard addition method: $6.44 \cdot 10^{-4}$ mol·L⁻¹,
 $5.79 \cdot 10^{-4}$ mol·L⁻¹.

Conclusions

The electrochemical behaviour of Zen was studied by recording cyclic voltammograms (CV) at a glassy carbon electrode (GCE) in the potential range -600 to 1400 mV (vs. the Ag/AgCl 3 M reference electrode).

The CV of zearalenone, recorded at GCE in DMSO in the presence of 0.1 mol·L⁻¹ TBAP supporting electrolyte, showed two oxidation peaks and a reduction one. The height of the three peaks from the CV of Zen increased with each addition of analyte solution indicating that these signals are due to the investigated compound.

The existence of Faradayic diffusion controlled processes was indicated by the linear dependence of the maximum peak current on the scan rate square root, for both the reduction peak situated around -300 mV and the oxidation peak with potentials about +600 mV. The conclusions regarding the proposal of redox mechanisms are under completion.

In the present work a simple and rapid method was developed for Zen determination by differential pulse voltammetry (DPV) employing the oxidation signal situated at potentials around +1100 mV, because its height showed the highest variation with the analyte concentration. Based on the experimental results the optimum operational parameters of the method were established to be: scan rate, $v = 25$ mV·s⁻¹, pulse amplitude, $\Delta E = 150$ mV, pulse period, PP = 200 msec, pulse width, PW = 21 msec and

sample width, SW = 18 msec. The peak height varied linearly with the analyte concentration having different slopes in two concentration ranges:

- in the concentration range $9.2 \cdot 10^{-5} - 5.1 \cdot 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ the equation of the calibration curve was: $I_{pa} = 71.179C - 4.322$ $r = 0,9986$

- in the concentration range $5.77 \cdot 10^{-4} - 1.68 \cdot 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ the maximum peak intensity varied linearly with concentration up to $1.48 \cdot 10^{-3} \text{ mol} \cdot \text{L}^{-1}$ there upon the dependence was flattening; the equation of the calibration curve was: $I_{pa} = 25.4C + 14.455$; $r = 0.9926$.

The developed method was applied with good results to the determination of Zen concentration in corn, barley and maize. Nevertheless, this method lack of sensibility and specificity, being more appropriate for fundamental chemistry research concerning the oxidation-reduction equilibrium and for the preparation of the oxidation/reduction compounds of Zearalenone.

2.3. LC-MS assessment of Zen by using ^{13}C enriched Zearalenone as internal standard

The use of compounds enriched with different stable isotopes (^{13}C , ^{18}O , ^{15}N , ^2D etc) for quantitative or semi-structural (excepting the stereochemistry resolution) studies aroused a particular interest in mass spectrometry. While the mass spectrum of the natural compound is formed as two or three mass peaks from the natural isotopes of the most abundant, uniform enrichment of stable nuclei allows its replacement by an isotopic cluster. The isotopic cluster is composed of five to ten peaks or even more, which is a real signature of the enriched compound. This is a remarkable advantage to easily distinguish the product of interest in a mixture, containing a large number of unknown compounds.

Other advantages of using enriched internal standards are due to the physical and chemical proprieties almost identical of the enriched standard and the analyte. Only mass spectrometry enables the differentiation between isotopologues¹ and, knowing the quantity of internal standard, the content of the analyte can be calculated. In contrast, a structurally different internal standard (which is still widely used) have distinct physical and chemical properties and, therefore may be the cause of errors or inaccuracies. The loss of analyte during different stages of purification and / or extraction are completely compensate by identical losses of the same isotopologues; while structurally different

¹ Isotopologues are molecules that differ only in their isotopic composition. Simply, the isotopologue of a chemical species has at least one atom with a different number of neutrons to the parent.

internal standard may not be extracted and / or purified to the same level and therefore be less appropriate. It is also important that the internal standard and the analyte be mixed properly before any stage of extraction and purification.

In addition to the information from the mass spectrometric analysis of the analyte, the internal standard must have a retention time and, more largely, chromatographic properties similar to those of the analyte (polarity, stability at temperature and under certain conditions, analytical performance of ionization for LC-MS coupling, etc.). Therefore, the chromatogram of an internal standard co-eluent with the analyte, ensure correct attribution of mass spectrum of the analyte, even if the analyte is at traces levels. Our team has published several articles concerning the production and use of mycotoxins uniformly enriched with stable isotopes for their dosage in biological samples (Bravin et al., 2007, 2008; see annex 1).

2.3.1. Mass spectrometry - brief reminder

Mass spectrometry (MS) is a physical analysis to detect and identify molecules of interest by measuring their mono-isotopic mass. In addition, it helps to clarify the chemical structure of molecules by fragmentation. Its principle lies in the gas phase separation of charged molecules (ions) according to their mass/charge (m/z) ratio.

This analytical technique is one of the most sensitive, currently is used for the study of biological or chemical samples. Indeed, it allows both the identification but also the semi-structural analysis (outside configurations) of unknown compounds, because the ions can be fragmented, cut into smaller entities. The fragmentations respect specific laws of gas phase chemistry; the study of these fragments allows determining a part of the structure of these ions. Due to a linear range from 3 to 7 orders of magnitude, mass spectrometry offers the possibility of a quantitative analysis on a wide scale.

Until now, the identification could be done by comparing a mass spectrum with banks spectra characteristic of known molecules. More recently, when using high-resolution analyzers (TOF, magnetic sector, FT-ICR, Orbitrap) mass spectrometry allows to accurately measure the exact mass of mono-isotopic ion and to deduce its molecular formula.

In most cases, the studied compounds are natural compounds, e.g. having a natural isotopic cluster formed by few peaks. In fact, each atom has, by definition, one or more isotopes of different masses. Indeed, the proportion of each isotope found in a mass spectrum is the natural characteristic of the presence of certain atoms and their number in

the analyzed ion (especially Cl, Br, which are natural isotopes M and M +2 significant amounts). Other structural rules, as the rule of the nitrogen (the parity of the mass is measured according to the ratio of the number of atoms that has a nitrogen molecule) allows, from the precise study of the spectrum peaks distribution of a natural compound, to determine its formula.

2.3.2. Zearalenone determination in different matrices

The main objective of this study focused on the use of ^{13}C enriched Zen as internal standard, in order to measure the natural Zen contained in different types of biological samples, ranging from extracts of fungi cultures on ^{13}C enriched cereals, to the plasma or urine of Zen treated rats. Initially we had in the laboratory only 10%- ^{13}C Zen, but furthermore we were able to use, also, the 39% - 81%- ^{13}C Zen.

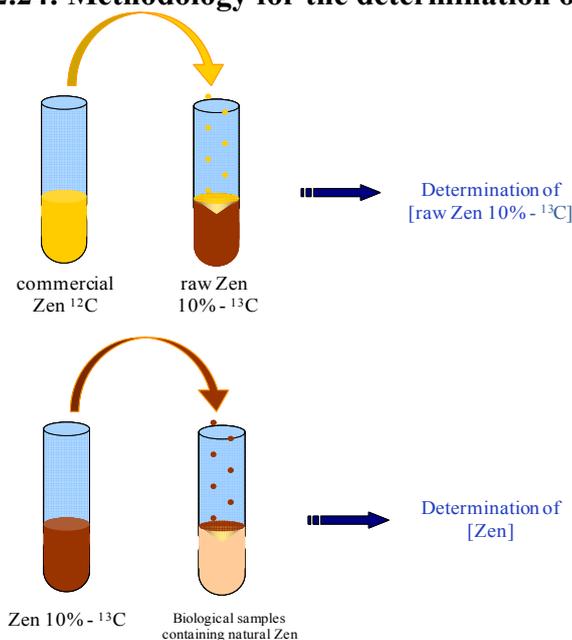
Frederique Bravin within her Ph-D thesis had as one of the main research directions: the use of uniform ^{13}C isotopical labelling within toxicological and metabolic studies. The next method had been developed and validated (for more details see Frederique Bravin's Ph-D thesis) and we have used it on the determination of zearalenone within rat and chicken biological samples (plasma, liver, urine).

2.3.2.1 Determination of Zen in extracts of cereal

The secondary objective here was to determine the concentration of Zen ^{13}C in extracts of cultures of *Fusarium culmorum* grown on grain wheat enriched with 10% of ^{13}C (strain G5G7). Such extracts will be called thereafter "raw extracts" because they contain, in addition to the Zen, a mixture of other unidentified compounds some of which might be mycotoxins.

The determination of these levels will allow us to use these solutions as internal standards for the determination of natural Zen content within biological samples (figure 2.24).

Fig. 2.24: Methodology for the determination of Zen.



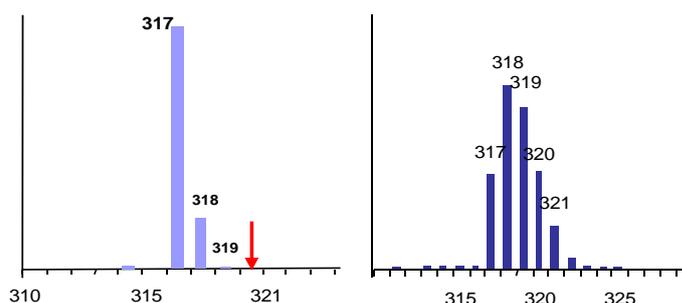
As a first step, ¹³C Zen solutions are measured using commercial ¹²C Zen as internal standard. In a second step, the solutions of ¹³C Zen are used as internal standards for the determination of natural Zen content within biological samples.

Methodology

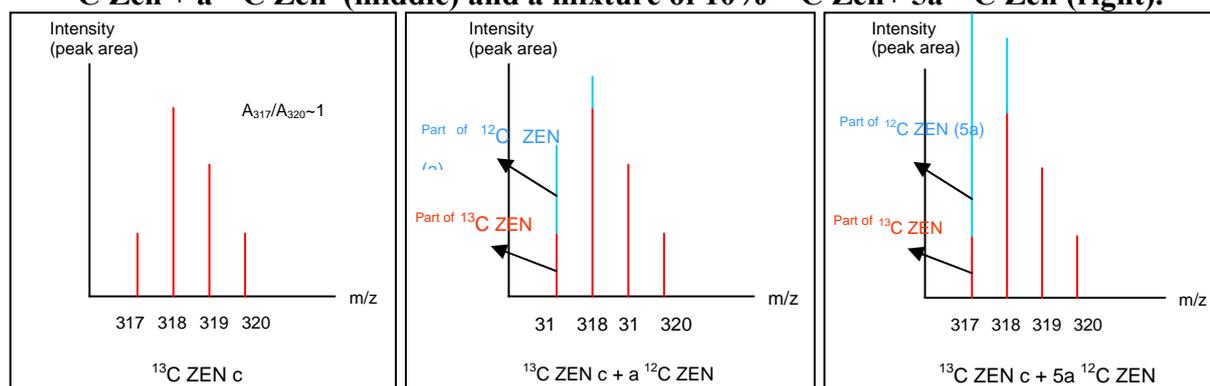
To begin, we used a solution of commercial ¹²C Zen as internal standard to calculate the unknown concentration of ¹³C Zen within the raw extracts (concentration “c”). To do this, we recorded the spectra of the solution of unknown concentration “c”, and a mixture of internal standard (10⁻⁴ M) and enriched solution (c).

The method of quantification of such additions has permitted access to the concentration of Zen in the extracts. A quantity “a” (0.02 nmol) and “5a” (0.1 nmol) of Zen ¹²C were added in each raw extract containing the ¹³C Zen. Analyzes were made by mass spectrometry to exploit the isotopical cluster.

The raw extract solution containing 10%-¹³C enriched Zen, the mass spectra of the ¹²C internal standard (IS) and of the ¹³C Zen (the analyte, here) are overlapping (figure 2.25, right). To determine what part is due to the standard and that due to the analyte, we used the intensity of the peak at m/z 320 which is null for the IS and non- null for the analyte. This peak intensity is the same in the spectrum of the mixture and in the ¹³C Zen alone. Thus the ratio of intensities I_{317(13C)} and I_{320(13C)} must be the same in both spectra.

Fig. 2.25. Experimental mass spectra of ^{12}C Zen (left) and the 10%- ^{13}C Zen (right).


In the mixture ^{12}C and ^{13}C spectrum, the peak at m/z 317 due to the ^{13}C enriched Zen is determined using the peak at m/z 320 (due only to ^{13}C) and the ratio of intensities of I_{317}/I_{320} spectrum of Zen enriched in ^{13}C . Indeed, this ratio is constant for a given concentration of ^{13}C . The share due to the ^{12}C Zen is deduced by subtraction (blue in figure 2.26).

Fig. 2.26. Simulations of the mass spectrum of 10%- ^{13}C Zen (left), a mixture of 10%- ^{13}C Zen + a ^{12}C Zen (middle) and a mixture of 10%- ^{13}C Zen + 5a ^{12}C Zen (right).


The red spectra correspond to the part of spectra linked to 10%- ^{13}C Zen. As for the blue part at m/z 317 and m/z 318, corresponds to the natural Zen contribution in the spectra of the natural Zen and 10%- ^{13}C Zen mixture.

Normally the peak area at m/z 320 is constant with or without the addition of ^{12}C Zen. If this is not the case, it is necessary to normalize the other peaks areas by the peak area at m/z 320.

Thus, the following calculations provide access to the concentration of Zen ^{13}C :

$$\text{Total area}^2 (\text{ZEN } ^{13}\text{C} + a \text{ZEN } ^{12}\text{C}) - \text{Total area}^3 (\text{ZEN } ^{13}\text{C}) = ^{12}\text{C ZEN normalized area}$$

$$^{12}\text{C ZEN normalized area} \leftrightarrow a$$

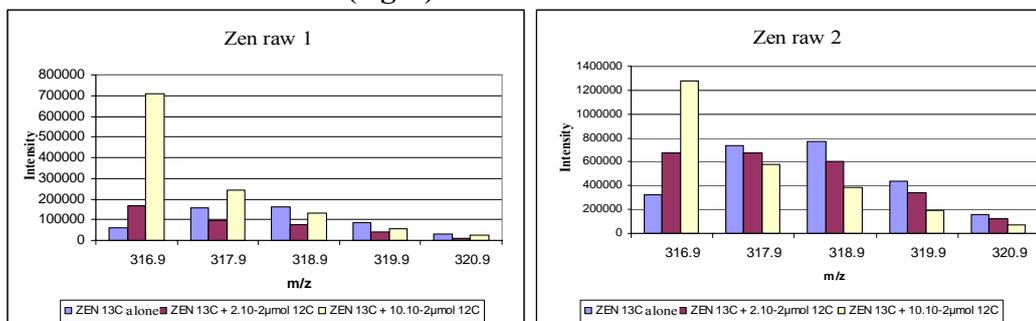
$$^{13}\text{C Zen c area} \leftrightarrow c$$

² normalized on the 320 peak area of ZEN ^{13}C
³ normalized on the 320 peak area of ZEN ^{13}C

Results and discussions

Two samples of 10%-¹³C Zen were measured using the standard additions technique previously detailed: the raw extracts were noted as "raw 1" and "raw 2".

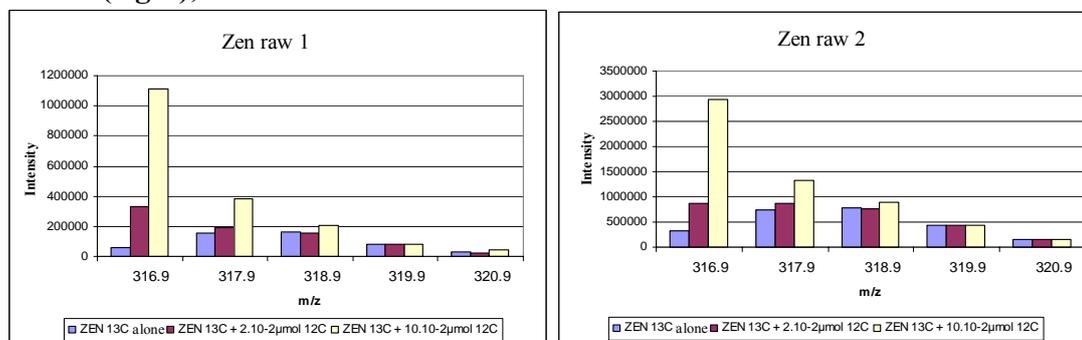
Fig. 2.27: Mass spectra of ZEN 10% ¹³C-1 with gross adding ZEN ¹²C (left) and without addition of ZEN ¹²C (right).



The spectrum of each raw extract was performed (in blue) and the spectrum of the mixture of the raw extract with an addition of 0.02 µM ¹²C Zen (red) and the spectrum of the mixture of the raw extract with an addition of 0.1 µM ¹²C Zen (yellow).

It was found preferable to normalize these spectra on the peak at m/z 320. Indeed, whatever the amount of ¹²C Zen added, the intensity of the peak at m/z 320 does not vary because the contribution of ¹²C ZEN peak m/z 320 is negligible (fig. 2.26).

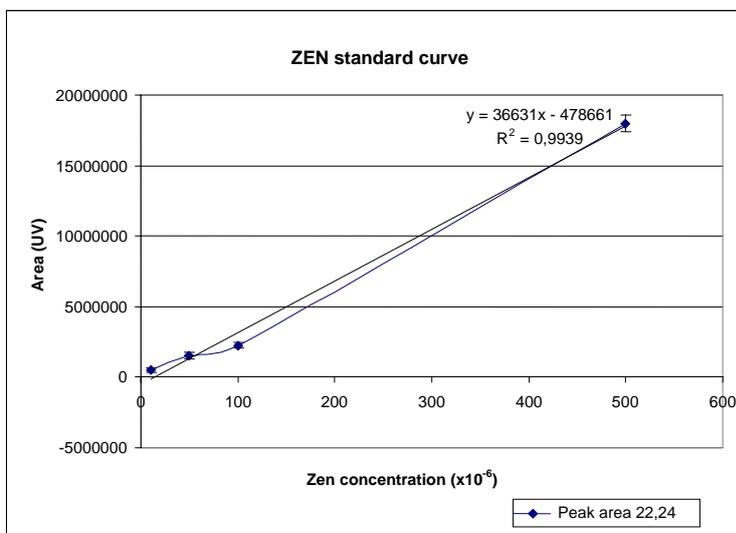
Fig. 2.28: Normalized mass spectra of the 10%-¹³C Zen, raw extract 1 (left) and raw extract 2 (right), with and without ¹²C Zen added.



The spectrum of each raw extract was performed (in blue). The spectrum of the raw extract with an addition of 0.02 µM ¹²C Zen (red) and the spectrum of the raw extract with an addition of 0.1 µM ¹²C Zen (yellow) have been normalized by adjusting the m/z 320 peak intensity on the m/z 320 peak intensity of 10%-¹³C enriched Zen spectrum.

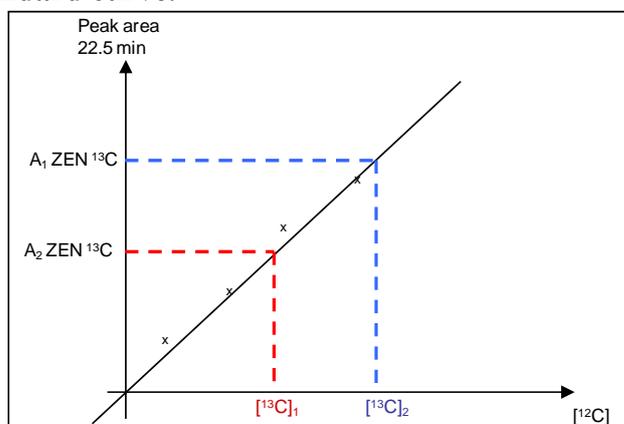
With a range of concentrations of ¹²C Zen (10 µM; 50 µM; 100 µM; 500 µM), we obtained the calibration curve of the area of the UV signal as a function of concentration (fig. 2.29).

Fig. 2.29: Standard curve of commercial ^{12}C Zen. The extended area is from UV absorbance signal at 280 nm.



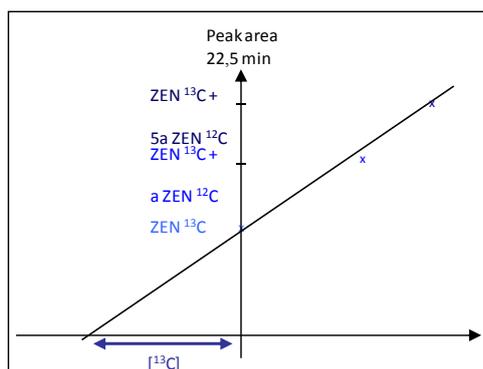
Using this UV standard curve we can calculate the unknown concentrations of the two samples referring to the ^{13}C samples 1 and 2 signals areas:

Fig. 2.30: Determination of 10%- ^{13}C Zen contained in the raw solutions 1 and 2, using a ^{12}C Zen standard curve.



We also verified the assay by the method of standard additions by HPLC-UV; the principle is the same as before, except that the curve is shifted:

Fig. 2.31: Illustration of the 10%-¹³C Zen determination by the method of standard additions.



The results of both methods of dosing, with MS additions (AD), and HPLC with and without additions are summarized in Table 2.5.

Table 2.5: Measuring the concentration of 10%-¹³C Zen starting from solution with or without ¹²C Zen. Analytical techniques used here are mass spectrometry and HPLC-UV (280 nm).

Concentration of ¹³ C Zen (μM)	Determined by MS starting with AD of 0.02 μM ¹² C Zen	Determined by MS starting with AD of 0.1 μM ¹² C Zen	Determined by HPLC starting with AD of ¹² C Zen	Determined by HPLC starting without AD of ¹² C Zen
Zen ¹³ C (1)	868 ± 95	943 ± 72	911 ± 49	892 ± 43
Zen ¹³ C (2)	381 ± 57	367 ± 31	396 ± 23	369 ± 19

Conclusion

Whether it is assessed by additions or a direct HPLC assay, the amounts of Zen determined in extracts 1 and 2 are about 0.9 mM and 0.4 mM, respectively.

2.3.2.2. Determination of natural Zen contained in extracts of treated animals

Materials and methods

Biological samples:

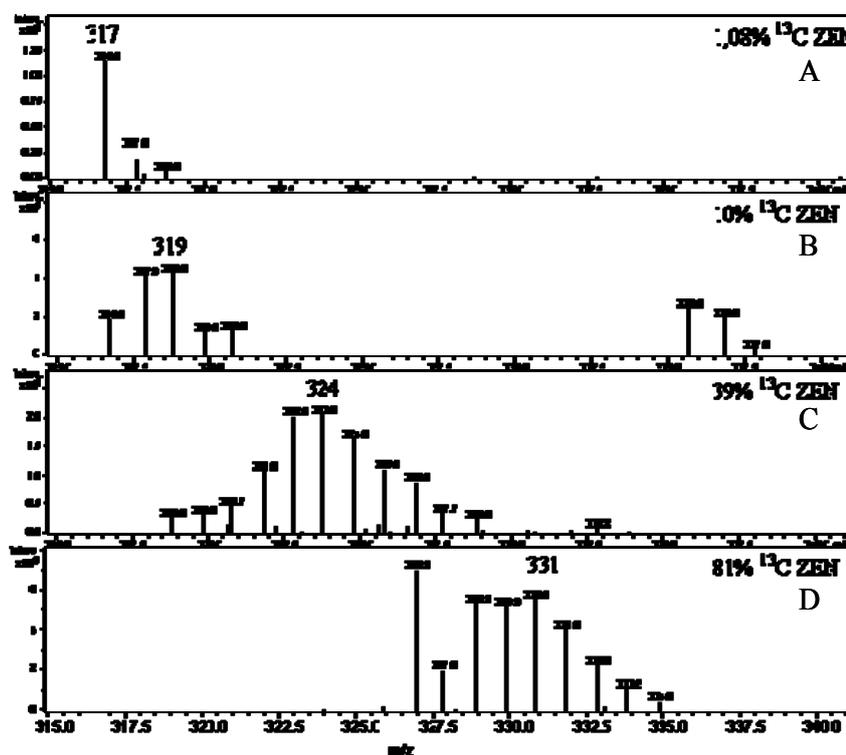
The method was used for the determination of zearalenone in biological samples from Zen treated rat and chicken. These treatments are part of a larger experiment detailed within the chapter 3 of the present work.

Details of post-treatment of biological extracts:

For the purification and treatment of samples, we followed the protocol described in the article by Songsermsakul et al., 2006 (see chapter 4 for more details). A 10 μM of internal standard (81% -¹³C Zen) were then spiked into each of these samples before any stage of purification or concentration.

Even if at the beginning we have developed the method of determination of zearalenone in biological samples using enriched 10% ^{13}C Zen, once obtained the 39% and 81% ^{13}C enrichments we followed the same steps described above. The next examples of zearalenone assessment using enriched molecules have been done using the 81% ^{13}C Zen because a higher isotopic enrichment, the mass isotopic cluster (mass between m/z 327 and m/z 336) is totally separated from the natural ^{12}C Zen (mass signal at m/z 317) on a mass spectra (fig.2.32).

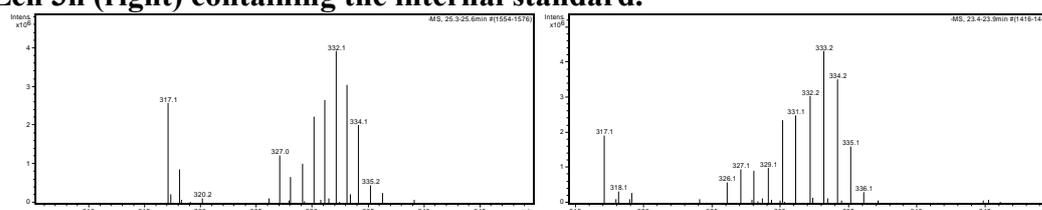
Figure 2.32: Mass spectra of natural Zen (A) and enriched 10% (B), 39% ou 81% with ^{13}C .



Results and discussions

Determinations were made by LC-MS using the standard conditions of gradient and ionization:

Fig. 2.33. Mass spectra examples of plasma samples of rat C-Zen 3h (left) and S9 rat C-Zen 3h (right) containing the internal standard.



The isotopic mass between m/z 327 and m/z 336 corresponds to the internal standard 81% of ZEN- ^{13}C . The signal at m/z 317 corresponds to the ZEN naturally present in the biological sample.

As we can see from the figure 2.33 mass spectra, the isotopic cluster shows the presence of the internal standard introduced. In both cases the signal of internal standard and the natural Zen contained in the sample (m/z 317) are separated so we can easily calculate the amount of ZEN by calculating the two signals intensities ratio:

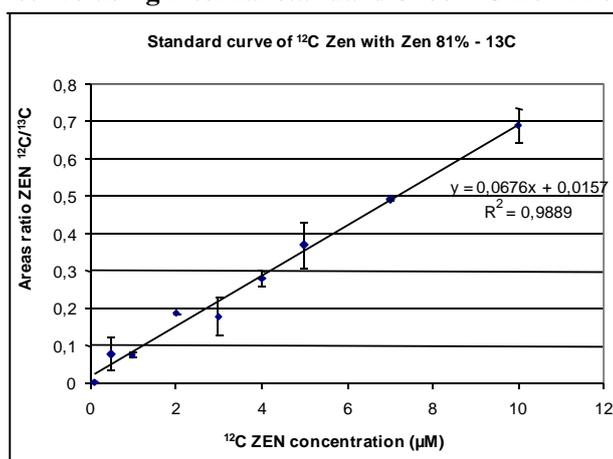
Table 2.6. Examples of ZEN determination in organic extracts of rat and chicken treated with Zen and/or barbiturates using 10 μ M 81%-¹³C Zen, (n = 2).

	[ZEN] (μ M)
S9 (rat)	
C-ZEN 3h +50 nmoles ¹³ C	1.4 \pm 0.2
Plasma (rat)	
C-ZEN 3h +20 nmoles ¹³ C	2.1 \pm 0.5
C-ZEN 6h +10 nmoles ¹³ C	1.5 \pm 0.3
Plasma (chicken)	
PB-ZEN 24h + 20 nmoles ¹³ C	0.2 \pm 0.04
ZEN 3d + 20 nmoles ¹³ C	0.4 \pm 0.08

The samples have all been treated with glucuronidase, the amounts of Zen dose corresponding to total amounts of Zen in the free samples. In fact some of that Zen may be in the glucuronised form.

To be sure of the ¹²C Zen and ¹³C Zen concentration ratio linearity in a biological sample, we added a constant amount of 81%-¹³C Zen (10 μ M) in an extract from chicken control plasma and an increasing amount of ¹²C Zen (from 0.1 to 10 μ M). A single step purification immuno-affinity column was performed to remove proteins not eliminated during the centrifugation.

Fig. 2.34: Calibration curve using internal standard 81%-¹³C Zen in chicken plasma matrix.



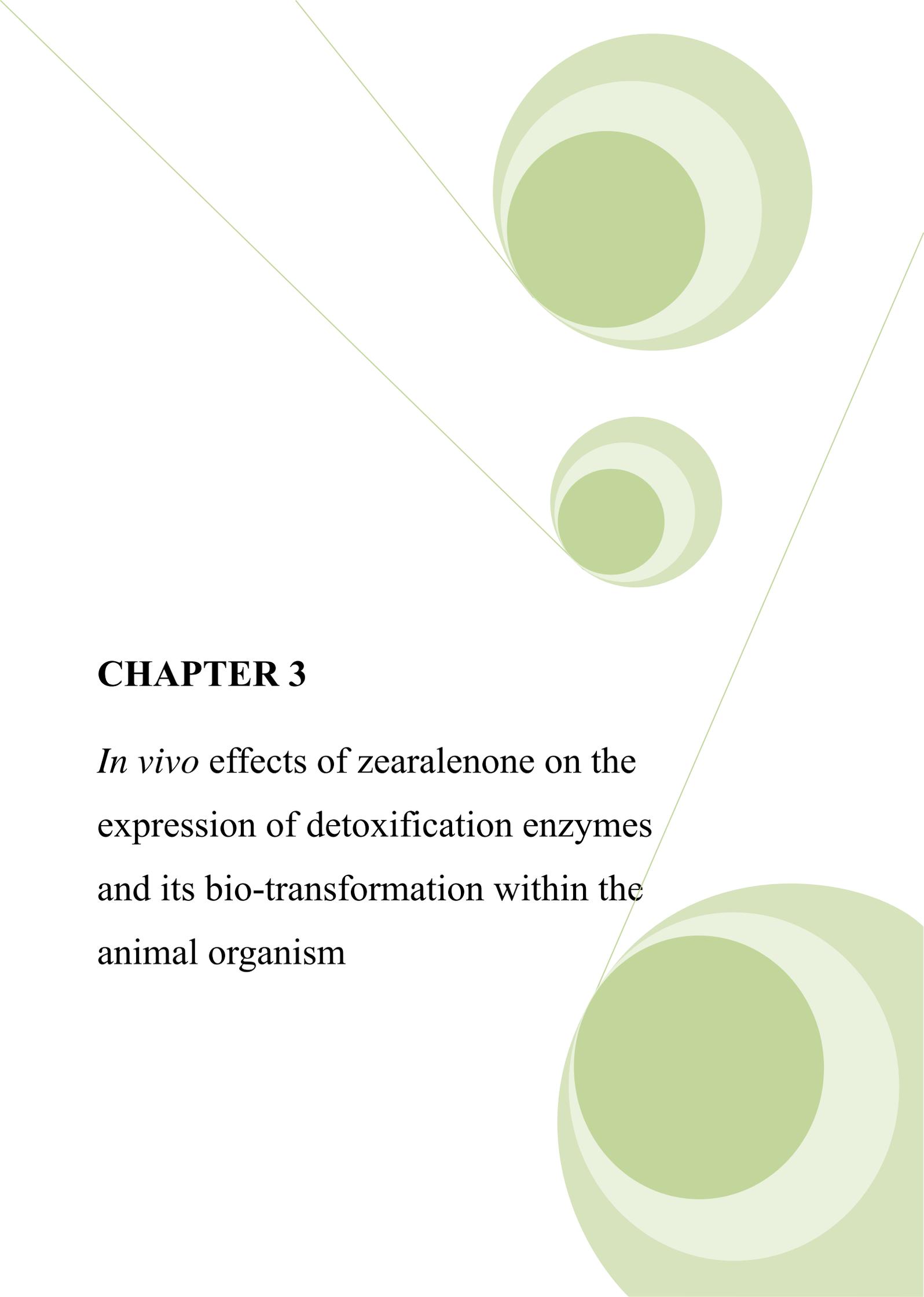
The ratio of areas of the isotopic cluster for ¹²C Zen by 81%-¹³C Zen in extracts of chicken plasma. The amount of 81%-¹³C Zen is constant (10 μ M) and the amount of ZEN ¹²C increases from 0.1 to 10 μ M, (n = 2).

As shown in figure 2.34, the ratio of the ^{12}C Zen areas and 81% ^{13}C Zen isotopic cluster areas is proportional to the amount of ^{12}C Zen spiked within the samples. This fact ensures us of the linearity and allows us to validate the determinations made using 81% ^{13}C ZEN. The matrix effect does not interfere with the quantification, regardless the amount of naturally Zen present in the sample.

Conclusions

The example of this assay allows us to affirm that the use of a ^{13}C enriched internal standard is a reliable technique for quantitative analysis.

Furthermore, this principle can be implemented in terms of quality for studies of metabolism and transport, as we have shown that the presence of a mass isotope can facilitate the identification of a metabolite.

The page features a decorative graphic consisting of three overlapping green circles of varying sizes, arranged in a vertical line. Two thin green lines intersect at the top left and extend diagonally across the page, framing the circles and the text. The circles are composed of concentric layers of different shades of green, creating a 3D effect.

CHAPTER 3

In vivo effects of zearalenone on the expression of detoxification enzymes and its bio-transformation within the animal organism

3.1. *In vivo* effects of Zearalenone on enzymes expression (cellular signalization)

In order to establish the zearalenone (*Zen*) *in vivo* effects on the detoxification enzymes we performed several experiments: on rat, a classical animal model for the biological studies and with a large documentation available in the literature on the xenobiotics effects and in particularly on *Zen* effects, and on chicken, one of the species considered most resistant and “insensitive” to Zearalenone presence. Rat models are often used because they are easy to manipulate, as well as because of the important liver activity (an indicator is the liver/body weight proportion that is approximately of 8%), nevertheless poultry are of an important agro-alimentary and economical importance. The animals were treated with zearalenone and classical P450 reference inducers, as described below. Despite its non-steroidal structure, *Zen* activates estrogen receptors resulting in functional and morphological alterations in the reproductive organs. In rats, for female subjects: hyperestrogenism, vulvovaginitis, vaginal and rectal prolapses, ovarian abnormalities, pregnancy loss and infertility; and for males: fertility rate reduction, lower total and gel free volumes of semen, with lower total motile sperm were reported (Gaumy et al., 2001). These results are from chronic exposure of about 28 days of treatment. In order to check the modifications of enzymes involved in the detoxification of xenobiotics we exposed rats / chickens to 25mg/Kg *Zen* for short times, which can be consider a high dose upon consideration for chronical studies, but on the other hand this dose can be consider in the range of usual employed dose treatments with classical CYP inducers (20-100 mg/Kg *i.p.*, for 3-methylcholanthrene or phenobarbital as an example).

Complementary approaches have been done on rats and chickens treated with *Zen* compared to classical detoxification enzymes inducers, measuring:

- a) changes in rats mRNA expression of 25 genes coding for proteins of xenobiotics detoxification using qRT-PCR approach;
- b) changes in the quantities of *Zen* and its metabolites found in the liver of chickens and rats treated with *Zen*, as well as in the chicken muscle;
- c) modifications of the nutritional values of chicken muscles treated with *Zen*;
- d) changes in detoxification enzymes activities;

are detailed in the following chapter.

Material and methods

Material and chemicals

Material: Bio-Rad iCycler iQ5 Real Time PCR System (Certified GeneTool, INC, California, USA); LC system (HPLC-DAD, Shimadzu Corporation, Japan), LC-MS coupling consisted of a LC 1100 series system, (Agilent, CA, US) coupled with a Ion Trap Mass spectrometer (Esquire HCT) using an ESI interface operating in positive or negative mode (Bruker Daltonics, MA, USA), Gas-chromatograph Clarus 500 - Perkin-Elmer (Waltham, MA, USA).

Chemicals: Total Protein Kit Micro-Lowry, Paterson's Modification, GenElute™ Mammalian Total RNA Kit, zearalenone, α -/ β - zearalenol, α -/ β - zearalanol, NADPH, acetonitrile, ammonium acetate, glucose-6-phosphate and MgCl₂ were purchased from Sigma (St-Louis, MO, USA). RT2Profiler™ PCR Array First Strand Kit was from SuperArray (Bioscience Corporation, USA). All reagents were of the highest purity available.

Methods:

Enzymes quantification in the microsomal fractions

Protein contents in microsomal suspensions were determined using the Sigma's Total Protein Kit using bovine protein standard. P450 was measured as described by Omura and Sato (Omura and Sato, 1964)

Quantitative RT-PCR

The RT-PCR was done using rat liver samples. The total RNA from liver samples was isolated using Sigma's GenElute™ Mammalian Total RNA Kit. The RNA extract was prepared and the mRNA was assessed using the RT2Profiler™ PCR Array First Strand Kit and following the provided protocol. Customized 96 well PCR plates for the 25 chosen genes and 2 reference genes (GAPDH and β -Actin), 5 control wells were used in order to eliminate the incorrect (or irrelevant) results; each 96 wells plate was used for 3 samples. The quantification was made by using a Bio-Rad iCycler iQ5 Real Time PCR System (Certified GeneTool, INC, California, USA).

Determination of zearalenone and its metabolites

Rat (urine, plasma and liver) and chicken (meat, plasma and liver) samples have been assessed as described within the chapter 2, selective HPLC-MS assessment has been done using internal standard Zen ¹³C enriched.

Rat treatments

Male Sprague-Dawley rats (Iffa Credo, St Germain l'Arbresle, France) were housed and treated according to the French legislation in a facility authorized by the Ministry of Agriculture. Rats of initial average body weight of 200 g were studied within two experiments. They were fed with a standardized diet *ad libitum*.

First experiment - 3 Days treatment

In order to determination the CYP isoform(s) induced by the presence of zearalenone within rats, animals (3 rats per group) were treated for 3 days with zearalenone and classical inductors of P450; intra-peritoneally as follows: **1)** Control rats: 1mL /day saline; **2)** PB rats: 80 mg/Kg phenobarbitone natrium salt *i.p.* in saline; **3)** DM rats: 100mg/Kg dexamethasone *i.p.* in corn oil; **4)** β-NF rats: 100mg /Kg β-naphtoflavone *i.p.* in corn oil; **5)** Clof rats: 100mg /Kg clofibrate *i.p.* in corn oil; **6)** Zen IP rats: 25mg / Kg zen *i.p.* in corn oil and: **7)** Zen PO rats: 25mg / Kg zen *p.o.* in corn oil. The animals were sacrificed 24 hours after the last treatment.

Second experiment - associated treatments

This associated treatment had two main objectives: to determine the early effects of zearalenone (from 0 to 24 hours after treatment) and to observe the occurrence of different metabolites based on the *in vitro* observation of a new hydroxylated Zen metabolite formation (OH-Zen) involving PB-induced P450s (Bravin et al., 2009).

Animals (3 rats per group) were treated for 3 days; intra-peritoneally as follows: **1)** Control rats: 1mL /day saline; **2)** PB rats: 80 mg/Kg phenobarbitone natrium salt *i.p.* in saline; followed by a single intra-peritoneal treatment with 25mg / Kg Zen *i.p.* in corn oil. The animals were sacrificed at 3, 6, 10 or 24 hours after Zen single treatment.

Poultry treatments

Leghorn chickens were housed and treated according to the French legislation in a facility authorized by the Ministry of Agriculture at AFSSA Ploufragan. Chickens of 2 weeks old

were studied within one experiment, regardless to the chickens' sex. They were fed with a standardized diet *ad libitum*.

Third Experiment - In order to determine the CYP isoform(s) induced by the presence of zearalenone within chickens, animals (5 chickens per group) were treated for 3 days with zearalenone and classical inducers of P450; intra-peritoneally as follows: **1)** Control poultry: 1mL /day saline; **2)** PB poultry: 80 mg/Kg phenobarbitone natrium salt *i.p.* in saline; **3)** DM poultry: 100mg/Kg dexamethasone *i.p.* in corn oil; **4)** β -NF poultry: 100mg /Kg β -naphthoflavone *i.p.* in corn oil; **5)** Clof poultry: 100mg /Kg clofibrate *i.p.* in corn oil; **6)** Zen IP poultry: 25mg / Kg zen *i.p.* in corn oil and one joint treatment; **7)** PB-Zen poultry: 3 days treatment 80 mg/Kg phenobarbitone natrium salt *i.p.* in saline and single treatment with 25mg / Kg zen *p.o.* in corn oil. The animals were sacrificed at 24 hours after the last treatment.

3.1.1. Zearalenone effects on the P450 amounts.

Rat liver samples (about 0.2 g) were taken for qRT-PCR assessment and the rest of the liver was used for microsomal preparations. Microsomes were prepared from thawed liver tissue using ultracentrifugation according to the method described by Peyronneau et al. (1992). The resultant microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4) with 20% glycerol, frozen in liquid nitrogen and stored at -80°C until use. The poultry microsomes were prepared in the same way. No chicken liver samples were taken for qRT-PCR due to the lack of chicken primers on the specialized market. The P450 activities and zearalenone effects on classical inducers microsomal extracts are detailed within chapter 4.

3.1.1.1. Zearalenone effects on P450 amounts in treated rats

Results and discussions:

The total amount of P450-CO complex from rats treated for 3 days with zearalenone (*i.p.* and *p.o.*) are comparable with the control and oil treatments (table 3.1).

P450/b5 ratios from 3 days treated rats indicate significant induction of total amounts of P450-CO complex in the case of phenobarbital and dexamethasone treatments, which are a first indicator of treatments success (table 3.1.). It is well known that cytochrome b5 content is constant regardless to the xenobiotic treatments.

Table 3.1. P450, b5 and protein levels within rat liver microsomal preparation from 3 days treatment experiment

Rats Treatments	Conc P450 (nmol/ml)	Conc b5 (nmol/ml)	P450/b5	Prot. Conc mg/ml	nmol P450 / mg Protein
3 days treatment					
Control	12.3 ±0.9	6.3 ±0.5	1.95 ±0.19	9.0 ±0.6	1.38 ±0.07
Oil	4.3 ±0.3	3.0 ±0.3	1.44 ±0.19	2.8 ±0.7	1.56 ±0.52
Phenobarbital	27.7 ±1.2	9.3 ±0.5	2.96 ±0.13^{a,b}	13.5 ±1.8	2.05 ±0.39^a
Dexamethasone	15.3 ±1.2	5.3 ±0.5	2.88 ±0.11^{a,b}	5.9 ±0.7	2.62 ±0.14^{a,b}
Clofibrate	17.3 ±0.9	9.0 ±0.9	1.93 ±0.17^b	11.2 ±0.8	1.55 ±0.03
β-Naphtoflavone	18.3 ±0.5	9.0 ±0.8	2.04 ±0.18^b	9.6 ±1.5	1.90 ±0.33^a
Zearalenone IP	15.7 ±1.7	8.7 ±0.9	1.81 ±0.06^b	11.7 ±1.2	1.34 ±0.12
Zearalenone PO	20.7 ±1.7	11.3 ±0.5	1.82 ±0.09^b	17.6 ±2.2	1.17 ±0.10
Joint treatments					
Control	26.7 ±2.6	10.9 ±1.9	2.44 ±0.19	22.7 ±0.2	1.18 ±0.10
C-Zen 3h	5.2 ±0.6	1.5 ±0.3	3.55 ±1.15	5.5 ±0.1	0.95 ±0.11
C-Zen 6h	10.3 ±0.6	5.0 ±0.6	2.05 ±0.37	12.4 ±0.1	0.84 ±0.04
C-Zen 10h	8.2 ±0.1	3.4 ±0.3	2.45 ±0.24	9.8 ±0.2	0.84 ±0.01
C-Zen 24h	19.5 ±0.2	9.7 ±0.1	2.01 ±0.02^c	19.3 ±0.1	1.01 ±0.01
PB	29.5 ±0.1	10.4 ±0.1	2.84 ±0.02	18.5 ±0.1	1.59 ±0.02
PB-Zen 3h	16.0 ±0.2	4.0 ±0.3	4.03 ±0.26^d	9.4 ±0.1	1.70 ±0.03
PB-Zen 6h	17.6 ±0.1	5.0 ±0.1	3.49 ±0.05^d	12.9 ±0.1	1.37 ±0.01
PB-Zen 10h	10.0 ±0.7	3.1 ±0.1	3.27 ±0.23^d	9.2 ±0.1	1.09 ±0.09
PB-Zen 24h	21.7 ±1.0	7.0 ±1.2	3.12 ±0.42	15.5 ±0.1	1.40 ±0.06

The presented results are on pooled microsomal samples and are the results of 3 determinations. The statistical analysis has been done using the Student t-Test; ^a - significant differences ($p \leq 0.05$) related to 3days Control treatment; ^b - significant differences ($p \leq 0.05$) related to 3days Oil treatment; ^c - significant differences ($p \leq 0.05$) related to joint Control treatment; ^d - significant differences ($p \leq 0.05$) related to joint PB treatment

P450/Protein ratios indicate significant induction of the total amounts of P450-CO complex in the case of PB and DM treatments, but as well for the β-NF. Beta-naphtoflavone, phenobarbital and dexamethasone are considered as classical inducers of CYP1A, CYP2A, B, C and CYP3A, respectively (Correia, M.A., 1995; Waxman et al., 1983; Waxman and Azaroff, 1992; Sidhu and Omiecinski, 1995; Hoen et al., 2000).

The total amounts of P450-CO complex from joint treated rats with zearalenone (control and PB) are comparable with the control and PB treatments, respectively.

Joint treated rats P450/b5 rapport indicates significant induction of total amounts of P450-CO complex in the case of phenobarbital, which are confirming the results from the 3 days experiment (table 3.1.). The results of the control and PB treatments are not identical with the first experiment results because of the inter-individual variability and/or because of a better control of the administrated treatments substances homogeneity. In the first

experiment Zearalenone standard have been dissolved directly within corn oil, resulting in a colloidal solution; in contrast for the second experiment Zen been first dissolved with a minimum quantity of alcohol and then mixed with the corn oil, resulting in a homogeneous solution.

3.1.1.2. Zearalenone effects on the P450 amounts of treated chickens

Results and discussions

In the case of poultry treatments we observed weight variations, as a first step for characterization of zearalenone effects (table 3.2). The dexamethasone treated poultries have been losing weight, but an increase of liver weight can be observed. Liver weight/Final weight ratio in the case of DM poultry is almost the double of control and oil poultry ratio. The β -Naphthoflavone treated poultry had a smaller total gain and increased weight liver then the control and oil poultry, which are not statistically sustained.

Table 3.2. – Total weight and liver weight variation of treated poultry

Poultry treatments	Initial weight (g)	Final weigh (g)	Total gain (g)	Liver weight (g)	Ratio Liver weight/ Final weight
Control	221.2 \pm 21.4	259.2 \pm 22.0	38.0 \pm 6.2	6.0 \pm 0.5	2.30%
Oil	218.0 \pm 25.2	255.0 \pm 29.9	37.0 \pm 6.7	5.7 \pm 0.8	2.24%
Phenobarbital	251.6 \pm 15.3	290.8 \pm 18.1	39.2 \pm 3.0	7.3 \pm 0.5	2.53%
Dexamethasone	251.8 \pm 31.6	236.6 \pm 29.5	-15.2 ^a \pm 7.3	10.1 ^a \pm 1.6	4.25 ^a %
β -Naphthoflavone	223.2 \pm 30.6	237.5 \pm 32.1	14.3 ^a \pm 4.0	7.7 \pm 1.3	2.58%
Clofibrate	218.6 \pm 7.6	250.4 \pm 15.5	31.8 \pm 8.6	6.7 \pm 0.6	2.69%
Zearalenone	222.7 \pm 32.1	259.8 \pm 38.3	37.2 \pm 10.1	6.8 \pm 1.1	2.60%
PB – Zen 24h	222.8 \pm 35.5	257.0 \pm 41.8	34.2 \pm 8.3	7.1 \pm 0.8	2.79%

The statistical analysis has been done using the Student t-Test; a - significant differences ($p \leq 0.05$) related to Control/Oil treatments.

Liver weight/final weight ratio in the case of β -NF poultry is equivalent with the ratio of control and oil poultry. No other differences, related to total weight and liver weight, were observed between the control/oil poultry and the PB, Clof, Zen and PB-Zen 24h treatments.

The prepared microsomes from poultry livers have shown the next results concerning CYP assessment presented within table 3.3.

Table 3.3. – P450, b5 and protein levels within chicken liver microsomal preparation

Poultry Treatments	Conc P450 (nmol/ml)	Conc b5 (nmol/ml)	P450/b5	Prot conc (mg/ml)	nmol P450/mg prot
Control	4.5 ±0.6	14.3 ±1.3	0.31 ±0.07	29.9 ±0.7	0.15±0.02
Oil	2.8 ±0.7	7.5 ±0.6	0.37 ±0.12	17.9 ±0.9	0.16±0.04
Phenobarbital	7.2 ±0.7	14.1 ±0.9	0.51 ±0.05^{a,b}	31.8 ±1.1	0.23±0.03^{a,b}
Dexamethasone	8.1 ±0.6	12.0 ±0.2	0.68 ±0.06^{a,b}	32.1 ±2.0	0.25±0.02^{a,b}
Clofibrate	4.2 ±0.1	12.0 ±0.8	0.35 ±0.02	32.8 ±0.9	0.13±0.01
β-Naphtoflavone	17.2 ±0.3	12.6 ±0.7	1.36 ±0.07^{a,b}	27.6 ±1.8	0.62±0.02^{a,b}
Zearalenone	5.5 ±0.4	14.1 ±0.7	0.39 ±0.01	37.5 ±1.9	0.15±0.01
PB – Zen 24h	6.6 ±0.6	15.3 ±1.1	0.43 ±0.04^a	37.3 ±1.7	0.18±0.02

The presented results are on pooled microsomal samples and are the results of 3 determinations. The statistical analysis has been done using the Student t-Test; a - significant differences ($p \leq 0.05$) related to Control treatment; b- significant differences ($p \leq 0.05$) related to Oil treatment.

Chicken microsomal extracts P450/b5 and P450/protein ratios are even 8 times lower for then rat microsomes. Similar results have been reported for untreated broiler chickens by Nebbia et al. in 2003 and for treated Ring-Necked pheasants with β-naphtoflavone, phenobarbital and clofibrate by Giorgi et al. in 2000.

The P450/b5 and P450/protein ratios are not statistically different for Zen compared with the control treatment. In the case of PB-Zen treatment a diminution of the P450/b5 ratio and P450/protein ratios can be observed if compared with PB treatment, which is not statistically sustained.

P450/b5 ratios from 3 days treated chickens indicate significant induction of the total amounts of P450-CO complex in the case of β-naphtoflavone treatment, as well as in the dexamethasone and phenobarbital treatments, but less than β-naphtoflavone; the same tendency can be observed from P450/protein ratios.

3.1.1.3. Zearalenone effects on the P450 amounts – Conclusions

Upon short duration treatment with 25 mg Zen/ Kg body weight, rats and chickens P450/b5 and P450/protein ratios are not statistically different if compared with control treatment. Nevertheless, the treatment success is confirmed by the 3 days treated rats and chickens, with the classical inducers: phenobarbital, dexamethasone and β-naphtoflavone, which are having a significant induction of the total amounts of P450-CO complex.

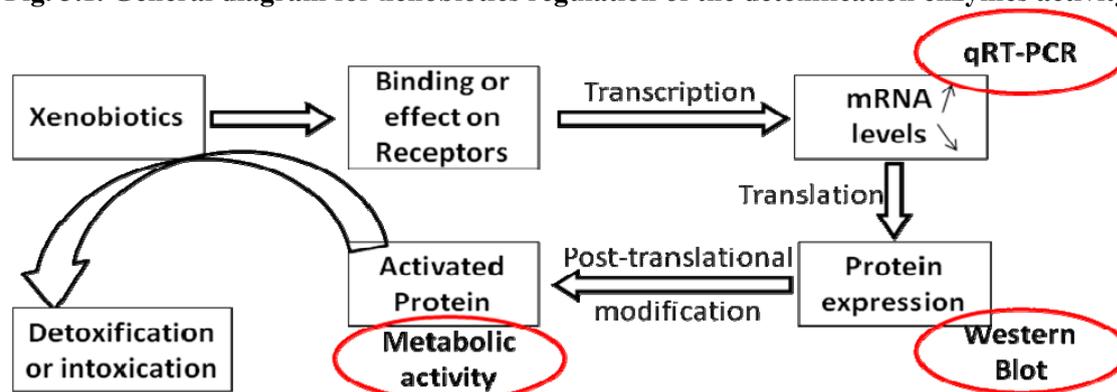
Differences can be seen even at this level between the rat and chicken metabolism. Even if the chicken and rat microsomal extracts are having P450/b5 similar ratios, the P450/protein ratio is 8 times lower for chicken then rat.

3.1.2. Determination of the expression of mRNA encoding the detoxification enzymes, by quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

To assess the *in vivo* Zen effects on the detoxification enzymes in rats, we choose the quantitative real time RT -PCR technique for mRNA assessment. We determined the mRNAs expression encoding 25 enzymes of interest (ABCB1/P-gP: P-glycoprotein, ABCC1,2/MRP-1,2: multidrug resistance associated protein 1,2, ABCG2/BCRP: breast cancer resistance protein; CYP: cytochrome P450 1A2, 1B1, 2B2, 2C, 2C7, 2D22, 2E1, 3A1, 3A2; CYP19: aromatase; HSD17B: 17-beta hydroxysteroid dehydrogenase; HSD3B: 3-beta hydroxysteroid dehydrogenase; UGT: UDP – glucuronosyl transferases; COMT: catechol-*O*-methyl transferase; HIF1B/ARNT: Aryl Hydrocarbon Receptor Nuclear Translocator; NR3A1/ ESR1: Estrogen Receptor; NR1C1/PPARA: Peroxisome Proliferative Activated Receptor Alpha; NR2B1/RXRA: Retinoid Xenobiotic Receptor Alpha; NR1I2/PXR: Pregnane Xenobiotic Receptor), which are usually involved in xenobiotic detoxification (see Annex 1 for more details concerning the chosen detoxification genes).

Zen effects on CYPs can be evaluated at different levels: mRNA expression, protein expression or activity levels using specific substrate of each CYP isoform. One can consider that such attempts to correlate mRNA expression and CYP activities remains hazardous since several factors are involved between messenger expression and protein activities (figure 3.1.).

Fig. 3.1. General diagram for xenobiotics regulation of the detoxification enzymes activity



The xenobiotics are interacting with receptors which are transcript resulting in a increase of mRNA levels. The mRNA expression, by translation implicates the protein expression. Never the less, even if the protein expression level is increased, due to the protein post-transcriptional modification or the lack of it, the metabolic activity might not be present. If the protein is activated this could result in a detoxification or intoxication process by its interaction with the xenobiotic that triggered the signalization path.

In addition, one must take into consideration that *in vivo* CYP protein turnover is generally in the range of 15-20 hours, which can lead to a delay between the two

phenomena. RT-PCR is a specific analytical method of mRNA, compared to enzymatic activities, done on pooled microsomal extracts and using substrates consider as specific of a CYP family, which have diminished responses for other families of CYPs. Moreover several factors are involved in the enzymatic expression (maturation and proteolytic degradation for example) which can also be under-, or over-expressed, modifying not only the amount of expressed protein but also the amount of active protein (normal protein + heme + reductase + electron transfer). One could say that a Western Blot protein assessment would be more appropriate, but we considered that this technique detects the presence of proteins and it does not reflect the actual metabolic activity.

Quantitative RT-PCR protocol

The mRNA expression for the 25 detoxification enzymes of interest was performed on rat liver samples. RNA was isolated using the GenElute™ mammalian total RNA kit (Sigma Aldrich). Total RNA concentration and purity were then determined by measuring absorbance at 260 nm and 280 nm. The A260/A280 ratio ranged between 1.8 and 2. A sample of 0.5 µg of total RNA was converted to cDNA with random primers in a total volume of 10 µl using RT² first strand Kit (Superarray Bioscience Corporation, Frederick, USA). The cDNA was diluted with distilled water to a volume of 100 µl. 0.4 µM was used for each primer set in a specific RT² Profiler PCR Array, according to the manufacturer's protocol. Customized 96-well PCR plates for the 25 chosen genes, 2 reference genes (GAPDH and β-Actin), a housekeeping gene panel and Genomic DNA control primer set, were used. The efficiency of the RT² first strand Kit was tested according to the manufacturer's protocol using: a housekeeping gene panel, a Genomic DNA control primer set and replicate Reverse Transcription Controls. Quantification was performed using a Bio-Rad iCycler iQ5 Real Time PCR System (Certified GeneTool, INC, California, USA). The thermal cycling conditions comprised an initial *Thermus aquaticus* polymerase activation step at 95°C for 15 min, 50 cycles at 95°C for 15 s, 65°C for 1 min and a dissociation stage in order to identify primer-dimer and specific amplification.

Relative expression values were calculated as $2^{-\Delta Ct}$, where ΔCt is the difference of Ct (threshold cycle) values for genes of interest and the housekeeping gene (GAPDH or β-actin HPRT). The parameter Ct (threshold cycle) is defined as the fraction cycle number at which the fluorescence generated by SYBR Green® dye-amplicon complex formation passes a fixed threshold above baseline. Gene expression was evaluated using the Ct value from each sample. A Ct value of 35 was taken as the detection limit; we considered

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that higher expression level too low to be applicable as previously reported (Bousquet et al., 2009). The mRNA levels variation, were expressed comparatively (fold difference) to those found in Control rats, arbitrarily set at the value of 1. The result which presents an up-regulation (fold up-regulation exceeding the control value of 1) was equal to the fold difference. A down regulation (fold-down regulation) was considered if the fold difference was smaller than the control value of 1 and calculated as: -1/fold difference.

3.1.2.1. Zearalenone and classical P450 inducers effects on mRNA gene expression

In order to have a complete view of the genes mRNA regulation in the presence of Zen or classical P450 inducers, we represented the mRNA expression variations (fold down/up-regulation) for liver samples from the rat first experiment in table 3.5.

Table 3.4. Gene's regulation for Zen and P450 classical inducers treatments

No	Gene	Rat 3 days treatments					
		Zen IP	Zen PO	PB	DM	β-NF	Clof
1.	ABCB1/Pg-P	6.45*	4.22*	-3.70**	-3.53*	-1.85	1.47
2.	ABCC1/MRP-1	1.55	1.28	-1.20	-1.75	1.09	-1.27
3.	ABCC2/MRP-2	-1.34	1.40	1.23	-1.32	-9.66*	-1.17
4.	ABCG2/BCRP	-1.95	-113.43*	-3.00*	-18.81*	1.07	-1.10
5.	CYP1A2	-1.45	-1.21	-3.90	-2.16	18.20*	-1.76
6.	CYP1B1	1.46	1.75	-1.37	-1.45	66.81*	-1.83*
7.	CYP2B2	-1.09	-1.18	29.03*	4.52*	-2.18*	1.79
8.	CYP2C	-2.27*	-5.22*	-1.10	-1.20	-1.81	2.12
9.	CYP2C7	2.15*	7.28*	-1.66	-1.25	-1.93	-1.16
10.	CYP2D22	-1.01	1.06	-2.27**	1.57	-1.16	1.31
11.	CYP2E1	2.44	-1.53	-1.04	2.16	-1.31	1.76
12.	CYP3A1	2.32*	1.91	3.58*	27.67*	1.22	1.94
13.	CYP3A2	2.02*	1.07	1.91	3.25*	-1.02	1.52
14.	CYP 19	2.28	1.31	1.64	1.08	-1.14	-1.27
15.	HSD17B1	-1.23	1.46	-1.77	-2.71	-1.83	-1.59
16.	HSD17B2	4.12*	-1.15	1.53	-11.62***	-1.05	1.38
17.	HSD3B	2.12	-1.08	-1.35	1.41	1.41	1.02
18.	UGT1A1	1.65	1.52	1.04	2.14	5.26*	1.29
19.	UGT2B	2.86	-2.01	1.70	228.07*	397.09*	20.25*
20.	COMT	1.82	-2.23	-1.14	-1.18	1.23	-1.29
21.	HIF1B /ARNT	2.00*	2.07*	-1.29	-1.54	-1.47	-1.14
22.	NR3A1/ESR1	1.34	1.17	-2.41**	-1.39	-4.30*	-1.71
23.	NR1C1 /PPARA	-1.12	1.31	-2.19*	-2.30*	-1.91	-1.87
24.	NR2B1/RXRα	1.25	1.12	-1.23	1.92	-1.66	-1.40
25.	NR1I2 /PXR	-2.15	-1.72	-2.83*	-1.15	-3.48*	-2.55*

The RT-PCR was done using rat liver samples. Total RNA of liver samples was isolated using Sigma's GenElute™ Mammalian Total RNA Kit; mRNA was assessed using the RT2Profiler™ PCR Array First Strand Kit and quantification was done by using a Bio-Rad iCycler. In order to control the results validity, we used the positive and negative controls; and we looked at the melting curves in order to observe any kind of abnormalities. The current results are the average values of 3 to 6 assessments. A significant "Fold Up- or Down-Regulation of Test Sample /Control Sample" is considered from an absolute value of "2" (bold values). As control were used the liver samples from oil treatment. Statistical analysis (t-test) has been done (* for p<0.05, ** for p<0.01 and *** for p<0.005).

The Zen treatments are showing a positive regulation of P-gp mRNA (table 3.4 - line 1). The mRNA's levels for CYP1A2 (table 3.4 - line 5), 2B2 (table 3.4 - line 7), 3A1 (table 3.4 - line 12) had confirmed the treatments using classical inductors, β -NF, PB and DM respectively. Differential expression of CYP2C forms (inhibition) and CYP2C7 (stimulation) for Zen *i.p.* and *p.o.* treatments (table 3.4 - lines 8 and 9).

Whereas Zen treatments (*i.p.* and *p.o.*) are showing a positive regulation of P-gp mRNA (figure 3.1., table 3.4.), PB and DM treatments are negative regulators and β -NF and Clofibrate have no significant effects (Table 3.4).

Zen treatment has showed no variation for MRP1 whereas the rat β -naphthoflavone treatment showed a negative regulation for MRP1 mRNA (10-fold down regulation). Zen treatment led to no significant variations of the mRNA levels of CYP1A2, CYP1B1, CYP2B2, CYP2D22 and CYP2E1. In opposite, CYP3A1 and CYP3A2 messengers are positively regulated under Zen treatment. Statistically sustain fold up values were obtained for Zen IP treatment. The mRNA detections for CYP1A2, 2B2, 3A1 confirmed the treatments using classical inductors, β -NF, PB and DM respectively, by a significant positive regulation of 18, 29 and 28 folds, respectively. In the case of CYP2C mRNA regulation, under Zen treatments (table 3.5.) CYP2C7 expression increased, even if the unspecific total 2C pool (CYP2C) was negatively regulated.

For the HSD17 β 2 expression the Zen IP treatment showed a small statistically positive regulation, DM treatment an important down regulation and for the other treatments no significant changes have occurred. UGT1A1 messenger was positive regulated by all the treatments, but were only significant in the case of β -NF and DM treated rat liver. UGT2B mRNA was regulated in a different way by Zen, in function of the treatment administration way: *i.p.* (positive regulation) and *p.o.* (negative regulation), results being statistically unsustainable. Nevertheless, UGT2B mRNA expression was positive regulated by β -NF and DM treatments (very high values, statistically significant).

The ESR1, PPARA, RXRA and PXR receptor mRNAs expression, were not modified contrary as expected or observed within *in vitro* tests (Sun et al, 2004; Ding et al., 2006) for Zen treatment. PXR and RXRA mRNAs expression was not modified upon *in vivo* treatment using PB and DM (Videmann et al., 2008). Only for ARNT expression, a small positive but statistically sustained regulation, was observed after Zen treatments, regardless to the administration way.

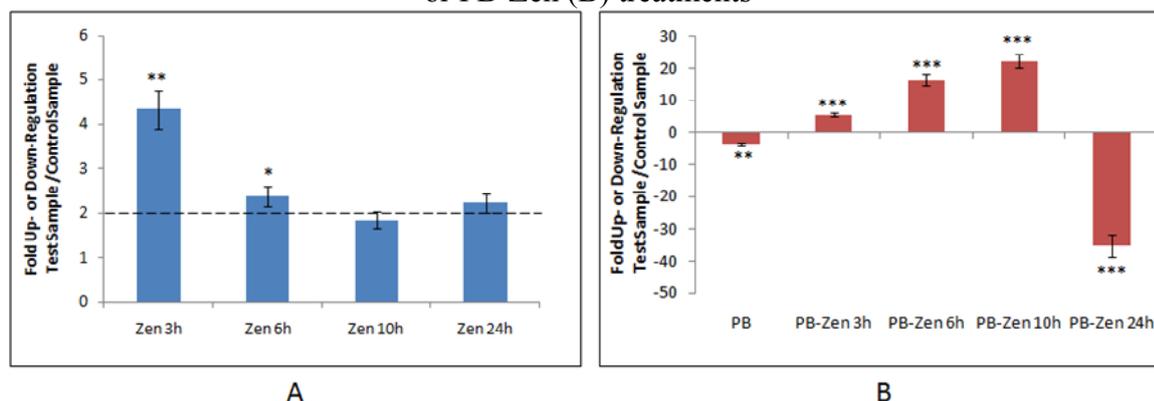
3.1.2.2. Early effects of zearalenone on mRNA gene expression

Results and discussions

The associated treatment (second rat experiment) has two main objectives: to determine the early effects of zearalenone (from 0 to 24 hours after 25 mg/kg *i.p.* treatment) and the occurrence of different metabolites based on an *in vitro* observation of a new metabolite formation. The results for liver samples from the second experiment, consisting in coupled treatments of Zen, are presented in figures 3.1 to 3.4.

The P-gp regulation is showing a different evolution in the case of Zen treatments than in the joint treatments, PB-Zen (fig. 3.1). For the zearalenone treatment a positive regulation can be observed with a diminution within the 10 hours after single treatment (early effects) and an augmentation at 24 hours, confirmed in the case of 3 days treatment (table 3.5).

Figure 3.1. Rat liver P-gp mRNA relative expression to untreated animals upon Zen (A) or PB-Zen (B) treatments



The RT-PCR mRNA determination was done using liver samples from rats control (A) or PB (B) after 3h, 6h, 10h and 24h single *i.p.* treatment with Zen; treated as describe within Material and Methods and Table 3.5. The current results are the average values of 3 to 6 assessments. Zen treatments values are represented with blue colour; PB-Zen treatments values are represented with red colour. A significant “Fold Up- or Down-Regulation of Test Sample /Control Sample” is considered from an absolute value of “2”. As control were used the liver samples from oil treatment. Statistical analysis (t-test) has been done (* for $p<0.05$, ** for $p<0.01$ and *** for $p<0.005$).

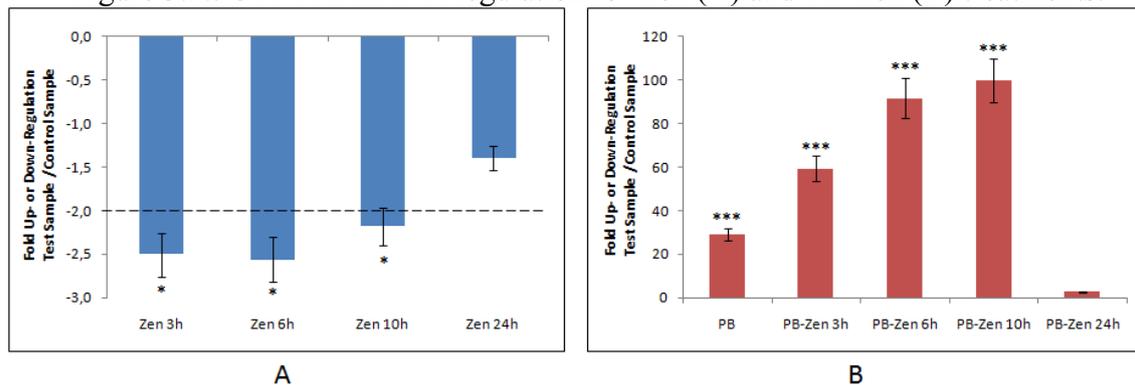
For the PB-Zen a stronger effect can be observed (a higher positive regulation than Zen treatment alone), as well as different time evolution, the early effects are showing an increase of the gene expression followed by a 24 hours 30 fold negative regulation.

An early small, but significant, negative regulation was observed for Zen treatment: from 3h to 10h for CYP2B2 (figure 3.2) and a general positive regulation for the CYP3A isoforms (figure 3.3).

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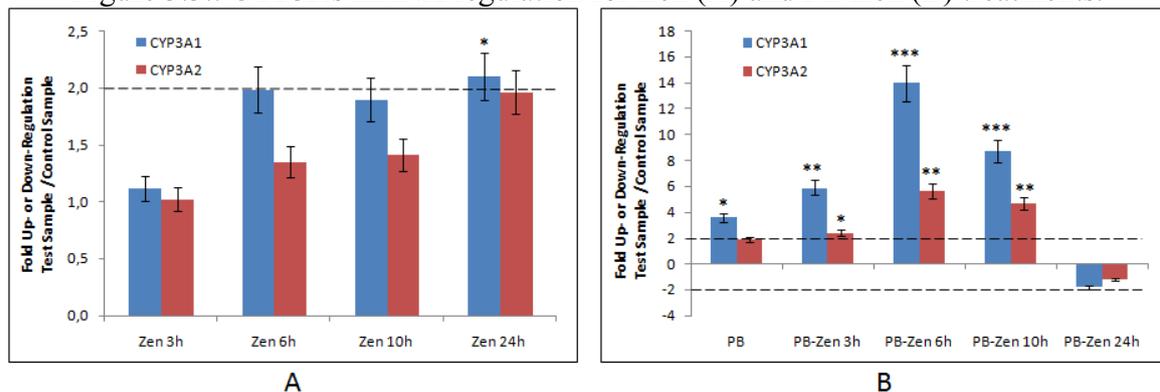
Another interesting fact can be observed for the CYP2B2 gene expression, were even if the Zearalenone is inducing a negative regulation and the PB a positive one, the PB-Zen joint treatment shows a higher positive regulation as an early effect (3 to 10 hours after Zen administration). The CYP3A's are showing the same tendency in both cases, with a higher positive regulation for PB-Zen. The Zen effect could be potentiated by PB treatment and the increase levels of certain CYPs, like CYP2 family and CYP3A.

Figure 3.2.: CYP2B2 mRNA regulation for Zen (A) and PB-Zen (B) treatments.



The RT-PCR mRNA determination was done using liver samples from rats control (A) or PB (B) after 3h, 6h, 10h and 24h single treatment with Zen; treated as describe within Material and Methods and Table 3.5 and the same protocol of data analysis has been used as described within figure 3.1 footnote. As control were used the liver samples from oil treatment. Statistical analysis (t-test) has been done (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.005$).

Figure 3.3.: CYP3As mRNA regulation for Zen (A) and PB-Zen (B) treatments.

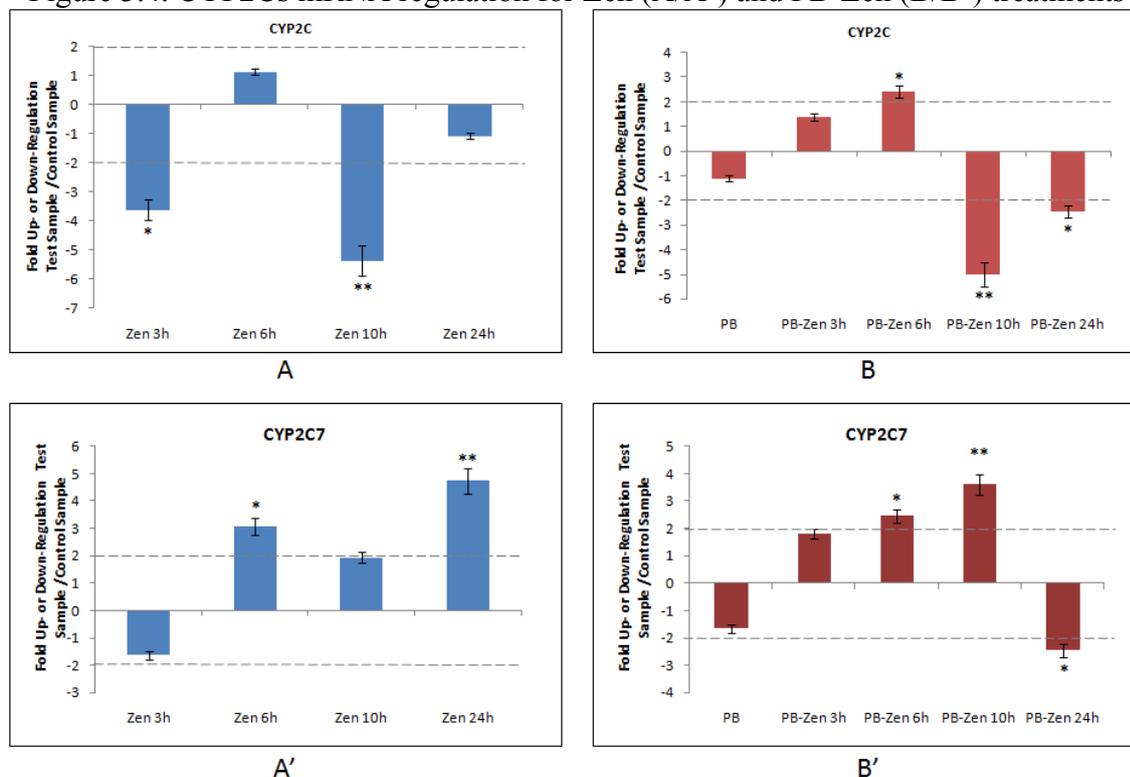


The RT-PCR mRNA determination was done using liver samples from rats control (A) or PB (B) after 3h, 6h, 10h and 24h single treatment with Zen; treated as describe within Material and Methods and Table 3.5 and the same protocol of data analysis has been used as described within figure 3.1 footnote. CYP3A1 values are represented with blue colour; CYP3A2 values are represented with red colour. As control were used the liver samples from oil treatment. Statistical analysis (t-test) has been done (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.005$).

The CYP 2C and 2C7 mRNAs are showing variable regulations (fig. 3.4.). An antagonist regulation appears at 10h and 24h after zearalenone treatment and at 10h for PB-Zen

treatment, also observed in the 3 days treatment. For CYP 2C mRNAs regulation no significant differences can be observed between Zen and PB-Zen treatments.

Figure 3.4. CYP2Cs mRNA regulation for Zen (A/A') and PB-Zen (B/B') treatments



A – CYP2C mRNA relative variation for liver samples from rats control after 3h, 6h, 10h and 24h single treatment with Zen; B – CYP2C mRNA relative variation for liver samples from rats PB after 3h, 6h, 10h and 24h single treatment with Zen; A' – CYP2C7 mRNA relative variation for liver samples from rats control after 3h, 6h, 10h and 24h single treatment with Zen; B' – CYP2C mRNA relative variation for liver samples from rats PB after 3h, 6h, 10h and 24h single treatment with Zen; treated as describe within Material and Methods and Table 3.5 and the same protocol of data analysis has been used as described within figure 3.1 footnote. As control were used the liver samples from oil treatment. Statistical analysis (t-test) has been done (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.005$).

Zearalenone *in vivo* treatments positive regulation and early effects on P-gp mRNA expression suggests that P-gp might be implicated within Zearalenone detoxification. P-glycoprotein implication in Zen transport has been determined in Caco2 cell lines by Videmann et al., in 2008. Previous publications reported that chemical treatment of rats with classical inducers (PB, DM, β -NF and Clof) have shown no consistent induction of P-gp mRNA regulation (Salphati and Benet, 1998; Brady et al., 2002). Our results are showing that the P-gp (ABCB1) mRNA is regulated differently in function of the treatment administrated: the PB and DM treatments negatively; the β -NF and Clof are not having significant effects.

In contrast to previous reports on chronic Zen exposure, we did not observe, in our short-term acute exposure experiments, noticeable modifications of receptor expression

(NR3A1/ESR1; NR1C1/PPARA; NR2B1/RXRA and NR1I2/PXR). Nor were such modifications observed upon *in vivo* rat treatments with classical CYP inducers, in contrast to the reported *in vitro* receptor involvement into CYP3A expression, as an example (Staudinger et al., 2003). These differences can also be explained by the differences between the *in vitro* determinations and the more complex *in vivo* experiments concerning the becoming of Zen and classical P450 inductors compounds, linked to the receptors mRNA expression.

The Zearalenone effects on the CYPs are not described in literature; not being describe whether it is an effect on CYP genes expression or directly on the enzymes. Our results are showing a small negative regulation of CYP2B2 mRNA and a small positive regulation for CYP3A family mRNAs after single Zearalenone treatment. The differences of regulation for this two genes mRNAs are between Zen single treatments and joint PB-Zen treatments; with an important stimulatory effect for the coupled treatments.

The Zearalenone effects on CYP2C family mRNA regulation are differentiated between CYP2C general negative tendency and the CYP2C7 positive regulation. The CYP2C family mRNA expression has showed no major difference between Zen and PB-Zen treatments.

A special interest has been paid to CYP2C7, because it is considered a homolog of human CYP2C8 (Nelson, 1999), an isoform which is implicated in the formation of a new hydroxylated Zen metabolite (Bravin et al., 2009).

A transposition of these results to human level, can encounter several problems especially for the P450 enzymes homologies. The rat CYP2B2 is considered a homologue of human CYP2B6 (National Center for Biotechnology Information – www.ncbi.nlm.nih.gov). The rat CYP3A1 and 3A2 are considered a homolog of CYP3A4 and 3A5 respectively (National Center for Biotechnology Information – www.ncbi.nlm.nih.gov). Problems are encountered when trying to establish the human homology for CYP2C7. Several publications are showing that CYP2C7 is homolog to CYP2C8 (Nelson, 1999) or to CYP2C9 (HUGO Gene Nomenclature Committee – www.genenames.org) and to CYP2C19 (HUGO Gene Nomenclature Committee – www.genenames.org and National Center for Biotechnology Information – www.ncbi.nlm.nih.gov). By doing a multiple sequence alignment using CLUSTAL 2.0.8 we have found approximately the same level of identity (68 to 71% of identity) between rat CYP2C7 and human CYPs 2C8, 2C9 and 2C19, respectively.

3.1.2.3. Effects of zearalenone on mRNA gene expression - Conclusions

The important stimulatory effect of Zearalenone on the P-gp mRNA (ABCB1) expression; could suggest the idea that P-glycoprotein might be implicated in the detoxification of Zearalenone; such P-gp transport of Zen has been recently determined in Caco2 cell lines (Videmann et al., 2008). Zearalenone presence is inducing early and rapid metabolic answer, especially for CYP2C7, which could have an important role within Zen's detoxification pathway in rats.

An influence of Zearalenone treatments on the CYP2B2 and 3A isoforms expression and activities can be observed. *In vitro* researches done on HepG(2) cells showed that Zen could induce the expression of the CYP3A4 gene transcription through activating SXR, possibly by affecting the other substrates of the CYP3A4, especially affecting the metabolism of drugs in the body (Sun JH et al, 2004).

In opposition to the previous reports on chronic exposure, we did not observe in these short term acute exposures to Zen, noticeable modifications of receptor expression (ESR1, PPARA, RXRA and PXR). Such expected modifications were also not observed upon rat treatments with classical CYP inducers in contrast to receptor involvement into CYP3A expression as an example (Staudinger et al., 2003). These differences also can be explained by the differences between the *in vitro* determinations and the *in vivo* (a more complex system) becoming of Zen and classical inductors compounds, linked to the receptors mRNA expression. A difference of a few hours exists between mRNA increased expression and enzymatic activities. Moreover several factors are involved in the enzymatic expression (maturation and proteolytic degradation for example) which can also be under or over-expressed, modifying not only the amount of expressed protein but also the amount of active protein (normal protein + heme + reductase + electron transfer).

3.2. Zearalenone presence and bio-transformation in the living animal

The strong estrogenic effects of zearalenone and its metabolites and the legislated maximal dietary levels prompted the necessity to determine amounts between 10 and 100 µg in foods and forages. Very sensitive methods (up to ppb) are compulsory for the determination in body fluids and human and animal tissues, for a good determination of the risk level and in the studies of zearalenone metabolism (Fink-Gremmels, 2008). Because of the strong native fluorescent activity of the zearalenone and its metabolites, HPLC methods using fluorescence detector are currently used as enough sensitive and efficient in terms of separation, but only after zearalenone extraction from the sample (Krska and Fresenius, 2001; Visconti and Pascale, 1998; Fazekas and Tar, 2001). The use of gas chromatography is limited by the need to derivatize the phenolic hydroxyl groups; in consequence, only GC/MS determinations have been used largely and deliver data you can rely on (Marques et al., 1998). The thin Layer Chromatography (TLC) and Enzyme-Linked ImmunoSorbent Assay (ELISA) (classical or „Open sandwich” (Suzuki et al., 2007)) are frequently used to determine zearalenone and its metabolites. The facile and robust traits of TLC determinations and its costs are higher than the other methods, but its sensitivity and selectiveness are clearly inferior. ELISA determinations offer sensitiveness comparable to the fluorescent detection. Because of the potential of cross reaction of the antibodies with the matrix components, it is desirable to confirm the results using other techniques, just to avoid false positive, erroneous results, or results with overestimation. Due to the same reason, the simultaneous determination of zearalenone and its metabolites is not possible by ELISA. In general and independent of the used method of detection, or of the sample matrix, difficult protocols are required for the extraction, cleaning and enrichment of the sample, in order to reach a minimal level necessary to determine zearalenone and its metabolites. This is why the very long and laborious liquid/liquid extraction has been replaced by solid phase extraction (SPE) with inverse aminopropyl phase and absorbent immunoaffinity materials or with MycoSep columns containing a mixture of charcoal, ions exchanging resins and other materials (Zollner et al., 2007; Hartmann et al., 2007; Filho Edson et al., 1997; Zollner and Mayer-Helm, 2006; Maragos and Appell, 2007). During the past years methods have been developed for zearalenone determination by laser-induced fluorescent capillary electrophoresis; these methods are capable to analyse between 5 ng/g to 500 ng/g in corn samples, but they are very expensive (Rosenberg et al., 1998).

The literature describes several chromatographic methods for zearalenone and its metabolites' separation, identification and determination from biological samples. Most times, zearalenone is determined alone in cereal grain samples (Rosenberg et al., 1998; Pallaroni et al., 2002 a,b; Pallaroni and von Holst, 2003 a,b; Schneweis et al., 2002; Krska et al., 2003; Pallaroni and von Holst, 2004; Royer et al., 2004; Berthiller et al., 2005). Zearalenone metabolites α -Zal and β -Zal were determined by several methods from fat and kidney samples (Joos and Van Ryckeghem, 1999), from cow muscle and liver (Horie and Nakazawa, 2000), chicken liver (Fang et al., 2002). Other methods are dedicated to the separation and determination of Zen, α -Zol and β -Zol from beer (Zollner et al., 2002) and fish samples (Lagana et al., 2003). There are papers published which describe the separation and determination of Zen, α -Zol, β -Zol, α -Zal and β -Zal from river water (Lagana et al., 2001), pig urine and tissue (Jodlbauer et al. 2000), cattle urine (Launay et al., 2004), eggs (Sypecka et al., 2004) and milk samples (Sorensen and Elbaek, 2005). The latter methods used Zan as internal standard, but it has two major inconveniencies: it is a natural metabolite of zearalenone and it has the same elution as Zen, if C18 columns are used (Sforza et al., 2006). Few are the liquid chromatography methods presented in the literature for zearalenone and all its metabolites separation from biological samples such as cattle urine (van Bennekom et al., 2002), pig liver, urine and muscle (Zollner et al, 2002; Kleinova et al., 2002; Zollner et al, 2003) and cattle liver, plasma and urine (Songsermsakul et al., 2006).

The zearalenone biotransformation within the animal organism is important in order to have a chart of the zearalenone metabolic pathway. But also because of the fact that studies of physical and chemical properties of zearalenone imply that this compound presents ideal characters for a large diffusion in the tissues (see chapter 1) The important differences of sensibility of different species concerning the effects of zearalenone could in part result from the different yields for the formation of different metabolites and from metabolism kinetic differences (Malekinejad H et al., 2006)

3.2.1. Zearalenone and its metabolites in treated rats

3.2.1.1. Assessment of the Zearalenone within rat organism (blood, urine and liver samples)

Sampling of urine, blood and liver have been done in order to determine the bio-transformation of zearalenone in the rat organism. Samples from the second experiment on rats were analysed in order to measure Zearalenone and its metabolites.

Samples of urine have been analysed in order to determine the elimination of zearalenone and of its metabolites. Urine sampling has been done using metabolic cages. The urine samples have been collected between 0 and 24 hours after the Zen treatment. Urine samples have been determined (table 3.6) as described in chapter 2.

Because the samples have been incubated for 15h at 37°C with glucuronidase, the obtained results are the total amount of Zen and its metabolites present in the samples. LC-MS assessment has been done. Selective Zen assessment has been done using as internal standard Zen ¹³C enriched.

The obtained values have varied from 1 to 10.4 µg/ml for Zen, from 1.6 to 6.1 µg/ml for α-Zol and from 0.7 to 2.0 µg/ml for OH-Zen (see table 3.6) upon Zen single treatment.

Plasma from the Zen and PB-Zen treatments were assessed in order to determine the Zearalenone presence within blood circulatory system and to observe the presumed presence of OH-Zen and other reduced metabolites.

Table 3.6. Assessment of Zen and its metabolites in samples of Zen treated rats

Treated rat samples		Zen	α-Zol	OH-Zen
PLASMA		ng/ml	ng/ml	ng/ml
C-Zen 3h		1124 ± 142	n.d.	n.d.
C-Zen 6h		1479 ± 83	n.d.	n.d.
C-Zen 10h		241 ± 124	n.d.	n.d.
C-Zen 24h		35 ± 3	n.d.	n.d.
PB-Zen 3h		748 ± 313	n.d.	n.d.
PB-Zen 6h		1070 ± 543	n.d.	n.d.
PB-Zen 10h		420 ± 109	n.d.	n.d.
PB-Zen 24h		148 ± 32	n.d.	n.d.
LIVER		µg/g	µg/g	µg/g
C-Zen 3h		1216 ± 203	25 ± 4	n.d.
C-Zen 6h		564 ± 114	98 ± 14	17 ± 2
C-Zen 10h		576 ± 94	112 ± 18	34 ± 5
C-Zen 24h		187 ± 20	30 ± 4	14 ± 1
URINE	mL	ng/mL	ng/mL	ng/mL
C-Zen 0-3h	3.0	10382 ± 2076	2316 ± 463	879 ± 176
C-Zen 3-6h	3.0	5535 ± 1107	1823 ± 365	2022 ± 404
C-Zen 6-10h	7.0	974 ± 195	1605 ± 21	652 ± 130
C-Zen 10-24h	7.0	3601 ± 720	6119 ± 1224	744 ± 149
PB-Zen 0-3h	3.0	2785 ± 557	413 ± 83	832 ± 166
PB-Zen 3-10h	6.5	1243 ± 249	2014 ± 403	1565 ± 313
PB-Zen 10-24h	7.0	795 ± 159	2321 ± 464	413 ± 83

Plasma, liver and urine samples, from rats control or PB after 3h, 6h, 10h and 24h single *i.p.* treatment with Zen, were used. The samples have been prepared and assessed as described in the chapter 2. The results are a mean of 3 samples assessment results, done on a LC-MS; the average standard deviation of the results is also represented.

Plasma samples have been assessed (table 3.6) as described in chapter 2. Similarly to the urine samples, plasma samples have been deglucuronidated and the obtained results are the total amount of Zen and its metabolites present in the samples. LC-MS assessment has been done. Selective Zen assessment has been done using as internal standard Zen ^{13}C enriched. Only Zearalenone amounts were determined. The plasma analyses have been limited because of the limited volume of certain samples.

Liver samples were analyzed (table. 3.6) starting from 10ml of S9-liver extracts (about 2.5 mg of liver) as described in chapter 2. Liver samples have been deglucuronidated and the obtained results are the total amount of Zen and its metabolites present in the samples. LC-MS assessment has been done. Selective Zen assessment has been done using as internal standard Zen ^{13}C enriched.

3.2.1.2 Zearalenone becoming within rats' organism - Conclusions

Zearalenone is rapidly eliminated in the urine within the first 6 hours after administration; about **60%** ($47.8 \pm 14.3 \mu\text{g}$) of the total amount of urinary eliminated Zen ($79.8 \pm 23.9 \mu\text{g}$). Interesting is the fact that the peak of metabolites elimination (α -Zol and OH-Zen) is between 6 and 24 hours after administration, about **81%** ($54.1 \pm 16.2 \mu\text{g}$) of a total amount of urinary eliminated α -Zol ($66.5 \pm 19.9 \mu\text{g}$) and about **58%** ($10.6 \pm 3.1 \mu\text{g}$) of a total amount of urinary eliminated OH-Zen ($18.5 \pm 6.0 \mu\text{g}$), respectively. The overall urinary eliminated Zen and metabolites represents about **4%** of the administrated Zen.

Plasmatic presence of Zearalenone is in the early time after administration, a peak of concentration ($1.48 \pm 0.1 \mu\text{g/ml}$) was observed after 6h of Zen administration. No other metabolites were observed.

The highest Zearalenone liver concentration ($1.2 \pm 0.1 \text{ mg/g}$) was observed 3 hours after administration, fact that is certifying the direct implication of liver within Zen detoxification pathway and which is in concordance with the observed early hour presence within plasma and urine. The peak of metabolites (α -Zol, $0.11 \pm 0.1 \text{ mg/g}$, and OH-Zen, $0.03 \pm 0.01 \text{ mg/g}$) presence within liver is between 6 and 10 hours after treatment.

3.2.2. Zearalenone bio-transformation within poultry organism

Meat quality is influenced by several nutritional factors. These factors are directly related to the quality of feedstuff used in the animal daily diets. One of the most important parameters that require quality control is the amount of mycotoxins in feeds; between them we can find Zearalenone, as well.

Meat quality is of high importance for human nutrition. In order to determine the quality of meat several determinations are necessary: the standard chemical composition (ether extractives, crude protein, etc.) and, to have a more precise approach, the fatty acids profiles. Nevertheless, the zearalenone and its metabolites occurrence within chicken meat could be consider an important factor that might need to be determined in order to certify meat quality.

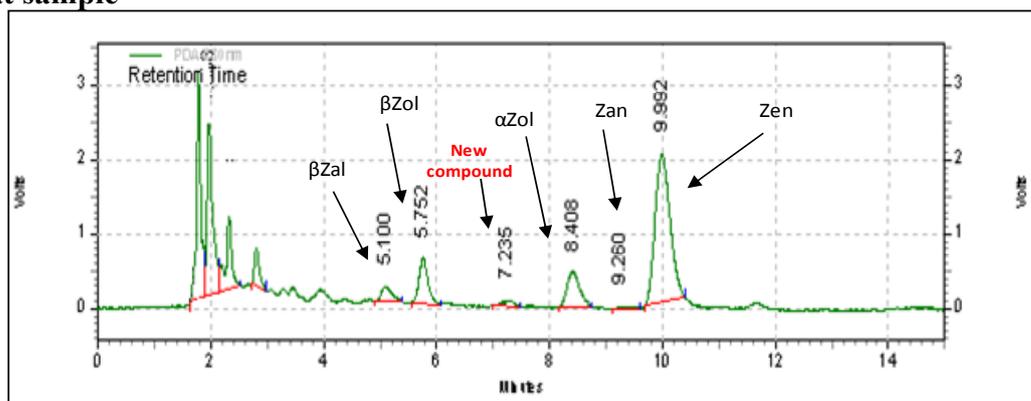
3.2.2.1. Assessment of the Zearalenone in chicken organism (blood, meat and liver samples)

Sampling of the chickens' pectoral and the left leg muscles was done, resulting in 40 meat samples, 5 samples per treatment. Plasma and liver samples have been also collected.

Analysis of Zearalenone has been done for Zen-treated chickens in meat, blood and liver samples, prepared as described in chapter 2 and using the validated HPLC-UV method.

In the chicken *muscle sample* only free zearalenone metabolites can be found; we have done assessment of Zen and its metabolites using glucuronidase/arylsulfatase in order to release the glucuroconjugated zearalenones. The same results have been obtained using or not glucuronidase/arylsulfatase (table 3.7). A similar situation is described by Peter Zollner et al. in 2002, when analyzing samples of pig muscle, exposed to zearalenone contaminated oats.

Figure 3.5. Example of a chromatographic separation of Zen 3 days treated chicken meat sample



The samples have been prepared as described in the chapter 2. The chromatographic separation has been done on a reverse-phase Hypersil Gold (C18), 150 x 4,5 mm, column; using an isocratic separation with water:acetonitrile:methanol (45:8:47,v/v/v) with a content of 50 mM ammonium acetate. The total flow was of 1ml/min, column temperature of 40°C and the chromatographic separation total time of 11 minutes. The DAD detector lambda was of 280 nm.

No α -Zearalanol has been found in the muscle samples, but the presence of new compound can be observed at a retention time of about 7.24 min. This t_R is not corresponding to any of the used standards: Zen ($t_R = 10$ min), Zan ($t_R = 9.26$ min), α -Zol ($t_R = 8.40$ min), β -Zol ($t_R = 5.75$ min), α -Zal ($t_R = 8.20$ min), β -Zal ($t_R = 5.10$ min). A HPLC-MS analysis has been done and the new compound is not an OH-Zen (molecular mass of 334), because its molecular mass is 217.

Table 3.7. Zen and its metabolites assessment in treated chickens muscle and liver samples

	Muscle (ng/g dry sample)		Liver (μ g/g)	
	Zen IP	PB-Zen	Zen IP	PB-Zen
Zen	35.72 \pm 6.12	22.98 \pm 8.86	3.01 \pm 0.32	3.69 \pm 0.77
Zan	1.72 \pm 0.68	0.56 \pm 0.30	-	-
α-Zol	13.42 \pm 2.50	4.08 \pm 1.11	26.46 \pm 2.22	5.48 \pm 0.22
New metabolite	21.45 \pm 11.51	2.74 \pm 1.15	-	-
β-Zol	11.47 \pm 3.95	5.48 \pm 1.29	23.69 \pm 2.05	9.57 \pm 0.95
β-Zal	5.61 \pm 0.94	7.10 \pm 1.60	-	-

The results are a mean of 5 samples assessment results done in duplicate on a HPLC - DAD using a C18 column. Standard curves have been done for all metabolites, HPLC-MS selective assessment have been done in order to be shore of the identified peaks

This could be a Zearalenone degradation (cleavage) product. Another cleavage product, see fig. 3.6, with a molecular mass of 274 has been observed in the in vitro metabolic test on the *Clonostachys Rosea* IFO 7063 by Takahashi-Ando N. et al. in 2002. More research

is required to characterize this new compound; a possible structure is presented in figure 3.7.

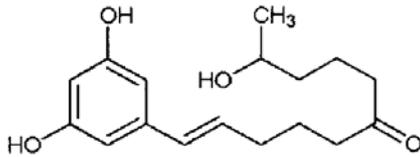


Fig 3.6 – Zearalenone cleavage product of 274 molecular mass (Takahashi-Ando N. et al., 2002)

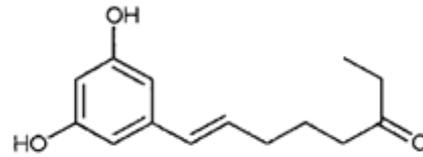


Fig 3.7 –Presumed Zearalenone metabolite of 217 molecular mass

A longer exposure to zearalenone determines an increasing of the zearalenones accumulation levels within chickens' meat.

Plasma sample of Zen IP and PB-Zen 24h treated chickens were analyzed as described in sample preparation in chapter 2 and only Zen could be assessed. For Zen IP treated poultry plasma samples we obtained an average concentration value of 6.62 ± 1.04 ng/ml of Zen. In the case of PB-Zen 24h treated poultry plasma samples we found 3.40 ± 0.54 ng/ml of Zen. A 91 ng Zen/ml in poultry plasma been reported upon 100 mg Zen/kg bw for 8 days (Maryamma KI et al., 1992; Oswald and Taranu I; 2008).

In chicken *liver samples* the occurrence of α - and β -Zol can be observed, but not of OH-Zen metabolite in difference to rat (table 3.6). Also a smaller level of Zearalenone was found in the liver samples, if compared with its metabolites that could imply the idea that a rapid direct elimination of Zearalenone occurs or a very rapid metabolisation process. In chicken, the administration of a diet containing 100 mg/kg zearalenone for 8 days, followed by exposure to 10^9 dpm/kg [3 H] zearalenone revealed that the kinetics of the toxin is rapid, tissue half-life ranging from 24 to 48 hours and a concentration peak was reached in the liver 30 minutes after administration (Mirocha CJ et al., 1982).

Similar results have been reported when feeding poultry on a contaminated diet with 100 mg zearalenone /kg b.w., for 8 days. The samples have been analyzed by GC-MS and only Zen (0.681 μ g/g), α -Zol (1.200 μ g/g) and β -Zol (662 μ g/g) were found in the chicken liver (Mirocha CJ et al., 1982). Comparable levels of Zearalenone have been reported in articles published on chickens meat (170 ng/g, upon 20 days administration of 10 mg Zen/kg bw) and liver (207 ng/g, upon 20 days administration of 10 mg Zen/kg bw) but not all zearalenone metabolites have been determined (Maryamma KI et al., 1992; Olsen M et al., 1986; Danicke S et al., 2001; Oswald and Taranu I; 2008). Most of the

reported results on poultry are from the 80s' and the research results concerning the zearalenone metabolism and tissue distribution remain incomplete.

The levels of α -Zol (13.42 $\mu\text{g}/\text{kg}$, Table 3.7) are higher than the JEFCA accepted level (2 $\mu\text{g}/\text{kg}$) and lower than FDA accepted levels (150 $\mu\text{g}/\text{kg}$) in the muscle samples. Nevertheless, due to the lack of regulation and reported researches concerning Zen and its metabolites co-occurrence in the meat and its consumption effects on human health, we considered that the current chicken muscle obtained upon acute Zen treatments is not suitable for human consumption. Moreover, the α -Zol concentration levels (26.46 $\mu\text{g}/\text{g}$) in the liver samples from the Zen treatments are much higher than the accepted concentration levels by JEFCA (0.01 $\mu\text{g}/\text{kg}$) and FDA (0.3 $\mu\text{g}/\text{kg}$).

3.2.3. Effects of Zearalenone on chicken meat quality

Materials and methods

Instrumentation

KjeltecTM 2300 and SoxtecTM 2055 (FOSS – Tecator – Hilleroed, Denmark) were used. A Gas-Chromatograph with FID detector Clarus 500 system from Perkin-Elmer (Waltham, MA, USA) with N_2 carrier gas and capillary chromatographic column (BPX 70, 60m) was also used. Grindomix GM 200 knife mill from Retsch (Haan, Germany) was used for dried sample grinding.

Chemicals and Solvents

A standard mixture of fatty acids: PUFA No.2 (Animal Source) from Sigma (Deisenhofer, Germany) was used. Methanol, hydrochloric acid, sulphuric acids and sodium hydroxide (all analytical reagent grades) were supplied by Sigma (Deisenhofer, Germany). Ultrapure Milli-Q water was used.

3.2.3.1. Determination of the gross chemical composition of the broiler meat

The following determinations were performed on the 40 chicken muscle samples: dry matter, according to SR ISO 6496:2001, crude protein by Kjeldhal method using a KjeltecTM 2300, in agreement with SR ISO 13325:1995, ether extractives using SoxtecTM 2055, in agreement with SR ISO 6492:2001, gross ash (insoluble residue in hydrochloric acid), residue insoluble in hydrochloric acid, according to STAS 10542/1:1986 on meat broiler samples.

Table 3.8. Gross chemical composition of chickens' meat

Sample Type	Humidity	Total Protein	Ether Extractives (E.E.)	Gross ash
	g/100g sample	g/100g dry sample	g/100g dry sample	g/100g dry sample
Control	73.09 ±2.44	75.76 ±1.94	13.19 ±2.39	4.28 ±0.26
Oil	73.86 ±0.70	73.96 ±2.80	14.99 ±3.29	4.26 ±0.33
Phenobarbital	74.32 ±1.10	74.30 ±2.32	13.94 ±2.52	4.29 ±0.09
Dexamethasone	72.89 ±0.66	72.22 ±3.41	17.26 ±3.82	4.25 ±0.36
β-Naphtoflavone	73.39 ±0.81	73.04 ±1.74	15.20 ±2.02	4.16 ±0.19
Clofibrate	74.07 ±0.40	74.33 ±1.54	14.20 ±1.19	4.29 ±0.25
Zearalenone	73.34 ±1.12	72.98 ±3.78	15.78 ±4.03	4.21 ±0.32
PB – Zen 24h	74.38 ±0.62	74.69 ±1.47	14.32 ±1.89	4.20 ±0.42

The results are the mean of 5 samples assessment results done in duplicate. Statistical analysis has been done using the Student t-Test; no significant differences at $p \leq 0.05$ been found.

The zearalenone treatments (Zen IP or PB-Zen) determined no statistically sustained changes, linked to the standard chemical composition, compared to the Control, Oil or classical inducer treatments. In the case of DM poultry, a higher Ether Extractives level and decreased Humidity and Total Protein levels can be observed, if compared with the Oil treated poultry.

3.2.3.2. Assessment of fatty acids profiles

The fatty acids were determined by transforming the fatty acids of the sample into methyl-esters, using an acidified methanol solution (2% sulphuric acid in methanol); followed by their separation in the Capillary Chromatographic Column (BPX 70, 60m). The fatty acids were identified and quantified using a standard mixture of fatty acids on a Perkin-Elmer gas chromatograph with FID detector and nitrogen gas carrier according to SR ISO 5508: 1990.

In order to see if the acute dose of Zearalenone, used in the current treatments, has determined changes in the fatty acids profile; the fatty acids composition was assessed on the 40 samples of chicken muscle. The table below shows the average results of the fatty acids assessment in chicken muscle.

Table 3.9. Fatty acids profiles within chickens' meat

Fatty acids	Control	Oil	PB	DM	β -Nf	Clof.	Zen IP	PB-Zen 24h
	g/100g EE.							
Myristic	0.50 ± 0.08	0.39 ± 0.07	0.47 ± 0.03	0.41 ± 0.09	0.51 ± 0.11	0.43 ± 0.04	0.46 ± 0.10	0.51 ± 0.05
Palmitic	22.71 ± 1.08	20.85 ± 1.94	22.62 ± 1.17	22.57 ± 0.92	22.03 ± 1.47	21.89 ± 0.57	22.32 ± 1.90	21.56 ± 0.98
Palmitoleic	4.53 ± 0.44	3.69 ± 1.40	4.55 ± 0.89	3.72 ± 0.70	4.14 ± 0.64	3.97 ± 0.42	4.82 ± 0.71	4.70 ± 0.56
Stearic	7.51 ± 0.81	7.52 ± 1.69	7.91 ± 1.22	10.11 ± 2.01	7.70 ± 0.92	8.06 ± 1.59	7.28 ± 1.04	6.98 ± 0.55
Oleic	33.53 ± 1.53	30.00 ± 3.86	33.08 ± 2.46	31.35 ± 2.37	33.45 ± 1.07	31.41 ± 4.75	35.15 ± 5.92	34.72 ± 2.96
Linoleic	27.24 ± 0.99	32.21 ± 4.43	26.82 ± 2.25	27.07 ± 0.97	27.98 ± 3.31	29.49 ± 1.53	25.46 ± 7.44	27.56 ± 1.59
Linolenic	1.44 ± 0.15	2.23 ± 0.68	1.50 ± 0.16	1.32 ± 0.11	1.12 ± 0.09	1.36 ± 0.06	1.39 ± 0.21	1.50 ± 0.11
Arachidonic	2.54 ± 0.48	3.11 ± 1.52	3.05 ± 1.08	3.47 ± 1.22	3.06 ± 0.27	3.36 ± 1.70	3.12 ± 0.73	2.45 ± 1.65
Unsaturated/ Saturated Fatty Acids Ratio	2.26	2.48	2.23	2.02	2.31	2.29	2.33	2.44

The results are a mean of 5 samples assessment results done in duplicate on a GC Perkin-Elmer with capillary column (BPX 70, 60m). The Unsaturated/Saturated Fatty Acids Ratio was calculated on the average values of the assessed fatty acids. Statistical analysis has been done using the Student t-Test; no significant differences at $p \leq 0.05$ have been found.

The zearalenone treatments (Zen IP or PB-Zen) determined no statistically sustained changes, linked to the fatty acids profile, compared to Control, Oil or classical inducers treatments. For the Dexamethasone treatment a diminution of the Unsaturated/Saturated Fatty Acids Ratio was noticed, due especially to the increase of the Stearic Acid level (Saturated fatty acid) and to the decrease of Linoleic and Linolenic Acids (polyunsaturated acids).

The effects on the nutritional proprieties of meat are not well outlined in part because of the experiment timescale, only of 4 days; only a tendency can be observed.

3.2.2.3 Zearalenone becoming within poultry organism – Conclusions

From a nutritional point of view the muscle chicken samples were not altered by the presence of zearalenone; but the occurrence of Zen and its metabolites in chicken meat made it unsuitable for human consumption. To our knowledge, no official regulation has been made concerning the accepted levels of Zen and/or its metabolites admitted levels in poultry meat. Nevertheless, EU policy relies on the fact that the effects of a long consumption of meat produced/contaminated with growth promoters (e.g. α -Zol) on the human organism are not properly documented, very few long-term tests having been

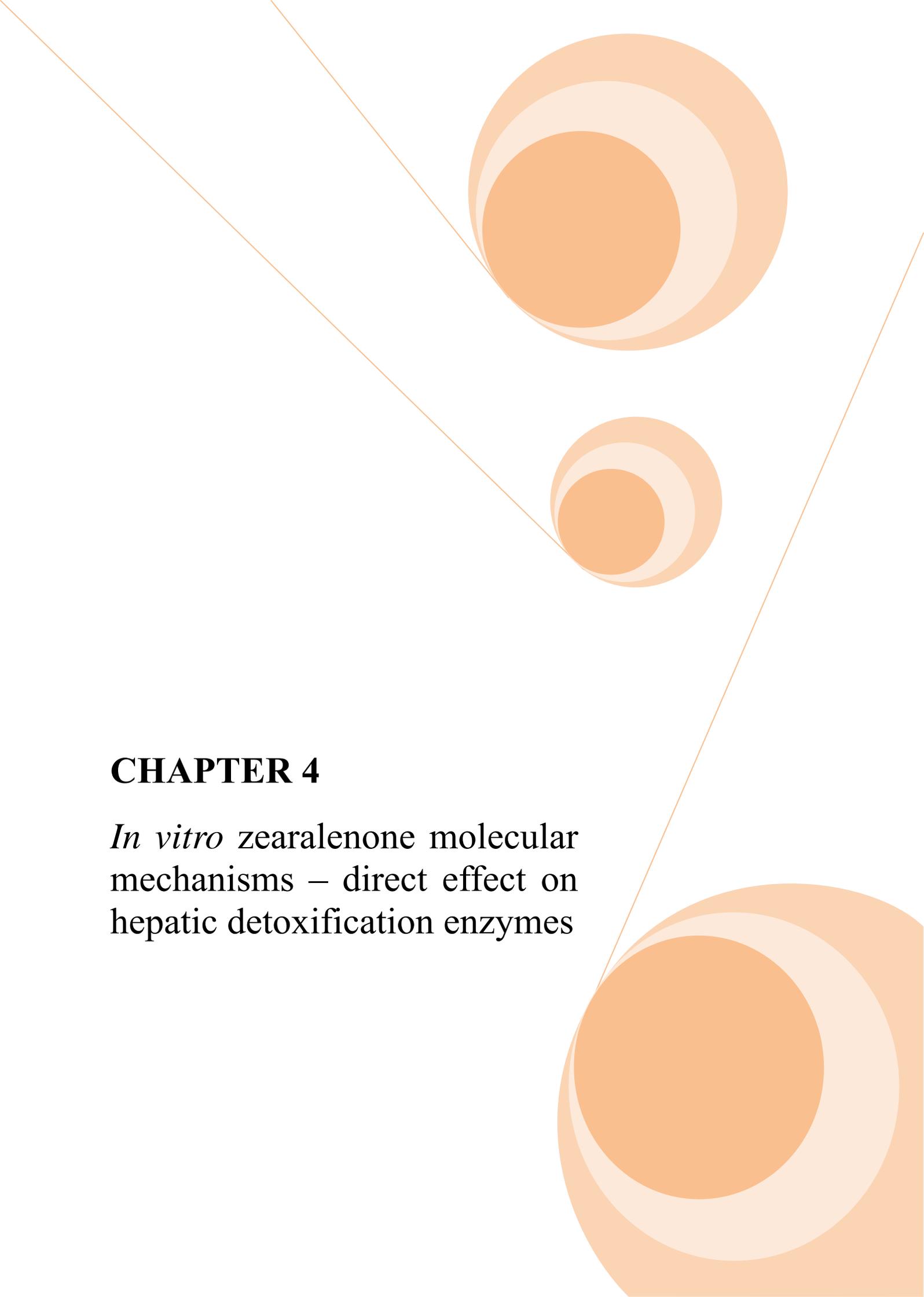
conducted on rats, dogs or monkeys. The Joint FAO/WHO Expert Committee on Food Additives (JEFCA), in contrast with the European Commission, proposed in 1987 an accepted maximum ingested amount of 0.5 µg/kg body mass, which corresponds to a residual level in the liver of 10 µg/kg or 2 µg/kg in the muscles, which are α-zearalenol levels which do not produce hormonal disturbances. The Food and Drug Administration (FDA) set the accepted highest levels of α-zearalenol at 150 µg/kg in muscles, 300 µg/kg in the liver, 450 µg/kg in kidneys and 600 µg/kg in fat from cattle.

Based on the results of a study on the optimization and validation of a HPLC method for the separation and determination of zearalenone and its metabolites in broiler biological samples, presented in chapter 2; the method responds to the requirements to determine zearalenone and its metabolites at ng/g (µg/kg) level in broiler biological samples, allowing the quality control of the samples. Zen and its metabolites can be separated with a proper resolution in 12 minutes using a Hypersil™ Gold C18 column (150 mm × 4.6 mm), using the mobile phase 50 mM AcAm : ACN : MeOH, with 45 : 8 : 47 (v/v/v) ratio, at mobile phase flow rate of 1 mL/min and chromatographic column temperature of 40°C. In the chicken muscle sample only free zearalenone metabolites were found; a similar situation is described by Peter Zollner et al. in 2002, when analyzing samples of pig muscle, exposed to zearalenone contaminated oats. In the dried muscle sample from Zen IP treatment we determined the presence of 35.72 ng/g Zen, 1.72 ng/g Zan, 13.42 ng/g α-Zol, 11.47 ng/g β-Zol and 5.61 ng/g β-Zal. The levels of α-Zol (13.42 µg/kg) are higher than the JEFCA accepted level (2 µg/kg) and lower than FDA accepted levels (150 µg/kg) in the muscle samples. Nevertheless, due to the lack of regulation and reported researches concerning Zen and its metabolites co-occurrence in the meat and its consumption effects on human health, we considered that the chicken muscle obtained by acute Zen treatments is not suitable for human consumption.

In plasma samples only Zen could be assessed (6.62±1.04 ng/ml for Zen IP treatment); the Zen metabolites were not detectable. In liver we only found Zen, α-Zol and β-Zol in concentrations of: 3.01 µg/g, 26.46 µg/g and 23.69 µg/g, respectively for Zen IP treatment; no zearalanones were found. The α-Zol concentration levels (26.46 µg/g) are much higher than the concentration levels accepted by JEFCA (0.01 µg/g) and FDA (0.3 µg/g)

The results of zearalenone and its metabolites assessment within plasma and liver PB-Zen treated chickens are much lower than the PB-Zen 24h treated rats, about 40-50 times

lower. In the PB-Zen chicken plasma we determined a Zen concentration of 3.4 ± 0.5 ng/ml and in the PB-Zen 24h rat plasma, a concentration of 148.2 ± 31.8 ng/ml. Large differences have also been observed for liver assessment of zearalenone: 3.7 ± 0.8 $\mu\text{g/g}$ for chicken liver and 187.0 ± 20.2 $\mu\text{g/g}$ for rat liver; and of α -zearalenol: 5.5 ± 0.2 $\mu\text{g/g}$ for chicken liver and 29.7 ± 3.6 $\mu\text{g/g}$ for rat liver. Another difference is the occurrence in chicken liver of β -zearalenol (9.6 ± 0.9 $\mu\text{g/g}$) and in rat liver of hydroxy-zearalenone (14.2 ± 1.3 $\mu\text{g/g}$).

The background features three large, overlapping orange circles of varying sizes, each with a lighter orange gradient. Two thin orange lines cross the page diagonally, one from the top-left to the bottom-right, and another from the top-right to the bottom-left, intersecting near the center.

CHAPTER 4

In vitro zearalenone molecular mechanisms – direct effect on hepatic detoxification enzymes

4.1. Assessment of Zearalenone metabolism on different species and expressed human P450

The implication of P450s and HSDs is important to be determined in order to have a wider view of the enzymes involved in zearalenone detoxification pathway and to understand the metabolic response and species specificity. Until now zearalenone metabolism studies made on mammals concerned only α/β -ZOLs, α/β -ZALs or ZAN. The formation of a new hydroxylated metabolite was reported only in moulds extracts (El-Sharkawy et Abul-Hajj, 1988), without proving its existence within animal organism. A more recent study have showed the formation of a hydroxylated Zen metabolite in rats (Pfeiffer et al., 2007). We paid a special interest, since 2005, to the formation of this new metabolite and its occurrence in the animal organism (more details about its structure and characterization can be found in Frederique Bravin thesis, presented in December 2008).

Material and method:

Rat and chicken microsomal extracts were prepared from thawed liver tissue using ultracentrifugation according to the method described by Peyronneau et al. (1992). The resultant microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4) with 20% glycerol, frozen in liquid nitrogen and stored at -80°C until use.

In vitro zearalenone metabolism:

The profile of zearalenone metabolism was assessed using *in vitro* incubation of different types of poultry, rat, human and other species microsomes (at a concentration of 1nmol P450/ml) and adding a known quantity of zearalenone ($50\mu\text{M}$).

Incubations were made at 37°C for 30 minutes, using a NADPH generating system (1 mM NADP + 10 mM Glucose-6-Phosphate, 1 UI de G6PDH), $100\mu\text{M}$ MgCl_2 and 0.1 M phosphate buffer (pH 7.4). The reaction was stopped with acetonitrile, placed a few minutes at -20°C , centrifuged at 7500 g and the supernatant was injected in a HPLC-DAD with a C18 column in order to separated and identify the formed metabolites.

The incubations made at pH 4.5 have been done using the same methodology; only the used buffer was 0.1 M citrate buffer (pH4.5) and the generating system replaced with $50\mu\text{M}$ NADPH.

In vitro estradiol and testosterone metabolism:

Similar incubations have been made like in the case of the *in vitro* zearalenone metabolism assessment at pH 7.4, with the differences that instead of zearalenone we used $200\mu\text{M}$ of estradiol or testosterone.

X-RODs and BFC O-dealkylase activities assessment:

In 96 wells Costar black plates we determined the X-ROD and BFC O-dealkylase kinetics for different microsomes at 1 μ M, in phosphate buffer pH7.4 and using as non-fluorescent substrates X-ROD or BFC at 100 μ M. The fluorescence of the samples was determined using a Fluorimetre Tecan GENios (extinction of 530 nm and emission of 550 nm) at different times after the start of reaction (the adding of 0.5mM NADPH) up to 30 minutes, every 3 minutes. The fluorescence measurements were made at 37°C, with agitation before each determination. Control wells (without NADPH) and standard wells (resorufin/HFC standards) were assessed with every determination.

4.1.1. Zearalenone metabolism on treated rats

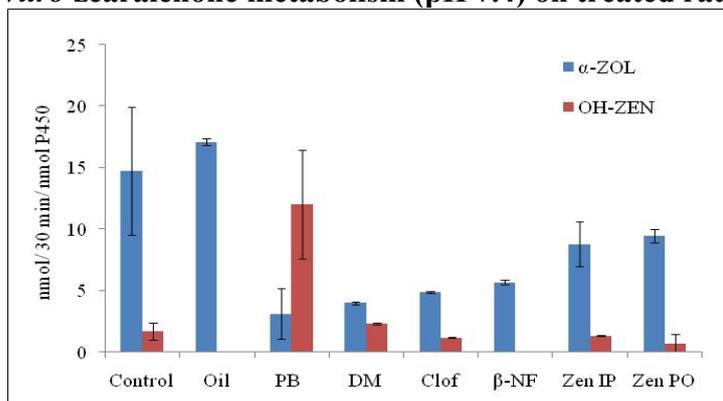
In order to assess zearalenone metabolism in the treated rats, the microsomes were prepared from rat liver samples, treated as described in chapter 3 – First experiment: Rat 3 days experiment. The rats have been treated with Zen and classical CYP inducers.

4.1.1.1 P450 mono-oxygenases activities measured at pH 7.4 on treated rats

Results and discussions:

In rat microsomes incubation at pH 7.4, we observed the formation of a new metabolite that is formed in important quantities especially with PB treated rats microsomes.

Fig 4.1 - *In vitro* zearalenone metabolism (pH 7.4) on treated rat microsomes

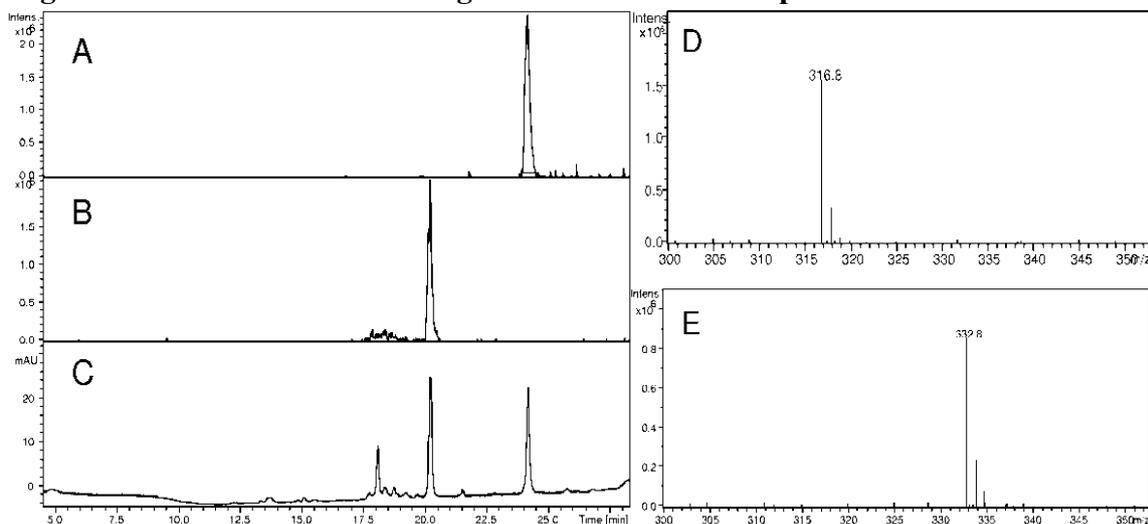


Current results are an average of 3 distinct incubations done in duplicate. Standard curves have been done for all commercially available metabolites, HPLC-MS selective assessment have been done in order to be shore of the identified peaks. The OH-Zen was assessed using Zen standard curve assuming it has a close extinction coefficient.

The formation of this new metabolite, named OH-Zen, was first observed by Frederique Bravin, within incubation of Zen with phenobarbital microsomes. This new compound has different retention time that α -Zol, which is also formed at pH7.4. The OH-Zen has an m/z

of 332.8, using ESI mass spectrometry in negative mode, corresponding to a mono-hydroxylated metabolite of Zen (M+16).

Figure 4.2: HPLC-UV chromatograms and HPLC-MS spectra of Zen and OH-Zen



(A) HPLC-MS chromatogram's of extracted ions m/z 316,8 (ZEN) and (B) m/z 332,8 (OH-Zen) ; (C) HPLC-UV at 280 nm; (D) MS spectra of a 23,5 min peak corresponding to Zen and (E) a 20 min peak corresponding to OH-Zen.

In order to determine if one or several P450 isoforms are implicated in OH-Zen (20 min peak) formation and taking into account the RT-PCR results (presented in chapter 2), which imply the idea that rat CYP2Cs are involved in zearalenone metabolism, we have selected several rat 2C family cytochromes: 2C6, 2C11, 2C12 and 2C13 (Sigma), to use for *in vitro* incubation with Zen. Unfortunately, we could not study the activity of 2C7 form, which was shown by RT-PCR research to be the most active, because it was not commercially available. 60 minutes incubation was done using the classical conditions, 50 μ M Zen and 1 μ M of each P450 at pH7.4, and the supernatant was directly analyzed by LC-MS.

We also note that several forms of OH-Zen are present in different incubations. Up to 5 different forms of hydroxy-zearalenone (the OH groups are formed on different carbons of Zen aliphatic part; structural studies been made by Frederique Bravin and presented in her thesis) are present. The CYP2C6, 2C11, 2C12 and 2C13 produce different forms of mono-hydroxylated Zen metabolites. In order to compare their implication in the formation of the OH-Zen (RT=20.7 min), which is formed specifically on microsomes of PB treated rat, we compared the level of its formation regardless of the other forms (table 4.1).

Table 4.1: Metabolisation level of Zen in OH-Zen (RT = 20.7 min) by different forms of expressed CYP2C forms

Rat	pmol OH-Zen (RT = 20.7 min) /nmol P450/min	pmol hydroxylated metabolites (333 m/z) /nmol P450/min	% OH-Zen (RT = 20.7 min) / total hydroxylated metabolites (333 m/z)
PB	167	167	100
2C6	6	37	15
2C11	40	193	21
2C12	0	27	0
2C13	9	21	43

The third column represents the percentage of produced OH-Zen by the rat PB microsomes or rat expressed forms, related to the total hydroxy metabolites (m/z 333) formed by 2C forms. We did not verify the linear formation of OH-Zen by the expressed forms. We have determined the level of OH-Zen formation at 60 minutes of incubation assuming the MS response is identical to that of Zen.

The 2C11 form metabolizes better Zen to OH-Zen, then the other forms. If we take into account the sum of hydroxylated metabolites (m/z 333), rat CYP2C11 produces 10 time higher quantity of hydroxylated metabolites (all the forms) then CYP2C13.

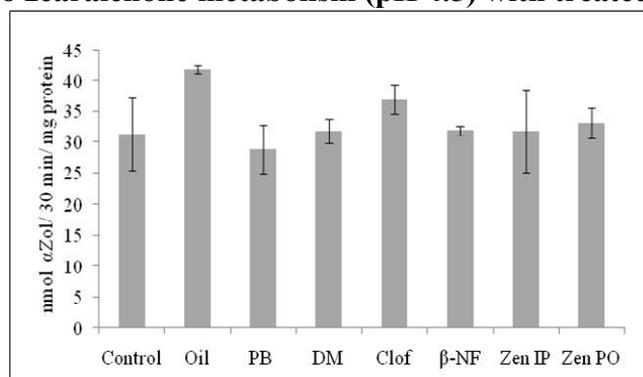
This result is in concordance with the one obtained with the expressed human forms; the human CYP2C8 being responsible (more than others human forms) for the formation of this OH-Zen (46 pmol/nmol P450/min) (table 4.4).

We note the fact that none of the tested expressed rat CYP2C formed specifically OH-Zen, like in the case of the PB rat microsomes and of the *in vivo* experience; forming several hydroxylated metabolites. Also, neither of the tested expressed forms, had an activity comparable with the PB treated rat microsomes. It is necessary to test CYP2C7 activity, which was not possible to procure when the present research was done and which is possible to be responsible for OH-Zen formation in rat.

4.1.1.2 *In vitro* zearalenone metabolism at pH 4.5 on treated rats

Results and discussions

Fig 4.3 - In vitro zearalenone metabolism (pH 4.5) with treated rat microsomes



The results are an average of 3 distinct incubations assessment done in duplicate. Standard curves have been done for α -zearalenol, using commercial standard.

Concerning the incubation at pH 4.5 it can be observed that the different rat treatments are not influencing the α -Zol formation. Responsible for the formation of α -ZOL and β -ZOL at pH4.5 are the steroids dehydrogenases, 3α and 3β HSD (Malekinejad et al., 2006). No important influences of *in vivo* treatments on the zearalenone metabolism at pH4.5 seem to occur in rats.

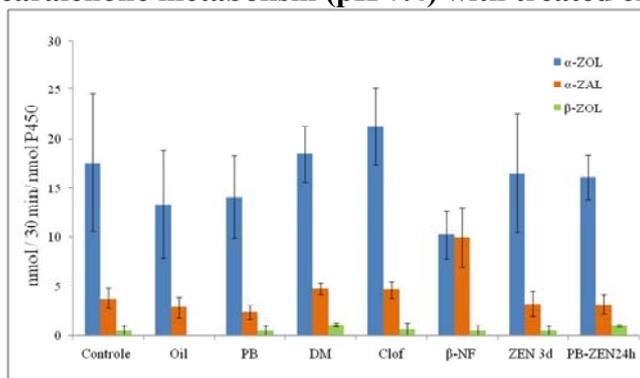
4.1.2. Zearalenone metabolism in treated poultry

4.1.2.1 P450 mono-oxygenases activities measured at pH 7.4 in treated chickens

Results and Discussions

To observe zearalenone metabolisation by chicken P450 enzymes, we have done microsomal incubations in the presence of 50 μ M Zen at pH 7.4 (using Phosphate Buffer 0.1M), using chicken microsomes from the Third experiment – Poultry treatment, presented in chapter 3.

Fig 4.4 - *In vitro* zearalenone metabolism (pH 7.4) with treated chicken microsomes



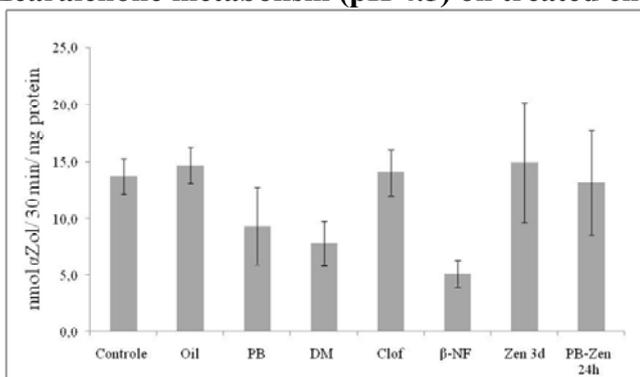
Current results are an average of 3 distinct incubations done in duplicate. Standard curves have been done for all metabolites; HPLC-MS have been done in order to quantify the α -Zol and α -Zal because with the used HPLC method the two metabolites have the same elution time.

Microsomes incubation at pH7.4 shows no significant changes for Zen and PB-Zen24h treatment, compared to the Control/Oil metabolic activity. Concerning the classical P450 inductors treatments, changes in metabolites profile can be observed for β -NF treated poultry microsomal extracts, with a diminution of α -Zol and an increase of α -Zal formation.

4.1.2.2 *In vitro* zearalenone metabolism at pH 4.5 on treated chickens

Results and Discussions

Fig 4.5 - *In vitro* zearalenone metabolism (pH 4.5) on treated chicken microsomes



Current results are an average of 3 distinct incubations done in duplicate. Standard curves have been done for α -zearalenol, using commercial standard.

Zearalenone *in vitro* metabolism at pH4.5 is dominated by α -Zol formation, with an important decrease for the PB, DM and β -NF treated poultry microsomal extracts, implying the idea that PB, DM and β -NF in poultry could influence the zearalenone

metabolism at pH4.5. Zearalenone treatments are having the same level of formed α -Zol as the Control/Oil treatments.

4.1.2.3. Zearalenone metabolism in treated poultry – *Conclusions*

Differences can be observed between the metabolic profile of chicken and rat microsomes. α Zal and β Zol formation occurs during chicken *in vitro* Zen metabolism at pH 7.4, but they are not present in rats. In the case of rat microsomes we can observe the formation of OH-Zen, which does not occur in the chicken microsomes. The total average metabolites formation for poultry is about 4.9 ± 0.8 nmol/30 min/mg protein, which is about 4 times lower than the rat total average metabolites formation (18.5 ± 8.1 nmol/30 min/mg protein).

Zearalenone *in vitro* metabolism at pH 4.5 is dominated by α Zol formation, but 2 to 4 times lower levels can be observed for chicken treatments, if compared to rat. PB, DM and β -NF in poultry could influence the zearalenone metabolism at pH4.5; this effect has not been observed in treated rats.

4.1.3. Zearalenone metabolism in human microsomes, expressed human P450 and other species microsomes

To observe zearalenone metabolism by P450 enzymes, we have done microsomal incubations in the presence of $50\mu\text{M}$ Zen at pH 7.4 (using Phosphate Buffer 0.1M), using human microsomes, expressed human P450 and other species microsomes, obtained from different sources: human microsomes were purchased from Xenotech; expressed human 2 family cytochromes were provided by Tebu-Bio (human cytochromes Cypex and human CYP-reductase expressed in *Escherichia coli.*), the other expressed CYPs were commercially available (e.g. BD Bioscience); the animal species were procured from commercial sources or from other laboratories (e.g. pig microsomes were from the Laboratory of Toxicology, INRA-Toulouse).

4.1.3.1. Zearalenone metabolism in human and other species microsomes

Results and discussions:

The P450 mono-oxygenases activities measured at pH 7.4 under the same incubation conditions, rat PB microsomes are 10-fold more active than the humane female microsomes. The human male microsomes do not form any detectable OH-Zen quantities (table 4.2). The difference between OH-Zen quantities produced by the human microsomes (male and female mixture) and the exclusively male or female microsomes could be explained by a higher content of female microsomes than male ones in the group of human microsomes. Other compounds have also been formed during these incubations, but we focused on OH-Zen, α - and β -Zol formation.

Table 4.2: *In vitro* metabolism of zearalenone at pH7.4 with human microsomes and other animal species (mammals) microsomes

	α -ZOL	β -ZOL	OH-ZEN
Human Female ¹	3.3±0.5	0.3±0.1	0.4±0.1
Human Male ²	3.6±0.3	n.d.	traces
Human ³	5.3±0.9	2.6±0.3	1.4±0.4
Baboon	2.2±0.7	0.6±0.4	7.7±4.6
Primate	3.3±0.4	n.d.	8.1±0.4
Cyno LM	4.0±0.4	n.d.	8.4±4.0
Pig	7.4±0.6	10.0±1.8	n.d.
Chicken	17.5 ±7.0	0.5±0.4	n.d.
Dog	2.8±0.3	n.d.	8.1±0.5
Rabbit	2.5±0.4	1.9±0.4	2.2±0.2
Rat	14.73±5.24	n.d.	1.68±0.69
Mousse	41.3±11.5	n.d.	n.d.

The Zen and its metabolites levels were determined after 30 minutes incubation, using 1 μ M of cytochrome P450, 50 μ M of Zen and 1mM NADPH generating system within phosphate buffer at pH7.4. The results are expressed in nmol /nmol P450 /30 min at 280nm wavelength (n=4, «n.d.» signify not detected and « traces » signify detected but under quantification level). The human microsomes utilized hereby are a pool of male and female microsomes.

An interesting fact that arises from the obtained results, presented hereby, is the formation of OH-Zen as a major compound in the Baboon, Primate, Cyno LM and Dog. The pig microsomes metabolize Zen to α and β -Zol; no OH-Zen formation was observed. The

¹ XenoTech LLC lot 0410044

² XenoTech LLC lot 0610050

³ The lot of human microsomes used here consisted of male and female microsomes.

mouse microsomes are metabolizing very well Zen to α -Zol; no β -Zol or OH-Zen metabolites were determined.

The *in vitro* metabolism of zearalenone at pH4.5 (using Citrate Buffer 0.1M) was determined using the same microsomal extracts of human and other species.

Table 4.3: *In vitro* metabolism of zearalenone at pH4.5 with human microsomes and other animal species microsomes

	α -ZOL	β -ZOL
Human	n.d.	n.d.
Baboon	4.9±1.2	0.9±0.2
Primate	4.9±0.6	0.7±0.1
Cyno LM	2.0±0.6	n.d.
Pig	n.d.	n.d.
Chicken	13.7±1.6	n.d.
Dog	7.2±1.1	n.d.
Rat	31.3±5.9	n.d.
Mouse	25.5±2.6	n.d.

The Zen and its metabolites levels were determined after 30 minutes incubation, using 1 μ M of cytochrome P450, 50 μ M of ZEN and 500 μ M NADPH within citrate buffer at pH4.5. The results are expressed in nmol/mg prot/30min at 280nm wavelength (n=4, « n.d. ». means not detected).

4.1.3.2. Zearalenone metabolism on expressed human P450

Results and Discussions

In order to determine which human isoform(s) is involved in OH-Zen formation we have made incubations of Zen with different human P450 isoforms. Due to the fact that incubation of Zen with rats PB microsomes leads to the formation of important quantities of OH-Zen (RT=20 min), we made incubations of 50 μ M Zen with 1 μ M of different isoforms of human P450 induced by the treatment with barbiturics: forms 1 (CYP1A1, 1A2), forms 2 (2D6, 2C19, 2E1, 2A6, 2C8, 2C9, 2B6, 2C18) and forms 3 (3A4 et 3A5).

The incubation time has been of 60 minutes in order to obtain sufficient quantities of metabolite. We want to specify that the reaction of OH-Zen (RT=20 min) formation using rats PB, as well as the expressed forms, is not linear. The rats PB microsomes were more efficient, 30 minutes are sufficient to obtain a detectable quantity of OH-Zen.

The incubation was analyzed by LC-MS, the level of Zen metabolism by human expressed forms was not sufficient to be explored only from the UV signals (280nm): the obtained peaks are too small to be taken into account without a mass confirmation of the formed compounds.

Table 4.4: Concentrations of P450s and total proteins of expressed forms of families 1, 2 and 3 humans used for the Zen incubations

Expressed P450s	Humans expressed forms		Obtained metabolite after 60min of Zen incubation
	[P450] nmol/mL	[proteins] mg/mL	OH-Zen pmol/nmolP450/min
2D6	3.8	26.8	Traces
2C19	4.7	15	Traces
2E1	5.5	16.2	Traces
2A6	3	10.1	Traces
2C8	9.1	6	46 ± 4
2C9	2.1	16.3	Traces
2B6	3.3	12.2	Traces
2C18	5.9	8.6	Traces
3A4	3.9	14.8	3.7 ± 0.3
3A5	1.4	11.7	Traces
1A1	4.2	23.5	n.d.
1A2	1	6	n.d.

The OH-Zen turnover was calculated starting from the OH-Zen (RT=20 min) peak obtained after 60 minutes of incubations of Zen at pH7.4. The use of a Zen external standard permitted the assessment of formed metabolite (n=4).

The human forms 3A4 are partially metabolizing Zen to OH-Zen. The 1A1 and 1A2 does not form OH-Zen. Only the 2C8 and 3A4 forms led to enough OH-Zen to be quantified. . 2C8 seems to favour the formation of a metabolite that we have determine as the hydroxy-Zen (RT=20 min). The conversion level of this metabolite is about 1% (related to the initial amount of Zen) or a turnover of about 40pmol/nmolP450/min. It was determined (UV quantification at 280 nm) by the integration of OH-Zen peak and of a Zen external standard, which allowed us to calculate the quantity of formed OH-Zen. We assumed, based on the identity of the aromatic part of Zen and OH-Zen structures, that both

compounds have the same extinction coefficient. Our results have been confirmed by the mass spectrometry analysis of the formed metabolite with an m/z 332.8 in negative mode. The mass spectrometry analysis showed the presence of a compound with a mass of m/z 333 for all the CYP2 family (between 0.2 and 0.3% of metabolism related to Zen initially present), but the 2B6, 2C8 and 2C19 produced this hydroxy more than the other forms (~1%). In order to confirm Zen metabolism by human 2C8 forms, Frederique Bravin, has done incubation of 50 μ M ZEN in the presence of human CYP2C8 or rat PB microsomes and of a substrate of 2C8 forms: Paclitaxel[®] (commercial name of taxol) at 20 μ M. The conclusion was that Paclitaxel influenced the formation of OH-ZEN, which confirmed the implication of human 2C8 in its formation.

4.1.3.3 Zearalenone metabolism on human microsomes, expressed human P450 and other species microsomes - *Conclusions*

The female microsomes are metabolizing Zen to OH-Zen in small quantities; the male human microsomes are not at all metabolizing Zen to OH-Zen. Nevertheless, we observed that the two types of human microsomes are metabolizing Zen to α - and β -ZOL, which is in agreement with the literature (Gaumy *et al.*, 2001).

In rat microsomes incubation at pH 7.4, we observed the formation of a new metabolite that is formed in important quantities especially with PB treated rats microsomes. In order to see its distribution and formation in other species' microsomes, we have made the same incubations with microsomal extracts from different species. Almost all the species are producing this metabolite in different proportions. The primates and the dog are producing the most important quantities of OH-Zen, a turnover of about 8 nmol/nmol P450/30 min. Interesting is the fact that the mouse and pig are not forming this metabolite *in vitro*.

Concerning the incubation at pH4.5 it can be observed that the different rat treatments are not influencing α -Zol formation, more research must be done at a lower protein concentration in order to be sure of these results. Differences can be observed between species; noteworthy is the non-metabolism of pig microsomes at pH4.5 and the occurrence of β -Zol in Primate and Baboon microsomes.

The CYPs 2C8 and 3A4 are responsible for the formation of OH-Zen, CYP2C8 in a more impotent manner (46 vs. 3,7 pmol OH-ZEN/nmol P450/min). Female microsomes also metabolize ZEN to OH-Zen, contrary to the male microsomes (13 vs. traces pmol OH-

ZEN/nmol P450/min). The last result is in agreement with a more important activity of 2C8 isoform, specified on the microsomal extract, for female microsomes than for the male microsomes: the hydrolase specific activity of 2C8 was 1.8 times more important in the female microsomes than in the male ones (XenoTech LLC group 0410044 for females and 0610050 for males).

4.2. Interactions of Zearalenone and its metabolites on the enzymatic metabolism of reference compounds

In order to determine the Zearalenone effect on the P450 metabolic activity and the isoform(s) implicated in the detoxification of Zen, we used a fluorimetric metabolic activity assessment method. The method principle is the quantification of fluorescence changes; it is important that the substrate and the metabolite have not the same fluorescence (table 4.5).

Table 4.5 – Non fluorescent substrates and CYPs implicated in their metabolisation

Substrates (non fluorescent)	Metabolites (fluorescent)	Implicated mammalian CYP
Benzyloxyresorufin	Resorufin	CYP 3 family
Methoxyresorufin	Resorufin	CYP 1 family
Ethoxyresorufin	Resorufin	CYP 1 family
Pentoxyresorufin	Resorufin	CYP 2 family
7-BFC	7-HFC	CYP 3 family

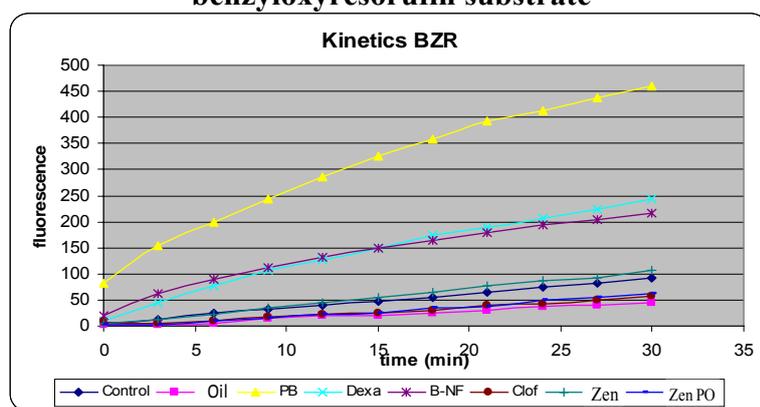
The determination been done as describe within the Material and Method part of the current chapter, using extinction and emission wavelengths of 530 nm and 550 nm, respectively. In these conditions neither NADPH nor Zen are fluorescent; non-interfering with fluoresces determination for the formed metabolites.

Resorufin analogues provide a convenient and sensitive fluorimetric method to assess changes in xenobiotic metabolism *in vitro* (Lubet et al., 1990; Crespi et al., 1997). The benzyloxyresorufin O-debenzylase activity (considered to be CYP3A-specific), methoxyresorufin O-demethylase activity (considered to be CYP1A2-specific), ethoxyresorufin O-deethylase activity (considered to be CYP1A1-specific), pentoxyresorufin O-depentylase activity (considered to be CYP2B-specific), the BFC O-

debenzylase activity (considered to be CYP3A4-specific) were studied on microsomal extracts (Pohl et al., 1980; Nerurkar et al., 1993; Hari Kumar et al., 2006; Renwick et al., 2001; Donato et al., 2004).

An example of kinetics assessment is presented in the next graph (Fig. 4.6).

Fig 4.6 - Determination the metabolic activity cytochrome P450 by fluorimetry, using benzyloxyresorufin substrate



96 wells costar black plates were used to determine the BROD O-dealkylase kinetics of treated rats' microsomes at $1\mu\text{M}$, in phosphate buffer pH7.4 and using as non-fluorescent substrates benzyloxyresorufin at $100\mu\text{M}$. The fluorescence of the samples was determined as describe within Material and Methods chapter. Control wells (without NADPH) and standard wells (resorufin standards, in the presence of PB microsomes) were used. Also the resorufin stability in the presence of different microsomes has been done.

The same kinetics was made for all the substrates and then we have calculated the metabolic activities for each substrate and each type of microsomes. The initial velocity was calculated in the linear part of the kinetics curves, usually between the 3 and 6 minutes points of the kinetics curves.

4.2.1. X-ROD and 7-BFC metabolism in treated rats microsomes

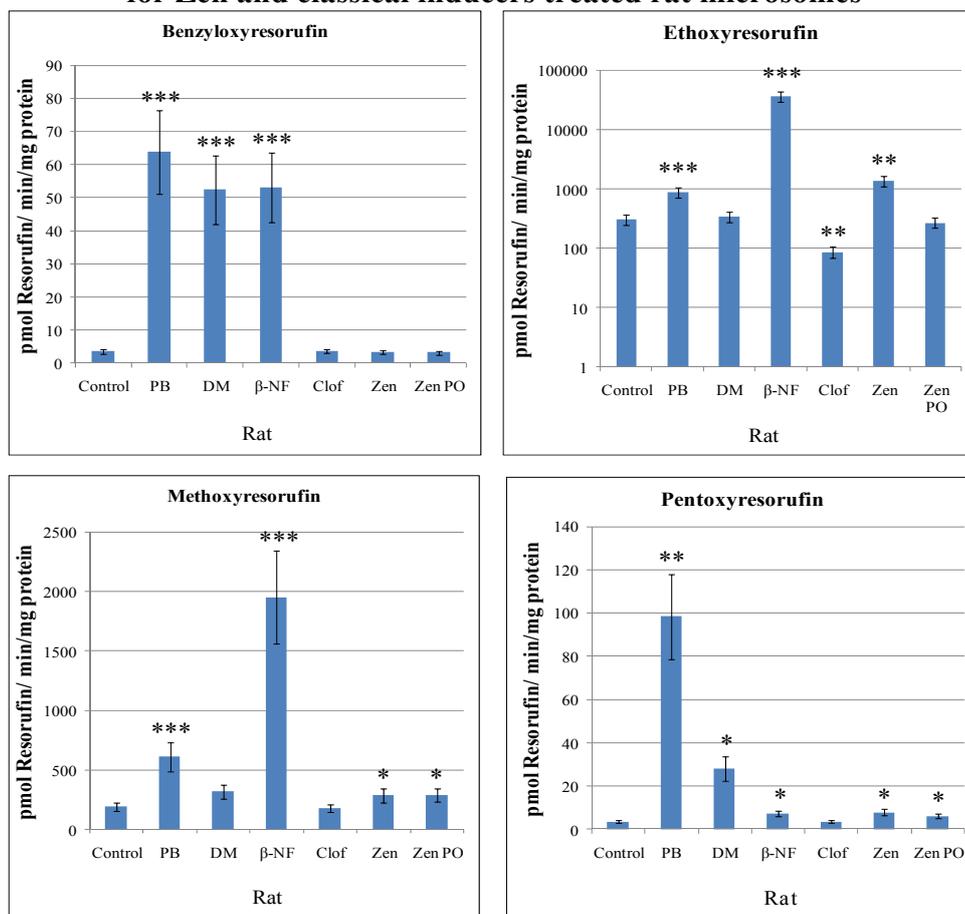
4.2.1.1. Metabolic activity of resorufin derivates (X-ROD) on Zen and classical inducers treated rat microsomes

Results and Discussions

The results from the X-ROD O-dealkylase assessment in the microsomal extracts of treated rats (from the first experiment presented in chapter 3) are presented in the next graphs (Fig. 4.7).

The microsomes Zen IP are showing a significant ethoxyresorufin and methoxyresorufin O-dealkylase activities that is “following” the microsomes of rat treated with β -Naphthoflavone. This led us, to think that the Zearalenone could be an activator of the CYP1A. Nevertheless, in the case of pentoxyresorufin O-depentylase activity zearalenone treatments are showing higher activities, statistically sustained, if compared to the control. This could imply the idea that CYP2 family might be implicated within the zearalenone metabolism.

Fig 4.7 - X-ROD metabolic activity profile for Zen and classical inducers treated rat microsomes



The results are an average of 3 distinct assessments done in duplicate. Fluorimetric assessment by kinetics curves determination, from 0 to 30 minutes. The formation of the fluorescent metabolite (metabolic activity) was determined within the linear part. A standard curve has been done using resorufin standards. Statistical analysis has been done using the Student t-Test; significant differences * for p<0.05, ** for p<0.01 and *** for p<0.005 related to Control.

Microsomes Zen PO are showing a very low activity concerning the X-ROD; only in the case of methoxyresorufin the same level of activity can be observed for the two zearalenone treatments.

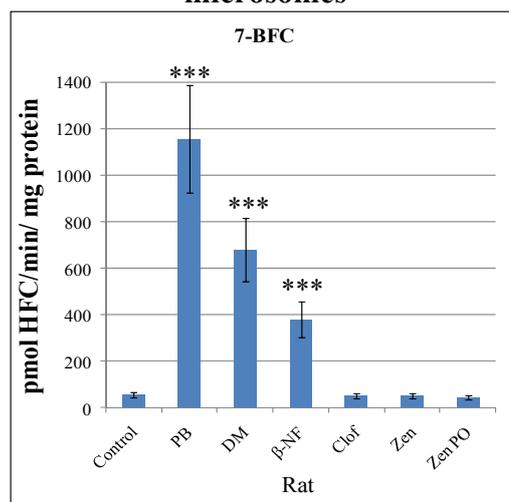
The microsomes from the rats treated with β -Naphthoflavone (inductor of CYP 1A) have a very high metabolic activity for ethoxyresorufin and methoxyresorufin. The two types of resorufin derivatives are specific for the forms CYP 1 family.

4.2.1.2. Metabolic activity of the 7-Benzyloxy-4-(trifluoromethyl)-coumarin (7-BFC) on Zen and classical inducers treated rat microsomes

Results and Discussions

7-BFC is metabolized to 7-HFC by Cyt 3A, that is one of the substrates with a very good specificity for the enzymes 3A. The results from the BFC O-dealkylase assessment on microsomal extracts of treated rats (from the first experiment presented in chapter 3) are presented in the next graph (Fig. 4.8).

Fig 4.8. 7-BFC metabolic activity profile for Zen and classical inducers treated rat microsomes



Fluorimetric assessment by kinetics curves determination, from 0 to 30 minutes. The formation of the fluorescent metabolite (metabolic activity) was determined within the linear part. A standard curve has been done using 7HFC standards. Statistical analysis has been done using the Student t-Test; significant differences * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.005$ related to Control.

Microsomes Zen IP and Zen PO are having the same metabolic activity in 7-BFC like the Control microsomes. These results are suggesting the idea that the zearalenone is not an inductor of CYP 3A.

The rat treated microsomes with PB, Dexa and β -NF are showing the higher metabolic activity on the BFC. This fact being in agreement with the fact that PB is a good inductor of all the types of P450, that DM is a specific inductor of the forms 3A and that β -NF induces especially the forms 1A, and in a smaller percentage 3A.

4.2.1.3. X-ROD and 7-BFC metabolism in treated rats microsomes - *Conclusions*

X-ROD O-dealkylase activity assessment highlights two major ideas: that zearalenone might induce the CYP 1 family, idea that arises from the EROD and MROD O-dealkylase activities of Zen IP treatments microsomes; and that zearalenone might also induce the CYP 2 family, idea that arises from the PROD O-dealkylase activities for Zen treated rats, regardless of the treatment way.

Zen treated microsomes are having the same metabolic activity in BFC as the control microsomes, suggesting the idea that the zearalenone might not be an inductor of CYP 3A.

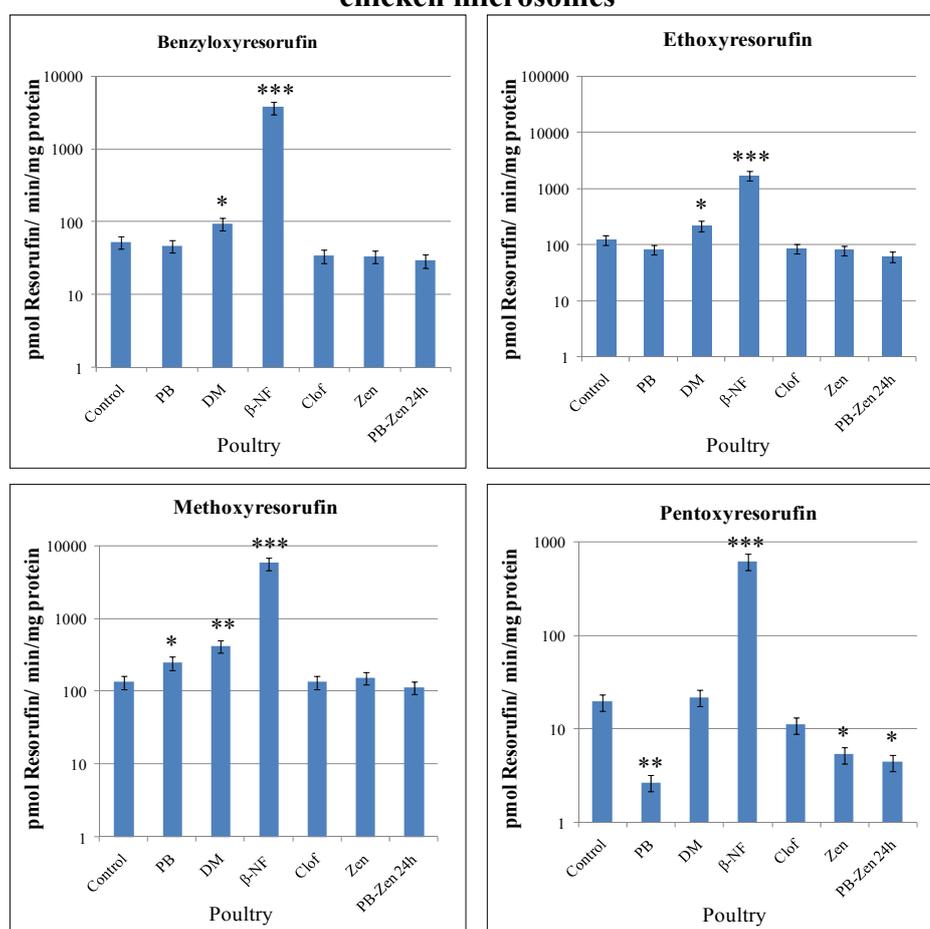
4.2.2. X-ROD and 7-BFC metabolism on treated poultries

4.2.2.1. Metabolic activity of resorufin derivates (X-ROD) on Zen and classical inducers treated chicken microsomes

Results and Discussions:

The results from the X-ROD O-dealkylase assessment on microsomal extracts of treated chickens (from the third experiment presented in chapter 3) are presented in the next graphs (Fig. 4.9).

Fig 4.9 - X-ROD metabolic activity profile for Zen and classical inducers treated chicken microsomes



The results are an average of 3 distinct assessments done in duplicate. Similar methodology was used as describe for the rat X-ROD O-dealkylase activity assessment (see fig. 4.7). Statistical analysis has been done using the Student t-Test; significant differences * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.005$ related to Control.

Zen chicken treatments are showing a general smaller X-ROD activity, compared to control activity level, but are statistically sustained only for PROD activity. Pentoxoresorufin is considered as a marker of CYP2B in mammals. Only two cytochromes from CYP2 family, have been identified, cloned and even purified in liver of

chicken, which are 2H1 and 2H2 (Hansen et al., 1989; Hobbs et al., 1986; Sinclair et al., 1990). Similar reports concerning the fact that PB is not enhancing in pheasant liver PROD activity has been reported by Giogi et al. in 2000.

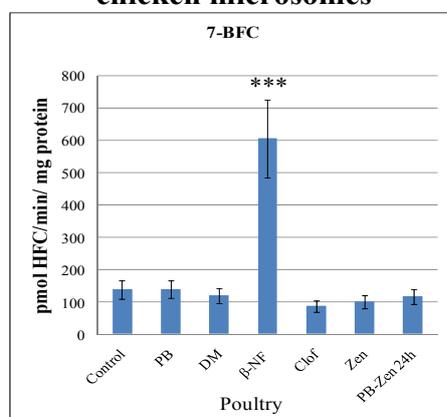
Verbrugge et al. in 2001, has reported that hepatic microsomal X-ROD activity of the untreated chicken (*Gallus domesticus*) is below the level of detection; nevertheless, the chickens treated with β -NF have shown important activities for EROD, MROD and BROD. Nebbia et al. in 2003, has showed that in untreated chickens (crossbred for meat production) important EROD, MROD and BROD activities have been observed, comparable to treated rats. β -NF is known as a classical inducer of CYP1A in rodents. The very high activities of β -NF observed in the treated chickens could imply the idea that chicken metabolism is dominated by CYP1A subfamily. Until now P450 1A4 and 1A5 have been purified and characterized in chicken; which were not classified as orthologous of mammalian P450 1A1 and 1A2 (Mahajan and Rifkind, 1999).

4.2.2.2. Metabolic activity of the 7-Benzyloxy-4-(trifluoromethyl)-coumarin (7-BFC) on Zen and classical inducers treated chicken microsomes

Results and Discussions

7-BFC is metabolized to 7-HFC by Cyt 3A, that is one of the substrates with a very good specificity for the enzymes 3A. The results from the BFC O-dealkylase assessment on microsomal extracts of treated chickens (from the third experiment presented in chapter 3) are presented in the next graph (Fig. 4.10).

Fig. 4.10 – 7-BFC metabolic activity profile for Zen and classical inducers treated chicken microsomes



Similar methodology was used as describe for the rat 7-BFC O-debenzylase activity assessment (see fig. 4.8) Statistical analysis has been done using the Student t-Test; significant differences * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.005$ related to Control.

7BFC is considered as a specific substrate for CYP3A subfamily in rats, especially for CYP3A4. In chicken the most important cytochrome from 3A subfamily is CYP3A37, cloned and analyzed by Ourlin et al. in 2000. CYP3A37 is considered as an orthologous of human CYP3A4 and regulated especially by PB and DM.

Poultry microsomes metabolic activity on 7-BFC is not showing any differences between zen treatments, classical inductors and control/oil extracts; excepting the β -NF microsomes that are showing a very high metabolic activity.

4.2.2.3 X-ROD and 7-BFC metabolism on treated poultries - *Conclusions*

Zen chicken treatments are showing a general lower X-ROD *O*-dealkylase activity, compared to the control level of activity, but are statistically sustained only for PROD activity. One could conclude that in chickens zearalenone might be interfering with the CYP2 family; more researches in this direction must be done.

Table 4.6 – General overview of the results of the X-ROD and 7-BFC *O*-dealkylase activities for chicken and rat microsomes

Treatments		Control	PB	DM	β -NF	Clof.	Zen
Total P450							
Chicken	nmol P450	0.15±0.02	0.23±0.03	0.25±0.02	0.62±0.02	0.13±0.01	0.15±0.01
Rat	/ mg protein	1.38±0.07	2.05±0.39	2.62±0.14	1.90±0.33	1.55±0.03	1.34±0.12
Benzyloxyresorufin <i>O</i>-debenzylase activity							
Chicken	pmol Resorufin/	53 ±5	47 ±5	94 ±9	3738 ±374	34 ±3	33 ±3
Rat	min/ mg protein	3 ±1	64 ±7	52 ±5	53 ± 6	3 ±1	3 ±1
Etoxyresorufin <i>O</i>-deethylase activity							
Chicken	pmol Resorufin/	121 ±10	82 ± 8	217 ±22	1704 ±170	86 ±9	80 ± 8
Rat	min/ mg protein	308 ±31	898 ±90	349 ±35	37623 ±380	87 ±8	1397 ±140
Pentoxyresorufin <i>O</i>-depentylase activity							
Chicken	pmol Resorufin/	20 ±3	3 ±1	22 ±2	622 ±60	11 ±1	5 ±1
Rat	min/ mg protein	3 ±1	98 ±1	28 ±1	7 ± 1	3 ±1	8 ±1
Methoxyresorufin <i>O</i>-demethylase activity							
Chicken	pmol Resorufin/	134 ±14	246 ±25	418 ±40	5741 ±600	135 ±14	154 ±15
Rat	min/ mg protein	188 ±20	608 ±50	314 ±30	1954 ±500	175 ±20	283 ±30
7BFC <i>O</i>-dealkylase							
Chicken	pmol HFC/	137 ±14	138 ± 46	119 ±19	604 ±39	87 ±11	99 ±11
Rat	min/mg protein	58 ±6	1157 ±100	680 ±70	380 ±40	53 ±5	52 ±4

The highest values obtained for the X-ROD and 7-BFC *O*-dealkylase activities for chicken and rat microsomes, respectively, are bold.

The observed very high X-ROD and 7-BFC *O*-dealkylase activities of β -NF treated chickens could imply the idea that chicken metabolism is dominated by CYP1A subfamily.

The poultry microsomes metabolic activity on 7-BFC is not showing any differences between zen treatment, classical inductors and control/oil extracts; excepting the β -NF microsomes that are showing a very high metabolic activity.

In order to have a general overview of the X-ROD and 7-BFC O-dealkylase activities of poultry with regards to rat we present in the table 4.6 a recapitulative of the results obtained.

The BROD O-debenzylase activity for poultry Control treatment showed higher activity (approximately 10 times higher) compared to rat Control treatment. Similar differences can be observed for zearalenone, β -naphthoflavone (microsomes from poultry treated with β -NF is having the highest BROD O-debenzylase activity if compared to the other poultry treatments) and clofibrate treatments. For phenobarbital and dexamethasone treatments no differences concerning BROD O-debenzylase activity, can be observed between poultry and rats.

The EROD O-deethylase activity for poultry Control treatments are smaller than the rat Control treatments. Similar differences can be observed for all the chicken treatments (excepting Clof treatments), compared to the similar treatments in rats. In poultry Zen treatment is having the same EROD O-deethylase activity as the Control treatments, instead in rats Zen IP is having higher, statistically sustained, EROD O-deethylase activity. The highest EROD O-deethylase activities are having also the β -NF treatments, in chicken as well as in rat.

Concerning the MROD O-demethylase activity similar profiles can be observed for poultry and rats. Zearalenone chicken treatments are not showing any differences compared to Control treatment. β -NF treatments are showing the highest MROD O-demethylase activity, but also PB and DM are showing statistically sustained O-demethylase activity increase.

The PROD O-depentylase activity for Control chicken treatment is smaller (10 to 100 times smaller) than the rat similar treatment. The general PROD O-depentylase activity in chickens is smaller than in rats, only β -NF treatment is having similar PROD O-depentylase activity bought in poultry and rat. An interesting fact can be observed in poultry, Zen and PB treatments are showing diminished PROD activity if compared to control treatments.

4.3. Interactions of Zearalenone and its metabolites on steroid metabolism

Estradiol and Testosterone metabolism are very important in zearalenone researches, being well known the fact that zearalenone is involved in reproduction disorders.

This is why the profiles of Estradiol and Testosterone metabolisms were assessed using *in vitro* incubation of different types of microsomes and adding a known quantity of estradiol and testosterone, respectively.

Using the same method and reagents we have added to the previous incubation 20 μM of zearalenone to determine the inhibitory effect of zearalenone on Estradiol and Testosterone metabolisms.

4.3.1. Estradiol metabolism

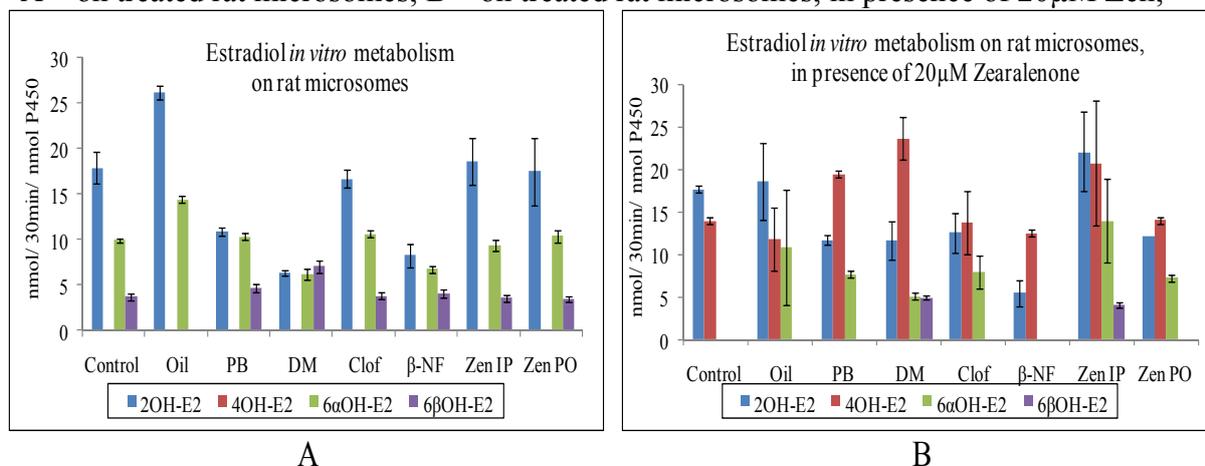
4.3.1.1. Estradiol metabolism on treated rats

Results and Discussions

In vitro estradiol metabolism was assessed using treated rat microsomes (2 μM total P450) from the first experiment (see chapter 3), with and without added zearalenone in order to determine the *in vivo* effect of Zen treatment on estradiol metabolism, as well as its direct *in vitro* effect. Incubations of 200 μM estradiol at 37°C, using the NADPH generating system (1 mM NADP + 10 mM Glucose-6-Phosphate, 1 UI de G6PDH), 100 μM MgCl₂ and 0,1M phosphate buffer (pH 7.4) have been done.

Figure 4.11 – Estradiol *in vitro* metabolism

A – on treated rat microsomes; B – on treated rat microsomes, in presence of 20 μM Zen;



Current results are an average of 3 distinct incubations assessment done in duplicate. Standard curves have been done for all metabolites, HPLC-MS assessment have been done in order to identify the 4OH-E2 and to distinguish the E2 metabolites from Zen metabolites, because the DAD detection is made both for E2 and

Zen metabolism at the same wavelength of 280 nm, nevertheless the E2 and Zen molecular masses are different of 272 and 318, respectively.

2OH-E2 is the main metabolite formed by all the rat microsomes (Fig. 4.11. A). There are no essential differences between the formed metabolites by Zen microsomes and by Control microsomes. Traces of 4OH-E2 have been observed for Control/Oil, PB, β -NF and Zen treatments, but it was not possible to be quantified due to the very small quantities observed.

Estradiol metabolism on rat microsomes is perturbed by the presence of 20 μ M Zen (Fig. B). Important diminution of 2OH-E2 level can be observed, but more important is the occurrence of the 4OH-E2, known carcinogen (Safe, 1998; Liehr et al., 1990; Liehr and Ricci 1996; Weisz et al., 1992). Zearalenone treatments are showing an important level of 4OH-E2 comparable the one for PB and DM microsomes. One can conclude that Zearalenone could be a carcinogenesis potentiator in rats.

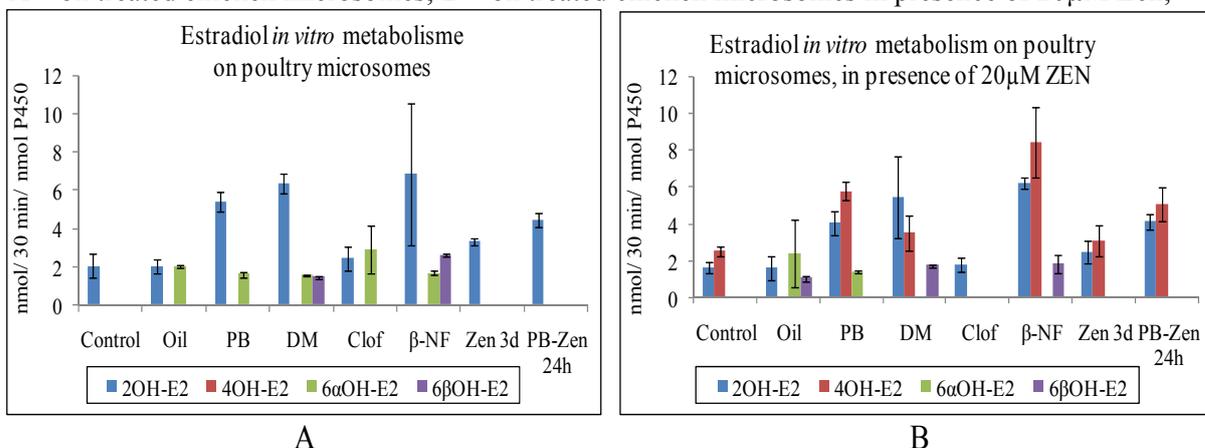
4.3.1.2. Estradiol metabolism in treated poultry

Results and Discussions

In vitro estradiol metabolism was assessed using treated chicken microsomes (2 μ M) from the third experiment (see chapter 3), with and without added zearalenone, in order to see the *in vivo* effects of Zen treatments on estradiol metabolism, as well as its direct *in vitro* effects.

Figure 4.12 – Estradiol *in vitro* metabolism

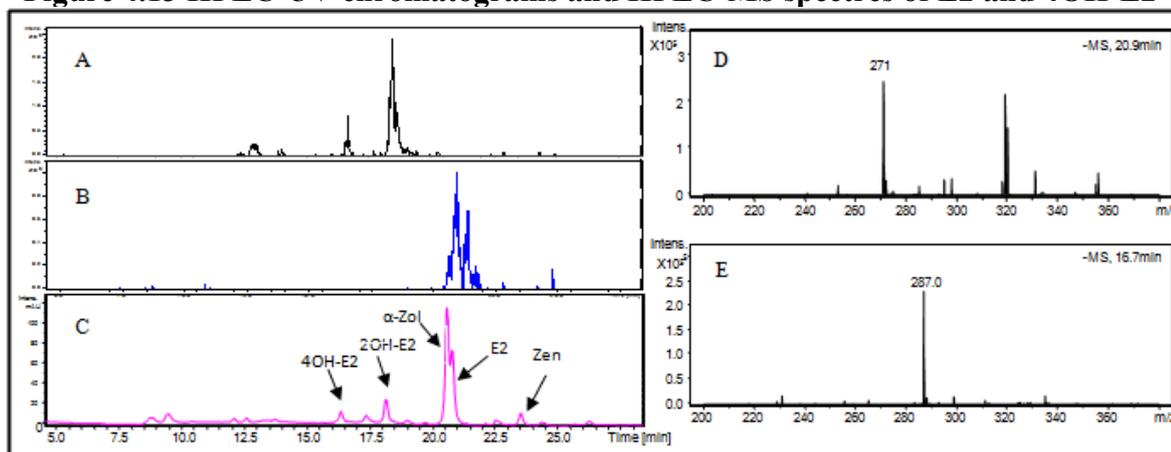
A – on treated chicken microsomes; B – on treated chicken microsomes in presence of 20 μ M Zen;



Current results are an average of 3 distinct incubations assessment done in duplicate. Similar methodology was used for the estradiol metabolism assessment on chicken microsomes as describe for rat microsomes (see fig. 4.11).

In order to be sure of the obtained results, especially concerning the occurrence of 4OH-E2 metabolites, incubation samples have been determined by LC-MS analysis (Fig 4.13). For chicken microsomes, Zen three days treatment is showing an increase of 2OH-E2 formation, if compared to the Control treatment, not sustained statistically. PB-Zen 24h chicken treatment is showing an intermediary 2OH-E2 level, between Zen and PB chicken treatments. Important metabolic activities can be observed for PB, DM and β -NF chicken treatments; increase that is observed not only by the increasing level of 2OH-E2 formation but as well as by the occurrences of other E2 metabolites like: 6 α OH-E2 and/or 6 β OH-E2. 2OH-E2 is the main metabolite formed by all the poultry and rat microsomes.

Figure 4.13 HPLC-UV chromatograms and HPLC-MS spectres of E2 and 4OH-E2



Example of incubation sample of estradiol on β -NF microsomes, in the presence of 20 μ M Zen: (A) HPLC-MS chromatogram's of extracted ions m/z 287 (OH-E2s) and (B) m/z 271 (E2) ; (C) HPLC-UV at 280 nm; (D) MS spectra of a 20,9 min peak corresponding to E2 and (E) a 16.7 min peak corresponding to 4OH-E2.

Estradiol metabolism in chicken microsomes is modulated by the presence of 20 μ M Zen. An important diminution of 2OH-E2 level can be observed, but more important is the occurrence of the 4OH-E2, known carcinogen. The highest levels of 4OH-E2 are observed for PB, DM and especially for β -NF microsomes. Zearalenone treatments are also showing an important level of 4OH-E2. One could conclude that Zearalenone is a potential carcinogen for poultry. Within the last years chickens and hens been used as animal models for the study of the human ovarian cancer (Johnson and Giles, 2006; Murdoch et al., 2005; Giles et al., 2006).

4.3.1.3. Estradiol metabolism in human and other species

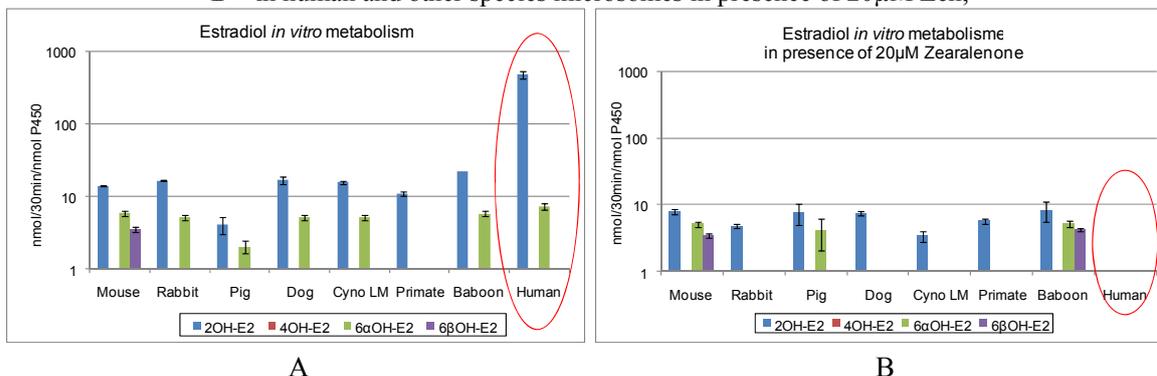
Results and Discussions

Microsomal extracts from several species were used in order to observe the inter-species variability and species specific effects of zearalenone on estradiol metabolism.

Figure 4.14 – Estradiol *in vitro* metabolism

A – in human and other species microsomes;

B – in human and other species microsomes in presence of 20 μ M Zen;



Current results are an average of 3 distinct incubations assessment done in duplicate. Similar methodology was used for the estradiol metabolism assessment as describe for rat microsomes (see fig. 4.11).

The *in vitro* estradiol metabolism using human, primates and other species microsomes is dominated by the occurrence of 2OH-E2 and 6 α OH-E2 (fig. 4.13 A). A very high level of 2OH-E2 formation can be observed for the human microsomes, which are having a metabolic activity of 482 \pm 53 2OH-E2 nmol/30 min/nmol P450.

The presence of 20 μ M Zen is drastically reducing the metabolic activities, especially for human microsomes which are totally inhibited (fig. 4.13 B), no estradiol metabolites are formed. On the other hand, pigs' microsomes *in vitro* metabolism seems not to be influenced by the presence of Zen. The other species are showing a diminished estradiol metabolic activity between 43 and 78 % inhibition of 2OH-E2 formation. No traces of 4OH-E2 formation were observed in microsomal extracts of these species, which casts a shadow of a doubt on our results. More researches must be done on microsomes of different species in order to monitor the occurrence of 4OH-E2 and inter-species differences.

4.3.1.4. Estradiol metabolism – Conclusions:

Estradiol metabolism is dominated by the formation of 2OH-E2 and 6 α OH-E2 for all the species microsomes that we have used. For chicken microsomes, Zen 3 day's treatment is showing an increasing of this 2OH-E2, if compared to Control treatment. For rat, there are no essential differences between the metabolites formed by Zen and Control microsomes.

A very high level of 2OH-E2 formation can be observed for the human microsomes, which are having a metabolic activity of 482 ± 53 nmol/30 min/nmol P450.

Estradiol metabolism on microsomes is disturbed by the presence of 20 μ M Zen. An important diminution of 2OH-E2 level can be observed, with an inhibitory effect between 43 and 100% 2OH-E2 formation for all the tested species microsomes, excepting pig microsomal extracts metabolic activity which is not influenced by the zearalenone presence.

4.3.2. Testosterone metabolism

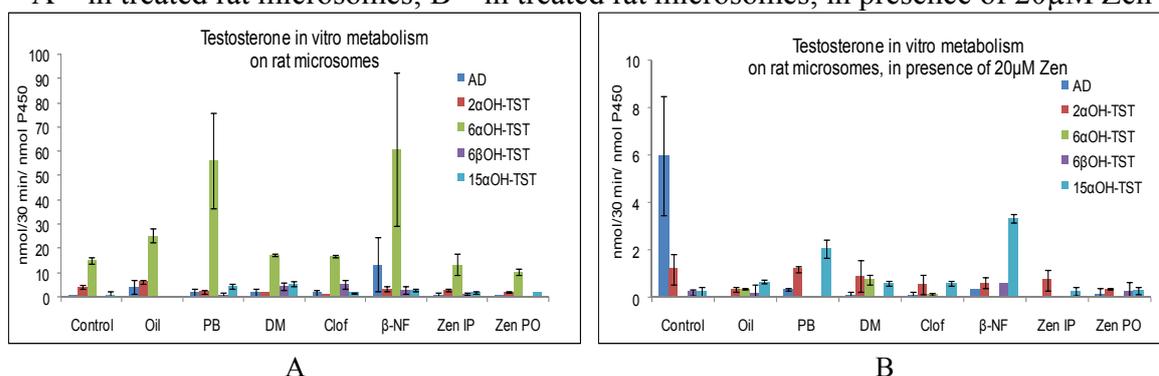
4.3.2.1. Testosterone metabolism in treated rats

Results and Discussions

In vitro testosterone metabolism was assessed using treated rat microsomes (2 μ M) from the first experiment (see chapter 3), with and without added zearalenone; in order to determine the *in vivo* effect of Zen treatments on estradiol metabolism, as well as its direct *in vitro* effect. Incubations of 200 μ M testosterone at 37°C, using the NADPH generating system (1 mM NADP + 10 mM Glucose-6-Phosphate, 1 UI de G6PDH), 100 μ M MgCl₂ and 0,1M phosphate buffer (pH 7.4) have been done.

Figure 4.15 – Testosterone *in vitro* metabolism

A – in treated rat microsomes; B – in treated rat microsomes, in presence of 20 μ M Zen



Current results are an average of 3 distinct incubations done in duplicate. Standard curves have been done for all metabolites, HPLC-MS selective assessment have been done. The DAD detection wavelength was of 252 nm, wavelength allowing detection of testosterone and its metabolites.

Rat TST metabolism is dominated by the occurrence of 6 α OH-TST.

In the case of testosterone metabolism, interesting for rat microsomes Zen IP, DM and β -NF, is the formation of 6 β OH-TST, which, in conformity with the published articles, is specific for CYP 1A and 3A (Tachibana S. and Tanaka M., 2001; Usmani K.A. et al., 2002). Another interesting thing is the high formation of 6 α OH-TST in PB and β -NF microsomes. The presence of 20 μ M Zearalenone, like in the estradiol metabolism, inhibits the metabolic activity of all microsomes, especially the formation of 6 α / β OH-TST.

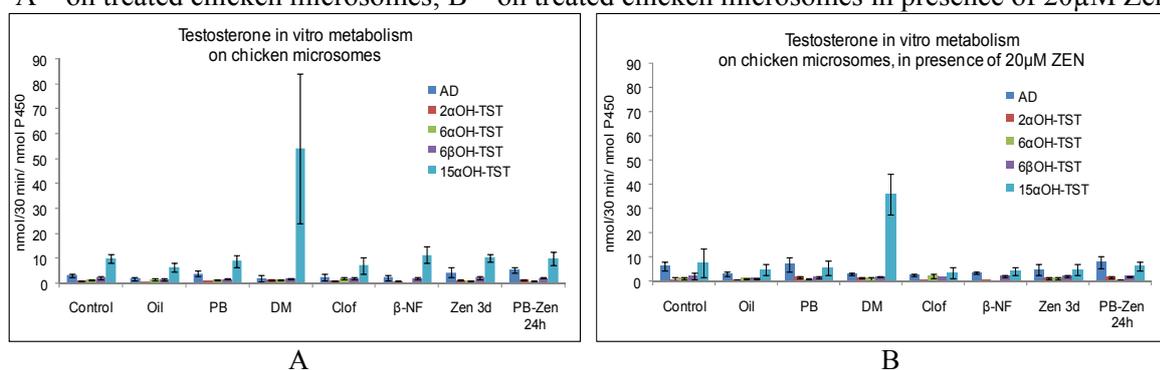
4.3.2.2. Testosterone metabolism in treated poultry

Results and Discussions

In vitro testosterone metabolism was assessed using treated chicken microsomes (2 μ M) from the first experiment (see chapter 3), with and without added zearalenone in order to determine the *in vivo* effect of Zen treatments on estradiol metabolism, as well as its direct *in vitro* effect.

Figure 4.16 – Testosterone in vitro metabolism

A – on treated chicken microsomes; B – on treated chicken microsomes in presence of 20 μ M Zen



Current results are an average of 3 distinct incubations assessment done in duplicate. Similar methodology was used for the estradiol metabolism assessment as describe for rat microsomes (see fig. 4.15).

Chicken TST metabolism is dominated by the formation of 15 α OH-TST. Testosterone metabolism profile on poultry microsomes is similar for Zen, PB-Zen 24h, PB, Clof and β NF treatments; no significant difference occurs if compared with Control microsomes. Only in the case of DM treated poultry microsomal extracts, an important increase of the *in vitro* formation of 15 α OH-TST was observed. Previous publications have reported the formation of 2 α , 2 β , 6 α , 6 β , 16 α , 16 β OH-testosterone and androstenedione in two experimental systems: *in ovo* and *in vivo* (Paolini et al., 1997; Giorgi et al., 2000; Ourlin et al., 2000) within different avian species, untreated or treated with PB, β NF and Clof. No previous reports on DM treated chicken liver metabolic testosterone activity have been published to our knowledge.

The presence of 20 μ M zearalenone presence has no effect on testosterone metabolism in chicken microsomes; a small general decrease of the metabolic activity was observed.

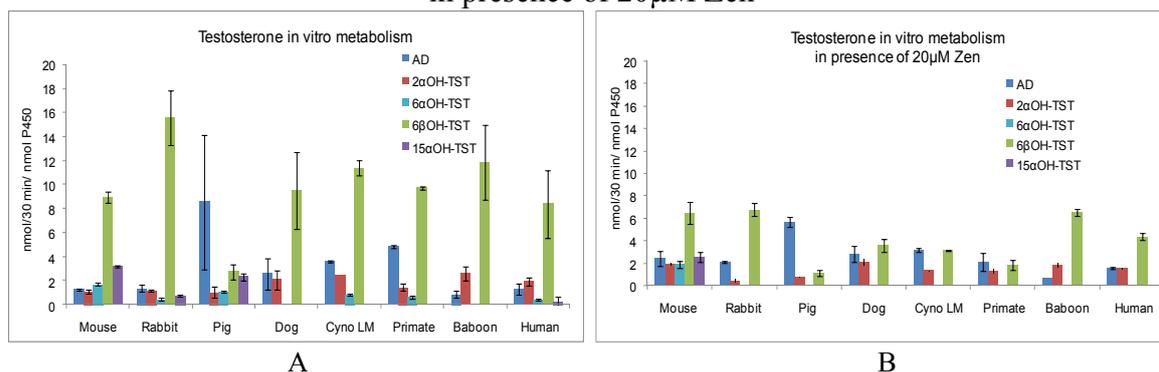
4.3.2.3. Testosterone metabolism in human and other species

Results and Discussions

Microsomal extracts from several species were used in order to determine the inter-species variability and species specific effects of zearalenone on the testosterone metabolism.

Figure 4.16 – Testosterone in vitro metabolism

A – in human and other species microsomes; B – in human and other species microsomes in presence of 20 μ M Zen



Current results are an average of 3 distinct incubations assessment done in duplicate. Similar methodology was used for the estradiol metabolism assessment as describe for rat microsomes (see fig. 4.15).

The *in vitro* testosterone metabolism in human microsomal extracts is dominated by the formation of 6 β OH-TST. 6 beta hydroxylation was shown to be the major route of NADPH-dependent oxidative metabolism with three steroid substrates: testosterone, androstenedione, and progesterone (Waxman et al., 1988). Similar metabolic profiles were observed for primates microsomes (Cyno LM, Primate and Baboon). The pig microsomes are showing a different testosterone metabolic profile, being dominated by 15 α OH-TST. The stereo selective testosterone oxidation profiles were different in human and pig microsomal extracts, fact that is not in agreement with the published reports on testosterone metabolism using human and pig hepatocytes (Donato et al., 1999). These differences might be due to the utilization of different enzymatic systems.

The 20 μ M Zen is having an inhibitory effect, of approximately 50%, on the metabolism of human, primates, dog, pig and rabbit. The mouse microsomal activity on testosterone doesn't seem to be influenced by the presence of Zearalenone.

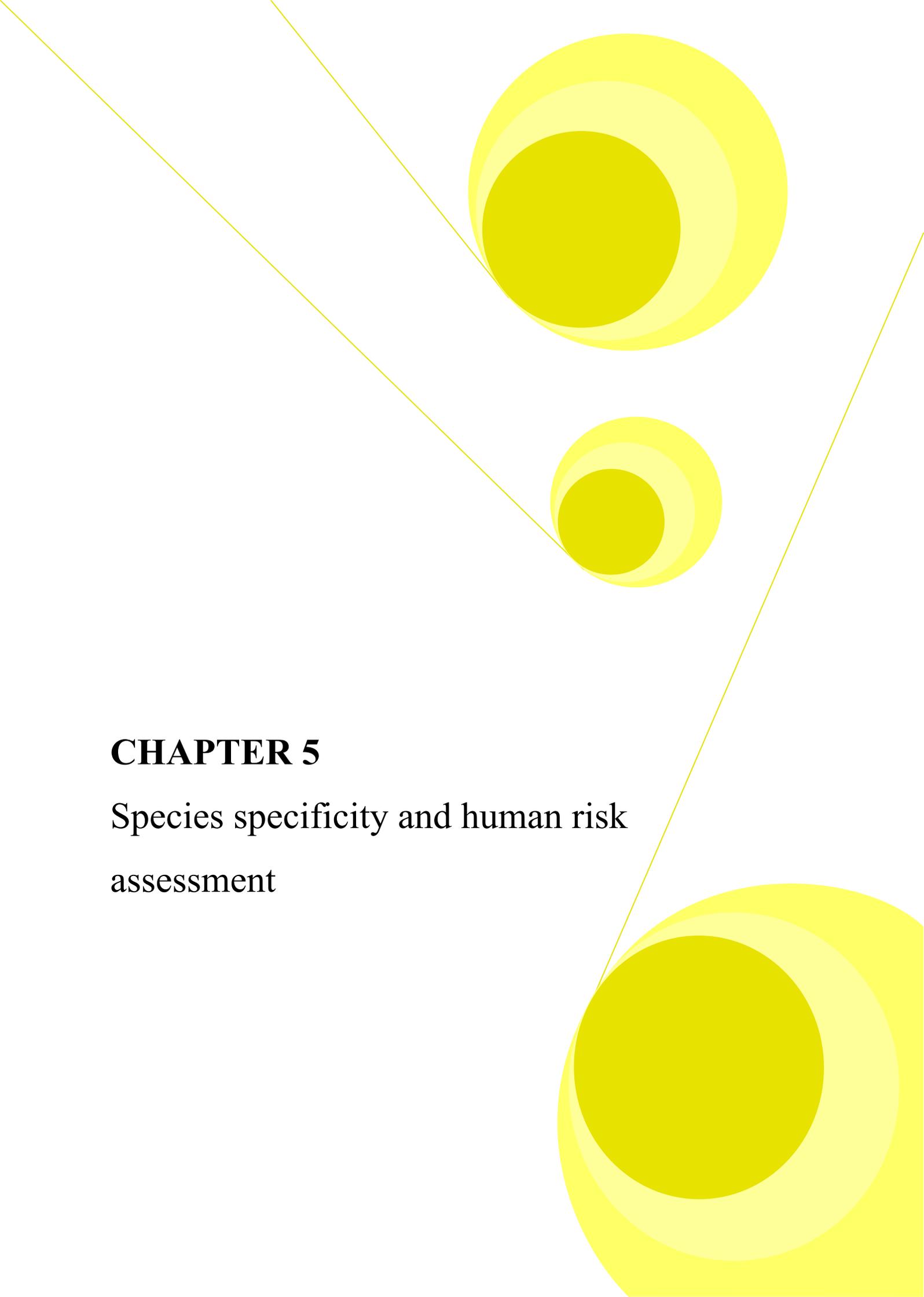
4.3.2.4. Testosterone metabolism – *Conclusions*:

Rat TST metabolism is dominated by 6 α OH-TST; chicken and pig TST metabolism is dominated by 15 α OH-TST; and human, rabbit and primates TST metabolism is dominated by 6 β OH-TST formation.

Using microsomes of Zen IP treated rat, we observed the formation of 6 β OH-TST that is specific for CYP 1A and 3A.

Testosterone metabolism profile in poultry microsomes is similar for Zen, PB-Zen, PB, Clof and β -NF treatments; no significant difference occurs if compared with Control microsomes.

The presence of 20 μ M Zearalenone inhibits the metabolic activity of almost all studied species microsomes, excepting the chicken microsomes which are not affected by zearalenone presence.

The page features a decorative graphic consisting of three sets of concentric circles in shades of yellow and gold, arranged in a descending staircase pattern from top-right to bottom-right. Two thin, parallel yellow lines run diagonally from the top-left towards the bottom-right, framing the central text area.

CHAPTER 5

Species specificity and human risk assessment

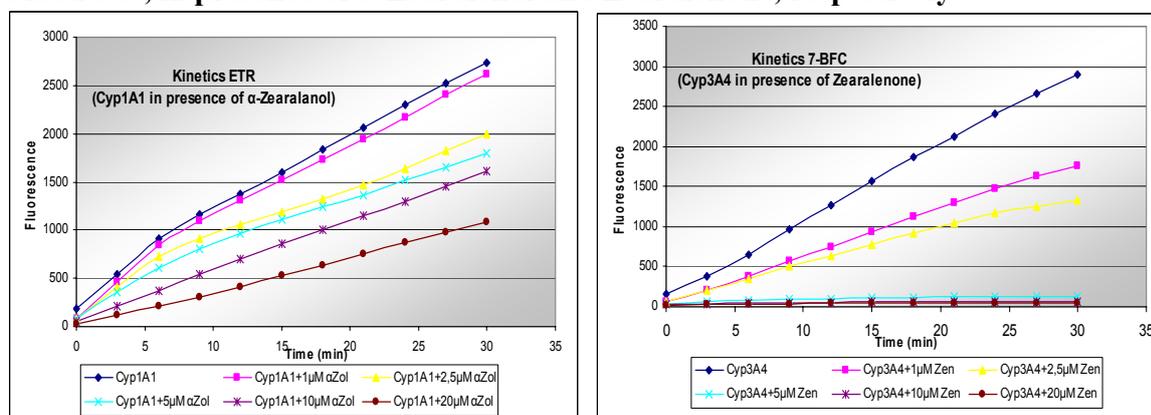
5.1. *In vitro* zearalenone metabolism effect on the metabolism of reference compounds and species specificity (IC₅₀ assessment)

The interaction of zearalenone and its metabolites on the detoxification enzymes (especially P450), is important to be determined in order to better understand a part of zearalenone metabolic pathway. The very important disparity of sensitivity between species to zearalenone and its metabolites effects could result also from the nature of the metabolic differences and reactivity toward targets such as steroid receptors, as well as from the structural conformation of zearalenone and its metabolites.

Using fluorimetry, we assessed the inhibition levels (IC₅₀ determination) of zearalenone and its metabolites on the metabolic activity of Ethoxyresorufin and 7-benzyloxy-4-(trifluoromethyl)-coumarin *O*-dealkylase. Several types of microsomes from different species (rat, pig and human) and treatments were used.

In the next graphs we present two examples of kinetics of ethoxyresorufin and 7-benzyloxy-4-(trifluoromethyl)-coumarin *O*-dealkylase activity, in the presence of α -Zearalenol and Zearalenone, respectively.

Fig 5.1 - Determination of the cytochrome P450 metabolic activity by fluorimetry, using ethoxyresorufin (left) and 7-benzyloxy-4-(trifluoromethyl)-coumarin (right) substrates, in presence of α -Zearalenol and Zearalenone, respectively



96 wells costar black plates were used to determine the ETR and 7-BFC *O*-dealkylase kinetics of human expressed forms at 1 μ M, in phosphate buffer pH7.4 and using as non-fluorescent substrates ethoxyresorufin and 7-benzyloxy-4-(trifluoromethyl)-coumarin at 100 μ M, respectively. The samples fluorescence was determined as within chapter 4. Control wells (without NADPH) and standard wells (resorufin and 7-HFC standards) were used.

This kinetic is typical for zearalenone and its metabolites, and is showing an inhibitory effect of zearalenone and its metabolites, in the case of ETR and 7-BFC metabolism, respectively.

5.1.1. Zearalenone metabolism effect on ethoxyresorufin O-deethylase metabolic activity

Resorufin analogues provide a convenient and sensitive fluorimetric method to assess changes in xenobiotic metabolism *in vitro* (Lubet et al., 1990; Crespi et al., 1997). The activity of ethoxyresorufin O-deethylase (considered to be CYP1A and 1B specific) was studied on microsomal extracts.

5.1.1.1. Zearalenone metabolism effect on ETR O- deethylase metabolic activity, using rat microsomes and human expressed forms

Ethoxyresorufin is a non-fluorescent substrate specific for CYP 1A forms. This is why we used microsomes of rat treated with β -Naphthoflavone (classical inducer of Cyp1A forms expression). Control and Zearalenone microsomes form rat treated with NaCl 9‰ and zearalenone intra-peritoneal and orally, respectively, were also used.

Results and Discussions

Table 5.1. Ethoxyresorufin O- deethylase IC₅₀s calculated values for different rat microsomes and human expressed forms

<i>Rat Microsomes</i>	pmol Resorufin/ min/nmol P450	IC ₅₀ Zen	IC ₅₀ α Zol	IC ₅₀ β Zol	IC ₅₀ Zan	IC ₅₀ α Zal	IC ₅₀ β Zal
<i>Control</i>	130 ± 37	10,0 ± 0,5	11,4 ^a ± 0,4	6,6 ^a ± 0,3	1,6 ^c ± 0,3	2,6 ^{b,c} ± 0,5	1,5 ^{b,c} ± 0,4
<i>β-Naphthoflavone</i>	4587 ± 430	8,5 ^d ± 0,5	4,3 ^{a,d} ± 0,2	2,3 ^{a,d} ± 0,4	4,6 ^{c,d} ± 0,6	5,7 ^d ± 1,4	1,6 ^b ± 0,2
<i>Zearalenone IP</i>	231 ± 63	6,0 ^d ± 0,5	2,3 ^{a,d} ± 0,4	4,7 ^{a,d} ± 0,6	14,3 ^{c,d} ± 1,4	7,4 ^{b,c} ± 1,6	4,7 ^{b,d} ± 0,3
<i>Zearalenone PO</i>	127 ± 40	1,7 ^d ± 0,4	6,8 ^{a,d} ± 0,5	5,2 ^a ± 0,9	4,2 ^{c,d} ± 0,6	7,6 ^{b,d} ± 0,2	2,1 ^{b,c} ± 0,5
<i>CYP 1A1(human)</i>	25942 ± 541	10,6 ± 1,3	10,3 ± 1,9	10,5 ^d ± 1,2	> 20 ^{c,d}	10,27 ^{b,d} ± 1,9	9,24 ^{b,d} ± 2,1
<i>CYP 1A2(human)</i>	1337 ± 154	1,5 ^d ± 0,5	6,2 ^{a,d} ± 0,5	2,5 ^{a,d} ± 0,2	3,5 ^{c,d} ± 0,7	3,2 ^c ± 0,7	> 20 ^{b,c,d}

^a Significant differences (p \leq 0.05) related to Zen IC₅₀ for each type of microsomes.

^b Significant differences (p \leq 0.05) related to Zan IC₅₀ for each type of microsomes

^c Significant differences (p \leq 0.05) between Zen and Zan, α Zol and α Zal, and β Zol and β Zal respectively; for each type of microsomes

^d Significant differences (p \leq 0.05) related to Rat Control microsomes

Zearalenone treatment showed no induction of the Ethoxyresorufin O- deethylase activity, compared to the control rat treatment. The CYP1A1 or β -NF IC₅₀s of different compounds are not showing important differences.

Rat Control microsomes have a higher inhibitory potency for zearalanone, α -zearalanol and β -zearalanol; an explanation of this could be the C₁₁- C₁₂ double bond presence / absence respectively. The C₁₁- C₁₂ double influences the C₇ cetone and OH groups' reactivity potentiating their reactivity.

The zearalenone *p.o.* microsomes are showing an increase inhibitory potency for zearalenone like in the case of CYP1A2.

Zen is a poor inhibitor of CYP1A1 forms, but shows high inhibition of CYP1A2 expressed forms. This is understandable because CYP1A1 is not found in liver, being a pulmonary tissue isoform.

5.1.1.2. Zearalenone metabolism effect on ETR *O*-deethylase metabolic activity, using rat, human and pig microsomes

In order to determine the inter-species differences concerning Zearalenone and its metabolites on the metabolic activity of ethoxyresorufin *O*-deethylase we used the same fluorimetric assay for microsomes of rat, human and pig. The obtained results are presented in table 5.2.

Results and Discussions

Table 5.2 Ethoxyresorufin IC₅₀ calculated values for different species

<i>Microsomes</i>	pmol Resorufin/ min/nmol P450	IC ₅₀ Zen	IC ₅₀ αZol	IC ₅₀ βZol	IC ₅₀ Zan	IC ₅₀ αZal	IC ₅₀ βZal
<i>Rat (Control)</i>	130 ±37	10,0 ±0,5	11,4 ^a ± 0,4	6,6 ^a ± 0,3	1,6 ^c ± 0,3	2,6 ^{b,c} ± 0,5	1,5 ^{b,c} ± 0,4
<i>Human</i>	134 ±17	5,4 ^d ± 0,4	1,9 ^{a,d} ± 0,2	2,5 ^{a,d} ± 0,4	9,9 ^{c,d} ± 1,9	18,5 ^{b,c,d} ± 0,2	13,5 ^{b,c,d} ± 2,2
<i>Pig (Control)</i>	580 ±82	> 20	> 20	> 20	> 20	> 20	> 20

^a Significant differences (p≤0.05) related to Zen IC₅₀ for each type of microsomes.

^b Significant differences (p≤0.05) related to Zan IC₅₀ for each type of microsomes

^c Significant differences (p≤0.05) between Zen and Zan, αZol and αZal, and βZol and βZal respectively; for each type of microsomes

^d Significant differences (p≤0.05) related to Rat Control microsomes

Rat control microsomes have a higher inhibitory potency for zearalanone, α-zearalanol and β-zearalanol.

Human microsomes are showing a higher inhibitory potency for zearalenone, α-zearalenol and β-zearalenol, in the human liver, from a metabolic point of view, the CYP1A are playing an impotent role, mainly the 1A2 isoform.

In the case of pigs microsomes IC₅₀s are over 20μM, suggesting that the pig CYP1A forms are not sensible to Zen presence.

5.1.2. Zearalenone metabolism effect on the metabolic activity of 7-benzyloxy-4-(trifluoromethyl)-coumarin O-debenzylase

5.1.2.1. Zearalenone metabolism effects on 7-BFC O-debenzylase metabolic activity, using rat microsomes

7-Benzyloxy-4-(trifluoromethyl)-coumarin is a non-fluorescent substrate specific for CYP 3A forms. Microsomes of rats treated with dexamethasone and of pigs treated with rifampicine (dexamethasone and rifampicine are inducing the expression of the CYP3A forms) were used. Control microsomes from rats and pigs, treated with NaCl 9%, and Zearalenone microsomes, from rats treated with Zearalenone (intra-peritoneal and orally), were also used.

Results and Discussions

Table 5.3. 7-BFC IC₅₀ calculated values for different rat microsomes

<i>Rat Microsomes</i>	pmol 7-HFC/ min/nmol P450	IC ₅₀ Zen	IC ₅₀ αZol	IC ₅₀ βZol	IC ₅₀ Zan	IC ₅₀ αZal	IC ₅₀ βZal
<i>Control</i>	219 ± 24	1,0 ± 0,30	3,0 ^a ± 0,2	1,3 ± 0,2	1,2 ± 0,2	4,6 ^c ± 0,4	1,6 ± 0,3
<i>Dexamethasone</i>	825 ± 137	0,5 ^d ± 0,1	2,2 ^{a,d} ± 0,2	4,0 ^{a,d} ± 0,4	3,4 ^{c,d} ± 0,5	2,5 ^{b,d} ± 0,3	1,6 ^{b,c} ± 0,6
<i>Zearalenone i.p.</i>	251 ± 58	0,6 ± 0,1	1,2 ^{a,d} ± 0,4	1,5 ^a ± 0,4	3,5 ^{c,d} ± 0,5	3,1 ^{c,d} ± 0,1	3,0 ^{c,d} ± 0,1
<i>Zearalenone p.o.</i>	231 ± 54	0,5 ^d ± 0,1	1,2 ^{a,d} ± 0,3	3,5 ^{a,d} ± 0,8	2,2 ^c ± 0,7	3,1 ^{c,d} ± 0,4	2,5 ^d ± 0,4

^a Significant differences (p≤0.05) related to Zen IC₅₀ for each type of microsomes.

^b Significant differences (p≤0.05) related to Zan IC₅₀ for each type of microsomes

^c Significant differences (p≤0.05) between Zen and Zan, αZol and αZal, and βZol and βZal respectively; for each type of microsomes

^d Significant differences (p≤0.05) related to Rat Control microsomes

For all the rat microsomes there is a strong inhibitory potency regarding Zearalenone.

The Control rat microsomes are showing a low inhibitory potency for α-, β-Zol and α-, β-Zal. In the case of the DM treatment, a low inhibitory potency can be observed for Zen and β-Zol. Zearalenone treatments have a low inhibitory potency for Zen and β-Zol, both for the i.p. and p.o. treatments.

As an overall conclusion, zearalenone and its metabolites are inhibitors of rat cytochromes 3A1 and 3A2.

5.1.2.2. Zearalenone metabolism effect on 7-BFC *O*-debenzylase metabolic activity, using pig microsomes

Results and Discussions

Table 5.4. 7-BFC IC₅₀ calculated values for different pig microsomes

Pig Microsomes	pmol 7-HFC/ min/nmol P450	IC₅₀ Zen	IC₅₀ αZol	IC₅₀ βZol	IC₅₀ Zan	IC₅₀ αZal	IC₅₀ βZal
<i>Control</i>	74 ± 2	0,90 ±0,10	2,65^a ±0,40	1,44 ±0,35	1,78^c ±0,69	1,72^c ±0,42	0,92^b ±0,20
<i>RIF</i>	225 ± 13	1,14 ±0,25	1,32^d ±0,38	3,51^{a,d} ±0,61	2,75^{c,d} ±0,47	0,70^{b,c,d} ±0,11	2,76 ±0,34

^a Significant differences (p≤0.05) related to Zen IC₅₀ for each type of microsomes.

^b Significant differences (p≤0.05) related to Zan IC₅₀ for each type of microsomes

^c Significant differences (p≤0.05) between Zen and Zan, αZol and αZal, and βZol and βZal respectively; for each type of microsomes

^d Significant differences (p≤0.05) related to Control microsomes

Discussion:

Pig microsomes are showing a higher inhibitory potency for Zearalenone and its metabolites in 7-BFC (consider specific of human CYP3A4) fluorimetric assay than in ETR assay, implying the idea of an important CYP3A induction in pigs and that CYP3A might be involved in pigs the in the detoxification (elimination) of Zearalenone and its metabolites.

5.1.2.3. Zearalenone metabolism effect on 7-BFC *O*-debenzylase metabolic activity, using microsomes with a high content of CYP3A.

The microsomes of different species/treatments that have an important content of CYP3A been compared. The results are presented in table 5.5.

Results and Discussions

Table 5.5. 7-BFC IC₅₀ calculated values for different microsomes with a high content of CYP3A

Microsomes	pmol 7-HFC/ min/nmol P450	IC₅₀ Zen	IC₅₀ αZol	IC₅₀ βZol	IC₅₀ Zan	IC₅₀ αZal	IC₅₀ βZal
<i>Human</i>	253 ± 57	1.0 ±0,3	1,9^{a,d} ±0,6	1,8^a ±0,6	3,2^{c,d} ±0,2	3,4^{c,d} ±0,4	3,3^{c,d} ± 0,2
<i>CYP 3A4(Human)</i>	469 ± 18	2,3^d ±0,2	2,7 ±0,1	2,7^d ±0,2	1,9^d ±0,2	2,2^d ±0,4	1,7^c ±0,2
<i>RIF (Pig)</i>	225 ± 13	1,14 ±0,25	1,32^d ±0,38	3,51^{a,d} ±0,61	2,75^{c,d} ±0,47	0,70^{b,c,d} ±0,11	2,76 ±0,34
<i>Dexamethasone(rat)</i>	825 ± 137	0,5^d ± 0,1	2,2^{a,d} ± 0,2	4,0^{a,d} ± 0,4	3,4^{c,d} ± 0,5	2,5^{b,d} ± 0,3	1,6^{b,c} ± 0,6

^a Significant differences (p≤0.05) related to Zen IC₅₀ for each type of microsomes.

^b Significant differences (p≤0.05) related to Zan IC₅₀ for each type of microsomes

^c Significant differences (p≤0.05) between Zen and Zan, αZol and αZal, and βZol and βZal respectively; for each type of microsomes

^d Significant differences (p≤0.05) related to Control microsomes

Even if the results are showing statistically sustained differences, from a physiological/biological point of view no important differences can be stated.

Nevertheless, these results are confirming that dexamethasone and rifampicine are inducers of CYP 3A1 for rats and CYP3A29 for pigs, respectively; orthologs of human CYP3A4.

5.1.3. In vitro zearalenone metabolism effect on the metabolism of reference compounds and species specificity (IC₅₀ assessment) - Conclusions

Zearalenone treated rats presented a higher inhibitory potency of the ETR *O*-deethylase metabolic activity for zearalenone, similar to the expressed Cyp1A2 human forms. One might say that CYP1A2 activity may be modified by zearalenone presence in rats.

Zearalenone treated rats presented a low inhibitory potency of 7-BFC *O*-debenzylase metabolic activity for Zen and β -Zol, similar to the DM (CYP3A classical inducer) treated rats. All the rat microsomes had an inhibitory potency regarding Zen. One might say that Zen interacts within CYP3A. This is in accordance with the observed influences of CYP3A mRNA expression and metabolic activity, on *in vivo* Zen treatments (see chapter 3 and annex 1) and the implication of CYP3A in Zen metabolisation to OH-Zen (Bravin et al., 2009)

Species differences (specificities) occurred upon *in vitro* zearalenone metabolism effect assessment on ETR and 7-BFC *O*-dealkylase metabolic activity.

Human microsomes have a higher inhibitory potency for Zen, α -Zol and β -Zol on ETR *O*-deethylase metabolism, in contrast to the rat microsomes which have a higher inhibitory potency for Zan, α -Zal and β -Zal. ETR *O*-deethylase metabolism on pig microsomes is not influenced by the presence of Zen or its metabolites (IC₅₀ > 20 μ M).

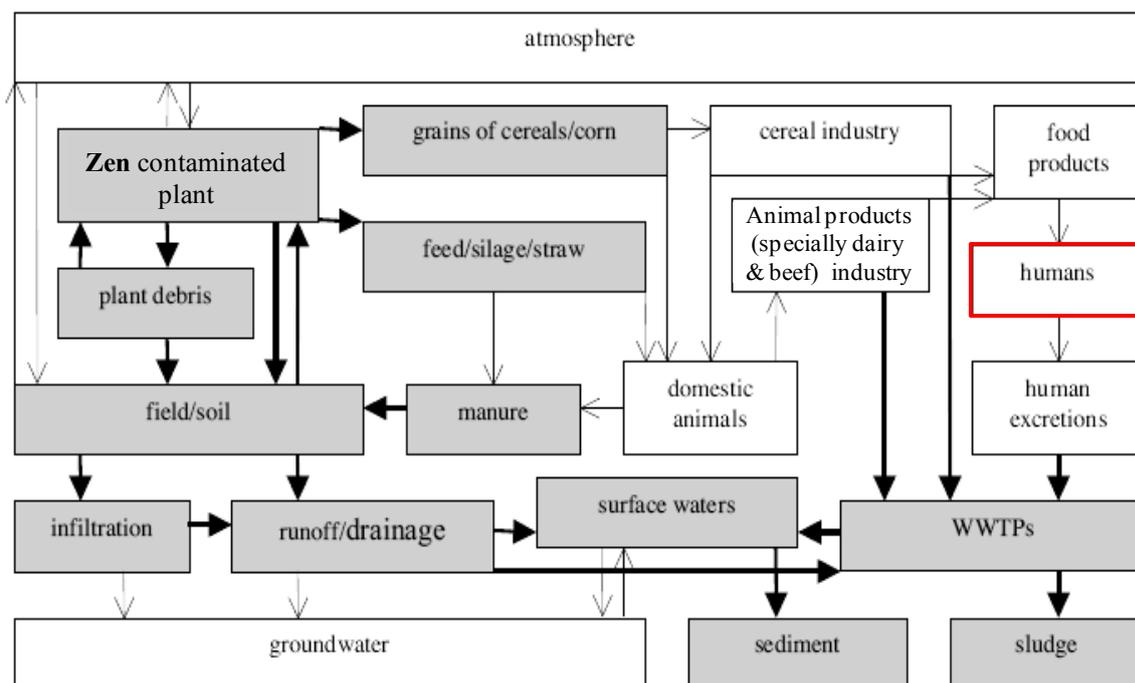
Human microsomes have a higher inhibitory potency for Zan, α -Zal and β -Zal on 7-BFC *O*-debenzylase metabolism. Pig and rat microsomes showed similar IC₅₀ ranking as following: IC₅₀ Zen \leq IC₅₀ Zan \leq IC₅₀ β Zol \leq IC₅₀ β Zal \leq IC₅₀ α Zol \leq IC₅₀ α Zal.

5.2. Zearalenone and its metabolites effect on human – risk assessment

5.2.1. Zearalenone risk assessment – general overview

Hepatocellular adenomas and pituitary tumours were observed in long-term studies of carcinogenicity in mice. However, these tumours were observed only at doses much in excess of the concentrations that have hormonal effects i.e. at levels 8–9 mg/kg of body weight or more. JECFA (2000) concluded that these tumours are a consequence of Zen estrogenic effects. Zen did not induce gene mutations in bacteria or recombination in yeast. However, Zen induced sister chromatid exchanges and chromosomal anomalies *in vitro*, and DNA-adducts as measured by ³²P-postlabelling in mice. Data available on Zen gene-toxicity do not allow an adequate evaluation of its genotoxic potential and its mechanism of action in inducing chromosomal anomalies and DNA-adducts in mice. JECFA (2000) concluded that the safety of Zen could be evaluated on the basis of the dose that had no hormonal effects in pigs, the most sensitive species, and established a Provisional Maximum Tolerable Daily Intake of 0.5 µg Zen/kg of body weight. This decision was based on the NOEL of 40 µg/kg of body weight per day obtained in a 15-day study in pigs and the lowest observed effect level of 200 µg/kg body weight per day in this study.

Fig.5.2. Hypothesized environmental distribution of Zen and its metabolites



Grey boxes and thick arrows indicate possible relevant compartments and pathways, respectively, and are subject to primary investigation (Bucheli et al., 2005)

Risk assessment, based on exposure and hazard evaluation, needs to take into account Zen transfer in the organism, and has to evaluate all contamination sources (Kuiper-Goodman, 1990). Bucheli et al., in 2005, had summarized the current limited knowledge on the presence of estrogenic mycotoxins in the environment and hypothesized the environmental distribution of Zen and its metabolites (figure 5.2.).

Dänicke et al., in 2008, attempted to evaluate the potential exposure of the consumer with Zen via foodstuffs of animal origin obtained from animals fed a contaminated diet containing 50 µg Zen per kg feed (table 5.6).

Table 5.6. Evaluation of human exposure to zearalenone via foodstuffs of plant and animal origin by assuming a similar contamination of a wheat batch which is either used directly as foodstuff or as feedstuff (Dänicke et al., 2008)

Zearalenone			
Tolerable daily intake (TDI):			
• TDI [µg/kg bw/d]	0.2 ²		
• TDI [µg/d] (70 kg bw)	14		
Contamination of food or feed (88 % dry matter):			
• Wheat as foodstuff [µg/kg]	50		
• Wheat as feedstuff [µg/kg]	50		
Foodstuff intake necessary for reaching the TDI:			
• Foodstuff of plant origin [g/d]	280		
• Foodstuff of animal origin after feeding the contaminated wheat:			
	Carry over ³	Intake [kg/d]	"Filter-factor" ⁴
Kidney (pig) ⁵			
Liver (pig) ⁵	0.295	0.9	3
Liver (Laying hen) ⁵	0.005	56	200
Muscle (pig) ⁵			
Back fat (pig) ⁵			
Egg (Laying hen) ⁵	0	-	
Milk (cow) ⁶	0	-	

¹ Scientific Committee on Food 2002, available at: <http://europa.eu.int/>

² Scientific Committee on Food 2000, available at: <http://europa.eu.int/>

³ Maximum experimentally determined carry over

⁴ "Filter-factor" = Foodstuff intake from animal origin necessary for reaching the TDI: Foodstuff intake from vegetal origin necessary for reaching the TDI

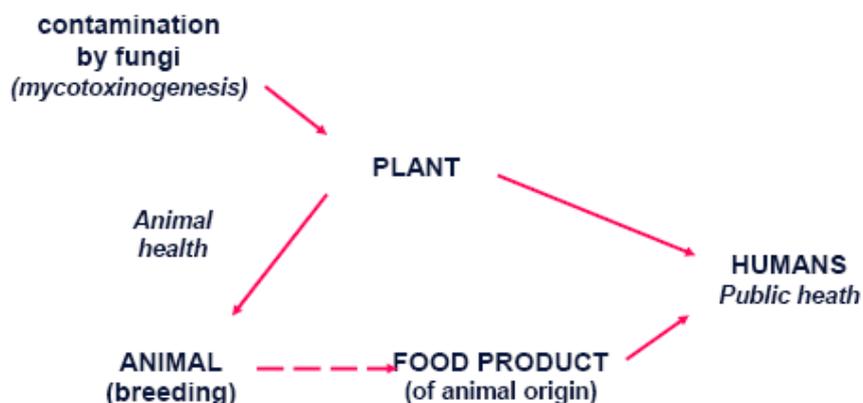
⁵ Carry over factor = toxin plus metabolite concentration in foodstuff: toxin concentration in feedstuff (As carry over factors were calculated from the sum of the toxin and of the metabolites the differences in their toxicity is not accounted for)

⁶ Carry over rate = toxin excretion with the milk: toxin intake with the diet (at a dry matter intake of 20 kg/d and a milk yield of 40 kg/d)

Moreover, the worst case scenario was completed by assuming the highest experimentally determined carry over factors or rates. This scenario was compared with the exposure of humans via a contaminated foodstuff of plant origin having a Zen concentration similar to the contaminated feedstuff. These worst case scenarios do not considered eating habits (food basket) which are normally used to derive maximum residue levels in foodstuffs. Their intention was to demonstrate the insignificant contribution of contaminated feedstuffs to the total exposure of the consumer.

In 2009, within an oral presentation at the 2nd FEED-SEG Symposium (Parma, Italy), I. Oswald stated that mycotoxins high toxicity doesn't mean a high risk and that the link between toxicity and risk is the exposure factor ($\text{risk} = \text{toxicity} \times \text{exposure}$). Starting from this remark and the fact that the danger of mycotoxins is not only due to the mycotoxins themselves but also to their metabolites, which can be produce in the fungi but also in the host (bio-activation), we have tried to do a more developed human risk assessment of Zearalenone with regard to the human food chain (Fig.5.3) and human eating habits (see chapter 1).

Fig. 5.3 Mycotoxins in food chain (Oswald, 2009)

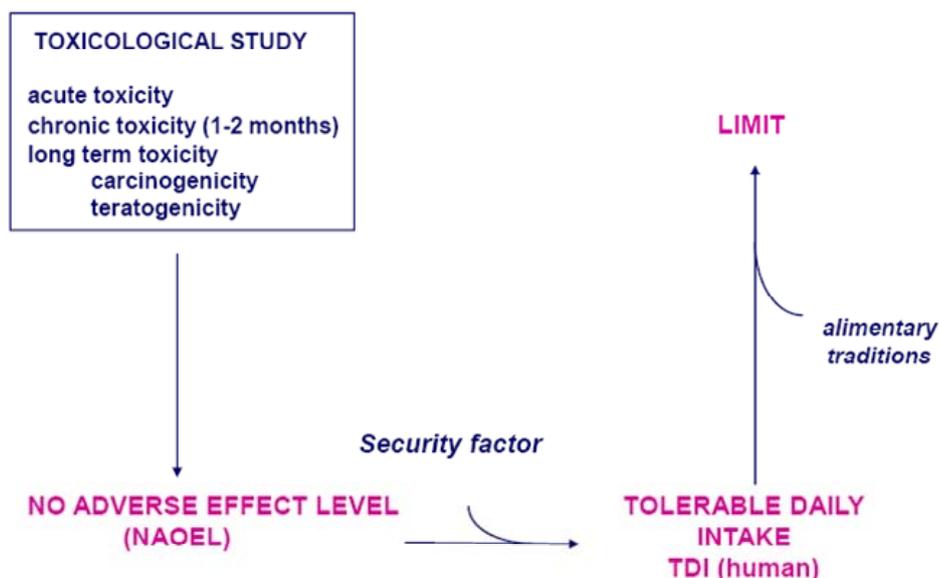


In order to calculate the maximum level of Zearalenone in human, we started from the NOAEL values (NO Adverse Effects Level) obtained in animals multiply by a security factor.

Calculation of the security factor (Oswald, 2009) has been done as followed:

1. A factor **10** to take into consideration the **inter-individual variability**
2. A factor **10** to take into consideration the **inter-species variability** (human is considered to be 10 times more susceptible than the most susceptible species)
3. A factor **10 to 50** to take into account, when they are demonstrated, the **irreversible effects** such as carcinogenic or mutagenic effect

Fig. 5.4. Determination of the maximum levels for mycotoxins in human (JECFA, 2001)



5.2.2. European zearalenone risk assessment

First of all, we calculated the Tolerated Daily Intake (TDI) starting for a value of NOAEL of 20 µg/kg bw/day (JEFCA, 2000) for the most sensible specie, the pig, more precisely gilts. We have used a security factor of 1000 (10 for inter-individual variability × 10 for inter-species variability × 10 considering that Zen is neither carcinogenic nor mutagenic) and we obtained a human TDI of 20 ng Zen/kg bw/day.

Taking in to account the European daily intake calculated in function of the contamination level of cereals/vegetables and the alimentary habits (see chapter 1), of 25 ng Zen/kg bw/day, we can observed that the calculated tolerated intake is below the estimated total intake. Thus it is an unacceptable Zen daily intake. Moreover, Zen daily intake due to the consumption of meat products, calculated using the carry-over factor of 0.295 for pig liver (reported by Dänicke et al., in 2008) and an average daily meat consumption of 0.45 kg/person (adult person over 15 years old, with a body weight of 60 kg), as well as the European feedstuff maximal Zen concentration of 200 ng/kg, is of 4 ng/kg bw/day.

An overall total daily intake for Europeans, taking in the account the eating habits, is of 29 ng/kg bw/day, value that is 45% higher than the TDI value.

The calculated maximal limit for the current TDI (20 ng Zen/kg bw/day), considering an average cereal consumption of 250 g/day for an adult (15 years or more) of 60 kg body weight, is of 5 µg/kg. European rules (No. 466 of 8/03/2001, OJ L77, No. 472, 12/03/2002, OJ L75; No. 257, 12/02/2002, OJ L41) for cereals and derived products are

Species specificity and human risk assessment

seating a maximum accepted limit of **50 µg/kg**, which is **10 times higher** than the current calculated maximal limit of 5 ppb.

5.2.3. Worldwide zearalenone risk assessment

To have a general overview we have done a worldwide risk assessment, following the same calculation and logic pathway as presented above. The results are presented within the table 5.7.

Table 5.7. - Worldwide zearalenone risk assessment, in function of the eating habits

<i>World regions</i>		<i>Europe</i>	<i>North America</i>	<i>South America</i>	<i>Africa</i>	<i>Asia</i>
Zen conc. in feedstuffs	µg/kg	1800	14410	5850	8000	840
Animal Meat Products						
Zen daily intake ^a	ng/kg bw/day	4	32	13	18	2
Cereals						
Eating habits ^b	g/day	252	328	273	348	462
Zen daily intake ^b	ng/kg bw/day	25	37	36	41	56
Maximal limit in cereals						
Calculated ^c	µg/kg	4.8	3.5	4.4	3.5	2.6
Current regulation ^d	µg/kg	50	3000	200	200	Not detectable
Tolerated Daily Intake	20 ng Zen/kg b.w./day					
Animal Meat Products and Cereals						
Zen total daily intake	ng/kg bw/day	29	69	49	59	58
Zen daily intake % of TDI	%	145	345	245	295	290

^a calculated upon an average meat products consumption of 0.45 kg/day, a carry-over factor of 0.295, for adults over 15

years old with an average body weight of 60 kg

^b values reported by JEFCA in 2000.

^c calculated upon Cereals/Vegetables eating habits

^d the lower values for Maximal limit in cereals FAO, 2004

It can be observed that the North Americans are the most exposed to the zearalenone, with a total daily intake of 69 ng Zen/kg bw/day, which is 245% higher than the calculate tolerated daily intake. Moreover, Africans and Asians are similarly exposed (58-59 ng Zen/kg bw/day) to zearalenone, exceed by 190% the calculate tolerated daily intake, followed by the South Americans with 49 ng/kg bw/day, value that is 145% higher than the TDI value, even if their eating habits are different. By far the less exposed to zearalenone are the Europeans with 29 ng/kg bw/day, value that is 45% higher than the TDI value. This fact could be partially due to the more restricted regulation concerning the use of

contaminated cereals and meat products. All those values are showing an unacceptable continuous human risk exposure.

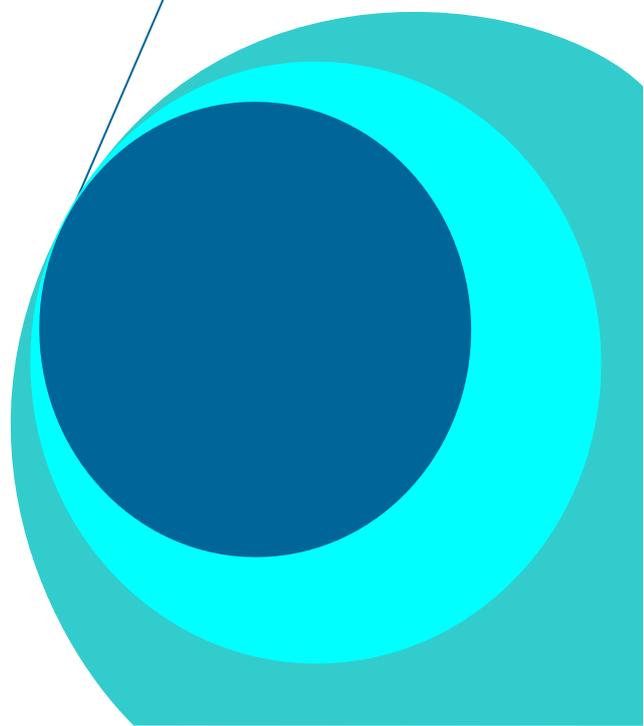
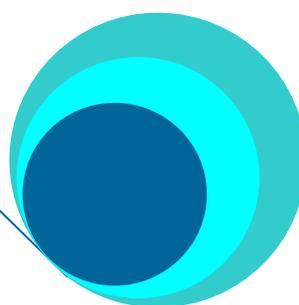
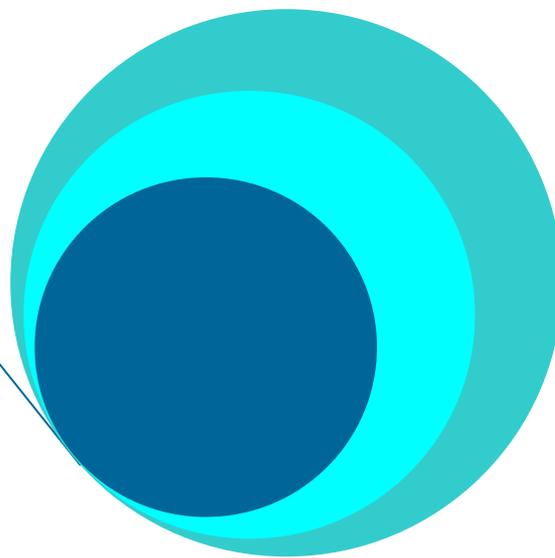
The calculated maximal limits in cereals function of the cereals eating habits are by far smaller (between 10 to 1000 folds) than the limits stated by FAO in 2004. Only in Indonesia the accepted limit is below the limit of detection. Upon our calculation we consider that new regulations concerning the zearalenone accepted levels in cereals must be developed. **An acceptable maximum level of zearalenone in cereals could be of 5µg/kg, if not “below the limit of detection”.**

Moreover, if we are taking in to account Zen bio-activation and the utilization of α -Zol in the North America as cattle growth promoter (under the commercial name of Ralgro[®]) the calculated daily intake of Zen and its metabolites and the overall effect on the human health might substantially increase. Considering the highest accepted value (American Food and Drug Administration) of **150 µg α -Zol/kg** within the cattle meat (upon treatment with Ralgro[®]) and including it in our risk assessment calculation scheme we obtained a value of α -Zol daily intake from meat consumption of **1150 ng/kg bw/day**; dose that is 6 times higher than the LOAEL (Low Observed Adverse Effect Level) determined by JEFCA of **200 µg/kg bw/day (using a security factor of 100)** and the TDI of **0.2 µg/kg bw/day** calculated by SFC (**using a security factor of 100**). In Europe the utilization of α -zearalenol drug for veterinary use is banned, as well as the import of cattle meat from USA or Canada.

In our risk assessment study we did not take into account the occurrence of all Zen metabolites, the human exposed to zearalenone and/or its metabolites due to the consumption of Zen contaminated water (Bucheli et al., 2005) and the respiration of Zen contaminated air (Jarvis and Miller, 2005), especially occupational exposure (e.g. poultry houses; Wang et al., 2008). These two new contamination pathways, even if they can be considered minor from a toxicological point of view, could have an increase impact on the exposure level to mycotoxins.

Mycotoxin risk is difficult to be controlled by humans, because of: the natural and unpredictable risk (humans do not control mycotoxin synthesis); the association between different mycotoxins (multiple risk) and the underestimation risk due to lack of knowledge in this field. Moreover, the mycotoxin risk should be considered in relation with other risks (microbiological, chemical...).

CONCLUSIONS



Many exogenous compounds are found in our closest environment, in our diet or in the air. These are often small organic molecules which can be toxic beyond a certain threshold and trigger immune reactions (allergies) or organic disorders (genetic mutations, cancer, hormonal disorders or regulation ...). **Zearalenone**, a secondary metabolite biosynthesised by several *Fusarium* strains, is a non-steroidal estrogen that frequently contaminates cereal crops worldwide (Bennett and Klich, 2003). Zen resists to most common treatments occurred during food manufacturing. Despite its non-steroidal structure, it binds to estrogenic receptors resulting in functional and morphologic alterations of steroidogenic organs. It interacts not only with both types of estrogen receptors (Celius et al., 1999; Shier et al., 2001; Yu et al., 2004; Takemura et al., 2007), but also with the substrates for a number of hepatic enzymes. Zen is well-absorbed and is able to enter into the cells of various targets. The important disparity concerning the effects of Zen on animal species could in part result from the differences in their hepatic enzymes profile (Gaumy et al., 2001; Cavret and Lecoecur, 2006). Zen metabolism is complex, dominated by conjugation reactions (generally considered as detoxification pathways) and reduction reactions which correspond to biological activation (Gaumy et al., 2001).

Our main objectives were to elucidate the effects of zearalenone on the detoxification enzymes (especially CYPs P450) and to understand Zearalenone effects on different species. In the available literature the effect of Zen on the expression and activity of detoxification enzymes is limited to *in vitro* experiments. To our knowledge *in vivo* researches has not been reported. In order to establish the zearalenone *in vivo* effects on the detoxification enzymes we performed several experiments: - on rat, the classical animal model for the biological studies on the xenobiotics effects and in particularly on Zen effects, and: - on chicken, one of the species considered as the most resistant to zearalenone effects and also through its human consumption, a source of exposition to zearalenone.

To elucidate the effects of zearalenone on the detoxification enzymes (especially CYPs P450) and the understanding of Zen effects on different species we tried to answer to several key questions.

✓ **Can we develop new technological tools to track traces of zearalenone and its derivatives in biological matrices?**

The strong estrogenic effects of zearalenone and its metabolites and the legislated maximal dietary levels prompted the necessity to determine amounts between 10 and 100 µg/kg in food and feed. Very sensitive methods (up to ppb) are compulsory for the determination in body fluids and human and animal tissues, for a good determination of the risk level and in studies of zearalenone metabolism (Fink-Gremmels, 2008).

In order to answer to this question several approaches have been done:

a) A HPLC-DAD method for the separation and quantification of zearalenone and its metabolites was optimised and validated. Using the optimised and validated HPLC method the concentrations of zearalenone and its metabolites in chicken meat, plasma and liver samples were determined. The method responds to the requirements to determine zearalenone and its metabolites at ng/g level in chicken biological samples, allowing the quality control of the samples; it could also be a useful instrument in the studies of zearalenone metabolism.

b) Until now there are no references related to the oxidation of Zen by means of common oxidative reagents. For this reason the oxidation-reduction equilibriums involving Zen and Ce (IV) were studied and the voltametric behaviour of Zen was established, too. Upon our results one can conclude that the oxidation of Zen depends on the ratio between the reagents Zen and Ce (IV) in the reaction system.

We studied **electrochemical behaviour** of Zen by cyclic voltammograms at a glassy carbon electrode in the potential range -600 to 1400 mV (vs. the Ag/AgCl 3 M reference electrode). Upon these results a simple and rapid method was developed for Zen determination by differential pulse voltammetry (DPV) employing the oxidation signal situated at potentials around +1100 mV, because its height presented a linear variation with the analyte concentration. The developed method was applied with good results to determine Zen concentration in corn, barley and oat.

c) The mass spectrum of a natural compound is formed of a main mass peak accompanied by one or two minor peaks arising from the natural isotopes. The uniform enrichment allows the replacement of the main mass peak signal by a cluster composed of five to ten signals depending of the enrichment percentage, which constitute a real specific signature of the enriched compound. The ¹³C enriched Zen as internal standard was used as model compound in order to measure the natural Zen concentrations in different types of biological samples, ranging from extracts of fungi cultures growth on ¹³C enriched

cereals, to the plasma or urine of Zen treated rats. Our results allow us to affirm that the use of a ^{13}C enriched internal standard is a reliable technique for quantitative analysis. Furthermore, this principle can be implemented in terms of quality for studies of metabolism and transport as we have shown that the presence of a mass isotope cluster can facilitate the identification of a metabolite.

Yes, not only we can, but we have developed several analytical techniques: HPLC-DAD, DPV and LC-MS (using enriched ^{13}C Zen as internal standard) to track traces of zearalenone and its derivatives in various biological matrices, like cereals, liver, meat, plasma, and urine samples.

✓ **Can we characterize the zearalenone metabolism and bio-transformation within the animal organism?**

In order to determine the zearalenone metabolisation and bio-transformation, using the developed analytical methods, we assessed zearalenone and its metabolites within biological samples from treated rat and chicken with zearalenone and classical inducers.

Zearalenone transformation within the rat organism has shown that Zen is rapidly eliminated in the urine within the first 6 hours after administration, 60% of urinary eliminated Zen. Interesting is the fact that we identified in the rat urine and liver a NEW metabolite (OH-Zen), previously proven to be formed upon *in vitro* Zen incubation with PB treated rat microsomes (Bravin et al, 2009). The peak of metabolites (α -Zol and OH-Zen) elimination is between 6 and 24 hours after administration, 81% of urinary eliminated α -Zol and 58% of urinary eliminated OH-Zen, respectively. Plasmatic presence of Zearalenone is higher in the early time after administration, with a peak at 6h after Zen administration. The highest Zearalenone liver concentration was observed 3 hours after Zen administration certifying the liver direct implication within Zen detoxification. The peak of metabolites (α -Zol and OH-Zen) presence within liver is between 6 and 10 hours after treatment.

Zearalenone transformation within the chicken organism had two major directions: its effect on the chicken muscle quality and its biotransformation within the chicken. From a nutritional point of view the chicken muscle samples were not altered by the presence of zearalenone; but the presence of Zen and its metabolites in chicken meat makes it unsuitable for human consumption. In the chicken muscle sample only free zearalenone metabolites were found; similar situation on pig muscle been described by Peter Zollner

Conclusions

et al. in 2002. In the dried muscle sample from Zen IP treatment we determined the presence of Zen, Zan, α -Zol, β -Zol and β -Zal. In plasma samples only Zen could be assessed. In liver Zen, α -Zol and β -Zol were found, α -Zol having the highest concentration. Differences have been observed between rat and chicken liver: much higher concentrations of zearalenone and α -zearalenol and the presence of 8-hydroxy-zearalenone were determined in rat liver, whereas β -zearalenol was determined in chicken liver.

The zearalenone bio-transformation within the animal organism is showing species specificity. In rats we observed a rapid urinary elimination of Zen (the first 6 hours after administration) and its metabolites (between 6 and 24 hours after administration); resulting in an overall urinary elimination of Zen and metabolites of about 4% of the administrated Zen. The zearalenone concentration within plasma and liver of Zen treated chickens is 40-50 times lower than in the treated rat samples. The presence of Zen and its metabolites in chicken muscle has been determined; making it unsuitable for human consumption. The in vivo presence of a new zearalenone hydroxy-metabolite has been showed.

- ✓ **Does zearalenone affect the hepatic detoxification enzymes? If yes, which are its in vivo effects and transformation, and its molecular mechanism?**

The **P450 total concentrations** were determined and differences have been observed between the treated rat and chicken total amounts; the P450/Protein ratio is 8 times lower in chicken than in rat. Differences were also observed between the **in vitro Zen metabolic profile** at pH 7.4 of chicken and rat microsomes; consisting in the α Zal and β Zol formation in chicken and the formation of OH-Zen in rat. The total average metabolites formation in chicken is about 2 times higher than in rat, implying the idea that chickens are more active from a metabolic point of view, possibly explaining thus the so-called “insensibility” of the poultry to zearalenone presence. The *in vitro* determination of the **X-ROD and 7-BFC O-dealkylase metabolic activity** on treated rat and chicken microsomes highlighted that zearalenone might induce the CYP 1 and 2 families.

The **in vivo Zen effects on the detoxification enzymes** in rats, was assed using the quantitative real time RT -PCR technique for mRNA assessment. The mRNAs expression encoding 25 enzymes usually involved in xenobiotic detoxification were determined. An

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important stimulatory effect of Zearalenone on the P-gp (ABCB1) mRNA expression has been determined, suggesting the idea that P-glycoprotein might be implicated in the detoxification of Zearalenone; P-gp transport of Zen has been recently determined *in vitro* on Caco2 cells (Videmann et al., 2008). Cytochrome mRNA expression been corroborated with the protein enzymatic activity. Zearalenone induce early and rapid metabolic answer, especially for CYP2C7, which could have an important role within Zen detoxification path in rats. The influence of Zearalenone on the CYP2B2 and 3A mRNA expression and activities was determined.

Zearalenone interactions with the **steroid metabolism** have been determined on several types of microsomes from different species (rat, pig and human) and treatments. Estradiol metabolism is dominated by the 2OH-E2 and 6 α OH-E2 formation and is perturbed by the Zen presence, resulting in a diminution of 43-100 % of 2OH-E2 level for almost all the tested species microsomes, excepting pig microsomes. More important is the formation of the 4OH-E2, known carcinogen, in rat and poultry microsomes. Inter-species differences were also observed for testosterone metabolism: the rat TST metabolism is dominated by the 6 α OH-TST; the chicken and pig TST metabolism is dominated by 15 α OH-TST; and human and primates TST metabolism is dominated by 6 β OH-TST, formation. Within zearalenone treated rat microsomes is predominantly formed 6 β OH-TST, specific for CYP 1A and 3A. Zearalenone inhibits the metabolic activity of almost all studied species microsomes, excepting chicken microsomes, which are not affected by its presence.

Yes, zearalenone affects the hepatic detoxification enzymes. We base our statement upon the results obtained in vitro and in vivo. Resorufin derivates O-dealkylase metabolic activity is perturbed upon Zen acute treatment. Zearalenone presence is interfering with the steroid metabolism resulting in a diminution of testosterone total metabolic activity and the occurrence of 4 hydroxy-estradiol (considered as highly carcinogenic) in rat and chicken microsomal extract. Zen affects in vivo the mRNA expression of the detoxification enzymes. Corroborating all our results one could conclude that P-gP might be implicated in zearalenone detoxification, while the rat CYP2C7 (homolog of human CYP2C8) is implicated in the formation of the hydroxylated Zen metabolite. Rat cytochromes 1A2, 2B2 and 3A1/2 are also affected by the zearalenone presence, but not at a gene regulatory level more likely at the post-transcriptional level.

✓ **Are there species specificities linked to Zen metabolism? If yes, are humans undergoing a risk?**

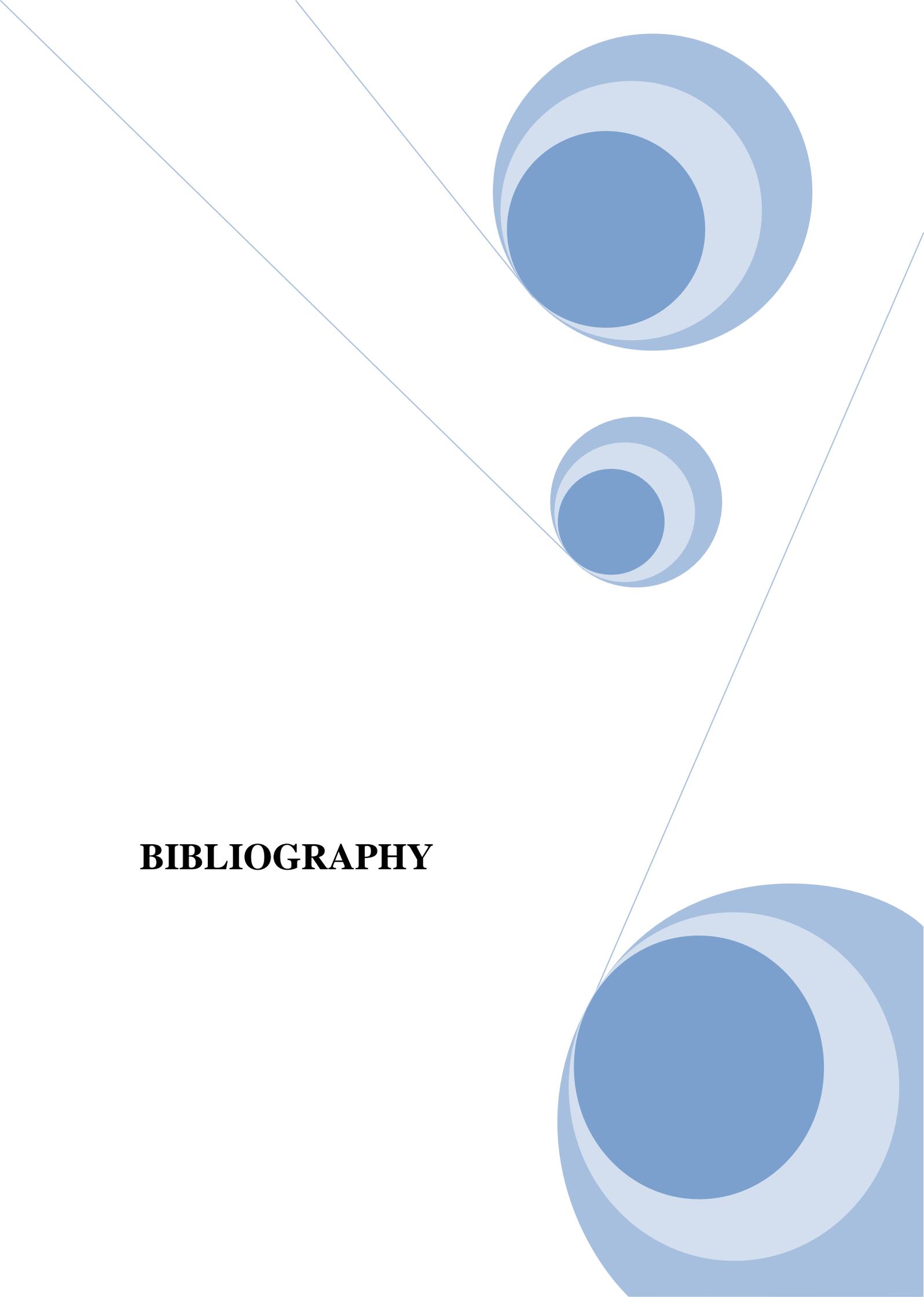
Differences have been seen first between the treated rat and chicken total amounts of P450-CO complex; the P450/Protein ratio is 8 times lower in chicken than in rat. Differences were also observed between the *in vitro* Zen metabolic profile at pH 7.4, estradiol and testosterone metabolism of chicken and rat microsomes

In order to have a clearer image of interspecies disparities we used fluorimetry, to assess the **inhibition levels (IC₅₀)** of Zearalenone and its metabolites on the ETR and 7-BFC *O*-dealkylase metabolic activity. Several types of microsomes from different species (rat, pig and human) and treatments were used. Species differences (specificities) were determined. Human microsomes have a higher inhibitory potency for Zen, α -Zol and β -Zol on ETR *O*-deethylase metabolism, in contrast to the rat microsomes which have a higher inhibitory potency for Zan, α -Zal and β -Zal. ETR *O*-deethylase metabolism on pig microsomes is not influenced by the Zen or its metabolites presence (IC₅₀ > 20 μ M). Human microsomes have a higher inhibitory potency for Zan, α -Zal and β -Zal on 7-BFC *O*-debenzylase metabolism. Pig and rat microsomes showed similar IC₅₀ ranking.

A **worldwide risk assessment** of human zearalenone exposure was done taking in to account the eating habits. The North Americans were found to be the most exposed to the zearalenone, with a total daily intake of 345% TDI (tolerated daily intake), Africans and Asians are similarly exposed to zearalenone, with a total daily intake of 290% TDI, followed by the South Americans with a total daily intake of 245% TDI, even if their eating habits are different. By far the less exposed to zearalenone are the Europeans with a total daily intake of 145% TDI. This fact could be in part due to the more restricted regulation concerning the use of contaminated cereals and meat products.

Also maximal limits in cereals were calculated in function of the cereals eating habits and are by far smaller (between 10 to 1000 folds) then the stated limits by FAO in 2004. According to our calculation we consider that new regulations concerning the accepted levels of zearalenone in cereals must be developed.

Species specificities linked to Zen metabolism have been stated all along the present work. A worldwide risk assessment of human zearalenone exposure, taking into account the eating habits was done, showing an important and constant human health risk. Also the necessity of regulation changes concerning the acceptable maximum level of zearalenone in cereals arises; a 5 μ g Zen per kg we consider to be acceptable.

A decorative graphic on the right side of the page. It features three blue circles of varying sizes, each composed of concentric circles in different shades of blue. Two thin, light blue lines intersect at a point, forming a V-shape that frames the circles. The circles are positioned at the top, middle, and bottom of the right side.

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