UNIVERSITA' DEGLI STUDI DI PARMA

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Ciclo XXV

Masked mycotoxins: occurrence, metabolism and role *in planta*

Coordinatore:
Chiar.mo Prof. Davide Barbanti

Tutor:
Chiar.mo Prof. Gianni Galaverna

Dottorando: Andrea Dall'Erta

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General introduction

i. Mycotoxins

Mycotoxins are toxic secondary metabolites produced by a wide range of different filamentous fungi, frequently occurring in different foodstuffs. These metabolites constitute a chemically heterogeneous group of molecules that are classified together only on account of their toxic activity towards humans and livestocks. The production of a particular mycotoxin is generally confined to a relatively small number of fungal species and may be species- or even strain-specific[1]. Molds are ubiquitous in nature and are universally found where environmental conditions are favorable to their growth. Fungi are present in soil and plant residues left in field after harvest, and they are spread by wind, insects, and rain; for these reasons they are often found in foods together with their associated mycotoxins. Of the thousands of existing mycotoxins, a few hundred are frequently found in food and even less can be considered as a challenge in terms of food safety. At the farm level, mold growth can result in reduced crop yields and livestock productivity stemming from illness or death due to consumption of contaminated feed. In food manufacturing, mycotoxins are characterized by high thermal stability and are generally not significantly degraded by conventional food processing. From the analytical point of view, mycotoxin detection is complicated due to limitations in analytical methodologies. In the marketplace, mycotoxins represent a hurdle to international trade; their presence in foods and feeds actually led to an increase of regulations concerning these products. Those commodities not accomplishing legal limits should be removed from the market. When present in foods in sufficiently high levels, these fungal metabolites can have toxic effects ranging from acute (i.e. liver or kidney damage), to chronic (i.e. liver cancer), mutagenic and teratogenic effects; the resulting symptoms range from skin irritation to immunosuppression, birth defects, neurotoxicity and death. Among the most important mycotoxins, aflatoxin B1 (AFB1) and ochratoxin A are well-known carcinogenic agents in humans, while fumonisins and patulin have been often associated with cancer-related diseases in mammals. Deoxynivalenol and other trichothecenes as well as AFB1 are likely to exert immunosuppressive effects and fumonisin B1 (FB1) may contribute to neural tube defects. Renal disfunction due to ochratoxin A exposure (suspected in Balkan endemic nephropathy) is also a significant problem, especially as this could exacerbate impaired renal function in individuals with diabetes and other chronical diseases. There is also uncertainty related to the effects of chronic, low-level, long-term exposure to single and/or multiple mycotoxins, which may show synergic effects. Mycotoxins can be classified according to the main target organ as reported in table 1.

***Table 1**: Classification of mycotoxins according to toxicological effects.*

Effect	Mycotoxins
Hepatotoxic	Sporidesmin Aflatoxins Luteoskyrin Cyclochlorotine Rubratoxins Sterigmatocystin
Nephrotoxic	Ochratoxin Citrinin
Neurotoxic	Penitrema Patulin Citreoviridin
Citotoxic	Trichothecenes
Hestrogenic	Zearalenone
Haemorrhagic and circulatory toxins	Ergot alkaloids

Some mycotoxins can have many toxic effects on human and animal health, as reported in Table 2.

***Table 2**: Mycotoxins and principal toxic effects.*

Mycotoxins	Principal effects
Aflatoxins	Liver diseases (hepatotoxic, hepatocarcinogenic); carcinogenic and teratogenic effects; hemorrhages (intestinal tract, kidneys); reduced growth rate; immune suppression.
Ochratoxins	Nephrotoxic; carcinogenic; mild liver damage; enteritis; teratogenic effects; poor feed conversion; reduced growth rate; immune suppression.
Fumonisins	Pulmonary edema; equine leukoencephalomalacia; nephro and hepatotoxic; immune suppression.
Trichothecenes	Digestive disorders (vomiting, diarrhea, feed refusal); reduced weight gain; hemorrhages (stomach, heart, intestine, lung, bladder, kidney); edema; oral lesions; dermatitis; blood disorders; infertility; degeneration of

	bone marrow; slow growth; immune suppression.
Zearalenone	Estrogenic effects; edema of vulva; prolapse of vagina; enlargement of uterus; atrophy of testicles; atrophy of ovaries; enlargement of mammary glands; infertility; abortion.
Ergot alkaloids	Nervous or gangrenous syndromes; digestive disorders (vomiting, diarrhea, feed refusal); reduced weight gain; convulsion; abortion.
Citrinin	Nephrotoxic; teratogenic; hepatotoxic.
Patulin	Mutagenic; genotoxic; neurotoxic; immune suppression.

Environmental factors affect mycotoxin occurrence in raw and stored commodities. Mycotoxin production by fungi is strictly related to optimal temperature and water activity. Typical values for toxin production by *Aspergillus*, *Penicillium*, and *Fusarium* spp. in culture are provided in Table 3.

***Table 3*: Optimal conditions for mycotoxins production.**

Microorganism (mycotoxin)	Temp (°C)	a_w
Aspergillus flavus (aflatoxin)	33	0.99
Aspergillus ochraceus (ochratoxin)	30	0.98
Penicillium verrucosum (ochratoxin)	25	0.90 - 0.98
Aspergillus carbonarius (ochratoxin)	15 – 20	0.85 - 0.90
Fusarium verticilloides, F. proliferatum (fumonisin)	10 – 30	0.93
Fusarium graminearum, F. Culmorum (deoxynivalenol)	25	0.99
Fusarium graminearum	25 – 30	0.98
Penicillium expansum (patulin)	0 – 25	0.95 - 0.99

Traditionally, control of mycotoxin contamination in foods has been attempted through control of water activity, pH and quality check of incoming ingredients. Nowadays, innovative strategies are available for mycotoxin reduction all along the food chain, including the use of genetically modified crops with increased insect resistance and thus, lowered rates of fungal infection; improved management of grain ingredients and inclusion of controls for mycotoxins in food manufacturing such as Hazard Analysis and Critical Control Point (HACCP) plans.
A food-related classification of mycotoxins is reported below:

***Table 4**: The major mycotoxins and contaminated food.*

Mycotoxins	Food/feed
Patulin	Fruit juice, apple
Fumonisins	Maize, maize-products
Aflatoxin B1-B2-G1-G2	Hazelnuts, brazil nuts, pistachio nuts, peanuts, spices, nuts, dried fruit, cereals, oilseeds, milk
Aflatoxin M1	Milk
Trichothecenes	Cereals and cereal products
Zearalenone	Cereals and cereal products
Ochratoxin	Cereals, wine, grape juice, coffee, and pork, dried vine fruit, spices, liquorice

The acute toxicity of mycotoxins resulted in serious human health problems throughout centuries. Since the early 1960s, when the aflatoxins were found to be carcinogenic, the scientific community started to consider these compounds also as chronical or sub-chronical toxic agents for humans. The public health concerns resulting from mycotoxins in food, including metabolites in animal tissues deriving from the carry over from feeds, has prompted research efforts, focusing actually on analytical methods development. Analysis of mycotoxins along the food production chain is indeed essential to minimize the consumption of contaminated food and feed[2]. Among the different mycotoxins, deoxynivalenol and zearalenone are the most frequently found in cereals and derived products: their general characteristics are described below.

Deoxynivalenol

Deoxynivalenol (DON, vomitoxin) is the most common contaminant of grains and derived products Fig. 1). From the chemical point of view, it is an epoxy-sesquiterpenoid whose chemical name is 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-one. Its molecule contains 3 free hydroxyl groups which are directly associated with its toxicity.[3]

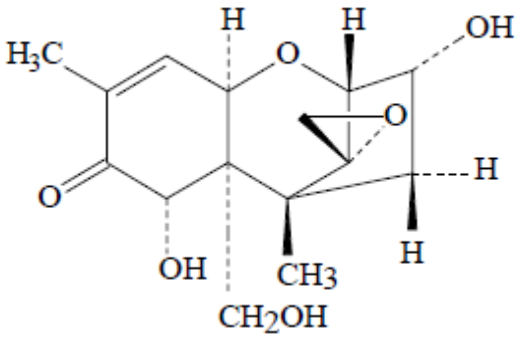

***Fig. 1**: Chemical structure of DON.*

DON is a biologically active compound that is able to disrupt cell signaling, differentiation and growth processes and macromolecular synthesis[4]. The effects of this mycotoxin on animal health are well documented and are localized at immune system and gastrointestinal tract. In particular, high doses of deoxynivalenol can cause acute effects such as diarrhea, vomiting, leukocytosis, hemorrhage, circulatory shock and ultimately death. At low doses, it can cause chronic effects characterized by anorexia, reduced weight gain, nutrients malabsorption, neuroendocrine changes and immunologic effects[5]. It is noteworthy that deoxynivalenol is highly stable during processing and cooking of food: for this reason human exposure to this mycotoxin can be very high. Surveys have shown that deoxynivalenol occurs predominantly in grains such as wheat, barley, oats, rye and maize and less often in rice, sorghum and triticale.
European commission fixed the maximum level of mycotoxin in cereal and cereal products with the Commission Regulation (EC) No 1126/2007 of 28 September 2007, [6] amending the already established Regulation (EC) No 1881/2006 (Table 5).

***Table 5*: Legal limits for DON contamination**

2.4	**Deoxynivalenol**	**Maximum levels (µg/kg)**
2.4.1	Unprocessed cereals other than durum wheat, oats and maize	1250
2.4.2	Unprocessed ddurum wheat and oats	1750
2.4.3	Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	1750
2.4.4	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 2.4.7, 2.4.8 and 2.4.9	750
2.4.5	Pasta (dry)	750
2.4.6	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
2.4.7	Processed cereal-based foods and baby foods for infants and young children	200
2.4.8	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	750
2.4.9	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	1250

Zearalenone

Zearalenone (ZEN, previously known as F-2 toxin) is a nonsteroidal oestrogenic mycotoxin. Its name was given as an acronym for G. zeae, resorcylic acid lactone, -ene (for the presence of the C-1' to C-2 double bond) and -one, for the C-6' ketone (Fig. 2). ZEN is biosynthesized through a polyketide pathway by a variety of *Fusarium* fungi, including *F. graminearum* (Gibberella zeae), *F. culmorum, F. cerealis, F. equiseti, F. crookwellense and F. semitectum*, which are common soil

fungi, widespread in temperate and warm climates and which are regular contaminants of cereal crops worldwide.

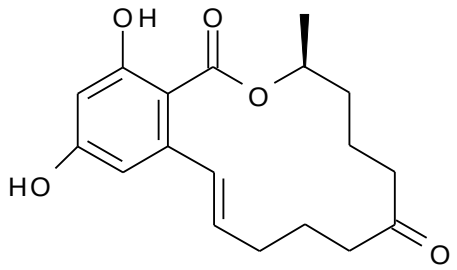

Fig. 2: Chemical structure of ZEN.

In mammals, the keto group at C-8 is reduced to two stereoisomeric metabolites of ZEN (α- and β-isomers). Fungi-producing ZEN contaminate corn and also colonize, to a lesser extent, barley, oats, wheat, sorghum, millet and rice. In addition, this compound has been detected in cereals products like flour, malt, soybeans and beer. Fungi of the genus *Fusarium* infect cereals in the field. Toxin production mainly takes place before harvesting, but may also occur post harvest if the crop is not properly handled and dried.[7]

The most important effect of zearalenone is on the reproductive system. Animal studies showed that zearalenone is fairly rapidly absorbed following oral administration and can be metabolized by intestinal tissue in pigs and possibly also in humans, with the formation of the previously described derivates (α- and β- zearalenol), which are subsequently conjugated with glucuronic acid. The proportions of these various products have been shown to vary considerably between species.

The ability of zearalenone to cause hyperestrogenism, particularly in swine, has been known for many years. Several of the closely related metabolites of zearalenone produced by *Fusarium spp.* also have similar properties, although few have been proven to occur naturally. Although swine have been found to be the most sensitive domestic animal to zearalenone, calves have been reported to show earlier sexual maturity, dairy cows have been reported to have vaginitis, prolonged oestrus and infertility and sheep are reported to become sterile.

Sub-acute and sub-chronic toxicity studies, of up to 14 weeks duration, have been completed using several species and results showed that most effects were due to the oestrogenic effects of zearalenone. The oestrogenic potency of this mycotoxin has been compared with other plant derived oestrogens in MCF-7 or T-47D breast cancer cells and results suggested that in comparison with 17-β-estradiol, it is one of the most potent natural xenoestrogens.

It has been concluded that there is limited evidence in experimental animals for the carcinogenicity of zearalenone, while the evidence for genotoxicity has been contradictory, although it has suggested that zearalenone is genotoxic in mice. However, these effects may be species dependant and further studies are required to confirm whether zearalenone should be considered as a potential human mutagen or carcinogen, although the association between zearalenone exposure and human diseases remains speculative at present[8].

European commission fixed the maximum level of zearalenone in cereal and cereal products with Commission Regulation (EC) No 1126/2007 of 28 September 2007, amending Regulation (EC) No 1881/2006 [6].

***Table 6*: Legal limits fixed for ZEN contamination.**

2.5	Zearalenone	Maximum levels (µg/kg)
2.5.1	Unprocessed cereals other than maize	100
2.5.2	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	350
2.5.3	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 2.5.6, 2.5.7, 2.5.8, 2.5.9 and 2.5.10	75
2.5.4	Refined maize oil	400
2.5.5	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50
2.5.6	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100
2.5.7	Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children	20
2.5.8	Processed maize-based foods for infants and young children	20
2.5.9	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	200
2.5.10	Milling fractions of maize with particle size ≤ 500 µm falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 µm not used for direct human consumption falling within CN code 1904 10 10	300

Biosynthesis of zearalenone

Zearalenone is biosynthesized through a polyketide pathway by a variety of *Fusarium* fungi, including *F. graminearum (Gibberella zeae), F. culmorum, F. cerealis, F. equiseti, F. crookwellense* and *F. semitectum*, which are common soil fungi, that are mainly found in temperate and warm countries and are regular contaminants of cereal crops worldwide. The biosynthetic pathway started with the condensation of one acetyl-CoA and five malonyl-CoA units, resulting in a hexaketide. The three reducing domains of PKS4 (PolyKetide Synthase) gene are deployed to various degrees during the different cycles of synthesis: no. 6 ketide remains unreduced, no. 1 and 3 ketides are processed by the KR (β-ketoreductase) domain resulting in hydroxyl groups, no. 5 ketide is processed and resulting in an enoyl group, while n. 2 and 4 ketides are reduced completely to alkyls by 3 enzyme domains. The formed hexaketide is then passed onto the non-reducing PKS13, functioning as a starter unit for further extension of the polyketide chain. PKS13 completes three iterations extending the chain by three additional ketide units, resulting in a nonaketide. This then undergoes two rounds of intramolecular cyclization reactions, resulting in formation of an aromatic ring and a macrolide ring structure with a lactone bond. The final conversion of the formed zearalenol to zearalenone is catalyzed by the isoamyl alcohol oxidase, ZEB1, which converts the macrolide bound hydroxyl group to a ketone. [9]

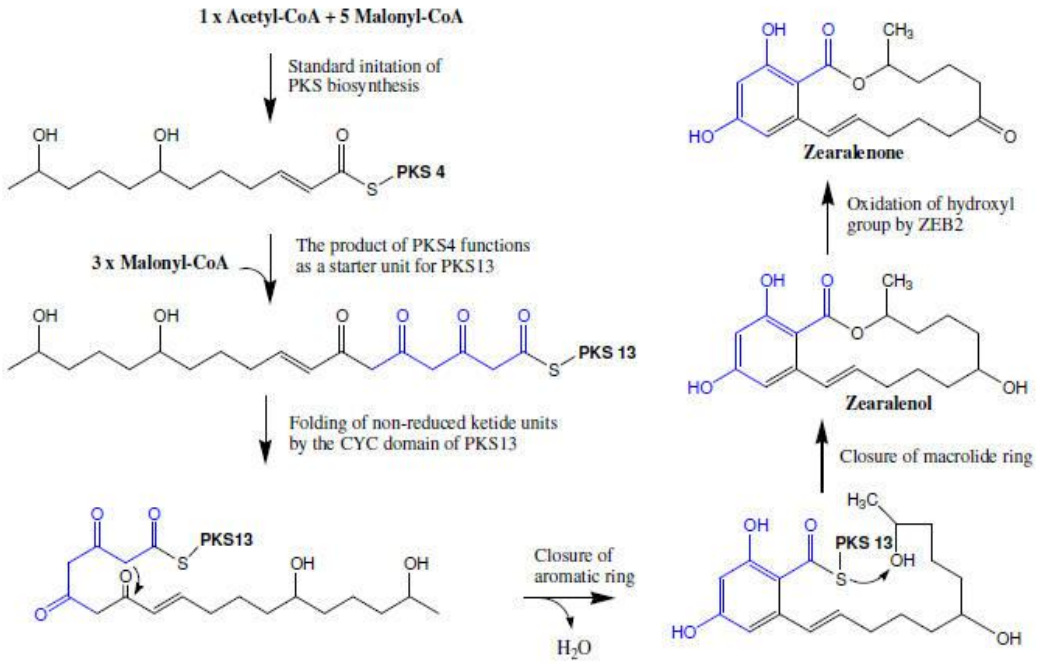

***Fig. 3*: Model for the zearalenone biosynthetic pathway.**

Occurrence

Zearalenone has been found worldwide in a range of cereals and other crops, including wheat, barley, maize, rice, oats, sorghum and some legumes. High levels have also been reported in bananas grown in India. The level of contamination in cereal crops varies widely depending on climatic conditions. For example, zearalenone was found in 11-80% of wheat samples collected randomly in Germany between 1987 and 1993. The mean yearly contents were 3-180 µg/kg and the highest level found was 8,000 µg/kg. There is evidence that cereal crops produced by 'alternative' or 'ecological' cultivation methods may develop higher levels of contamination than those produced by conventional methods.[10]

Zearalenone has also been found in processed foods, especially those produced from cereals, although its levels are usually low. Foods reported to be contaminated have included wheat and corn flour, bread, breakfast cereals, noodles, biscuits, snacks and corn beer. The metabolite β-zearalenol may be produced from zearalenone by yeast fermentation and so may occur in beer. Contamination with zearalenone does not seem to be the major problem in foods of animal origin. It has been found to be excreted into the milk of lactating cows, along with α- and β-zearalenols, but only when very high oral doses (6,000 mg) were used.

Average dietary intakes of zearalenone in humans have been estimated at 1.5 µg per day for the European diet and 3.5 µg per day for the Middle Eastern diet. Cereals are the major contributor of zearalenone in the diet. Data on the prevalence of ZEN throughout the world are summarised below. Despite these issues the data set is very useful in giving a general indication of which commodities and foods represent the greatest potential sources for human exposure.

Grains

Maize clearly represents a large potential contributor to ZEN exposure, with a relatively high incidence of contamination. The mean levels of contamination in maize, are clustered around concentrations between 10-100 μg/kg (13 studies), 200-500 μg/kg (8 studies) and below 6 μg/kg (9 studies). The maximum levels found in maize in these studies was rather high, being often above 1000 μg/kg. The mean levels of contamination in wheat and barley are clustered around concentrations of 10-100 μg/kg (8 studies) and below 5 μg/kg (7 studies), with a few above 100 μg/kg (5 studies). The maximum levels found in wheat and barley in these studies also tended to be lower, with few above 1000 μg/kg. These factors suggest that, in general, there is a lower level of ZEN contamination in wheat and barley than in maize. Marked differences in ZEN concentrations were found at the two maize locations and with the type of its fraction; however generally the levels in the chaff were substantially higher than kernels classified as either healthy or *Fusarium* damaged[11].

Edible oils, miscellaneous foods and spices

Tests made on edible vegetable oils have recently been evaluated in regard with ZEN contamination. Maize oil, derived from its germ, showed the higher average level of contamination than oil derived from soybeans or sunflower seeds[12]. Other edible oils tested by the same research group derived from olive, rapeseed, safflower, wheat germ, pumpkin kernel, peanut, walnut, grape kernel, sesame seed, linseed, and palm, all resulted (n=63) having no detectable ZEN or α-zearalenol (α-ZOL) levels. A wide variety of spices have also been examined for ZEN contamination, but generally they showed very low levels or the absence of toxin[13]. Recent data on the occurrence of zearalenone in paprika and chilies in Spain suggest that it may be prevalent in these spices, but at modest levels, as the highest positive sample contained 131 μg/kg. Recently 39 types of medicinal or aromatic herbs, purchased in Spain, were tested for multiple mycotoxins presence, including ZEN[14]. Of the 84 samples tested, 82 resulted contaminated and above the limit of detection (0.14 μg/kg), 40 contained ZEN toxin in a quantity between 0.3 and 5 μg/kg, 22 between 5.1 and 10 μg/kg, and for 20 samples the level was more than 10.1 μg/kg. [15]

In conclusion it is possible to summarize that zearalenone is widely distributed in a large number of foods with high concentration mean values. These data confirm the importance of studies on zearalenone directed to the improvement of analytical methods for its detection and aimed to a better comprehension of its effects on human health.

ii. Masked mycotoxins

It has been recently shown that, analogously to animals, also plants are able to counteract fungal invasion modifying the chemical structure of mycotoxins, producing different kind of metabolites. Among them, particular attention has been raised by so called masked mycotoxins, secondary metabolites produced by plants via conjugation mechanisms. Masked mycotoxins are so called because usually escape routine analysis on account of their different chemical behavior in comparison to their parent compounds (native forms).

In particular, these derivatives are mainly produced by plants via enzymatic transformations related to detoxification processes (phase II metabolites) and have been related to a resistance mechanism exerted by plants to counteract pathogen invasion. This detoxification process includes the conjugation of mycotoxins to polar substances such as sugars, amino acids or sulfate and subsequent storage of this metabolites in vacuoles of cells or conjugated to biopolymers such

as cell wall components. These structural modifications are in addition to the chemical transformations which may also occur during food processing and fermentation. Soluble low-molecular weight derivatives can be detected by appropriate analytical methods when their structure is known and analytical standards are available. On the contrary, masked mycotoxins bound or associated to solid matrices cannot be directly detected, as they have to be previously cleaved from the matrix by chemical or enzymatic treatment.[16]

The topic of conjugated or masked mycotoxins first caught attention in the mid-1980s because in some cases of mycotoxicoses, clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed. It has been hypothesized that the unexpected high toxicity could for instance be attributed to the occurrence of undetected, conjugated forms of mycotoxins that are hydrolysed to the precursor toxins in the digestive tracts of animals. High-performance liquid chromatography (HPLC) combined with tandem mass spectrometry (MS/MS) offers a powerful tool for the identification and characterization of mycotoxin conjugates.[17] Metabolites of both deoxynivalenol and zearalenone have been found to occur in cereals: in particular, deoxynivalenol-3-β-glucoside (the glycosylated form of DON), zearalenone-14-glucoside and zearalenone-14-sulfate are described in the following paragraphs.

Deoxynivalenol-3-β-D-glucoside

Deoxynivalenol-3-β-D-glucoside (D3G), a phase II plant metabolite of deoxynivalenol (DON), occurs in naturally *Fusarium* contaminated cereals (Fig. 3). This compound is far less active as protein biosynthesis inhibitor than DON, as demonstrated from *in vitro* tests with wheat ribosomes: thus, the glucosyl transfer reaction can be considered as a detoxification mechanism of DON in plant.

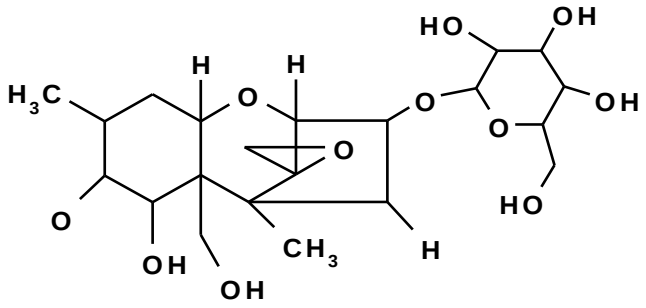

***Fig. 4*: Structure of Deoxynivalenol-3-glucoside.**

D3G can be formed by *Fusarium* infected plants as a response to DON production by fungi. The existence of a plant conjugate of DON, which might be responsible for partial resistance towards *Fusarium* head blight disease of wheat, was proposed in the mid-eighties. Miller at al. in 1983 was the first that observed an unusual behavior in plants[18]. In this work, corn plants were inoculated with *F. graminearum* and then it was observed that concentration of deoxynivalenol increased in the first six weeks from inoculation and decreased after this period. In the following year Young et al. observed that in the case of a yeast-raised product, made from contaminated wheat flour, the concentration of DON increased by over 100%, which is not observed in other investigated products. Data are reported in the following table.

***Table 7*: Deoxynivalenol variation during processing (Young at al.).**

		% change		
			relative to unclean wheat	
processing step	% soft wheat in product	realtive to flour used	processing only	processsing and diluition
biscuit flour to			-26	-26
low chlorine treated flour	100	-14	-36	-36
high chlorine treated flour	100	-14	-36	-36
cookie A	63	-35	-51	-69
cookie B	56	-8	-31	-61
cookie C	58	+10	-18	-52
cake flour to			-36	-36
low chlorine treated flour	100	+12	-28	-28
yeast doughnut A	16	+189	+86	-71
yeast doughnut B	18	+118	+40	-78
cake doughnut	22	-34	-58	-91
white cake	29	-4	-39	-82
cookie D	48	-20	-49	-76
bran to			+42	+42
hammermilled bran to	100	-27	+3	+3
wheatlbran blend to	100	+1	+4	+4
cooked blend to	82	-36	-33	-45
bran cereal B	82	-19	-44	-56
cooked bran to	76	+14	+18	-10
bran cereal A	76	-11	+6	-20

In 1984, Young et al. tried to explain these phenomena attributing the increase of DON after fermentation to the enzymatic conversion of some precursor into DON.[19] The most probable reason for this apparent increase was that the toxin had been metabolized by wheat plant to a

compound other than DON, which, under certain conditions, could be transformed back to DON. It was assumed that the conjugate form might be deoxynivalenol-3-glucoside (D3G). One of the most relevant characteristic of D3G and of all the other masked mycotoxins is their ability to escape routine analysis. The increased polarity of these forms in comparison with their precursor toxins makes them difficult to extract with the usual solvents and/or get lost in the cleanup process. Moreover, standards for these substances are not commercially available. This problem was solved in 1991 by Savard at al[20]., that chemically synthesized the glucosylated form of deoxynivalenol. In 1992 D3G was formed from DON treated maize cell suspension cultures.[21] After few years, D3G was for the first time detected in natural contaminated wheat and maize[22]. Since then, the worldwide occurrence of D3G in different cereal crops has been reported. The molar percentages of D3G/DON varied strongly in these studies, but reached maximum levels of 46%. Accordingly, considerable amounts of D3G were found in foodstuffs such as breakfast cereals, snacks[23] and beers[24]. Despite its frequent occurrence, the toxicological relevance of D3G in humans and animals has not yet been evaluated. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) stressed the possibility that D3G is hydrolyzed in the digestive tract of mammals. Although this assumption is not yet supported by *in vivo* data, a recent study showed that certain intestinal bacteria are capable of cleaving D3G to DON *in vitro*[12]. Numerous studies have examined the toxicokinetics of DON *in vivo*, revealing two major metabolic pathways: de-epoxidation by anaerobic bacteria and conjugation to glucuronic acid. De-epoxy deoxynivalenol (DOM-1), which is at least 50-fold less toxic than DON, is formed by anaerobic ruminal or intestinal microbes. DOM-1 can be excreted via the feces or it can be absorbed and detected in different biological samples of animals, like urine, plasma, and milk[25]. The ability to detoxify DON to DOM-1 in the upper gastrointestinal tract is considered a major cause for the differences regarding the susceptibility to DON among species. The main metabolic pathway of mammals to detoxify resorbed DON is glucuronidation, a phase II reaction which reflects one of the most important mechanisms to inactivate xenobiotics by enhancing their polarity and excretion. Studies in different animal species showed that deoxynivalenol-glucuronide (DON-GlcA) is the major DON metabolite in plasma and urine. In humans, the measurement of urinary DON and DON-GlcA levels has been used successfully as a biomarker to assess DON exposure[26]. In addition, the formation of DOM-1-glucuronide (DOM-1-GlcA) in urine of rats has recently been reported. The presence of characteristic metabolites in urine and in feces allows conclusions regarding the absorption and metabolism of mycotoxins. Studies determining the total recovery of orally administered DON in excreta of rats have been performed already in the 1980s. Depending on whether DON was applied in its pure form or as a radiolabelled compound, observed recoveries ranged from around 15 to 89% of the applied toxin dose, respectively. SO far D3G has not been considered in the regulatory limits for cereal-based food established by the European Commission for DON (European Commission, 2006). Yet, JECFA stated that D3G might be an important contributor to dietary DON exposure and emphasized the need of *in vivo* data concerning the absorption, distribution, metabolism and excretion (ADME), in order to evaluate the potential health risk of D3G.

Zearalenone-14-glucoside and Zearalenone-14-sulphate

Masked forms of zearalenone have also been found in plants as detoxification forms: in particular, zearalenone-14-glucoside and zearalenone-14-sulphate[27] (Fig. 4). The fate of these conjugate forms in plants is still not well understood: however, it is assumed that storage occurs either in vacuoles or by incorporation into the cell wall (as insoluble residue). Upon ingestion by animals and humans, masked mycotoxins can potentially be cleaved during digestion of the contaminated feed or food, releasing their toxic aglycones.

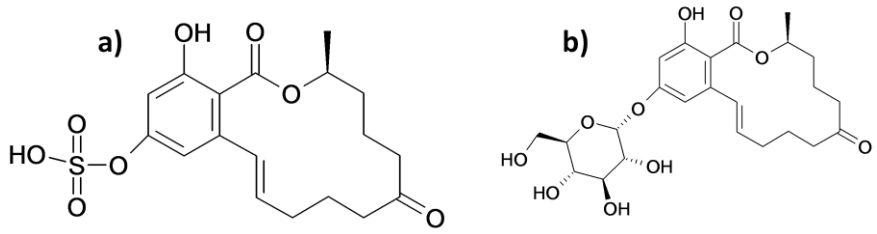

***Fig. 5*: Structure of Zearalenone-14-sulfate (a) and Zearalenone-14-glucoside (b).**

To date, only few studies have demonstrated the presence of these metabolites in crop plants. A survey of 10 wheat grain samples revealed the relative proportion of Z14G to ZEN to be, on average, about 27%. The second masked form deriving from ZEN is Z14S, also formed from detoxification pathway of plant. This compound was found in different commodities (wheat flour, whole-meal wheat bread, maize meal, biscuits, wheat) although in very low concentration, with the highest amount found at 6.1 µg\Kg in bran flakes. Several investigations are currently focused on the evaluation of the occurrence of these masked forms in different cereals and derived products. These surveys are aimed at understanding the significance of these forms both for plants as well as for human and animal health.

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Chapter 1: Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat

Introduction

1.1 Wheat

1.1.1 General characteristics

Wheat is certainly one of the most important cereal. It belongs to the *Graminacee* family (*Triticum* genus), which also includes other important cereals like rice, maize, oats, sorghum etc.. As far as the structural features are concerned, wheat is an herbaceous annual plant whose height may reach up to 1.2 m. The stems are erect and hollow inside except at the nodes. Stems' growth is not apical but produced by stretching of the tissues above the nodes (meristem), from which the leaves grow. Flowers are gathered in spikes. Each spike consists of a main axis or rachis on which the spikelets are laterally distributed. These consist of a main axis from which some filaments arise terminated by the glumes: these structures enclose the flowers until maturation starts. Moreover, flowers are protected by two bracts: the inner is called palea and the outer is called lemma. The latter is topped with a beard that gives the ear of wheat a feathery appearance. Wheat flowers don't have petals or sepals. Each female flower consists of an ovary from which two styles emerge, with two stigmas each. The male flowers have three stamens that can be gold, green or violet. The maturation of wheat produces the wheat grain, which is called caryopsis. The grain of wheat consists of the following parts:

- a protective coating or husk: it is commonly known as bran and consists mainly of fiber. It is completely removed when wheat is milled and flour is refined;
- external casings: the outer layer is called the pericarp, the central layer mesocarp and the inner layer epicarp. These layers are mainly composed of minerals, proteins and vitamins;
- the inner layers that are:
  - testa or tegmen, an intermediate layer between the outer casings and the endosperm or albumen. It consists mainly of oils and dyes;
  - endosperm or albumen: it is the internal layer of wheat grain and the one that represents the greater percentage of the whole grain (between the 80 and 90% of the gross weight). Albumen is formed by carbohydrates such as starch. Its function is to provide reserve substances for the growth of the new plant;
  - germ: it occupies the bottom of endosperm and it consists mainly of proteins, oil, enzymes and vitamins of group B. It is formed from the radical (embryonic root) and the plumule (embryonic leaf).

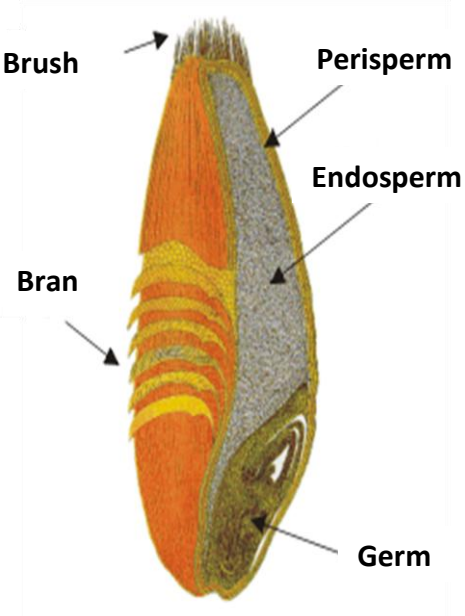

***Fig. 6*: main parts of wheat grain.**

Grain grows in the temperate regions of the world and also in the highland areas of tropics and subtropics. Russia, United States of America, China, India, France, Canada, Australia, Turkey, Pakistan, and Argentina produce the 80% of the total world wheat. The two most important crop species are *Triticum aestivum* (T. vulgare) and *Triticum durum* (Triticum durum): the first one constitutes about 90% of world wheat cultivation, while the latter represents only 10% of the total production. Cultivars of aestivum wheat may be classified as hard (vitreous endosperm) or soft (mealy endosperm); hard wheat is used in bread making, while the soft one in cakes, cookies, biscuits, and pastry production. In Europe most of durum wheat cultivation takes place in Italy; elsewhere it is grown in Australia, North Africa, Ethiopia, Russia, and North and South America. Durum wheat is characterized by a generally higher protein content. Upon milling durum wheat produces groats and semolina with large granules having sharp edges, while from bread wheat flours with rounded granules are obtained. Durum wheat is specially suited for pasta production (as well as bread), the soft one for bread or egg pasta. Its protein content is quite variable, from 7 to 18% (average 12%): proteins are mostly prolamines, fundamental constituents of gluten, which is formed during kneading and is responsible for dough viscosity, elasticity and cohesion, the most important properties for bread and pasta production. Upon milling the grain are separated into different constituents: bran, germ and semolina. The latter is the main ingredient used for pasta production. By milling it is also possible to obtain flour, used in noodles production. Durum wheat is used to make leavened bread but its gluten does not retain carbon dioxide to the same extent as bread wheat[1].

Table 7: **Wheat chemical constituents.**

	Durum wheat	**Aestivum wheat**
Macroscostituents	Value per 100 g	Value per 100 g
Edible part (%)	100	100
Water(g)	11,5	12
Protein (g)	13	12,3
Lipids (g)	2,9	2,6
Carbohydrates (g)	62,5	65,2
Starch (g)	53,9	56,3
Soluble sugars (g)	3,2	3,3
Total fiber(g)	9,8	9,7
Energy (kcal)	312	317
Energy (kJ)	1307	1327
Minerals	Value per 100 g	Value per 100 g
K (mg)	494	-
Fe (mg)	3.6	3,3
Ca (mg)	30	35
P (mg)	330	304
Mg (mg)	160	-
Zn (mg)	2.9	3,1
Cu (mg)	0.4	0,31
Se (µg)	3.8	-
Vitamins	Value per 100 g	Value per 100 g
Thiamine (mg)	0.43	0,42
Riboflavin (mg)	0.15	0,14
Niacin (mg)	5.7	5,4
Vitamin A (µg retinol eq.)	2.0	-[2]

The table below represents data about wheat trade from 2002 to nowadays and includes forecast for 2012/2013:

***Table 8*: Wheat market trade from 2002 to nowadays.**

	Production[1]	Supply [2]	Utilization	Trade [3]	Ending stocks [4]	World stock-to-use	Major exporters' stock-to-disappearance [5]
	(. . . . . million tonnes . . . . .)					(. . . percent . . . . .)	
2002/03	573.99	812.84	611.36	102.69	206.46	34.38	19.82
2003/04	561.51	767.97	601.26	103.31	164.40	26.60	17.65
2004/05	632.66	797.06	618.65	111.83	180.31	28.96	20.66
2005/06	625.58	805.88	622.89	110.30	178.72	28.53	21.02
2006/07	600.99	779.71	625.64	113.08	158.43	25.09	14.10
2007/08	611.29	769.82	628.40	111.77	141.70	21.97	12.86
2008/09	683.87	825.57	645.11	140.09	173.13	26.27	17.94
2009/10	685.66	858.79	658.94	130.59	199.24	30.05	21.54
2010/11	655.34	854.58	662.96	125.33	192.66	27.62	20.14
2011/12	699.44	892.09	697.64	146.98	189.23	27.53	18.24
2012/13	661.22	850.45	687.46	134.99	166.67	23.96	13.90[3]

(1) Production data refer to the calendar year of the first year shown.

(2) Production plus opening stocks.

(3) Trade data refer to exports based on a July/June marketing season for wheat and coarse grains and on a January/December marketing season for rice (second year shown).

(4) May not equal the difference between supply and utilization due to differences in individual country marketing years.

(5) Major wheat exporters are Argentina, Australia, Canada, the EU, Kazakhstan, Russian Federation, Ukraine and the United States; major coarse grain exporters are Argentina, Australia, Brazil, Canada, the EU, Russian Federation, Ukraine and the United States; major rice exporters are India, Pakistan, Thailand, the United States, and Viet Nam. Disappearance is defined as domestic utilization plus exports for any given season.

1.2 Wheat diseases

Wheat cultivation is affected by several diseases, which damaging wheat grain reduces yield and/or quality. The most important diseases include black point, ergot, common bunt, loose smut, and Fusarium head blight (scab). Fungi causing ergot and scab also produce toxins, which pose a health risk to humans and animals. Grain affected by black point, ergot, common bunt, or scab can be discounted or rejected at the elevator depending on the level of damage or contamination.

1.2.1 Black Point

Black point, also known as kernel smudge, is characterized by a brown to black discoloration of the embryo region of the grain. It is caused mainly by fungi of the genera *Alternaria* and *Bipolaris*, that live as saprophytes on crop residue but can also parasitize plants. Their spores are abundant in the air and infection of wheat heads takes place during wet weather which promotes sporulation and growth of the fungi. Black point is also favored by prolonged rainfall during grain maturation. The infection of the plant takes place from flowering stage to grain maturation. Expanding green kernels are most susceptible; however, because the causal fungi are saprophytic, premature grain senescence also favors black point. The disease adversely affects grain quality. Discolored grain has lower value because the milled flour contains dark specks.

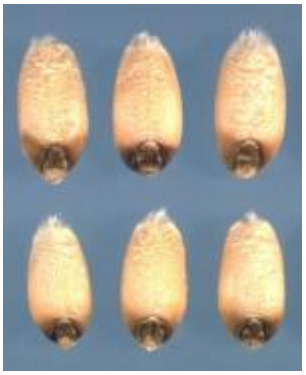

Fig. 7: Black point.

1.2.2 Ergot

Spores of ergot fungus, *Claviceps purpurea*, infect floral tissues on the head. Ergot is a potential threat in particular for hybrid wheat production because the open-floreted, male-sterile wheat lines used for hybrid seed production are often susceptible. Primary infections are caused by spores produced by fruiting structures on germinating sclerotia on the soil surface. Secondary infections originate from spores produced in honeydew exuding from infected florets. These spores are windblown, rain-splashed, or disseminated by insects to nearby wheat heads. Sclerotia present on the soil surface from the previous year's infections germinate in response to moisture by forming stromata (compact masses of specialized vegetative hyphae). Fruiting structures produced on the stromata release infective spores. Following infection of florets, the ovaries enlarge and are converted from base to tip into sclerotia (ergots). If heading of the grasses roughly coincides with that of the wheat, both crops may become infected. Wheat heads are most susceptible just before anthesis (stage in flowering at which anthers rupture and shed pollen). Cool, wet weather that accompanies or prolongs flowering in the grasses and wheat favors infection and honeydew formation. Not all the sclerotia in harvested wheat may have originated from infected wheat heads. Some may be from infected grasses or rye in the field and mixed in with the wheat, contaminating the grain and causing the wheat to be graded as ergoty. Sclerotia from grasses usually are more slender or flattened in shape than those produced in

Fig. 8: Wheat grain contaminated with ergot sclerotia also known as ergot bodies or ergots.

wheat, whereas those from rye are usually fairly large. Federal grain standards classify wheat as ergoty if the harvested grain contains more than 0.30 percent sclerotia. These low tolerances are necessary because the sclerotia contain several compounds (alkaloids) that are toxic to humans and animals. Because ergotism in livestock is a real threat, livestock producers might adopt a zero tolerance for sclerotia in either hay or grain feed.

1.2.3 Common Bunt

***Fig. 9*: Common bunt of wheat. The kernels are filled with masses of black spores.**

Common bunt, also known as stinking smut, is caused by two fungi, *Tilletia tritici* and *T. laevis*. Grain contaminated with bunt spores has a darkened appearance and gives off a pungent, fishy smell. Bunted grain can be discounted or rejected at the elevator. Livestock often refuse to eat it because of the smell and low palatability. Large masses of bunt spores are easily ignited and can cause explosions and fires during harvesting and in grain storage facilities. Workers handling bunted grain should wear dust masks because inhaling spores may create respiratory problems for people with allergies. Bunted grain produces off-white flour with an objectionable odor. Because it requires cool, moist soil conditions for infection, common bunt is less of a problem on spring wheat than on winter wheat. The disease cycle is similar to that of other covered smuts of small grains. When infected grains are harvested, the bunt balls break and contaminate the grain, combine, storage facilities, and soil with spores. In the field, the bunt spores can survive up to two years in arid regions. If infested seed is planted, the spores on the seed coat or in the soil germinate in response to moisture. The mycelium penetrates directly through the cuticle of the seedling coleoptile before emergence. Optimum temperatures for spore germination and mycelia infection of the coleoptile are 6-10°C. The mycelium grows within the terminal meristem (growing point) of the developing wheat plant and eventually colonizes the developing ovary where it displaces the seed tissues with spores. There are no signs or symptoms of infection until the head matures. Normally, tillers also are infected and entire plants, including heads, are smutted.

1.2.4 Loose Smut

***Fig. 10*: A loose-smutted wheat head with kernels transformed into olive-black masses of smut spores.**

Like common bunt, loose smut, caused by the fungus Ustilago tritici, also is a seed-borne disease of wheat. U. tritici is unique among the wheat smuts in that it is incorporated into the developing kernel and persists inside the grain. Affected grain shows no symptoms and outwardly appears healthy. Up to 40 percent yield loss can occur in individual fields. Unlike common bunt, the presence of *U. tritici* in grain has little effect on quality for milling or feed, but the infected grain should not be used as seed without first being treated with a systemic fungicide. The disease cycle starts when the infected seed is planted. Mycelium of *U. tritici* is present in all parts of the seed. When the infected seed germinates, dormant mycelium in the embryo becomes active and growth keeps pace with the growing point of the developing seedling. When the growing point terminates in a head, the fungus replaces the grain and other tissues with a mass of spores. Smutted

heads emerge slightly earlier than healthy heads. Spores from these heads are blown to healthy heads during flowering where they germinate and become established as dormant mycelium in the developing kernel. Infection is favored by wet, humid weather and a temperature range of 16-22°C. Other than symptoms, loose smut differs from common bunt in that it is an embryo-infecting pathogen that is carried inside the seed as dormant mycelium. Common bunt is a seedling-infecting pathogen that has spores that are carried on the outside of the seed coat or are present in the soil[4].

1.2.5 Fusarium Head Blight (FHB)

***Fig. 11*: Typical symptom of FHB infection.**

Fusarium head blight (FHB), also known as head scab, can be a devastating disease affecting all classes of wheat and other small grains. This fungal disease has the ability to completely destroy a potentially high-yielding crop within a few weeks of harvest. This desease is mainly caused by two fungi, *Fusarium Graminearum* and *Fusarium Culmorum,* it is promoted by frequent rainfalls, high humidity and heavy dews that coincide with the flowering and early kernel-fill period of the crop. Damage from FHB is multifold: reduced yields, discolored, shriveled "tombstone" kernels, contamination with mycotoxins, and reduction in seed quality. The disease also reduces test weight and lowers market grade. Difficulties in marketing, exporting, processing, and feeding scabby grain are experienced. The diffusion of *Fusarium* spp. varies depending on weather. *F. graminearum* in fact predominates in warmer, humid areas of the world such as the USA, while *F. culmorum* has been shown to be one of the predominant *Fusarium* species causing FHB in Europe, in fact it prefers to grow in the cooler maritime regions of the world. Recent surveys indicate an increase in *F. graminearum* over *F. culmorum* in several European countries. Temperatures above 25 °C and moist periods longer than 24 h promote infection by both *F. culmorum* and *F. graminearum* and wheat heads are most susceptible to infection at anthesis[5]. *F. graminearum* is also associated with stalk and ear rot of corn and may cause a root rot of cereals. The fungus persists and multiplies on infected crop residues of small grains and corn. The first symptoms of head scab blight include a tan or brown discoloration at the base of a floret within the spikelets of the head. As the infection progresses, the diseased spikelets become light tan or bleached in appearance. The infection may be limited to one spikelet, but if the fungus invades the rachis, the entire head may develop symptoms of the disease. The base of the infected spikelets and portions of the rachis often develop a dark brown color. When weather conditions have been favorable for pathogen reproduction, the fungus may produce small orange clusters of spores or black reproductive structures called perithecia on the surface of the glumes. Infected kernels are often shriveled, white, and chalky in appearance. In some cases, the diseased kernels may develop a red or pink discoloration. The chaff, light-weight kernels, and other infected head debris of wheat and barley, returned to the soil surface during harvest, serve as important sites of overwintering of the fungus. Continued moist weather during the crop growing season favors development of the fungus, and spores are windblown or water-splashed onto heads of cereal crops. Wheat and barley are susceptible to head infection from the flowering (pollination) period up through the soft dough stage of kernel development. Spores of the causal fungus may land on the exposed anthers of the flower and then grow into the kernels,

glumes, or other head parts. One of the major problem related to the infections is the accumulation of mycotoxins, which are frequently associated with the growth and invasion of cereal grains infected by *Fusarium*. The most common toxin associated with *Fusarium* spp.–infected grain is deoxynivalenol (a trichothecene mycotoxin, also known as vomitoxin as it causes nausea, vomiting and feed refusal in non ruminant animals and poses a threat to other animals and humans if exposure levels are high. The occurrence of mycotoxins in infected grain further increases grain losses caused by FHB. Numerous research and survey reports have described the worldwide occurrence and epidemic levels of scab during the past century. In the United States, scab was found in 31 of 40 states surveyed in 1917, with losses estimated at 288,000 metric tons, primarily in Ohio, Indiana, and Illinois. Scab caused an estimated loss of 2.18 million metric tons of winter and spring wheat throughout the United States in 1919. Extensive field surveys showed additional large yield losses from 1928 to 1937. In the 1980s, cool, wet weather in May and June led to epidemics in parts of Kansas, Nebraska, Missouri, Oklahoma, Iowa, southern Illinois, Indiana, and New York. In 1982, scab caused an estimated 4% reduction in total United States wheat production, or more than 2.72 million metric tons. Scab infections have been reported worldwide from wherever cereal crops are grown. Scab is endemic in China, the world's largest producer of wheat, where losses in excess of 1 million metric tons have been reported. The severe losses caused by scab in barley in South Korea in 1963 threatened some of the population with starvation. Argentinean epidemics affecting durum wheat occurred in the 1960s, 1970s, and 1980s. Epidemics in Canada and Japan also have been severe, resulting in extensive studies on the epidemiology and control of the disease[6].

1.3 FHB: prevention strategies

Given the severity of damages caused to plants, it is of great interest to find effective prevention methods against FHB. The most important step for the FHB prevention and consequent mycotoxins production, is to counteract plant contamination of *Fusarium* spp.. Several agricultural practices can influence the crop contamination, but even the best management of agricultural strategies cannot totally eradicate mycotoxin contamination. The main prevention methods are described below.

1.3.1 Crop rotation

Selection of the right crop rotation is an important factor to reduce the FHB development. Spores released from mature sporangia of Fusarium sp. are spread far and wide by wind, and then settle on the soil. They can stay in soil for a long period or grow on dead plant residues such as straw or stubbles, which increase the soil contamination level. Then conidiospores and ascospores can infect ears and leaves of the next crop after being spread by wind or rain-splash droplets. Crop residues are the most important source of inoculum for *F. graminearum*, which causes FHB and DON contamination. So, crop rotation and tillage are recommended to control plant contamination with *Fusarium* sp., but these agricultural practices are not always recognized as efficient. In a 4-year study,[7] it was observed that planting a crop other than wheat 2 years previous to planting a wheat crop significantly decreased the level of DON in wheat grain in one year out of

four (P=0.03). While the presence of maize residue appears to be an important factor contributing to DON contamination of wheat. However, there is no evidence that wheat following wheat is more at risk than wheat following a non-cereal crop since some pathogenic *Fusarium* species isolated from cereals can also have pathogenicity towards non-cereal crops .

1.3.2 Tillage

Several techniques are used for soil cultivation. One is ploughing, where the top 10–30 cm of soil are inverted at the opposite. Another practice is the so called no-till technique, in which seeds are drilled into the previous crop stubble with minimum changes of the soil structure. Removal, burning or burial of crop residues is likely to reduce *Fusarium* inoculum for the following crop, while it's important to note that minimum tillage instead of ploughing resulted in a 10-fold increase in DON content in the following wheat crop. In the same way, Steinkellner and Langer (2004)[8] noted that the deeper the tillage the lower was the concentration of *Fusarium* sp. in the soil. Dill-Macky and Jones (2000)[9] showed that no-till after wheat or maize increased DON levels in the following wheat crops compared to ploughing, but the effect of soil cultivation disappeared when the previous crop was soy beans.
Ploughing and twice power-harrowing (15 cm deep) both reduced the *Fusarium* infection of wheat, while direct sowing produced high mycotoxin contents in wheat kernels.

1.3.3 Soil fertilisers

Fertilisers can affect *Fusarium* sp. contamination of crops by altering the rate of residue decomposition, by acting on the rate of plant growth, and by changing the soil structure and its microbial activity. Nitrogen supply resulted in increased incidence of *Fusarium* infected grain[10].

1.3.4 Planting date

Fusarium contamination in cereal crop depends on both the susceptibility of the cultivar to infection and the occurrence of environmental conditions favorable for infection. Contamination is most likely when the crop flowering stage occurs at the time of spore release. Since the timing of events is a decisive factor for infection, any change in planting date or in ripening of the variety can significantly affects fungal infection and mycotoxin contamination. For wheat and barley, it is best to avoid planting varieties that mature late in the growing season. Winter varieties develop and mature earlier than spring varieties and consequently they have a reduced risk of *Fusarium* infection.

1.3.5 Breeding and transgenic approach for plant resistance

Plant breeding can be considered as the best solution for *Fusarium* control in susceptible crops. Lines have been produced to provide good resistance to *Fusarium* sp., but quality and agronomic properties were adversely affected thus preventing lines from being registered. Some current varieties already available provide higher *Fusarium* resistance than others. Quantitative trait loci (QTLs) for *Fusarium* resistance have been identified in wheat[11]. They are often coincident with genes controlling morphological plant characteristics. For instance, a correlation has been shown between the higher FHB resistance and taller varieties.
Anti-*Fusarium* proteins may exist which, if identified, could be used for a transgenic approach to limit the risk of fungal infection. Genetic engineering used to reduce mycotoxin risk is based on three approaches: enhance resistance to insect attack, increase plant resistance to infection and induce detoxification pathways or processes that inhibit mycotoxin production in the grain.

Transgenic maize expressing Bt-toxin targeted to the European corn borer is an example of the first approach, but it does not address the non-insect route of *Fusarium* entry such as in *Gibberella* ear mould. However, Hammond et al. (2004) indicated that Bt maize can reduce fumonisin levels in grains[12]. Although it has never been done in maize, an overexpression of specific antifungal proteins and metabolites or enhancement of the plant defense systems can be considered in the second approach. Preventing mycotoxin biosynthesis or detoxifying mycotoxin *in planta* is another extremely attractive and feasible approach.

1.3.6 Chemical control of Fusarium infection

Many fungicide have been tested against *Fusarium* sp. either *in vitro*, in the greenhouse or under field conditions. To be efficient, these substances must be totally lethal to *Fusarium* sp. otherwise they stimulate mycotoxin production, as it was shown by *in* vitro experiments. Another important study regards the effect of fungicides on soil mycoflora. In-fact these substances can have a negative impact on the soil saprophyte microbial biomass. The last problem regards the contamination of groundwater with chemical residues, that produces serious limitations on the utilization of chemical substances. For this purpose biological control of *Fusarim* sp. was improved. Microbial antagonists or competitors can be sprayed on plants at the flowering stage to eradicate or limit the growth of toxin producers. For example, *Bacillus subtilis* can inhibit the growth of fungi during their endophytic growth phase to eradicate or limit the growth of toxin producers. More research is needed to know the real possibility of microbial antagonists on the control of *Fusarium* sp. infection under practical conditions[13].

1.3.7 Insect control

Insects are involved in *Fusarium* infection of plants with two mechanisms: first, they compromise the external protection of grains and plant tissues, thus allowing the fungal hyphae to penetrate and have access to nutrients and second, they carry fungal spores.
Thus, control of insects by the use of genetically modified insect-resistant cereals has been suggested to reduce the fungal infection level, but preliminary results differ. In fact the results obtained by Munkvold et al. (1999) showed that transgenic Bt-maize hybrids decreased significantly *Fusarium* ear rot and the subsequent fumonisin contamination, while others have found that the Bt gene had only a slight effect on *Gibberella* ear rot and the subsequent DON contamination[14]. Differences in the efficiency of Bt-maize can be explained by the route of infection. *Fusarium* ear rot pathogens, which infect via physical damages caused by insects on grains, are more susceptible to the toxin produced by Bt-corn than *Gibberella* ear rot pathogens, which infect chiefly via the silk route. The other important external agent is the weed infestation that can harbor a wide range of *Fusarium* species. This explains why an high weed density tended to increase infection in wheat crops. However, the use of glyphosate to eliminate weeds has been shown to increase FHB in wheat and also to extend soil and root contamination as well as propague density probably by raising the amount of plant debris on soils.[15]

1.4 Fusarium toxins

Mycotoxins from *Fusarium* species have traditionally been associated with cereal growth in temperate climate areas, since these fungi require mild temperatures. However, extensive data indicate that Fusarium mycotoxins are widespread in cereals at a global scale.

Fusarium species are able to produce a wide range of mycotoxins having different structure and chemical behavior. Among these, the most important, from the animal health and productivity point of view, are trichothecenes, zearalenone, moniliformin and fumonisins.[16] Trichothecenes are a family of more than 180 cyclic sesquiterpenoids. The term "trichothecene" derives from trichothecin, which was one of the first substances identified from this family. All these compounds contain a common 12,13-epoxytrichothene skeleton and an olefinic bond with various side chain substitutions. They can be divided into four groups (types A, B, C, D) according to their characteristic functional groups. Non-macrocyclic trichothecenes include type-A and –B, that are the most common mycotoxins of this group. Type A, mainly represented by HT-2 toxin and T-2 toxin, are characterized by the presence of functional group other than a ketone at C8; type B, with the carbonyl function on C8, is most frequently represented by deoxynivalenol (DON), 3-acetyl-DON (3-Ac-DON), 15-acetyl-DON (15-Ac-DON), nivalenol (NIV) and fusarenon X (FUS-X).
These mycotoxins exert their toxicity at subcellular, cellular and systemic level. They swiftly penetrate cell lipid bilayers, thus allowing access to DNA, RNA and cellular organelles. These low molecular weight metabolites (approximately 200–500 Da) interact with the eukaryotic 60S ribosomal subunit and prevent polypeptide chain initiation or elongation. Trichothecenes inhibit protein synthesis by affecting polyribosomes to interfere with the initiation phase of protein synthesis. At the subcellular level, they inhibit protein synthesis and covalently bond to sulfhydryl groups.[17] All trichothecenes have in common a 9, 10 double bond and a 12, 13 epoxide group, but extensive variations exist concerning ring oxygenation patterns. Experimental evidences indicate that low to moderate dose acute oral exposure to trichothecenes causes vomiting, diarrhea and gastroenteritis; whereas higher doses cause severe damage to the lymphoid and epithelial cells of the gastrointestinal mucosa resulting in hemorrhage, endotoxemia and shock. Other targets of mycotoxins action include bone marrow and thymus, where damage can contribute to generalized immunosuppression. The diffusion of agricultural practices for cereal crops (wheat, barley and maize) such as "no-till farming" is leading to an increase of contamination due to thricothecenes. This particular system, that consists in planting crops without plowing and using herbicides to control weeds, permits to reduce soil erosion and the preservation of soil nutrients but, on the other hand, plant residues remained in field after harvest, cannot be degraded during soil tillage, allowing fungal growth. This last phenomena is also influenced by changing climate patterns.

1.4.1 Analysis of *Fusarium* toxins

a) Sampling

Sampling plays a crucial part in the reliability of data about mycotoxin occurrence, since their distribution in food commodities is highly heterogeneous. For this reason, sampling strategies are often considered at a regulatory level. While it can be assumed that mycotoxins in liquid samples are homogeneously distributed, contamination in crops is often concentrated in 'hot-spots''. The number of contaminated particles may be very low, but the contamination level within a particle can be very high. To obtain the same representativeness for batches of food products with large particle sizes, the weight of the taken incremental sample has to be larger than in cases of batches with smaller particle size.

b) Sample preparation and clean-up

Only a few analytical techniques, i.e. optical techniques based on IR spectroscopy, are capable of detecting mycotoxin contamination directly in cereal samples without the necessity of further sample preparation, such as solvent extraction. However, the application of such techniques is still limited to screening purposes due to a high matrix dependence and lack of appropriate calibration

materials. Analytical methods based on chromatography or immunoassays usually require solvent extraction to liberate the mycotoxin from the sample matrix, and subsequent clean-up of the extract to reduce matrix effects. Various combinations of solvents, sometimes with the addition of modifiers (e.g. acids, bases, etc.), are used for extraction, depending on the physicochemical properties of the mycotoxin, the sample matrix and the type of clean-up used afterwards.

For example, DON extraction is usually performed by mechanical shaking or blending with aqueous acetonitrile or aqueous methanol, although other solvents, e.g. water–PEG, chloroform–ethanol, and chloroform–methanol have also been used. It has been shown that the use of aqueous acetonitrile provides cleaner extracts than aqueous methanol. Accelerated solvent extraction (ASE), also known as pressurised liquid extraction (PLE) or microwave-assisted extraction (MAE), helps to speed-up and automates the extraction process and offers a robust and time-saving alternative to classical solvent extraction techniques. So far, the high cost of an ASE apparatus, however, has limited to a few laboratories the application of this technique in the field of mycotoxin analysis.

Supercritical fluid extraction (SFE), especially with supercritical CO_2 as an environmentally safe extraction medium, received a lot of attention in the 1990s. The extraction selectivity of the non-polar supercritical CO_2 is influenced by temperature and pressure and can be varied in a wide range by adding modifiers. This method has the same problem of the previous described technique; in fact the high investment and maintenance costs for SFE apparatus and the poor recovery obtained are, still, unsolved problems which limit the applicability of SFE method in routine analysis.

For further purification and analyte enrichment, liquid samples and extracts are predominantly submitted to various clean-up techniques. Although clean-up is not usually required for immunoassays, physicochemical methods commonly employ extensive clean-up procedures. The main procedures employed for clean-up in analysis of *Fusarium* toxins are liquid–liquid partitioning, solid-phase extraction (SPE), column chromatography, use of immunoaffinity columns (IAC) and multifunctional clean-up columns. Interfering lipids can, if necessary, be removed by extracting the sample extract with *n*-hexane or another non-polar solvent before further clean-up.

- Liquid–liquid partitioning can be conventionally employed by shaking the sample extract with an immiscible solvent in a separation funnel. For this type of clean-up, the aqueous phase can also be supported on a column packed with a solid hydrophilic matrix and the organic solvent percolated down the column to elute the purified toxin.
- Column chromatography can be performed on a variety of stationary phases (silica gel, aluminum oxide, Florisil, charcoal and C8 or C18 reversed-phases), depending on the adsorbent polarity range required. The most frequently employed column packing material for DON and ZEN is a mixture of charcoal, alumina, and celite. Modern SPE columns are a potential alternative to these conventional column chromatographic methods. SPE columns are generally delivered prepacked in disposable plastic cartridges and are also available with the adsorbents mentioned above. Gel-permeation chromatography has also been employed for clean-up during DON analysis.
- The application of IACs (Immuno-Affinity Columns) for purification before instrumental analysis has been very successful for several mycotoxins. IACs contain immobilised antibodies that exclusively retain a certain mycotoxin or mycotoxin class. Due to their high specificity, IACs produce cleaner extracts with a minimum level of interfering matrix components and excellent signal-to-noise ratios compared to less selective SPE sorbent materials. IACs have

been developed for most major mycotoxins and mycotoxin classes such as aflatoxins, ochratoxin A, trichothecenes, zearalenone and their metabolites.

- Mycosep columns contain a variety of adsorbents (e.g. charcoal, celite, ion-exchange resins, and others). The packing material is housed in a plastic tube between filter discs with a rubber flange on the lower end containing a porous frit and a one-way valve. The Mycosep column enables rapid sample purification – within 10 to 30 s. One of the major advantages of this column is the absence of the time-consuming rinsing steps required in solid-phase extraction. In addition, these columns present an optimal clean-up, in fact nearly all analytical interfering substances are retained on the column whereas molecules of interest are not adsorbed on the packing material. In combination with HPLC and GC, Mycosep columns are currently the most frequently employed commercial columns for clean-up of aflatoxins, trichothecenes, ochratoxins, zearalenone, moniliformin and patulin with recoveries higher than 80%.
- Mycotoxin specific molecularly imprinted polymers (MIPs) are also considered as a potential and cheaper alternative for clean-up, that, in contrast to IACs, does not suffer from storage limitations and stability problems regarding organic solvents.

Careful selection of the clean-up method is, however, essential for the effectiveness of an analytical method. Immunoaffinity materials are expensive and distinctly less feasible for multitoxin analysis, since they are highly specific for only one target mycotoxin. Some scientists even talk about ‘‘overkill’’ when using highly specific clean-up techniques, such as IACs in combination with liquid chromatography/mass spectrometry (LC/MS), since compound-specific detection stands in contradiction to the multi-analyte detection capabilities of MS. However, there are already combined immunoaffinity materials on the market that are specific to a wider range of mycotoxins.

It has been shown that, in many cases, the quality of the analytical result does not suffer when conventional SPE approaches are used. Of course, this also depends on the selectivity of the MS equipment itself. Single-stage MS in selected ion-monitoring mode might need selective clean-up to remove matrix interferences, while those interferences might not be visible with multi-stage MS in selected reaction-monitoring. However, matrix-induced signal suppression or enhancement should always be taken into consideration and can normally be omitted by clean-up of the extract or by using an appropriate calibration method (e.g. matrix-matched calibration standards, standard addition, or the use of adequate internal standards, i.e. isotope-labelled standards, etc.).

c) Analytical techniques

The currently used quantitative methods for the determination of regulated mycotoxins, such as fumonisins, zearalenone, type-A (e.g. T2-toxin) and -B trichothecenes (e.g. deoxynivalenol) in food and feed, mainly use immunoaffinity clean-up with high-performance liquid chromatography (HPLC) or gas chromatography (GC) in combination with a variety of detectors, such as fluorescence detection (FLD) with either a pre- or post-column derivatisation step, UV detection, flame ionisation detection (FID), electron capture detection (ECD) or mass spectrometry (MS).

Liquid chromatography/mass spectrometry (LC/MS) within the last 10 years, has become the universal approach for mycotoxin analysis, as many analytes are compatible with the conditions applied during separation and detection. Nevertheless, the breakthrough of this approach did not occur until the mid-1990s, when suitable interfaces, such as atmospheric pressure ionization, became accessible on a routine basis. Compared to conventional detection techniques, such as UV or fluorescence, mass spectrometry offers increased selectivity and sensitivity (although

fluorescence detection might be more sensitive for certain mycotoxins, e.g. zearaleone), unambiguous confirmation of the molecular identity of the analyte and the option to use isotopically labelled substances as internal standards. Furthermore, it is possible to investigate the molecular structure of metabolites and sugar conjugates and to omit time consuming and error-prone derivatization and clean-up steps. However, the reduction of the sample preparation inevitably emphasizes problem of LC/MS, relatively poor method accuracy and precision due to the irreproducible and unpredictable influence of co-eluting matrix components on the signal intensity of the analytes.

d) Fast screening methods

A single chapter is necessary to describe rapid methods based on immunochemical techniques which often have the advantage of not requiring any clean-up or analyte enrichment steps. ELISAs (Enzyme-Linked Immunosorbent Assay) have become routinely used tools for rapid monitoring of most mycotoxins, especially for the screening of raw materials. Although ELISA tests may show a high matrix dependence and possible overestimation of levels, the advantages of the microtitre-plate format are speed, ease of operation, sensitivity and high sample throughput. ELISA test kits are commercially available for most of the major mycotoxins. Immunosensors are emerging as a cost-effective alternative for screening and quantitative determination of mycotoxins. Array biosensors have been developed using competitive-based immunoassays for the simultaneous detection of multiple mycotoxins, including ochratoxin A, fumonisin B, aflatoxin B1, and deoxynivalenol, on a single waveguide surface by imaging the fluorescent pattern onto a CCD (charge-coupled device) camera. Other formats with fluorescence detection include automated flow-through immunosensors with enzyme-labelled mycotoxin derivatives. In recent years, interest in rapid membrane-based immunoassay methods, such as flow-through immunoassays and lateral flow devices (LFDs), has strongly increased due to the need for rapid on-site screening. Requiring no sample preparation other than an extraction step, LFDs allow qualitative or semi-quantitative determination of mycotoxins on one-step strip tests within a few minutes. The strong interest is furthermore reflected in the increasing number of commercially available test kits for field use, based mostly on direct competitive assays.

1.5 Durum wheat and Fusarium

As previously described, wheat can easily be affected by FHB, a devastating and severe plant disease who has the ability to destroy entire harvests. Its risk is mainly connected to the capacity of fungi to produce mycotoxins (deoxynivalenol), which are consequently found in food and feed. Durum wheat is particularly prone to fungal infection[18] and, in recent years some work displayed a occurrence of DON in this grain, in particular in 2010 Bensassi et al. displayed that 83% of analyzed samples, coming from Tunisia, were positive with averages ranging from 12.8±5% to 30.5±13.3% µg/g, [19] Lori et al. displayed similar results in samples collected in Argentina (55% of positive samples).[20] For this reason, a very hot research field is the study of resistance mechanism to identify FHB resistant durum wheat varieties.

1.6 Durum wheat market

The world wheat production in 2010 is fixed to 34.4 million of tons and the major production percentages is in Europe. In particular Italy is the main Durum wheat producing country with about four million tons produced in 2010. Importance of this grain in our country enhance if data about use were analyzed. Italy in 2010 use 6.23 millions tons of durum wheat almost entirely for semolina production (5.85 million tons). Semolina is the main ingredient for pasta production that is one of the "made in Italy" symbols all over the world. Italy is the major producer of pasta with about 3.1 millions of tons in 2009 and is also the most consumer of this food product with 26 kg/capita/year. All these data demonstrate the great importance of durum wheat in Italian food industry and why it is so important begin to investigate the health problems regarded this commodity.

1.7 Masking mechanisms of DON

Evidences are reported in literature to support the existence of a virulence factor of FHB. In fact Fusarium mutants with a disrupted trichodiene synthase (Tri5) gene, who is directly involved in trichothecene biosynthesis, still resulted pathogenic. However they exhibit a reduced virulence on wheat, since they are unable to spread from the infection site. Results of several studies indicate that the in vitro resistance of wheat cultivars toward DON is correlated with Fusarium head blight resistance in the field. The most probable detoxification pathway used by plant seems to be a metabolic transformation often followed by compartmentalization of DON within cells. An observed decrease in the concentration of DON, which occurred in Fusarium-infected wheat in the field, suggests that the toxin may be metabolized. Sewald et al. was the first to demonstrate that starting from DON, there is a possibility to conjugation of such mycotoxin with glucose[21]. In a maize suspension culture, using a radiolabeled DON, he observed the formation of D3G. Genome sequencing projects revealed the existence of a large number of genes in plants able to code for putative UDP-glycosyltransferases (UGTs), predicted to conjugate small molecules.

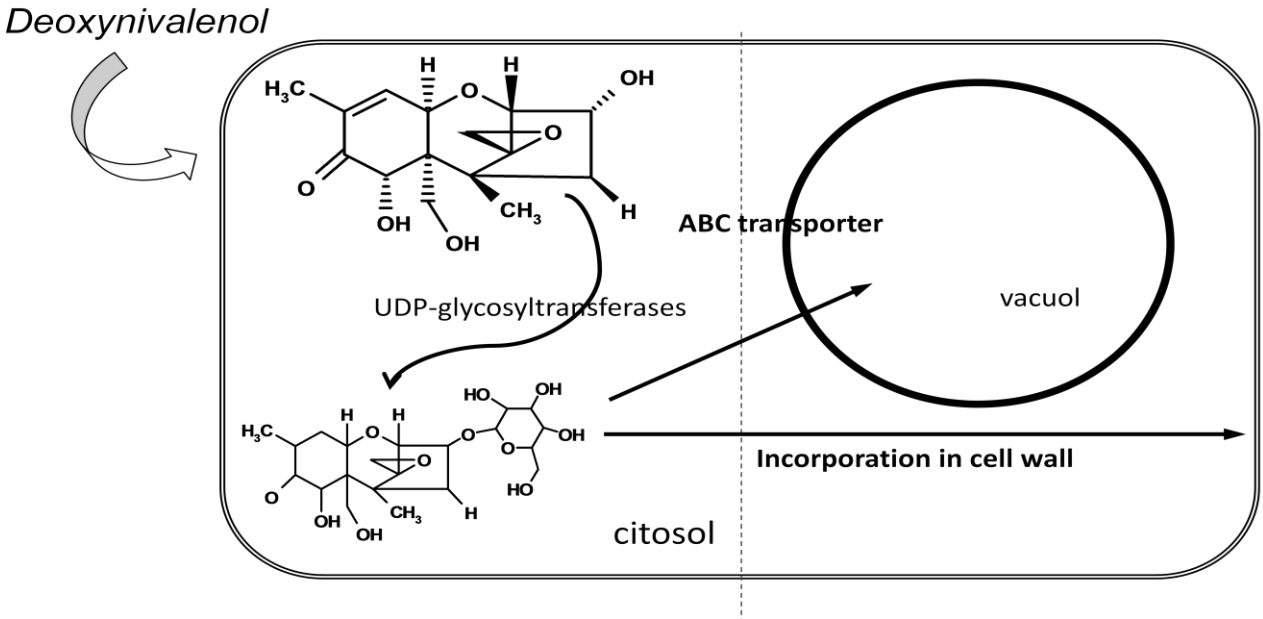

Fig. 12: Scheme of DON metabolism in plant cells as proposed by Poppenberger et al. in 2003.

Figure above displays the hypothesis made by Poppenberger et al. in 2003., In this work scientists isolated and characterized one gene from Arabidopsis thaliana encoding a UDP-glycosyltransferase able to detoxify deoxynivalenol. The enzyme catalyzed the transfer of glucose from UDP-glucose to the hydroxyl group at carbon 3 of deoxynivalenol. By using a wheat germ extract-coupled transcription/translation system it is been possible to demonstrate that this enzymatic reaction allowed to inactivate the mycotoxin. [22]After DON glucosilation this new, more polar compound, was able to be transported in vacuol by ABC transporter or incorporated in the cell wall, in order to reduce its toxic effects. D3G was first detected in naturally contaminated wheat and maize in 2005[23] and then, the worldwide occurrence of D3G in different cereal crops has been reported. In these studies the molar percentages of D3G/DON varied strongly, reaching maximum levels of 46%. This percentage could increase in the future as a consequence of plant breeding efforts to enhance Fusarium head blight resistance by introgression of resistance loci. Considerable amounts of D3G were found in foodstuffs such as breakfast cereals[24], snacks and beers[25]. All data collected in literature on D3G occurrence regard aestivum wheat, maize, oats, barley, malt and beer, but no data are available on its presence in durum wheat[26].

Aim of the work

The aim of the present work is to evaluate the occurrence of deoxynivalenol-3-β-D-glucoside in durum wheat. A new extraction method has been developed starting from that proposed by Zachariasova et al. in 2010 (QuEChERS method)[27]: the method has been optimized through an experimental design approach and applied for multiresidual analysis of mycotoxins by UPLC-ESI/MS instrumentation. A large number of naturally contaminated durum wheat samples (n=150) have been analyzed and the results statistically evaluated, in order to find possible correlations with characteristics of samples, such as harvest zone and diseases that can affect plants during their growth.

Materials and methods

1.8 Chemicals

Sodium acetate, sodium chloride, magnesium sulphate, methanol and acetonitrile were purchased by Sigma-Aldrich (Taufkirchen, Germany). All solvents were of HPLC grade. Ultra-pure water was in-house produced by using a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Mycotoxin standards deoxynivalenol (DON), deoxynivalenol ^{13}C (internal standard), deoxynivalenol-3-glucoside (D3G), 3-acetyldeoxinivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), nivalenol (NIV), fusarenone X (FusX), diacetoxyscirpenol (DAS), HT2 toxin (HT2) and T-2 toxin (T-2) were purchased from RomerLabs (Tulln, Austria). All stock solutions (25 µg/ml) were stored at -20°C and brought to room temperature before use. A working solution (2.5 µg/ml), containing all the target mycotoxins, was prepared by combining suitable aliquots of each standard. A Certified Reference Material (wheat) contaminated with DON has been purchased from RomerLabs (Tulln, Austria).

1.9 Sampling strategy and sample collection

A total number of 150 durum wheat samples (25 lines x 2 reps x 3 environments) were analyzed for DON,D3G, 3ADON and 15ADONcontamination. The samples belong to 25 genotypes collected from three different areas located in Northern-Central Italy, namely Argelato (Bologna, 44°39'03.52"N 11°20'34.47"E), Poggio Renatico (Ferrara, 44°46'22.68"N 11°25'46.58"E), and Falconara (Ancona, 43°38'05.59"N 13°21'23.24"E). The advanced lines were selected from different crosses (simple, three and four-way crosses) developed within the durum wheat breeding program of the seed company Società Produttori Sementi S.p.A. (PSB, Argelato, Bologna) in order to sample a good amount of genetic diversity in terms of pedigree (different parental lines used in each cross). Moreover, the lines were selected in order to cover a wide range of heading date (HD). According to the standard guide lines for durum wheat cultivation in each of the three area considered, durum samples were sown in 6 m^2 plot in November 2009 and the ordinary cultural practices were applied to control weeds and pests; no fungicide were applied. To provide different natural FHB selective pressures across the three field trials, the locations were selected based on a preliminary evaluation of the DON content of the selected lines by using dedicated commercial ELISA kit (Ridascreen® DON, R-Biopharm, Darmstadt, Germany). As to standard PSB breeding strategy, each lines was evaluated for FHB field incidence by using a 0-9 ranking score mainly based on visual score of FHB damaged plot, where “0” means FHB absence and “9” is 100% infected plot.

1.10 Trichothecenes extraction method

For toxin content evaluation, an amount of 50 g of whole mill flour of each line were prepared in a Cyclotec™ sample mill (FOSS Analytical A/S, Hillerød, Denmark) and after milling, samples were maintained at -20°C in plastic bags until analysis.

A modified QuEChERS method was applied for the extraction of target mycotoxins (Sospedra et al. 2010). In particular, 2 g of sample, weighted in a 50 ml centrifuge tube, were added with 1.2 g of NaCl and 7.5 ml of methanol:acetonitrile (80:20, v/v). After 1 min homogenization step by Ultraturrax IKA-T18 basic (IKA®-Werke GmbH & Co. KG, Staufen, Germany), the sample was centrifuged for 5 minutes at 3500 rpm (Universal 320 R Hettich, Tuttlingen Germany). The surnatant (1 ml) was transferred into a 2 ml vial and added by a proper amount of internal standard solution in order to get a final concentration of 0.5 mg/Kg. After solvent evaporation, the extract was re-dissolved in 300 µl of water/acetonitrile (80/20 v/v) and analysed by UPLC-ESI/MS. The extraction procedure was optimised by application of a Design of Experiment (DoE) approach, based on a Box-Behnken method. In particular, 4 factors (methanol and acetonitrile volume, NaCl and $MgSO_4$ concentration) were included, considering 3 levels for each factor.

1.10.1 UPLC-ESI/MS analysis

The Uplc-ESI/MS analysises were carried out with an Acquity UPLC separation system (Waters Co., Milford, MA, USA) equipped with an Acquity Single Quadrupole MS detector with an electrospray source. Chromatographic condition were as follows: Acquity UPLC BEH C18 column (1.7µm, 2.1x50 mm); 0.35 ml/min flow rate; 30°C column temperature; 5µl injection volume; gradient elution using 0.1 mM sodium acetate solution in water (eluent A) and methanl(eluent B), both acidified with 0.2% formic acid. Initial condition were set at 2% B for 1 min. After an isocratic step (6 min), eluent B increased to 90% in 9 min. After 3 min isocratic step (90 % B), the system was re-equilibrated to initial condition for 2 min. the total analysis ime was 22 min. The ESI source was operated in positive ionisation mode. MS parameters were as follows: 2.50 KV capillary volage; 30 V cone voltage; 120 °C source block temperature; 350 °C desolvation temperatue; 50 l/h cone gas; 850 l/h desolvation gas. Detection was performed using single ion monitoring mode for ions reported in table below.

***Table 9*: ion monitored in proposed method.**

Mycotoxin	**$[M+Na]^+$**	**$[M+H]^+$**
Nivalenol	335.3	313.3
Deoxynivalenol-3-glucoside	481.3	459.3
Deoxynivalenol	319.3	297.3
3-acetyldeoxynivalenol	361.4	339.4
15-acetyldeoxynivalenol	361.4	339.4
Diacetoxyscirpenol	389.4	367.4
T-2 toxin	447.5	424.5
HT-2 toxin	489.5	466.5

Matrix-matched calibration curves (calibration range 100-3,000 μg/Kg) were used for target analytes quantification. A good linearity was obtained for all considered mycotoxins (r^2>0.99). For all target compounds, limit of quantification (as S/N>10) and limit of detection (as S/N>3) were lower than 30 and 10 μg/Kg, respectively. Recovery experiments were performed for all the target compounds by spiking a blank wheat matrix at two different concentration leels (1 and 2.5 mg/Kg). Inter-day repeatability was evaluated over three days by spiking experiments at two different levels and analyses were performed in triplicate.

1.11 Experimental design – Multivariate optimization

To determine the effect of solvents and salts on the DON recovery in wheat, response surface methodology was applied with a Box-Behnken experimental design. The effects of four factors in a single block of 24 sets of test conditions and 3 central points were studied. The order of experiments was fully randomized. Three levels were attributed to each factor. Statistical analyses were performed with the software package Statistica v.8.0 (StatSoft. Inc. Tulsa, OK 71104. USA). The Pareto chart was used for data exploration. A second Box-Behnken experimental design was applied on the two main factors found to affect DON recovery in durum wheat. Also in this case, the order of experiments was fully randomized and three levels were attributed to each factor. A quadratic polynomial model was defined to fit the response:

$$Y = \beta_0 + \beta_1 X_{CH3OH} + \beta_2 X_{CH3CN} + \beta_{11}(X_{CH3OH})^2 + \beta_{22}(X_{CH3CN})^2 + \beta_{12} X_{CH3OH} X_{CH3CN} \quad \text{(Eq. 1)}$$

Where Y is the response expressed as DON concentration (μg/Kg) and β_0 is a constant coefficient of the model. The regression coefficients (β_1, β_2), (β_{11}, β_{22}) and β_{12}, estimated by multiple regression analysis, represent linear, quadratic and interaction effects of the model, respectively. Interpretation of the data are based on the signs (positive or negative effect on the response) and statistical significance of coefficients ($p < 0.05$). Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient). Descriptive statistics and ANOVA analysis ($\alpha = 0.05$) were performed by SPSS v.19 (IBM Italia, Milano, Italy).

Results and Discussion

1.12 Method development and validation.

At first, an efficient extraction method is needed in order to obtain a good recovery of the glucosilated metabolites as they are characterized by a higher polarity in comparison with native mycotoxins, thus potentially being lost during the common extraction and purification procedures. The extraction method was developed starting from the QuEChERS (Quick Easy Cheap Effective Rugged Safe) extraction protocol proposed by Zachariasova et al. in 2010.[27] The method is based on the use of mixed polar organic solvents and salts, which allow to disaggregate sample matrices and optimize analyte extraction and it was originally proposed for the analysis of pesticides. The application of the QuEChERS method have been recently proved to be very effective also for mycotoxin detection in foods. This method has many advantages over traditional techniques, high simple throughput, the use of smaller amounts of organic solvent and the use of no chlorinated solvents. QuEChERS extraction is a rapid and economic method for food contaminants extraction. This method is just applied for the analysis of various compounds (primarily pesticeds) in a several matrixes (no fatty and low fatty foods) and makes it possible the extraction of molecules with very different chemical characteristics. Given all these advantages it is considered the reference method for multiresidual analysis. The extraction procedure, usually employed for pesticides analysis, involved the use of acetonitril and formic acid solution as solvents and sodium chloride and magnesium sulphate as salts. This solutions allow a two-phase separation and the consequent recovery of all analytes in organic solvents. Classic methods also include the addition of primary secondary amines in order clean up the extract and allow a better determination of the compounds. On account of the polarity characteristics of the target compounds considered in this study, several modifications have been applied to the classical QuEChERS protocol. First of all, the use of primary secondary amines, used in the original protocol as sorbent to retain co-extractive impurities, was avoided in our case, since its sorbent effect could induce the retention of more polar mycotoxins such as D3G. Moreover, since Sospedra et al.[28] achieved the best recovery for DON using a mixture of methanol and acetonitrile as extraction solvent, our optimisation protocol was designed by considering methanol and acetonitrile as solvents and NaCl and $MgSO_4$ as salts. Extraction parameters were optimized using a *Box-Behnken experimental design*, including 4 factors (volume of methanol and acetonitrile, NaCl and $MgSO_4$ concentration) with 3 levels for each factor. The Box-Behnken design is widely used to perform a Design of Experiment and coupled with surface charts analysis, can easily provide the best conditions to carried out an experiment. This experimental design is an independent quadratic one in which does not contain an embedded factorial or fractional factorial design. In this design the treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require 3 levels for each factor. The designs have limited capability for orthogonal blocking compared to the central composite designs. The experimental design, considering all parameters and factors, designed 27 testing, in order to optimize the recovery of DON in wheat samples. Deoxynivalenol was chosen as target compound for the optimization procedure. However, the optimized protocol was then applied to D3G spiked samples in order to evaluate whether the recovery of this compound was satisfactory under the applied conditions. Analyses were performed on blank wheat samples spiked at a known DON level (500 μg/Kg). The table below shows the experimental design and the results derived from each run.

***Table 10**: Assignment of factors and levels of the Box-Behnken design*

Exp. N°	**Preliminar DoE - Factors**				**Final DoE - Factors**	
	NaCl (g)	**$MgSO_4$ (g)**	**CH_3CN (ml)**	**CH_3OH (ml)**	**CH_3CN (ml)**	**CH_3OH (ml)**
1	0.1	0.5	1.55	7	7	15
2	1	0.5	1.55	7	3	15
3	0.1	5	1.55	7	3	10,5
4	1	5	1.55	7	7	10,5
5	0.55	2.75	0.1	4	3	6
6	0.55	2.75	3	4	5	10,5
7	0.55	2.75	0.1	10	5	15
8	0.55	2.75	3	10	5	6
9	0.1	2.75	1.55	4	7	6
10	1	2.75	1.55	4		
11	0.1	2.75	1.55	10		
12	1	2.75	1.55	10		
13	0.55	0.5	0.1	7		
14	0.55	5	0.1	7		
15	0.55	0.5	3	7		
16	0.55	5	3	7		
17	0.1	2.75	0.1	7		
18	1	2.75	0.1	7		
19	0.1	2.75	3	7		
20	1	2.75	1.55	4		
21	1	2.75	3	7		
22	0.55	0.5	1.55	4		
23	0.55	5	1.55	4		
24	0.55	0.5	1.55	10		
25	0.55	5	1.55	10		
26	0.55	2.75	1.55	7		
27	0.55	2.75	1.55	7		

To better understand the optimal condition of the extraction, the results obtained were analyzed with surface charts that compared two factors at a time with DON recovery.

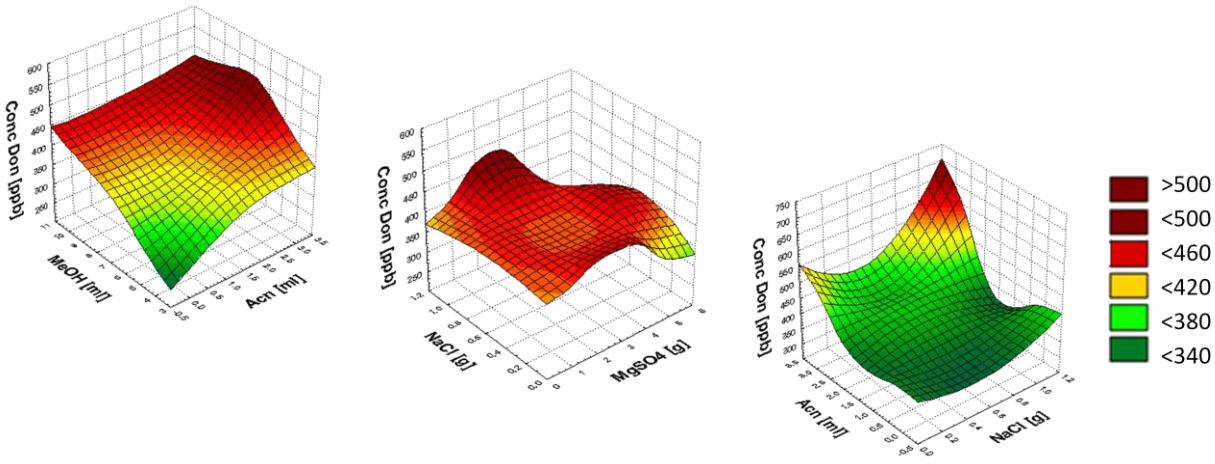

***Fig. 13*: surface charts that showing conditions to obtain the best recovery of DON.**

The standardized effect of the independent variables and their interaction on the dependent variable was investigated by Pareto chart, which quantify the main influence of the independent variables and interactions with their relative significance on the extraction procedure.

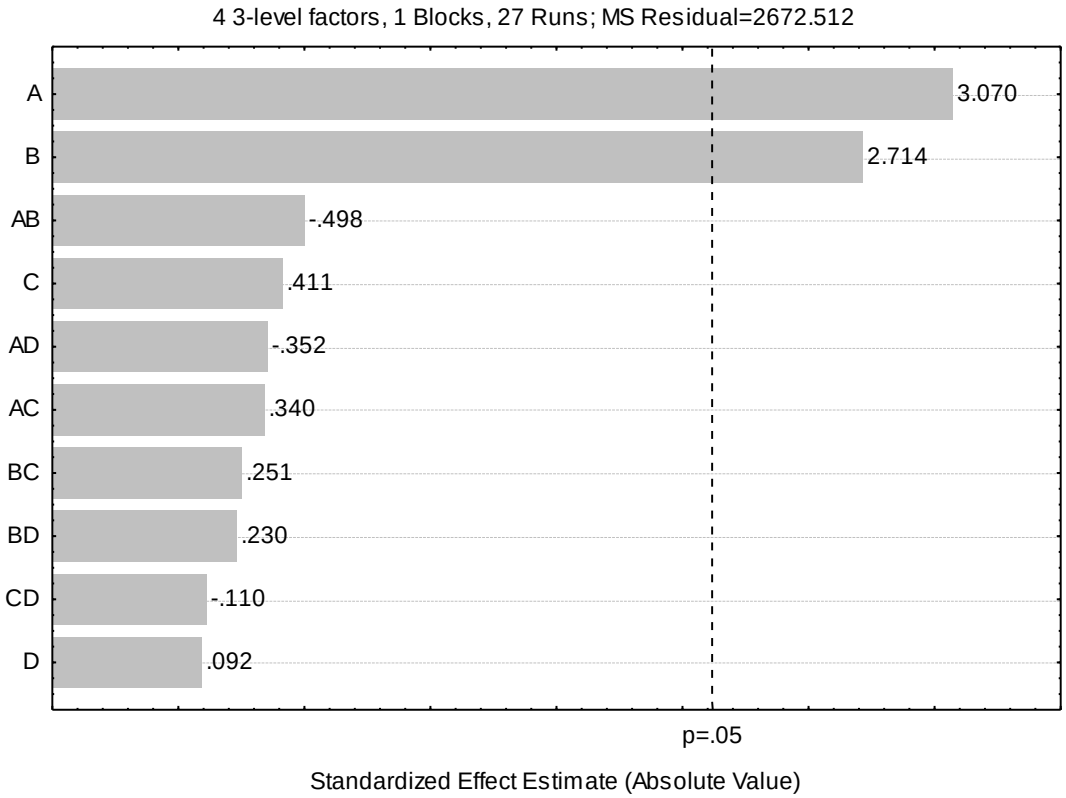

***Fig. 14*: Pareto chart obtained for the preliminary Box-Behnken design of experiment. Factors: A, methanol; B, acetonitrile; C, NaCl; D, MgSO4.**

A positive value for the estimated effects indicates an increase in the extraction yield if the variable increases to its higher level, while a negative value indicates that a better extraction yield is obtained at low levels of the variables[29].

The interpretation of the chart indicates that the factors A (methanol) and B (acetonitrile) are significant, while salts (factors C and D) are not significant. The positive value obtained for both solvents indicated that the extraction yield increases with the increase of these factors. Therefore, a new Box-Behnken design was carried out considering only these two factors as critical variables (Table 1). A Box-Behnken experimental design was used by neglecting all the interactions higher than second order in Eq. 1.

$$Y = \beta_0 + \beta_1 X_{CH3OH} + \beta_2 X_{CH3CN} + \beta_{11}(X_{CH3OH})^2 + \beta_{22}(X_{CH3CN})^2 + \beta_{12} X_{CH3OH} X_{CH3CN} \quad (1)$$

A total of 9 experiments were needed to estimate the model. A multiple regression analysis was performed to obtain the coefficient of the equation, which can be used to estimate the response (Eq. 2):

$$z = 182.87 + 0.61A + 2.49A^2 + 31.10\ B - 0.30\ B^2 \quad (2)$$

where z is the predicted DON concentration (μg/Kg), A is the methanol volume (ml) and B is the acetonitrile volume (ml).

The results of this limited set of experiments provided a statistical model used to optimize the extraction conditions. Duplicate extractions were carried out for each run, obtaining a relative standard deviation value lower than 0.5%. Both methanol ($p = 0.0005$) and acetonitrile ($p = 0.0000$) have been found to significantly affect the model as first order factors, while no significance was found for the second order factors.

The R^2 value (0.9937) for DON shows a good relation between the experimental and the predicted values of the response, while the adjusted R^2 (0.9874) indicates that only 1.26% of the total variance is not explained by the model. Accordingly, the non-significant value of lack-of-fit ($F = 1103.46$, $p = 0.000$) revealed that the quadratic model is statistically significant. The observed against predicted DON levels are reported in the following figure.

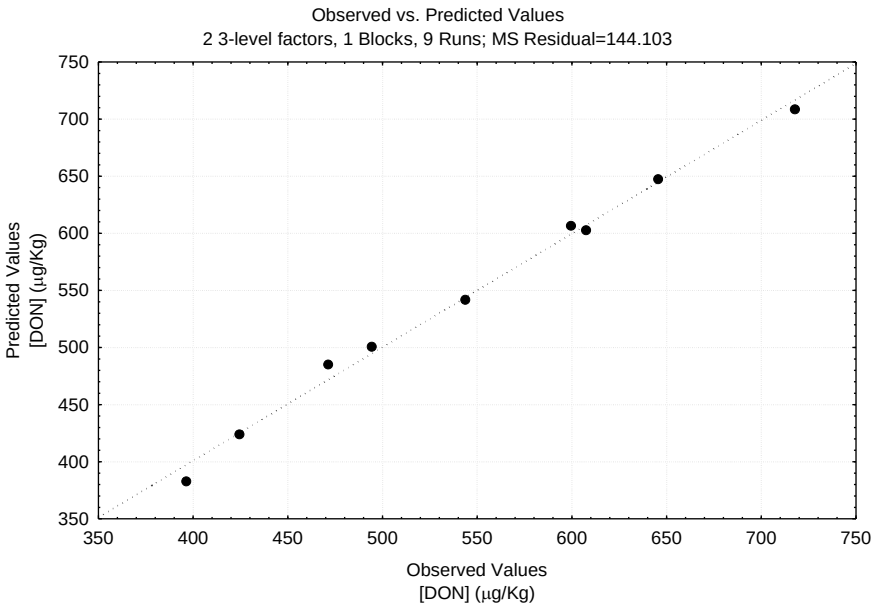

***Fig. 15**: Predicted vs observed DON concentration values plot, obtained by application of the final Box-Behnken design of experiment.*

Results obtained from each analysis were elaborated with surface charts that enabled us to calculate the optimal condition for the best recovery of mycotoxin during extraction, finding that the optimized volumes for acetonitrile and methanol were 10.5 ml and 5 ml, respectively. The addition of salts in the extraction mixture is very important since it ensures the disaggregation of the matrix thanks to the salting out effect. On the other hand, the lack of significance of these factors indicates that recovery efficiency is not affected by salt amount. Thus, salt concentrations were fixed in the protocol at low values in order to limit ion suppression effects during the mass spectrometric analysis.

The chosen parameters were used for the validation of the extraction procedure, which has been performed analyzing blank samples fortified with DON and D3G at two spiking levels: 1 and 2.5 mg/Kg. The method showed high recovery and reproducibility values, obtained by applying the optimized protocol to blank samples (n = 2), spiked with all the target toxins at 2 different concentration levels, as shown in table 2.

***Table 11:** Quality parameters obtained for the proposed method.*

	Spiking level: 1 mg/Kg			**Spiking level: 2.5 mg/Kg**		
	Mean (mg/Kg)	**CV%**	**Recovery (%)**	**Mean (mg/Kg)**	**CV%**	**Recovery (%)**
NIV	0.742	0.5	89.1	2.028	0.2	81.1
DON	0.800	2.4	98.4	2.130	2.3	85.2
D3G	0.779	3.0	92.1	2.230	3.4	89.2
3ADON	0.878	0.5	104.8	2.327	0.7	93.1
15ADON	0.923	0.1	105.3	2.348	0.2	93.9
T2	0.792	0.5	95.6	2.098	1.0	83.9
HT2	0.637	0.2	75.9	1.548	0.0	61.9

All the analyses were performed in duplicate. The method accuracy was also tested by measuring a Certified Reference Material (wheat) naturally contaminated by DON (declared concentration: 1062±110 μg/Kg), obtaining a recovery of 85±2% and a z-score of 1.45. A good recovery values have been obtained not only for DON, but also for D3G, especially in comparison with data reported in the literature.[21]. The use of a methanol/acetonitrile extraction mixture instead of a water/acetonitrile mixture allowed for the satisfactory recovery of the more polar glucoside. Indeed, as a possible explanation of this result, the use of a water/acetonitrile mixture resulted in a phase separation, due to the high salt concentration, and to the consequent transfer of analytes into the organic layer, transfer which is not quantitative for the highly polar D3G. On the other hand, the use of a methanol/acetonitrile mixture do not induce such a separation, thus allowing the quantitative extraction of the analyte.

For the analysis, an UPLC-ESI/MS method was developed starting from a previous method proposed by our group[30], which exploit the use of sodium acetate added to the eluent as cationization agent: this approach was found to be very efficient in enhancing the method

sensitivity using single quadrupole mass spectrometry. In order to avoid ion suppression/enhancement phenomena, the flow was diverted to waste during the initial conditioning step of the chromatographic separation, performed at 98% water. Moreover, isotopically labelled ^{13}C-DON was used as internal standard in order to correct possible matrix effect affecting the peak intensity.

The method, although developed by using a single quadrupole instrumentation, allowed for a satisfactory sensitivity, being the LOD values comparable to those obtained by means of a TOF-MS instrumentation[46]. This is an important point, since the developed protocols allowed for an accuracy and a sensitivity comparable to those achieved by using more expensive instrumentation which can be rarely available for routine analysis.

1.13 DON and D3G determination in durum wheat.

Using the developed method, occurrence of trichothecenes was assessed in 150 durum wheat samples produced in three different districts of northern Italy during the 2009-2010 growing season. Samples shows significant genetic variation being bred starting from different parental lines of wide diffusion in Italy and the three districts represent areas of intense durum cultivation in the northern part of the country. As far as mycotoxin contamination, all the samples were found positive for DON contamination, with a concentration varying between 47 and 3715 μg/kg. D3G was detected in 85% of the analyzed samples with a concentration range between 46 μg/kg and 842 μg/kg. Concerning the other *Fusarium* mycotoxins, NIV was found in 10 out of 150 samples, being the median and the maximum concentration 74.1 μg/Kg and 159.4 μg/Kg, respectively. The acetylated derivatives of deoxynivalenol have been also found: in particular, 3ADON was found in 86 out of 150 samples, being the median and the maximum concentration 134.0 μg/Kg and 202.6 μg/Kg, respectively; 15ADON was found in all the considered samples although at lower levels, being the median and the maximum concentration 91.7 μg/Kg and 243.5 μg/Kg, respectively. None of the other considered *Fusarium* mycotoxins have been detected in this sample set.

1.13.1 Statistical analysis

In order to verify the possible relationship between mycotoxins contamination and harvesting areas, the dataset was analysed by OneWay-ANOVA ($\alpha = 0.05$), showing that samples harvested in the Falconara area (code: 3) were characterized by a significantly higher amount of DON ($p = 0.000$) and D3G ($p = 0.001$) in comparison to those harvested in the other districts. The mean data as well as the contamination ranges for the considered area are reported in the figure below.

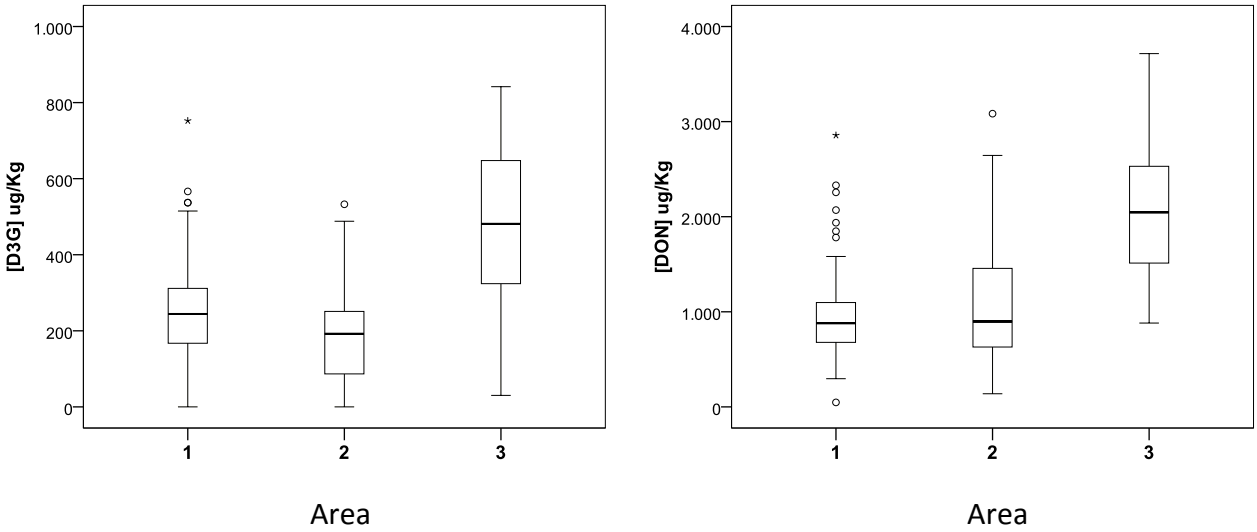

***Fig. 16*: Box plots obtained for DON and D3G occurrence (Area's codes: 1 Argelato, 2 Ferrara, 3 Falconara).**

It is noticeable that the DON mean value for samples harvested in Falconara was found to be 2055±220 µg/Kg, thus higher than the EU legal limit for raw wheat. This difference in contamination levels is mainly due to local microclimate conditions annually registered during the crop growing season, since Falconara and Poggio Renatico are usually characterized by particularly humid conditions during flowering and early stages of kernel development. As far as agronomical conditions are concerned, precropping systems were also different in the three considered areas. In particular, corn was used in rotation with durum wheat in Poggio Renatico and in Falconara, being this condition very favorable to FHB occurrence with respect to Argelato, where rape was used.

1.13.2 DON and D3G correlation to Fusarium Head Blight resistance in durum wheat.

The contamination data were then statistically elaborated by considering the 0-9 fusariosis ranking scores, as an index of the infection virulence. The ranking score summarizes the percentage of infected plants per plot and the infection severity in the plants. In particular, a non parametric Spearman's correlation test was performed (two tails, $\alpha = 0.05$) even in consideration of the relatively small sample number considered for this study. Besides DON and D3G concentrations, also the D3G/DON ratio has been considered. The significant correlations found for the dataset are reported in table below.

***Table 12**: Spearman's correlation values between contamination data and selected agronomical factors.*

		DON	D3G	D3G/DON	FHB ranking
DON	Rho	-	**.777****	**-.370****	-.034
	P		*.000*	*.000*	*.698*
D3G	Rho		-	**.218***	.116
	P			*.012*	*.188*
D3G/DON	Rho			-	**-.260****
	P				*.003*

As first, a positive correlation was found among DON and D3G concentrations, showing that high DON levels usually correspond to high D3G levels. In addition, a significant positive correlation was found among D3G/DON ratio values and FHB ranking scores, in agreement thus with what already reported by Lemmens et al.[31] for bread wheat. In that study, several bread wheat lines with different susceptibility towards FHB were inoculated with DON and analysed for DON and D3G occurrence. The reported findings suggested that in DON-resistant lines, a significant part of the applied DON was metabolized to D3G. Based on the close relation between the ratio of D3G to DON concentration in ears with the DON resistance data (R^2 = 0.84), the authors claimed that DON conjugation to glucose is the primary biochemical mechanism for resistance towards DON in bread wheat.

Accordingly[31], in our study the positive value indicates that plants with higher D3G levels compared to DON concentrations are also characterized by lower FHB symptoms obtained upon visual evaluation. Although the sample size considered in this study does not allow to draw general conclusions, the results herein described, when confirmed by further studies, indicate that D3G formation may be a possible detoxification mechanism exerted by the more FHB tolerant/resistant durum wheat lines as a response towards *Fusarium* infection.

These data thus open an important issue concerning the understanding of resistance mechanisms in durum wheat and can be relevant for practical breeding.

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Chapter 2: Study of D3G occurrence in greenhouse experiment

Introduction

After explaining the occurrence of fusarium mycotoxins in food and their relation with wheat diseases, this chapter will address the study of masking mechanism of DON. This specific mechanism involves Deoxynivalenol-3-β-glucoside (D3G), the masked form of DON originated by detoxification pathway in plants, who can be stored in plant cells, within vacuole and in wall cells, in order to reduce its toxicity. D3G production represents one of the main system of FHB resistance put in place from the plant. Since DON is the major virulence agent of FHB, its detoxification causes a reduction of FHB infection. The first studies on masked mechanism started with Lemmens et. al in 2005[50], whose proposed an experiment in which ninety-six double haploid lines, coming from a cross obtained by combining two lines named 'CM-82036' and 'Remus', were examined. These genotypes were divided in two groups: plants belonging to the first group were contaminated with a solution of DON, while the second ones were inoculated with *Fusarium* spp. Both inoculation and contamination were made on the flowering ears and induced typical FHB symptoms to the plant. This experiment involved only *aestivum* wheat genotypes, while in this chapter the durum wheat FHB resistance is considered.

2.1 FHB

FHB has been associated with 17 different fungal species, but *F. culmorum* and *F. graminearum* appear to play a predominate role in wheat infections in Europe and North America. *F. culmorum* is favored by the cooler environments of Western Europe and Canada, while *F. graminearum* is favored by the warmer, temperate zones of the United States.

2.1.1 Fusarium graminearum

Fusarium graminearum represents a double threat for wheat crops as it decreases grain yield and produces trichothecene mycotoxins, such as deoxynivalenol, which reduce the quality of the grain. Deoxynivalenol (DON), also known as vomitoxin, is the most commonly encountered *Fusarium* toxin in food and feed. Infection, caused by these fungi, induces shifts in the amino acid composition of wheat, resulting in shriveled kernels and contaminating the remaining grain with mycotoxins. [1]Despite several efforts to find resistance genes against *F. graminearum*, fully resistant varieties are not currently available. Research on the biology of *F. graminearum* is directed towards gaining insight into more details about the infection process and reveal weaknesses in the life cycle of this pathogen to develop fungicides that can protect wheat from scab infection. *Fusarium graminearum* infects plant via the floral tissues and can result in characteristic blighted symptoms on barley, oats and rice, as well as wheat. In addition, because of the ability to cause head blight, *F. graminearum* is one of the causative agents of stalk and ear rot of corn. Many other crops experience colonization by this cosmopolitan fungus, which makes it difficult to interrupt its life cycle through crop rotation.
Fusarium graminearum may survive from season to season on crop residues as a facultative saprophyte microrganism. Perithecia, its sexual reproductive structure, is produced on crop stubble during favorable environmental conditions. Mature ascospores are forcibly discharged from perithecia into the air and then dispersed by wind. They may be originated in multiple

geographic areas and be distributed over great distances. Macroconidia, the asexual spores, are produced on sporodochia and rely on splash dispersal[2].

***Fig. 17*: Macroconidia of Fusarium graminearum.**

2.1.2 Fusarium culmorum

Fusarium culmorum is a fungal plant pathogen and the causal agent of seedling blight, foot rot, ear blight, stalk rot, common root rot and other diseases. It can affect cereals, grasses, and a wide variety of monocots and dicots. In coastal dunegrass (*Leymus mollis*), *F. culmorum* is a nonpathogenic symbiont conferring both salt and drought tolerance to the plant, while it becomes dominant in cooler areas like north, central and western Europe. The fungus reproduces asexually by means of conidia, which represent the main mode of its dispersal[3]. Head blight is by far the most serious concern of *Fusarium* infection on pre-harvest wheat and other small grain cereals. The significance of *F. culmorum* in wheat production is attributed to both head blight and mycotoxin contamination of the grain coming from infected ears. Ear infection mainly occurs during anthesis and is favoured by wet weather or high humidity and warm temperatures. The main mycotoxins produced by *F. culmorum* are deoxynivalenol, nivalenol and zearalenone, which are a potential health hazard for both humans and animals. These compounds, especially deoxynivalenol, are believed to play an important role in disease development, since they are considered infection agents. [4]Available options of managing FHB include the use of fungicides, cultural practices, resistant cultivars and biological agents. However, no wheat cultivar is fully resistant to FHB, while fungicides are effective against natural infection up to 70%.[5]

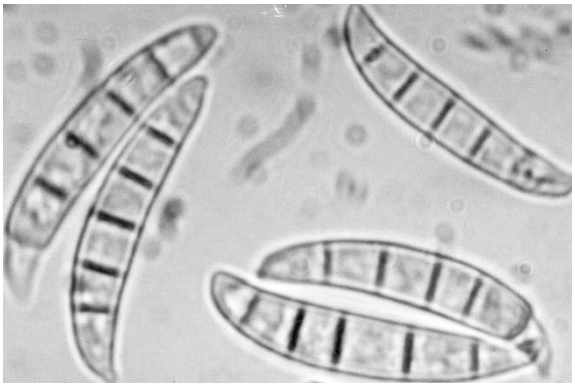

***Fig. 18*: Macro spores of Fusarium culmorum.**

2.2 FHB resistance of "Sumai 3" genotype

Studies made in China through the creation of a large number of wheat crosses and the consequent application of many breeding programs, allowed to identify a spring wheat cultivar called "Sumai 3" having one of the highest resistance to FHB[6]. Through the study of wheat DNA, three quantitative trait loci (QTL) were identified on chromosome arms 3BS, 6BL and 2DS as responsible of such mechanism. Moreover it was demonstrated that QTL on chromosome 3BS explained on its own up to 50% of the phenotypic variation and seems to be primarily associated with Type II resistance to FHB[7]. This because resistance to FHB can be classified into three types. Type I resistance means that plant is resistant to initial infection caused by spray inoculations. An alternative way to measure this mechanism is through the evaluation of FHB incidence, intended as the proportion of the infected florets or the proportion of infected spikes in a field plot in which inoculation is done by scattering infected grain on the ground. Type I resistance may be active and include defense reactions such as the activation of enzymes able to damage the fungal cell wall. Type II resistance is described by the ability of plant to counteract the spread of FHB symptoms in a point inoculated spike. Type II resistance is the predominant one identified in the majority of wheat cultivars. It is measured by counting the infected/total spikelets in an inoculated spike and then calculating the proportion of symptomatic spikelets as the final disease severity[8]. That score is also useful to measure Type I resistance, since it allows to evaluate phenotypic resistance of plants. When plant resists to the accumulation of DON in infected grains, it shows Type III resistance. The mycotoxin content is determined by quantifying the amount of deoxynivalenol in the infected grain.
FHB resistance of Sumai 3 genotype is the reason why it is often chosen as reference in studies on masking mechanism.[9,10]

2.3 Method of fungi determination

2.3.1 Plate count

Plate count is an indirect method used to detect fungi contamination. For this purpose the product to be examined is first mixed with a sterile physiological dilution fluid to separate and suspend the organisms. A series of tenfold dilutions is made from this initial suspension. Aliquot parts of these dilution stages are transferred to petri dishes and mixed with a nutrient medium which is still molten at first. When the agar has solidified the individual cells are fixed and they can multiply and form colonies in these positions during incubation. The number of colonies is determined and described as "number of moulds and yeasts per g of sample". The accuracy of the method depends on how far one is successful in completely separating all the microbial organisms from the substrate, in avoiding damage to the cells during the necessary manipulations and obtaining an even distribution of cells in the culture medium. Greater accuracy is obtained if the count is determined on several subsamples. [11]This method needs a long time to be carried out, in fact fungal growth in plate needs 7 to ten days to be usable for count.

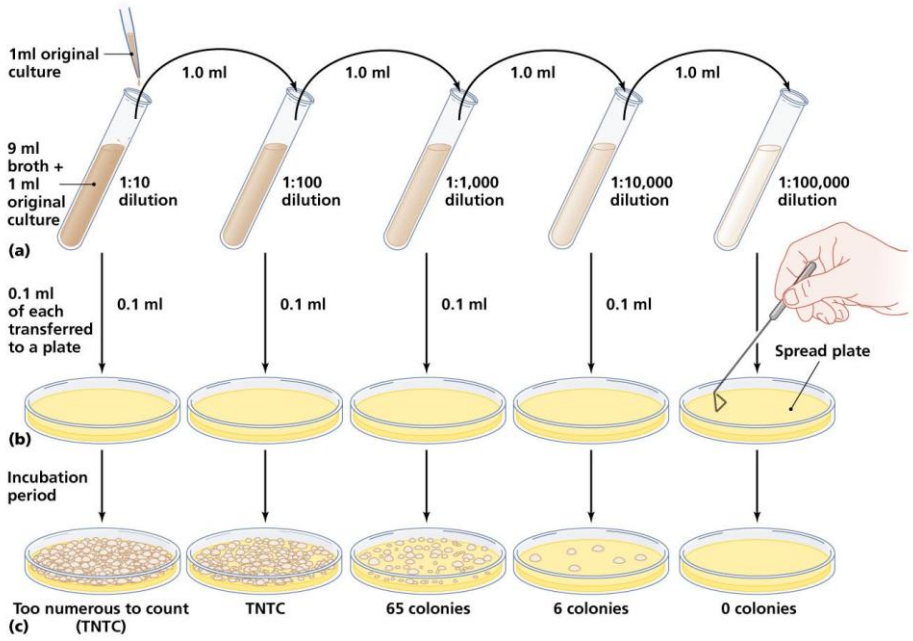

***Fig. 19*: Scheme of plate count protocol.**

2.3.2 Real-time PCR

Real Time PCR works like a standard PCR, except that a probe is designed to anneal between the primers. This probe is an oligonucleotide carrying a fluorescent marker on the 5' end and a quencher on the 3' end. The quencher suppresses the fluorescence of the marker because it is only a short distance from it (the probes are 20 to 30 bases long). During each PCR cycle, the probe anneals to the DNA between the primers, and is cleaved by the 5' -3' exonuclease activity of the DNA polymerase. The quencher and the fluorescent marker are not longer in close proximity, and so the fluorescence of the PCR augments with each cycle. The Real Time PCR instrument measures the increase in fluorescence. A threshold of fluorescence is chosen manually, taking care to lie in the exponential phase of the PCR The Real Time instrument calculates at which cycle (Ct) this threshold is crossed. The Ct is then compared to the Ct values of a standard curve with known yeast quantities, and the cell numbers of the samples are calculated. Actin was used to calculate cell numbers from viable yeasts and molds in yogurt and pasteurized food products.[12] SybrGreen dye was used for the realtime PCR assays, which intercalates with double stranded DNA and can be read by realtime PCR instruments in a similar way to TaqMan probes. It was shown that actin is a reliable gene for this type of work. However, the primers that were used were not specific for certain yeast species, they amplify many different yeasts to allow to measure a general contamination. This method is very fast but requires a very expensive instrument to amplify DNA and measures the increment of fluorescence.[13]

2.3.3 Evaluation of ergosterol content

Ergosterol assay is a rapid tool to assess the extent of fungal contamination in grains and feeds. This method is based on the quantification of ergosterol content, a primary fungal metabolite. A disadvantage of this method is the inability to discriminate the different fungal species present in the sample.[14]

2.4 Ergosterol

2.4.1 General description

Ergosterol is a major compound of cell membranes of fungi. This molecule has the same functions of cholesterol in animal cells. It is an unsaturated levorotatory alcohol with a cyclopentane-peridro phenanthrene core and formula $C_{28}H_{44}O$. This phytosterol is widely distributed in fungi and algae. Ergosterol is considered D2 pro-vitamin as if irradiated with UV it turns in D2 vitamin and other subproducts. Ergosterol (24-methylcholesta-5,7, trans 22-trien- 3 -ol) is the principal sterol of fungi in which it plays an essential role in cell membrane and other cellular constituents.

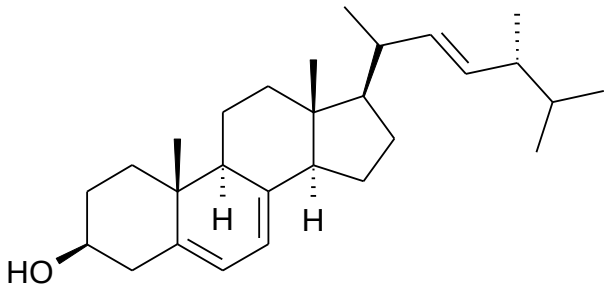

***Fig. 20*: Structure of ergosterol.**

This sterol is considered as a good marker of fungal contamination and of mycotoxin production as a primary metabolite of fungi and its clearly related to fungal growth.[15] All this characteristics makes this molecule a good index of fungal development in cereals and could be an early indicator of potential mycotoxin production. Therefore, it has been proposed as a global indicator of mycological quality of foods and feeds. One interesting characteristic of this compound is that it is not influenced by harsh physical treatment, allowing the detection of previous molds contamination. Consequently its levels are commonly used as quality parameters in ecological, industrial and agronomics environments. Moreover, significant correlations were found between ergosterol and the major mycotoxins (fumonisin B1, Zearalenone, Deoxynivalenol, Ochratoxin A and patulin) in cereals. Industries can take advantage from its detection for screening productions, prior to mycotoxin analysis. In cereals a concentration of 3 μg of ergosterol per gram is considered as the maximum acceptable level for maize, while for wheat 8 μg of ergosterol per gram is the retained value for certifying correct quality of the grains. On the other hand, when the amounts of ergosterol are upper than 8 μg/g on maize and 12 μg/g on wheat a doubtful quality of grains is suspected. For these reasons, several chromatographic methods have been proposed to assess the presence of ergosterol in crops. Most of them are based on UV absorption of this compound because its molecule absorbs ultraviolet (UV) light with a peak at 282 nm, a rare feature among

sterols, reducing the possibility of interference during chromatographic analysis. The strong absorbance at 282 nm has permitted the development of very sensitive methods for ergosterol assay, using reverse phase High Pressure Liquid Chromatography (HPLC)[16], which became the reference method in France. [17]Recently both gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry methods were developed to confirm the presence of ergosterol in samples analyzed. These techniques require a saponification step prior to quantification, so as to release esterified ergosterol from cytosolic lipid particles. This hydrolysis allows total ergosterol quantification. This amount is usually considered as the most sensitive marker of fungal biomass. After this saponification step, an extraction/purification procedure by solid phase extraction is required by some methods, whereas other authors described the use of a liquid/liquid purification/extraction. [18] Some discrepancies on results obtained by different techniques show that it is really important to elaborate or modify actual analytical techniques for rapidly quantify ergosterol with sufficient accuracy.

2.4.2 Ergosterol biosynthesis pathway

The ergosterol biosynthesis pathway is a complex route in which about 20 enzymes are involved. Some of these enzymes have been extensively studied as targets for the development of new drugs that interfere with parasite growth without severe effects on host cells. The carbon skeleton of the sterol molecule is derived from acetyl-CoA, with the exception to the presence of the C24 methyl group in its side chain. The first reactions in the biosynthetic pathway involve condensation of two acetyl-CoA units to form acetoacetyl-CoA, followed by the addition of a third unit to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is then reduced by NADPH to give mevalonic acid. These three steps comprise the mevalonate pathway and after these reactions, the next steps is represented by the isoprenoid pathway. Isoprenoids are the most diverse and abundant compounds present in nature and are essential components of all organisms due to a variety of roles in different biological processes. These compounds are involved in ergosterol synthesis starting from the conversion of mevalonate to isopentenyl diphosphate (IPP) subsequently, isomerization of IPP produces dimethylallyl diphosphate (DMAPP). After that, longer isoprenoids are formed by a consecutive condensation of IPP with DMAPP and geranyl diphosphate (GPP) to produce the 15-carbon isoprenoid compound known as farnesyl diphosphate (FPP). After the isoprenoid pathway, the next two reactions comprise the first committed step in sterol biosynthesis. They are catalyzed by squalene synthase. In the first reaction, presqualene pyrophosphate (PPP) is produced by the loss of an inorganic pyrophosphate, which is converted to squalene in the second reaction in presence of NADPH, an essential cofactor required to drive this conversion. In yeasts, squalene synthase is associated with the endoplasmic reticulum. After production of squalene, ergosterol biosynthesis continues with the synthesis of 2,3-oxidosqualene (or squalene epoxide) in a reaction catalyzed by the enzyme squalene epoxidase. This is the first step in the conversion of the 30-carbon chain squalene to the tetracyclic sterol skeleton. In a reaction that is considered to be one of the most complex part in the sterol pathway, 2,2-oxidosqualene cyclase cyclizes the intermediate 2,3-oxidosqualene to lanosterol, which is the precursor of all steroid structures. After the cyclization of 2,3-oxidosqualene to form lanosterol, several sequential transformations occur to form ergosterol in fungi. One of the earliest steps in the lanosterol pathway is the demethylation of the ring system at the C14 position in a two-stage oxidative reaction catalyzed by a cytochrome P-450-containing monooxygenase enzyme known as lanosterol-14α-methyl demethylase (C14α-demethylase). Removal of the 14α-methyl by C14α-demethylase generates a Δ8(14) unsaturated sterol with a double bond at the C14 position. This unsaturation needs to be removed to produce Δ5 sterols in two consecutive reactions catalyzed by

the enzyme Δ8(14)-reductase with NADPH as cofactor. However in some fungal species such as *Candida albicans* and also *L. amazonensis* and *T. cruzi*, it is metabolized by the C4-demethylase enzymes and Δ24-methenylase to form Δ14 fecosterol. After removal of the C4 and C14 methyl groups and the methenylation of the side chain, the next reaction in the sequence is the isomerization of the double bonds in fecosterol in a reversible reaction catalyzed by the Δ7-Δ8 isomerase that does not require cofactors such as NADPH. One of the most important stages of ergosterol biosynthesis is the addition of a methyl group at the C24 position in the sterol side chain. The methyl group is transferred from S-adenosyl-Lmethionine (SAM) to C-24 of Δ24 sterols to produce Δ24(28)-sterols in some reactions catalyzed by the S-Adenosyl-Lmethionine: Δ24-sterol methyltransferase (EC 2.1.1.43; 24- SMT). 24-MT is a 150 000 dalton membrane-bound protein that is present in plants, fungi and trypanosomatids, but is absent in mammalian sterol biosynthetic systems; thus it may constitute an interesting target for the development of antifungal and antitrypanosomal agents. [19]The synthesis of ergosterol is summarized in figure:

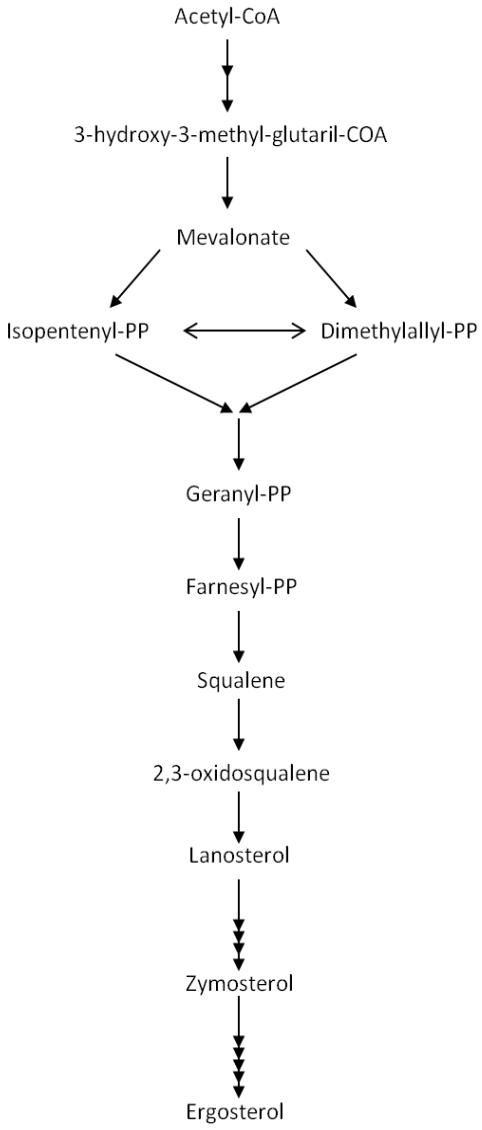

***Fig. 21*: Ergosterol biosynthetic pathway.**

Aim of the work

The study of masking mechanism of DON is an important step to better understand plant resistance machanism against FHB infection. At this purpose, a greenhouse experiment has been carried out using four durum wheat genotypes and one genotype of FHB resistant aestivum wheat. All genotypes have been contaminated with two *Fusarium* strains (*Fusarium graminearum* and *Fusarium culmorum*) and a standard solution of deoxynivalenol. In order to prove the absence of cross-contamination, a group of cultivated plants have not been contaminated and have been used as control. Samples have been collected after five, fifteen days from the contamination stage (flowering) and at the ripening stage. All the collected samples have been analyzed with the method described in chapter1 in order to quantify DON and D3G content in all of them. Statistical analysis have been applied to the obtained data, in order to find correlation between FHB, mycotoxin accumulation and D3G conversion capability of the different genotypes.

Materials and methods

2.5 Chemicals

Sodium acetate, sodium chloride, magnesium sulphate, methanol, exane, ethyl acetate, N,O-Bis-(trimetilsilil)-trifluoroacetammide (BSTFA) and acetonitrile were purchased by Sigma-Aldrich (Taufkirchen, Germany). All solvents were of HPLC grade. Ultra-pure water was in-house produced by using a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Mycotoxin standards deoxynivalenol (DON) was purchased from RomerLabs (Tulln, Austria). All stock solutions (1 mg/ml) were stored at -20°C and brought to room temperature before use. Conidial suspension of *F. graminearum, F. culmorum* (2×10^5 cfu/ml) were purchased by Department of Agricultural Science (Bologna, Italy). Ergosterol and colestanol standard were purchased by Sigma-Aldrich (Taufkirchen, Germany) and silica gel by Sigma-Aldrich (Taufkirchen, Germany).

2.6 Greenhouse experiment

Four *durum* wheat varieties (Svevo, Claudio, Kofa and Neodur) and one of *aestivum* wheat, carrying a major QTL for FHB (Sumai 3), have been selected for the greenhouse experiment. For every varieties 4 groups, each consisting of 5 plants, were considered. 3 groups of plants, at flowering stage, were artificially contaminated with 2 *Fusarium* strains (*F.graminearum* and *F.culmorum*) and a standard deoxynivalenol solution (1 mg/ml), while the fourth one (negative control) was mock inoculated with distilled water. All the contaminated groups, as well as the negative control, have been replicated four times. The whole greenhouse experiment was carried out in triplicate in order to allow three sampling at different growing stage. From the contamination phase, plants were collected after 5 days (P1), 15 days (P2) and at the end of ripening (P3). The total number of grown plants was of 1200.

2.7 Mycotoxins extraction method

All the collected ears were at first cooled with liquid nitrogen and then grinded in a A 11 basic Analytical mill (IKA®-Werke GmbH & Co. KG, Staufen, Germany). Milled samples were extracted and analyzed by UPLC/MS technique with the method previously described in chapter 1 to determine mycotoxins content.

2.8 Ergosterol analysis

All the collected ears were grounded with liquid nitrogen with an A11 basic analytical mill (IKA®-Werke GmbH & Co. KG, Staufen, Germany). For ergosterol evaluation 0.5 g of grounded samples were extracted with 10 ml of hexane and kept under stirring for 30 minutes. After this first step 300 µl of internal standard (colestanol 10 mg\Kg in hexane) and 1 ml of KOH methanolic solution 5% W\V were added to the extract. The hexane fraction was then separated on a silica gel packed column. After a first washing with 3 ml of hexane, useful to eliminate the more apolar compounds,

3 ml of ethyl acetate were added and the eluate collected. This fraction was finally derivatized with BSTFA and analyzed with GC-MS instrument.

2.8.1 GC-MS analysis

Samples were analysed with an Agilent Technologies (Santa Clara, CA) 6890 N gas-chromatograph coupled to an Agilent Technologies 5973 mass spectrometer using a DB5ms capillary column (0.25mm X 30m X 0.25um) Agilent Technologies (Santa Clara, CA). Helium was used as carrier gas, with a total flow of 13.5 ml/min. The injector temperature was set at 290 °C and the injection was performed in the split mode (ratio 10:1). Oven temperature increased from 240 C to 280 °C in 20 minutes. The final temperature was maintained for 5 min. The detector temperature was 290 °C and the MS acquisition mode was SIM (monitored m/z: 363.3, 445.4, 460.4, 468.4)

Results and discussion

2.9 Greenhouse experiment

This experiment was carried out at the research structures of Produttori Sementi Bologna (PSB), one of the oldest Italian seed company, which from the beginning focused its activity on breeding trying to match scientific research with the increasing request of innovation coming from agro-industrial sector. This company is partner of "From Seed To Pasta" project, just like the work described in this chapter. PSB supplied all structures and technologies to perform this experiment in which 4 *durum* wheat genotypes (Claudio, Kofa, Neodur and Svevo) and 1 of *aestivum* wheat (Sumai) were utilized. Sumai genotype was used as reference standard, since its well known resistance to FHB disease, thanks to the presence within its DNA, of QTL resistance gene. The selection of durum wheat genotypes was made on the basis of their high technological performance and it is been investigated if they possess the resistance to disease comparing with that of Sumai wheat genotype. Since the FHB resistance of these genotypes remains still unknown, having informations about that could be interesting to forecast harvesting yields and consequently pasta production. The experiment was arranged in a RCB (Randomized Complete Block) design with four replicates/variety and four treatments. All plants were sown individually and maintaining thirty days at low temperature (vernalization) to mimic winter period when the plant growth stops. After this step plants were moved on the greenhouse and maintained at conditions that mimic spring period at first and finally summer season. All plants were inoculated at the flowering stage (zadok 59-60). The inoculum was made on four adjacent spikelets of principal ear. Each spikelet was inoculated with 10 µl of conidial solution ($1x10^5$ conidia/ml) or standard solution of DON (0.828 mg/ml). This process consists in manual inoculum of plants with two *Fusarium* strains (*F. graminearum* and *F. culmorum*), a standard solution of deoxynivalenol and water (negative control samples). The first sampling has been performed after five days from the inoculum (zadok 68-69) and it was made with the aim to evaluate fungal growth status and the internalization of DON by plants. After fifteen days from the flowering stage (zadok 70-75) a second sampling was carried out to evaluate the conversion rate of deoxynivalenol to deoxinivalenol-3-glucoside, both in *Fusarium* inoculated samples as well as in those contaminated with the native toxin. When plants were ripe (zadok 92), the last sampling was made in order to collect all contamination data (mycotoxins content and conversion rate of DON to D3G). All samples were milled with liquid nitrogen, extracted and analyzed with the methods described in chapter 1.

2.10 Mycotoxins content

2.10.1 Fusarium contaminated samples

All inoculated samples resulted positive to deoxynivalenol and its masked form (D3G), while samples inoculated with water (negative control) were not contaminated. Samples of durum wheat inoculated with *Fusarium* strains showed a very high contamination level, ranging between

1.5 mg/kg and 31.7 mg/kg as regards *Fusarium graminearum* contaminated samples; relatively to the samples inoculated with the other *Fusarium* strain (*F. culmorum),* results fluctuated between 3.4 mg/kg and 21.4 mg/kg. These data revealed a high variability between the different wheat genotypes and fungal infection. Moreover, they probably depend on the different infection capacity and growth speed of fungi on the plants. Data obtained from analysis on Sumai genotype show a low contamination level of DON (1.7 mg/Kg - 5.6 mg/Kg) in *F. graminearum* contaminated samples and ranging between 2.6 mg/Kg and 4.7 mg/Kg in those inoculated with *F. culmorum* and a very low variability, probably due to the high resistance of this genotype to FHB. Data obtained from the first sampling are showed in the graphs.

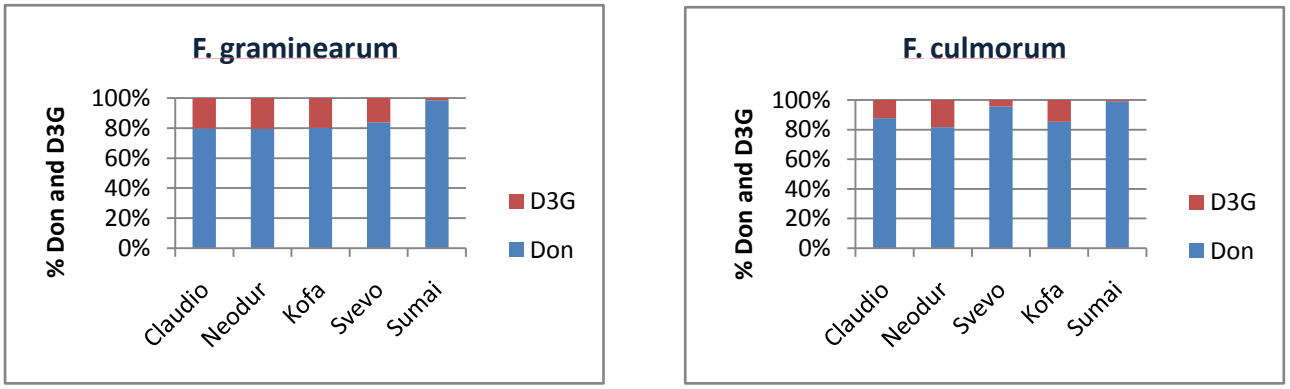

***Fig. 23*: % of DON and D3G in Fusarium strain inoculated samples after 5 days from the inoculum**

From the figure it is clearly evident that all the inoculated samples show a percentages of detoxification ranging between 2 and 15% . The low percentage referring to Sumai genotype, both in *F. graminearum* and in *F. culmorum* inoculated samples, is due to the low initial deoxynivalenol contamination. However, concentration of deoxynivalenol is higher in S1 samples, when compared to S2 ones. In fact, the concentration in durum wheat samples inoculated with *F. graminearum* ranges from 45.5 mg/kg to 158.2 mg/kg, while in *F. culmorum* inoculated samples, levels of contamination are between 40.9 mg/kg and 146.5 mg/kg. In this sampling, a high difference in contamination level is also detected, as well as in S1 sampling. Sumai shows again the lowest level of contamination, corresponding to 30 mg/Kg in *F. graminearum* inoculated samples and to 25 mg/kg in the *F. culmorum* inoculated samples. In graphics below the percentages of D3G and DON in S2 sampling are showed.

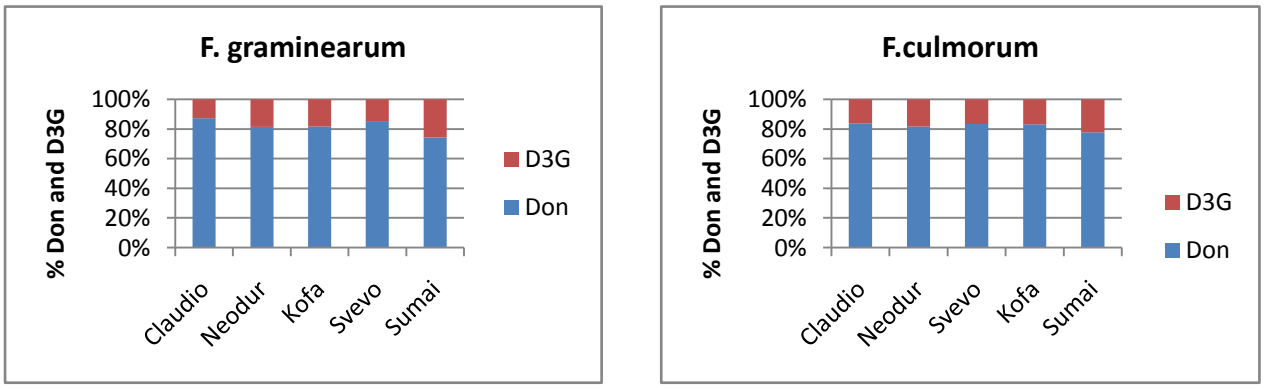

***Fig. 22*: % of DON and D3G in Fusarium strain inoculated samples after 15 days from inoculum.**

As it is possible to note, in these graphs data obtained in S1 were confirmed; indeed, all samples analyzed show a percentage of D3G conversion of about 15%. This value in Sumai samples resulted higher than that revealed in previous sampling and also compared to the other genotypes. This last data confirm the optimal FHB resistance of Sumai genotype. Comparing data obtained in S1 and S2, it is clear that the level of contamination increases from five to fifteen days after inoculum. This behavior is confirmed considering the deoxynivalenol contamination trend. Referring to D3G content, a different behavior is observed, as showed in the graph below, in which percentages of D3G from S1 to S2 are compared.

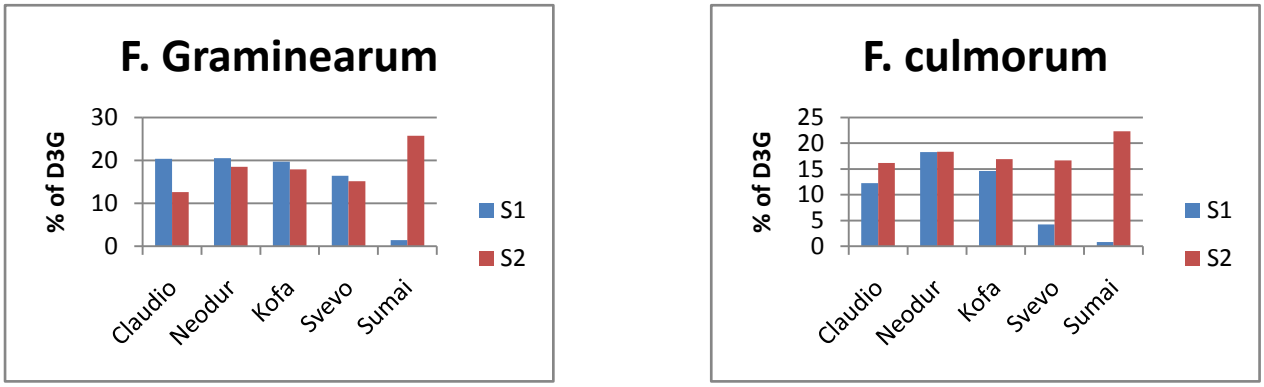

***Fig. 24*: Trend of D3G percentage from S1 to S2 in Fusarium inoculated samples.**

In samples contaminated with *F. graminearum* the percentages of D3G remain almost constant from S1 to S2, while in those inoculated with the other *Fusarium* strain, an increase from S1 to S2 in masked mycotoxin contamination was observed. This behaviour is probably due to the different response of the plant to fungal infection. For Sumai genotype it is clear that the percentage of D3G is higher in S2 than in S1 and this is due to the FHB resistance of this *aestivum* wheat genotype.

2.10.2 Deoxynivalenol contaminated samples

As regards samples contaminated with standard solution of deoxynivalenol, they generally showed a lower level of contamination than samples inoculated with *Fusarium* strains. This is probably due to the low concentration of standard utilized for contamination and problems of loss of the mycotoxin as a consequence of washout phenomena. The measured concentration of DON is very high (40 µl of a solution of 800 mg/Kg) which corresponds to 33.1 µg of deoxynivalenol inoculated in each principal ears. This amount of DON brings to a final concentration of mycotoxins equal to 800 mg/kg and in the analyzed samples this value ranges between 3.1 mg/kg and 19 mg/kg in S1 sampling (after five days from contamination) and between 2.9 mg/kg and 27 mg/kg in S2 sampling (after fifteen days from contamination). The percentages of conversion DON/D3G in two samplings are showed in the graph below:

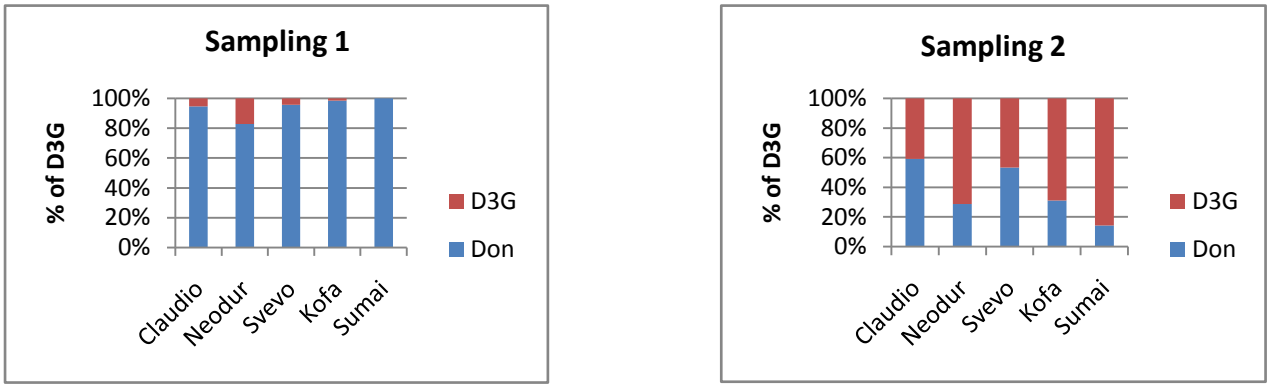

Fig. 25: % of DON and D3G in deoxynivalenol contaminated samples after 5 (S1) and 15 (S2) days from contamination step.

Graphs show a high increase of D3G percentages going from sampling 1 to sampling 2. Plants keep intact their mechanisms of resistance to FHB since no fungal infection was detected. This results confirm that the most important mechanism of resistance in plants is detoxification of DON to D3G. That fact also demonstrates that the toxin has been internalized and then metabolized by plants.

2.11 Ergosterol content

In order to evaluate fungal biomass, ergosterol content was evaluated in wheat samples collected after fifteen days from inoculum (S2 sampling). Ergosterol is a specific component of fungal cell membranes and its content is therefore correlated with the amount of metabolically active fungal biomass. Since the first application of ergosterol analysis in mycorrhizal research by (), the procedure has become increasingly popular and is now the most commonly used method for estimating fungal biomass in mycorrhizas. Results obtained for S2 analysis show that all treated wheat samples contain ergosterol, while non treated ones did not contain this compound. Results obtained are summarised in the table below:

***Table 13*: Ergosterol content in Fusarium inoculated samples.**

Sample	Fungi	mg/Kg ergosterol	Sample	Fungi	mg/Kg ergosterol
Claudio	F. graminearum	14.73	**Claudio**	F.culmorum	19.99
Claudio	F. graminearum	27.73	**Claudio**	F.culmorum	23.93
Claudio	F. graminearum	29.77	**Claudio**	F.culmorum	47.88
Neodur	F. graminearum	7.67	**Neodur**	F.culmorum	7.22
Neodur	F. graminearum	1.22	**Neodur**	F.culmorum	3.32
Neodur	F. graminearum	3.66	**Neodur**	F.culmorum	5.65
Kofa	F. graminearum	2.19	**Kofa**	F.culmorum	3.86
Kofa	F. graminearum	55.92	**Kofa**	F.culmorum	3.91
Kofa	F. graminearum	2.30	**Kofa**	F.culmorum	10.01
Svevo	F. graminearum	11.43	**Svevo**	F.culmorum	9.51
Svevo	F. graminearum	9.14	**Svevo**	F.culmorum	7.44
Sumai	F. graminearum	2.71	**Svevo**	F.culmorum	2.12
Sumai	F. graminearum	0.32	**Sumai**	F.culmorum	0.02
Sumai	F. graminearum	0.31	**Sumai**	F.culmorum	0.46

A great variability was observed in the data related to ergosterol content, which can be explained by a different development of the fungal biomass on the different plants, different development which is also related to mycotoxin accumulation. The graph below shows the relationship between mycotoxins contamination and ergosterol content:

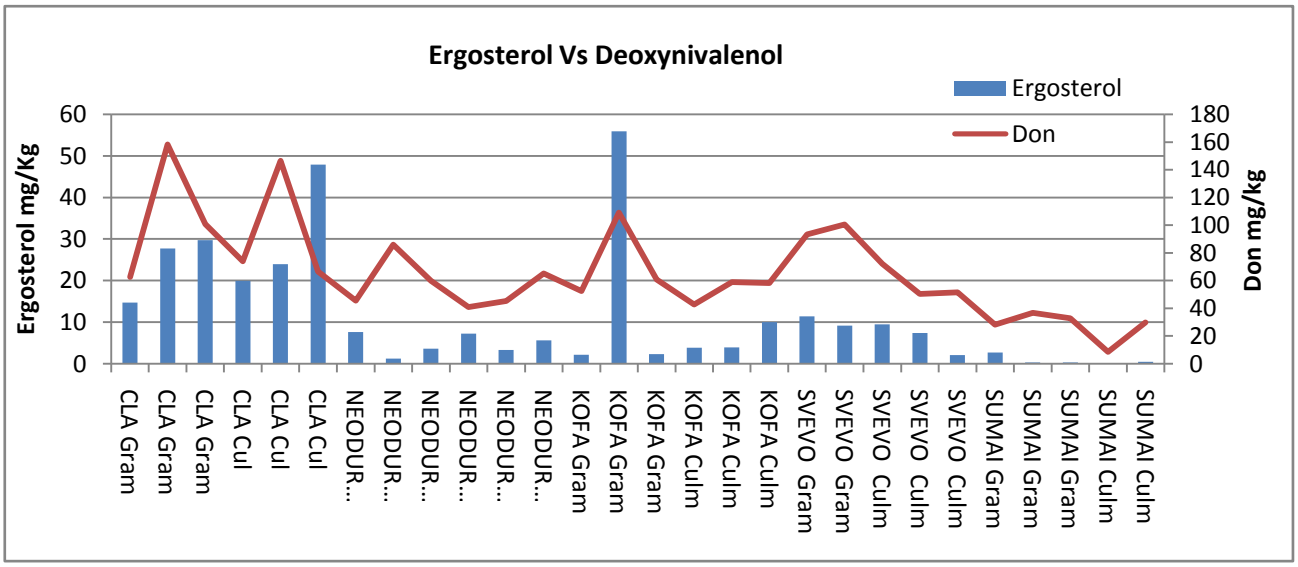

***Fig. 26*: Comparison between DON and ergosterol content in Fusarium inoculated samples.**

Examining the graph, it can be seen that when the ergosterol content increases, DON contamination increases too. This is an expected result because ergosterol is considered as a marker of fungal growth and the increase of its content corresponds to an increase of biomass and then probably of mycotoxin production. After the analysis of these first results a new statistical analysis was carried out, in order to reveal a possible correlation between ergosterol and mycotoxins content, wheat genotypes and type of fungi. This statistical elaboration showed a positive correlation between ergosterol content and deoxynivalenol contamination, confirming the behavior previously described (Fig 26). The absence of correlation between ergosterol and D3G makes sense because this molecule is produced by plant during the infection and not by fungi.

***Table 14*: Correlation between ergosterol content and mycotoxin contamination**

		Ergosterol	DON	D3G	D3G_DON
Ergosterol	Pearson Correlation	1	,609**	,175	-,693**
	Sig. (2-tailed)		,001	,372	,000
	N	28	28	28	28
DON	Pearson Correlation	,609**	1	,703**	-,698**
	Sig. (2-tailed)	,001		,000	,000
	N	28	28	28	28
D3G	Pearson Correlation	,175	,703**	1	-,133
	Sig. (2-tailed)	,372	,000		,500
	N	28	28	28	28
D3G_DON	Pearson Correlation	-,693**	-,698**	-,133	1
	Sig. (2-tailed)	,000	,000	,500	
	N	28	28	28	28

**. Correlation is significant at the 0.01 level (2-tailed).

Finally, the results concerning mycotoxins content have been recalculated in order to consider the different development of fungal biomass. At this purpose DON and D3G contamination data were weighed on ergosterol content. Data obtained after this elaboration were analyzed to investigate the possibility of a correlation between mycotoxin content, genotype and inoculated fungi.

***Table 15*: Correlation between mycotoxin content and inoculated fungi**

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	DON	1,448E11	1	1,448E11	168,074	,000
	D3G	3,735E9	1	3,735E9	156,785	,000
Intercept	DON	2,371E12	1	2,371E12	2752,152	,000
	D3G	1,033E11	1	1,033E11	4337,375	,000
Fungus	DON	1,448E11	1	1,448E11	168,074	,000
	D3G	3,735E9	1	3,735E9	156,785	,000
Error	DON	3,049E11	354	8,614E8		
	D3G	8,434E9	354	23824398,582		
Total	DON	2,863E12	356			
	D3G	1,170E11	356			
Corrected Total	DON	4,497E11	355			
	D3G	1,217E10	355			

a. R Squared = .322 (Adjusted R Squared = .320)

b. R Squared = .307 (Adjusted R Squared = .305)

Fungi show a positive correlation with DON and D3G contamination. Positive correlation means that different fungi produce different amount of mycotoxins in relation to their specific growth optimal conditions. D3G content depends on the response of the plant towards infection: if the plant is weakened as a result of higher fungal infection, probably also the defense mechanisms based on glycosilation are reduced and percentages of D3G are lower than those produced by a healthy plant.

In Table 16 the results of the statistical correlations between genotype and amount of accumulated mycotoxins are reported.

***Table 16*: Correlation between mycotoxins content and wheat genotype.**

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	DON	1,676E11	4	4,190E10	52,127	,000
	D3G	4,123E9	4	1,031E9	44,959	,000
Intercept	DON	1,002E12	1	1,002E12	1246,731	,000
	D3G	4,919E10	1	4,919E10	2145,654	,000
Cultivar	DON	1,676E11	4	4,190E10	52,127	,000
	D3G	4,123E9	4	1,031E9	44,959	,000
Error	DON	2,821E11	351	8,037E8		
	D3G	8,046E9	351	22924433,029		
Total	DON	2,863E12	356			
	D3G	1,170E11	356			
Corrected Total	DON	4,497E11	355			
	D3G	1,217E10	355			

a. R Squared = .373 (Adjusted R Squared = .366)

b. R Squared = .339 (Adjusted R Squared = .331)

Statistical analysis shows a positive correlation between mycotoxins content and wheat genotype. This fact is probably due to the different behavior of genotypes in relation to the fungal infection. A higher accumulation of DON is observed in some genotypes, whereas others seem to better contrast mycotoxin accumulation: this is probably due to different susceptibility of the plant to FHB infection and consequent mycotoxin production. On the contrary, D3G production is probably related to the detoxification ability of the different plants. Once the possible correlations between data obtained have been verified, a post-hoc test was made. Results are reported in the following table.

***Table 17*: Tukey test about DON contamination and genotype.**

Tukey HSD[a,b]

Cultivar	N	Subset 1	Subset 2	Subset 3	Subset 4
5	27	32151,5556			
3	34		55206,0882		
2	43		62322,3488		
4	71			81164,8169	
1	181				100119,4530
Sig.		1,000	,751	1,000	1,000

a. Uses Harmonic Mean Sample Size = 45.740.

b. Alpha = .05.

Wheat genotypes resulted separated into four groups. Each group comprises genotypes with similar content of deoxynivalenol. Cultivar 5 is Sumai, the other four Claudio, Kofa, Neodur and Svevo, respectively. It is important to note that sumai (*aestivum* wheat genotype) is the cultivar that presents the lowest concentration of Deoxynivalenol and this data confirms that it is the most resistant cultivar to FHB infection. The second less contaminated samples belong to Kofa and Neodur cultivars; then, there is Svevo and, finally, the most contaminated line, Claudio.

***Table 18*: Tukey test about D3G contamination and genotype**

Tukey HSD[a,b]

Cultivar	N	Subset for alpha = 0.05			
		1	2	3	4
5	27	10230,9549			
2	42		13178,0531		
3	33		13355,0858		
4	71			16336,9483	
1	179				20155,8439
Sig.		1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 45.670.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Tukey post-hoc test carried out considering D3G contamination and genotypes allowed to separate genotypes again into four groups, with the same composition previously described. This test showed that Sumai is once again the most FHB resistant genotype; it is noteworthy that this higher resistance is linked to a higher capability of deoxynivalenol conversion to D3G and this is recognized as one of the most important mechanism of resistance to FHB.

2.11.1 Sampling 3

Results concerning sampling 3 (at ripening) will be separately treated because S3 grains, unlike those collected in other sampling (S1 and S2), were at first separated from their glumes and then milled; this latter treatment removed bran, considered as the most contaminated part of the kernel. In this samples, artificially DON contaminated samples showed a very low mycotoxin content, probably due to the absence of its internalization by the plant. This result is due to the fact that, applying the toxin solution on the external surface of the spikelet, in the absence of fungal infection only a minimum part of the toxin is absorbed within the vegetable cells: when milled, also this fraction is highly reduced. Furthermore, the *Fusarium* inoculated samples were almost totally destroyed as the used strains have shown a very high virulence. The percentage of D3G found in *Fusarium* inoculated samples are showed in the following graphs.

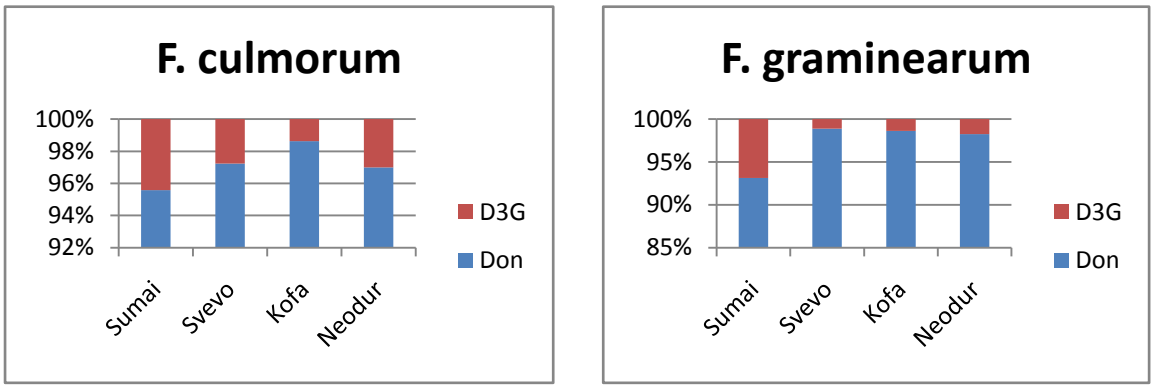

***Fig. 27*: Percentages of D3G in sampling 3.**

A very low percentages of D3G comprised between 2 and 5% in *F. culmorum* inoculated samples and between 1 to 7% in those inoculated with *F. graminearum* strains was observed. These data resulted lower than those obtained from S1 and S2 samplings, probably on account of the elimination of the most contaminated part of the kernels during ginning and milling processes. Sumai genotype showed again the highest capacity of DON detoxification. The final amount of mycotoxin detected in DON contaminated samples resulted between 40 ppb and 90 ppb. This fact confirms the hypothesis of no-internalization of DON by plant.

In conclusion this work confirmed the high resistance capability of the Sumai genotype, which shows a great capacity to detoxify DON to D3G. As far as the durum wheat genotypes are concerned, Kofa and Neodur showed a similar behavior compared to that of Sumai in DON contaminated samples, suggesting a partially FHB resistance of these lines; while the other genotypes evidenced a very low capacity to detoxify the mycotoxin. Analyzing the results obtained from *Fusarium* contaminated samples, it was confirmed that Sumai is the most resistant genotype, showing the lowest DON content; data about durum wheat lines indicated that Kofa and Neodur seem to be characterized by a good resistance to FHB, with a DON contamination level lower than the other lines. On the contrary Claudio was indicated as the most susceptible genotype to Fusarium infection.

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Chapter 3: Impact on human health of masked mycotoxins: preliminary data.

Introduction

In previous chapters masked mycotoxins and their importance in food chain were largely described. The occurrence of this toxins in many food products induced to investigate the real importance of this compounds for human health. Currently the fate of this masked forms during digestion remains still unknown. If masked myctoxins are degraded, their native forms are released and can subsequently increase the intake of toxic substance within the organism. While if these molecules remain intact during digestive processes, the research will be moved in the intrinsic toxicity of masked mycotoxins. The first part of this chapter is devoted to the in vitro and in silico evaluation of the toxicological aspects of masked mycotoxins (ZEN derivates) while the second part involved the study of fate of Z14G, Z14S and D3G during human digestion. At this purpose an in-vitro digestion and fecal fermentation experiments will be used.

3.1 Zearalenone

3.1.1 Effects on health

The acute toxicity of zearalenone is low and its toxic effects are related to the potent oestrogenic activity of the toxin itself and its metabolites. Zearalenone is metabolised in the gut of animals, especially pigs and potentially humans, forming α- and ß-zearalenols. These metabolites are then conjugated with glucuronic acids and may be more potent oestrogens than zearalenone itself. Zearalenone has been shown to cause hormonal effects on the reproductive systems of pigs and sheeps, which appear to be more sensitive than other animal species. Feeding zearalenone to female pigs at levels of up to 0.25 mg/kg produced slight inflammation of external sexual organs. Effects of higher doses (50 mg/kg) in the diet of pigs included abortion and stillbirths, while more moderate doses (10 mg/kg) caused reduced litter sizes and birth weights. Sheep are similarly affected and zearalenone is reported to be a cause of infertility in flocks in New Zealand. Dairy cows are also reported to develop reproductive abnormalities when the toxin is present in their diet. There is some evidences of similar effects in humans. Zearalenone was suspected to be the cause of an outbreak of early secondary breast development affecting girls from six months to eight years old in Puerto Rico between 1978 and 1981. A similar incident was reported in Hungary in 1997[1].

3.1.2 Metabolism

ZEN is rapidly absorbed after oral administration. Although the degree of its absorption is difficult to measure, due to extensive biliary excretion, it appears to be extensively absorbed in rats, rabbits and humans. The uptake in a pig after a single oral dose of 10 mg/kg b.w. was estimated to be 80–85%. Two major biotransformation pathways for ZEN in animals have been suggested. [2]These are:

1. Hydroxylation: resulting in the formation of α-ZOL (α-zearalenol) and β-ZOL, assumed to be catalyzed by 3α- and 3β-hydroxysteroid dehydrogenases (HSDs).

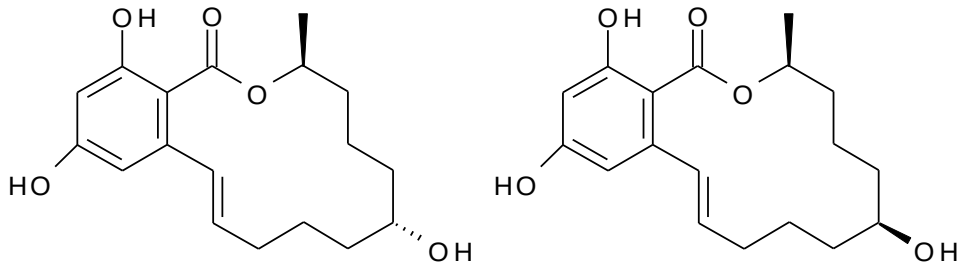


***Fig. 28*: Structure of α-ZOL and β –ZOL**

2. Conjugation of ZEN and its reduced metabolites with glucuronic acid catalyzed by uridine diphosphate glucuronyl transferases (UDPGT).

Earlier investigations of Ueno et al. (1983) showed that α-ZOL was a major metabolite in cultured hepathocytes of the rat, mouse, pig, cow and rabbit at pH 4.5 with either NADH or NADPH and at pH 7.4 with NADH, although at pH 7.4 with NADPH, β-ZOL was the predominant metabolite. In guinea pigs, both β-ZOL and α-ZOL were produced in roughly similar amounts irrespective of the pH and cofactor, while in hamsters β-ZOL was the major metabolite produced. [3]These findings indicated that there are two types of ZEN reductase differing in optimum pH. Olsen et al. (1987) also reported that ZEN was metabolized to α-ZOL and β-ZOL by sow intestinal mucosa in vitro, with the predominance of β-isomer[4]. Recently, Malekinejad et al. (2006) has reported differences between species in hepatic biotransformation of ZEN. The authors demonstrated that pigs seem to convert ZEN predominantly into α-ZOL, whereas in cattle β-ZOL is the dominant hepatic metabolite, while the sheep liver post-mitochondrial fraction converted ZEN mainly into a-zol. Even though significant differences were found in the metabolic profile of ZEN among animal species, very limited data are available for man. In pigs and probably in humans, ZEN is rapidly adsorbed after oral administration and can be metabolized by intestinal cells. In these cells, ZEN is degraded into α-ZOL, β-ZOL, α-ZAL (α-zearalanale) and β-ZAL, which are subsequently conjugated with glucuronic acid.[5]

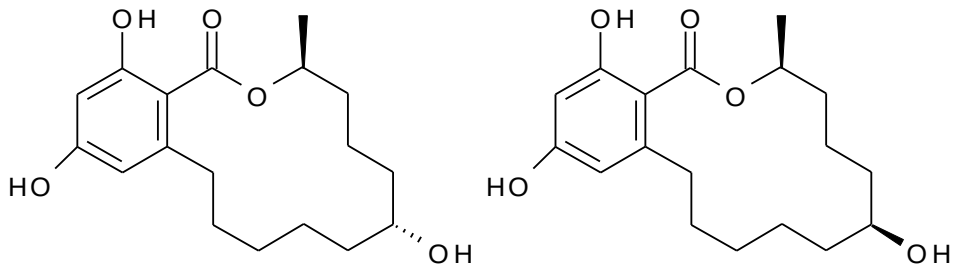


Fig. 29: Structure of α-ZAL and β –ZAL

High levels of some of these forms may be excreted in the urine as glucuronides by grazing sheep. Doll et al. in 2003[6] observed that ZEN would be significantly eliminated through bile and urine. ZEN, α-ZOL and β-ZOL concentrations in the urine of a male volunteer 6, 12, and 24 h after a single oral dose of 100 mg ZEN, were 3.7 and 3 µg/ml and not detected respectively after 6 h, 6.9, 6, and 2.7 µg/ml respectively after 12 h and finally 2.7, 4 and 2 µg/ ml after 24 h[7]. In ruminants, ZEA and its metabolites are detected in bile at respective rates of 68% for β -ZOL, 24% for α-ZOL and 8% for ZEN[8]. In subsequent studies it was observed that ZEN and its derivates concentrations in liver and bile increases after administration of a toxin dose[3]. Neither ZEN nor its metabolites are detected in muscles, kidneys, liver, bladder, dorsal fat of male bovine ingesting 0.1 mg ZEN/day/kg feed[5]. Metabolism of mycotoxins in animals may cause the manifestation of adverse effects. There may also be additional implications for carcass and milk quality if extensive transformation occurs within the digestive tract or within the tissues of animals[9]. The ovine metabolism of ZEN has been proposed to include the synthesis of at least five metabolites including ZEN, α-ZOL, β-ZOL, α-ZAL and β-ZAL[10]. The adverse effects of ZEN depend on the process of elimination. In pigs, as in sheep, ZEN is conjugated with glucoronic acid and in addition may be metabolized to α-Zol. However, Biehl et al. (1993) indicated that biliary excretion and enterohepatic cycle are important processes affecting the fate of ZEN. [11]It was suggested that the glucuronide of ZEN was substantially excreted in bile to be re-absorbed and metabolized further by intestinal mucosal cells, ultimately entering the liver and the systemic circulation via the portal blood supply. The reduced form of ZEN, zearalenol, has increased oestrogenic activity. A synthetic commercial formulation called zeranol (Ralgro) has been marketed successfully for use as an anabolic agent for both sheep and cattle. In 1989, zeranol was banned by the European Union, but it is still used in other parts of the world (Hagler et al., 2001)[12].

3.2 Estrogen receptor

Estrogens play key roles in development and maintenance of normal sexual and reproductive functions. In addition, in both men and women they exert a vast range of biological effects in the cardiovascular, musculoskeletal, immune, and central nervous systems. The most potent estrogen produced within the body is 17β-estradiol whose chemical structure is represented in the figure below:

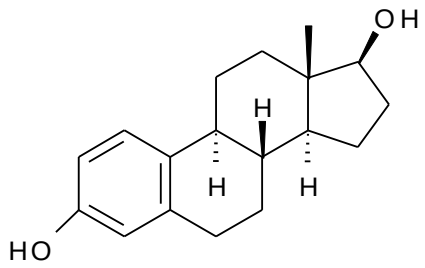


Fig. 30: Structure of 17β-estradiol.

Estrogen receptors (ERs1) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. In the late 1950s, the existence of a receptor molecule that could bind 17-estradiol was demonstrated by Jensen and Jacobsen[13]. The first ER was cloned in 1986.[14] This receptor was regarded as the only ER until a second ER was reported in 1996.[15]

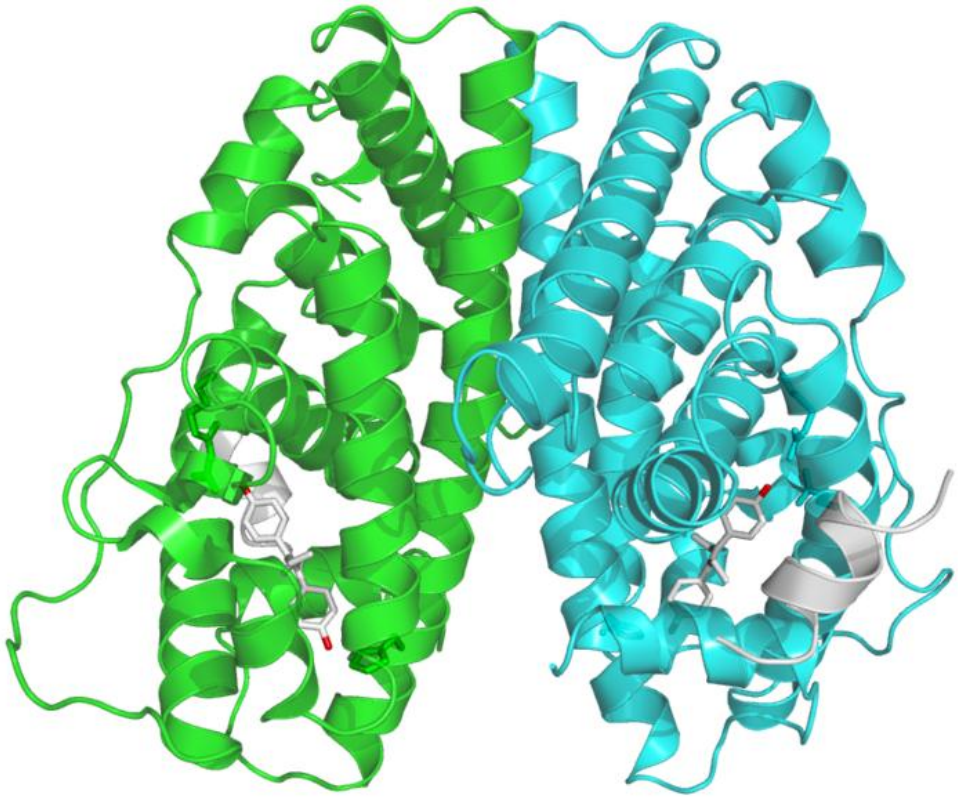


Fig. 31: Estrogen receptor 1 (ER-alpha).

Nowadays two receptors are known, whose names are ERα and ERβ. They show a high degree of similarity when compared at the amino acid level. Amino acids sequence identity between ERα and ERβ is approximately 97% in the DNA-binding domain and approximately 56% in the ligand-binding domain (LBD), whereas the N terminus is poorly homologous at 24%. Transcriptional activation by ERα is mediated by two distinct activation functions: the constitutively active AF-1, located in the N-terminal domain of the receptor protein and the ligand-dependent AF-2, located in the C-terminal domain of the receptor protein. ER seems to have a weaker corresponding AF-1 function and thus depends more on the ligand-dependent AF-2 for its transcriptional activation function.[16] The relative importance of the AF-1 and AF-2 activation functions depends on cellular and promoter context[17].

3.2.1 ER Expression

ERs can be detected in a broad spectrum of tissues. In some organs, both receptor subtypes are expressed at similar levels, whereas in others, one or the other subtype predominates. In addition, both receptor subtypes may be present in the same tissue but in different cell types. ER is mainly expressed in, for example, uterus, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, various regions of the brain, liver, and white adipose tissue. ER is expressed in, for example, colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelium, and certain regions of the brain. Likeness between 17-β-estradiol (natural ligand of extrogen receptor) and ZEN explains the similar activity on the human health, while is nowadays unknown the possible estrogen activity of ZEN animal derivatives (ZEN, α-ZOL, β-ZOL, α-ZAL and β-ZAL) and plant metabolite (ZEN-14-glucoside). The latter compound described is produced by plant as a detoxification mechanism. Z14G has probably a lower toxicity in plant respect to the native form, but its effects on human heath are still unknown.

3.3 Deoxynivalenol

3.3.1 Effect on health

DON induces pathophysiologic effects such as altered neuroendocrine signaling, proinflammatory gene induction, disruption of the growth hormone axis and altered gut integrity[71]. Toxicity of this mycotoxin mainly regards immune system and gastrointestinal tract. More precisely the acute effects of deoxynivalenol provoke diarrhea, vomiting, leukocytosis, hemorrhage, circulatory shock and ultimately death. At low doses, it can also cause chronic effects characterized by anorexia, reduced weight gain, nutrients malabsorpion, neuroendocrine changes and immunologic effects. All animal species evaluated to date are susceptible to DON according to the rank order of pigs > mice > rats > poultry ≈ ruminants. Differences in metabolism, absorption, distribution, and elimination of DON among animal species might account for this differential sensitivity [81].

3.3.2 Metabolism of DON

DON is, however, conjugated to glucuronides within liver and resultant metabolites are found in animal tissue and excreta. While the principal DON metabolite detected in urine and faeces of animals is de-epoxy DON which is produced via intestinal or rumen microbe activity rather than by liver or other organs. This reaction transforms DON to DOM-1 (Deepoxy deoxynivalenol) that is a detoxification form of this molecule, since the epoxide group represents the major responsible in toxicity of DON.[18] The de-epoxy metabolite of deoxynivalenol was identified in the urine and faeces of male Wistar rats given oral doses of deoxynivalenol at 8–11 mg/kg bw. The compounds were quantified by gas–liquid chromatography and identified by GC–MS [19]. In other study blood, urine, bile, and faeces were collected from pigs over 24 h after they were given [^{14}C]deoxynivalenol intragastrically at a dose of 0.6 mg/kg bw or intravenously at a dose of 0.3 mg/ kg bw. GC–MS analysis for deoxynivalenol and metabolites showed little metabolism or conjugation. About 95% of the administered dose was recovered as deoxynivalenol; the amounts recovered as conjugated deoxynivalenol or as other metabolites were not reported quantitatively[20].

3.4 Masked mycotoxins

In the last decade, several studies about *Fusarium* mycotoxins have been designed with the aim of elucidating the formation and the role of their masked forms in food. Masked mycotoxins are phase II metabolites formed by plants upon conjugation of parent mycotoxins to polar groups. Generally, these forms are not considered in risk assessment studies, due to their still unknown biological effects. However the concern about their potential effects on human health is growing, as these conjugates consistently co-occur with their parent compounds in food and feed[3]. Specifically, deoxynivalenol-3-glucoside (D3G), zearalenone-14-glucoside (Z14G) and zearalenone-14-sulphate (Z14S) are the most representative masked mycotoxins occurring in food commodities[21].

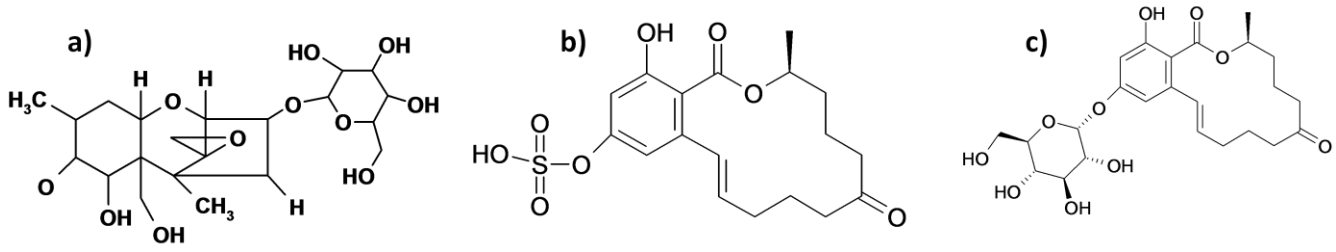


***Fig. 32*: Structures of a) D3G, b) Z14S, c) Z14G.**

These compounds could have direct toxicological properties and/or might be totally or partially hydrolysed to release their parent compounds after ingestion, thus leading to an increased exposure compared to the estimates made with conventional analytical approaches (which do not consider conjugates). Conjugation is known to be a detoxification process which occurs in plant that produces D3Gand Z14G. The first compound showed a high reduction capacity to inhibit protein synthesis in wheat in-vitro test. Z14G also exhibits a reduce estrogenic activity if compared to ZEN. No studies have been performed on bioavailability of masked mycotoxins. Although this assumption is not yet supported by *in vivo* data, a recent study showed that specific intestinal bacteria are partially capable of converting D3G into DON *in vitro*[22]. Study carried out by Berthiller et al. in 2011 showed a capability of some bacteria to hydrolyze D3G to DON. In this work Forty-seven different bacterial strains, isolated from guts, were examined towards their ability to hydrolyze D3G. Results displayd in this work clearly indicate that only few microorganisms have an hydrolyzing activity. *E. casseliflavus*, *E. faecalis*, and *E. gallinarum* liberated minor amounts of DON (1–8% after 8 h) from D3G. However, *E. cloacae, E. durans, E. faecium, E. mundtii* but also *L. plantarum* and *B. adolescentis* efficiently cleaved D3G, releasing up to 62% DON after 8 h. Enterobacter and Enterococcus are genera of bacter present in the intestine and responsible to various infections, while Lactobacillus generaare important as probiotic bacteria. This in vitro study suggests that DON,detoxifiedby the plant into D3G, may become partly bioavailable due to D3Ghydrolysis by bacterial -glucosidases in the colon. It seems impossible to predict to which extent hydrolysis occurs in each individual. Beside an intestinal microbiota, D3G hydrolysis may be also highly dependent on other factors, such as the kind of fermented milk products or abundant probiotic bacteria consumed together with D3G contaminated cereal products. This data suggest that most of the introduced D3G is hydrolyzed to native toxin. The role of the gut microbiota is becoming a key point for the investigation of xenobiotic bioactivity in humans and animals. The vast amount of different bacterial strains and associated enzymes in the human colon makes this gastrointestinal tract a remarkable bioreactor, able to chemically transform most of the

unabsorbed compounds ingested by humans. The overall toxic effect of mycotoxins is a function of their own bioactivity, but also of their bioavailability and their catabolism. As an example, rumen bacteria are often supposed to degradate mycotoxins to less toxic compounds[23]. An increasing number of reports about masked mycotoxins occurrence has been published so far, as recently reviewed by Berthiller et al[72]. In addition, analytical methods mainly based on a mass spectrometry multitoxin approach are now available[72]. Toxicological aspects related to masked mycotoxins are still to be clarified[73,24,25]. Several studies have been reported so far, dealing with in single strain *in vitro* model[73] or trials using Caco-2 cell lines[75]. Only one study has been reported about the *in vivo* metabolic fate in rats[76], while nothing is known about the catabolic fate of these compounds in humans. Mycotoxin conjugates, indeed, may represent a detoxification product, in the case that bound forms cannot be actually released in the digestive system. On the other hand, when partially or totally cleaved under gastrointestinal conditions, masked forms may exert the same toxic effects reported for their parent compound[73]. Finally, their own toxicity should also be considered, although very few is known so far about their potential bioactivity. For all these reasons, a classification of these conjugates under a toxicological perspective is very cumbersome and requires further investigation, leading to the necessity to include the occurrence of masked mycotoxins in risk assessment studies in order to avoid possible underestimation of the total exposure. This report is thus aimed to demonstrate whether the human microbiome is able to modify masked mycotoxins and to release parent compounds in the intestinal tract. For this purpose, the D3G, Z14G and Z14S stability under digestive conditions and microbial colonic fermentation has been investigated.

Aim of the work

In the first part of this chapter the estrogen activity of Z14G and Z14S will be studied and compared with the effects of 17-β-estradiol and zearalenone, in order to better understand the activity of these compounds on human. For this purpose Z14G and Z14S will be synthesized and purified in our laboratory to perform in-vitro tests. In the second part of this chapter will be investigate the fate of masked mycotoxins (D3G, Z14G and Z14S) after ingestion, during digestion and fecal fermentation. This work will be carried out in order to clarify the effect of digestion and intestinal fermentation on masked mycotoxins fate in human. Possibility of totally or partially hydrolysation of this compounds and subsequent release of their native forms after ingestion, would thus an increased human exposure to toxic effect of these mycotoxins.

Materials and Methods

3.5 Chemicals

Mycotoxin standard compounds (zearalenone, deoxynivalenol and deoxynivalenol-3-glucosyde) were from RomerLabs (Tulln, Austria). Zearalenone-14-β-Dglucopyranoside (Z14G) and Zearalenone-14-Sulfate were synthesized in our laboratory as reported below. All solvents (HPLC grade) were from Carlo Erba (Milan, Italy); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA). Salts were from Fluka (Chemika-Biochemika, Basil, Switzerland). 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosylbromide, tetrabutylammonium bromide and sulfuric acid were purchased from Sigma (Stuttgard, Germany). All chemicals for the preparation of the solutions mimicking the digestive juices (urea 98%, D-(+)-glucose 99.5%, D-glucuronic acid, D-(+)-glucosamine hydrochloride 99%, type III mucin from porcine stomach, uric acid, type VIII A R-amylase from barley malt, bovine serum albumin (BSA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, type III lipase from porcine pancreas, and bovine and ovine bile) were purchased from Sigma (Stuttgart, Germany). For fecal fermentation, L-cysteine hydrochloride monohydrate and Fe(II)-sulfate heptahydrate were purchased from AppliChem (Darmstadt, Germany). Bile salts, calcium chloride, (+)-arabinogalactan, tryptone, yeast extract, inulin, buffered peptone water, Dulbecco's phosphate buffer saline, casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, xylan from Birchwood, sodium hydrogen carbonate, potassium hydrogen phosphate, magnesium sulfate monohydrate, guar gum, Tween 80, and resazurin redox indicator were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.6 Zearalenone-14-glucoside: synthesis and characterization

Zearalenone-14-glucoside (Z14G) was synthesized according Zill et al[8], with slight modifications. Zearalenone (25 mg) was dissolved in chloroform and added of acetobromoglucose (2,3,4,6-tetra-O-acetyl-α-D-glucopyranosylbromide, 1g) and tetrabutylammonium bromide (252 mg) as transfer phase catalyst (molar ratio 1:30:10), both dissolved in 50 mM Cs_2CO_3. The mixture was magnetically stirred for 24 h at 30° C. Afterwards, the organic layer was collected; the aqueous layer was washed with chloroform and the organic layers were pooled and evaporated. The residue was redissolved in 0.1N NaOH and stirred for 5 h to allow Z14G deprotection. The reaction was checked by TLC (buthanol-ethanol-water 5:4:1). After neutralization with CH_3COOH, the final product was evaporated to dryness and the residue was dissolved in methanol and stored at -8° C. The reaction yield was calculated at 88%. The final product was characterized by ^{1}H- and ^{13}C-NMR, after dissolving 1 mg of the final product in CH_3OD (1 ml). The NMR spectra were consistent with those already reported by Kamimura et al.[9] and by Berthiller et al.[10], allowing for the univocal identification of the product as zearalenone-14-β-D-glucopyranoside.

3.7 Synthesis of Zearalenone-14-sulfate

In order to obtain Zearalenone-14-sulfate (Z14S) 200 µl of standard solution of ZEN (2000 mg/Kg) were added to an equal volume of H_2SO_4(98 % V/V) and the reaction vial was placed within an ice

bath for 2 hours. Once the reaction ended 1.5 ml of cool water were added and finally product was neutralized with a NaOH 5M solution. Z14S was then purified with preparative TLC and finally characterized with ITMS (Ion Trap Mass Spectrometry). MS^n experiments confirmed the presence of Z14S by using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA).

3.8 In vitro test on estrogen receptor

3.8.1 Yeast strain

For the estrogen assay a strain of *S. cerevisiae* Y190 (MATa, ura3-52, his3-200Δ, ade 2-101 trp 1-901, leu 2-3,112, gal4Δ gal80Δ, URA3:::GAL-lacZ, cyhr2, LYS2:::GAL-HIS3) has been used.

3.8.2 Plasmids

The strain Y190 was transformed with plasmids pGBT9-LBD-hERα and pGAD424-TIF2. The first one is an expression vector, in which was cloned the Ligand Binding Domain (LBD) of the human estrogen receptor hERα in frame with the DNA binding domain of Gal4 (Gal4-DBD). This plasmid also contains the TRP1 gene selective. In the second plasmid pGAD424 was cloned the coding sequence for the coactivator TIF2 in frame with the domain of activation of Gal4 (Gal4-AD). pGAD424 contains the gene LEU2 selective.
In vitro test on estrogen receptor were carried out by Biochemistry Department, University of Parma.

3.9 In-silico test on α-receptor

The model of α-receptor used for binding test is based on crystal structures of α-ER complexed with the endogenous ligand 17-β-estradiol (deposited in 2011, PDB code 2YJA, 1.80A˚resolution). The structure was processed using Sybyl 8.1 software (www.tripos.com) to check atom and bond type assignments. Amino- and carboxy-terminal groups were set as protonated and deprotonated respectively. Hydrogen atoms were computationally added and energy-minimized using the Powell algorithm. The docking of all considered compounds was performed with (the program) GOLD software, version 5.0.1 (CCDC; Cambridge, UK; www.ccd.cam.ac.uk). All crystallographic waters molecules and ligands were removed and 50 poses for each compound were generated. No constraints were set up and the explorable space was defined in a radius of 10 Å from the centroid of the binding pocket (x, y, z coordinates of 17-β-estradiol carbon #9 in PDB structure 2YJA). For each GOLD docking search, a maximum number of 100,000 operations were performed on a population of 100 individuals with a selection pressure of 1.1. Operator weights for crossover, mutation and migration were set to 95, 95, and 10, respectively. The number of islands was set to 5 and the niche to 2. The hydrogen bond distance was set to 2.5 Å and the vdW linear cut-off to 4.0 Å. Ligand flexibility options flip pyramidal N, flip amide bonds, and flip ring corners were

allowed. From the comparison of the crystallographic data and aimed to simulate the ligand binding pocket (LBP) microflexibility, we set as flexible the residues Phe404, Met421, Ile424, Phe425, His524, and Leu525. All poses generated by GOLD were subjected to a rescoring procedure using the scoring function HINT. Because positive and higher HINT scores correlate with more favorable binding free energies, HINT allows us to evaluate the thermodynamic benefit of a certain predicted protein–ligand complex. Tests were performed on ZEN and its metabolites (Z14G and Z14S) compared with 17-β-estradiol.

In-silico test on α-receptor were carried out by Molecular Modelling Laboratory, Department of Food Science, University of Parma.

3.10 In vitro digestion assay

The *in vitro* digestion experiments were performed using a procedure based on the protocol proposed by Versantvoort *et al*.[26] with some modifications. In Table below resumes constituents and their respective concentrations used for the preparation of the synthetic juices.

Table 19: Costituents of juices used for in-vitro gastrointestinal digestion .

	Saliva	**Gastric Juice**	**Duodenal Juice**	**Bile Juice**
Inorganic Solution	5 ml KCl 89.6 g/l 5 ml KSCN 20 g/l 5 ml NaH_2PO_4 88.8 g/l 5 ml Na_2SO_4 57 g/l 850 µl NaCl 175.3 g/l 10 ml $NaHCO_3$ 84.7 g/l	7.85 ml NaCl 175.3 g/l 1.5 ml NaH_2PO_4 88.8 g/l 4.6 ml KCl 89.6 g/l 9 ml $CaCl_2$ 16.65 g/l 5 ml NH_4Cl 30.6 g/l 3.25 ml HCl 37% g/g	20 ml NaCl 175.3 g/l 20 ml $NaHCO_3$ 84.7 g/l 5 ml KH_2PO_4 8 g/l 3.15 ml KCl 89.6 g/l 5 ml $MgCl_2$ 5 g/l 90 µl HCl 37% g/g	15 ml NaCl 175.3 34.15 ml $NaHCO_3$ 84.7 g/l 2.1 ml KCl 89.6 g/l 75 µl HCl 37% g/g
Organic Solution	4 ml urea 25 g/l	5 ml glucose 65 g/l 5 ml glucuronic acid 2 g/l 1.7 ml urea 25 g/l 5 ml glucosenamine hydrochloride 33 g/l	2 ml urea 25 g/l	5 ml urea 25 g/l
Other constituents	290 mg/l α-amilasi 15 mg/l uric acid 25 mg/l mucin	1g/l BSA 2.5 g/l pepsin 3 g/l mucin	9 ml/l $CaCl_2$ 16.65 g/l 1 g/l BSA 9 g/l pancreatin 1.5 g/l lipase	10 ml/l $CaCl_2$ 16.6 g/l 1.8 g/l BSA 30 g/l bile
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

The inorganic and the organic solutions must be prepared separately and augmented to 250 ml with bidistilled water. After mixing the inorganic and organic solutions, enzymes and other constituents are added to a selected volume and dissolved by heating to 37°C under stirring. If necessary, pH of each juice is adjusted to the appropriate interval using HCl 1M or NaOH 1M. Before each experiment, all digestive juices were heated at 37 ± 2°C. The digestion started by adding 3 ml saliva to 2 g of ground sample, followed by an incubation step of 5 min. Then, 6 ml gastric juice were added and the mixture was incubated for 2 hours. Finally, 6 ml duodenal juice, 3 ml bile and 1 ml bicarbonate solution (1 M) were added simultaneously to the mixture and a final incubation step of 2 hours was performed. During the *in vitro* digestion, the mixture was stirred by a magnetic stirrer (250 rpm) to obtain a gentle but systematic mixing of the matrix with the digestive juices. The pH of the chyme varied in the range 6.5 - 7. At the end of the experiment the digestion tubes were centrifuged for 15 min at 3500 rpm (Alc Centrifuge pk110, DJB Labcare Ltd, Newport Pagnell, Buckinghamshire, UK), yielding the chyme (the supernatant) and the digested matrix (the pellet).At the end of the experiment, the volume was adjusted to 2 ml adding 100 µl of bidistilled water before LC-MS/MS analysis.Each experimental set (D3G, Z14G, Z14S) was run in triplicate; a control sample was prepared for each set: the target compound was added of the inorganic and organic media without enzymes and let react as described above. A blank sample was also prepared by mixing all the digestive juices (inorganic, organic media and enzymes) in the absence of the target compound.

3.11 In vitro fecal fermentation assay

The fecal fermentation was performed according to Dall'Asta et al.[30], with slight modifications. The composition for 1 L of growth medium was 5 g of soluble starch, 5 g of peptone, 5 g of tryptone, 4.5 g of yeast extract, 4.5 g of NaCl, 4.5 g of KCl, 2 g of pectin, 4 g of mucin, 3 g of casein, 2 g of arabinogalactan, 1.5 g of $NaHCO_3$, 0.69 g of $MgSO_4xH_2O$, 1 g of guar, 0.8 g of L-cysteine $HClxH_2O$, 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 0.4 g of bile salt, 0.08 g of $CaCl_2$, 0.005 g of $FeSO_4x7H_2O$, 1 mL of Tween 80, and 4 mL of resazurin solution (0.025%, w/v) as an anaerobic indicator. The growth medium was sterilized at 121°C for 15 min in glass vessels (50 mL) before sample preparation. For the fecal slurry preparation, fresh fecal samples were collected from three healthy donors who did not have previous intestinal disease, were not treated with antibiotics for the previous 3 months before fecal collection. Samples were immediately stored in an anaerobic jar and then diluted with Dulbecco's phosphate buffer saline at 10% (v/v) and homogenized to obtain a 20% (w/w) slurry to be used as the fermentation starter. The final fermentation volume was 4 mL: 50% growth medium and 50% fecal slurry. A proper toxin amount was added in order to get a final concentration of 0.5 mg/L. The fermentation starter was introduced in the vessel containing sterilized growth medium, sealed with a rubber seal, and flushed through a double needle with nitrogen to create an anaerobic condition. Samples were then introduced to the vessel through the needle and incubated for 24 h at 37°C at 200 strokes/min in a Dubnoff bath (ISCO, Milan, Italy). After 30 min and 24 h of incubation, the fermentation samples were centrifuged, and 0.5 mL was filtered through a 0.2-mm nylon filter and stored at -80°C. All samples were fermented independently and all experiments were carried out in triplicate. Control samples were prepared by adding 10% acetonitrile to the fecal slurry before toxin addition, in order to stop any enzymatic activity, and let react for t = 0 min, t = 30 min and t = 24 min. Negative control samples were prepared by adding a proper amount of target toxin to the growth media without the addition of fecal slurry, and let react for 30 min and 24 h. A blank sample was also prepared by mixing growth media and fecal slurry in the right proportion without the addition of any toxins.

3.12 UPLC-ESI-MS analysis

The UPLC/MS analyses were carried out with an Acquity UPLC separation system (Waters Co., Milford, MA, USA) equipped with an Acquity Single Quadrupole MS detector with an electrospray source. Chromatographic conditions were the following: column, Acquity UPLC BEH C18 (1.7 µm, 2.1 x 50 mm); flow rate, 0.35 mL/min; column temperature, 30°C; injection volume, 5 µL; gradient elution was performed using 0.1 mM sodium acetate solution in water (eluent A) and methanol (eluent B), both acidified with 0.2% formic acid. Initial conditions were set at 2% B for 1 minute, then eluent B was increased to 20% in 1 minute. After an isocratic step (6 minutes), eluent B was increased to 90% in 9 minutes. After a 3 minutes isocratic step (90% B), the system was reequilibrated to initial conditions for 2 min. The total analysis time was 22 minutes. The ESI source was operated in positive ionization mode, with the exception of Z14S which was monitored under ESI- conditions. MS parameters were as follows: capillary voltage, 2.50 kV; cone, 30 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas, 50 Lh^{-1}; desolvation gas, 850 Lh^{-1}. A good linearity was obtained for all the considered mycotoxins (r^2 > 0.99). For all the target compounds, limit of quantification (LOQ) and limit of detection (LOD) were lower than 30 µg/L and than 10 µg/L, respectively. Recovery experiments were performed by spiking a blank chime or a blank fecal slurry at the target concentration level (0.5 mg/L). Samples were analysed using both single ion monitoring (SIR) and scan mode (m/z 100 – 600), as reported in table below.

***Table 20**: Monitored ions and ionisation parameters applied in UPLC-ESI-MS analysis.*

	Ionisation mode	Monitored ions (m/z)	Capillary Voltage (kV)	Cone Voltage (V)
D3G	ESI+	481.3, 459.3	2.5	30
DON	ESI+	319.3, 297.3	2.5	30
Z14G	ESI+	503.4, 341.4, 319.4	2.5	30
Z14S	ESI-	397.4, 317.4	2.5	30
ZEN	ESI+	341.4, 319.4	2.5	30

Matrix-matched calibration experiments were performed in the range 0.05 – 1.00 mg/L for the target analytes. Recoveries, LOD and LOQ were calculated for each mycotoxin in both chyme and fecal slurry, as reported in table below.

***Table 21**: Quality parameters of the UPLC/MS method.*

		D3G	DON	Z14G	Z14S	ZEN
Chyme	Recovery (%)	89%	95%	92%	88%	96%
	LOQ[#] (µg/L)	80	50	80	100	80
	LOD[§] (µg/L)	30	10	25	50	25
Fecal slurry	Recovery (%)	87%	93%	91%	85%	93%
	LOQ[#] (µg/L)	150	150	150	200	150
	LOD[§] (µg/L)	50	50	50	100	50

[§]LOD was calculated as signal to noise ratio> 3; [#]LOQ was calculated as signal to noise ratio > 10

3.13 Compound confirmation by ITMS analysis

Target compound confirmation was performed by MS^n experiment using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Experiments were performed in both positive and negative ionisation modes. Analyses were carried out using full scan, data-dependent MS^2 and MS^3 in the conditions reported in table below.

***Table 22**: Linear Ion Trap (ITMS) parameters applied for analysis.*

	Ionisation mode	**MS (m/z)**	**CID**	**MS^2**	**CID**	**Full Scan (m/z)**
D3G	ESI-	503 $[M+HCOO]^-$	11.5	457 $[M-H]^-$	15.0	125 – 600
DON	ESI-	341 $[M+HCOO]^-$	12.0	295 $[M-H]^-$	12.0	80 – 600
Z14G	ESI-	524 $[M+HCOO]^-$	22.0	-	-	75 – 600
Z14S	ESI-	397 $[M-H]^-$	22.0	-	-	90 – 600
ZEN	ESI-	317 $[M-H]^-$	22.0	-	-	85 – 350

Collected spectra were compared to those obtained for reference standards as well as with those reported in the literature.

Results and discussion

3.14 Synthesis of zearalenone derivates

3.14.1 *Z14*-glucoside

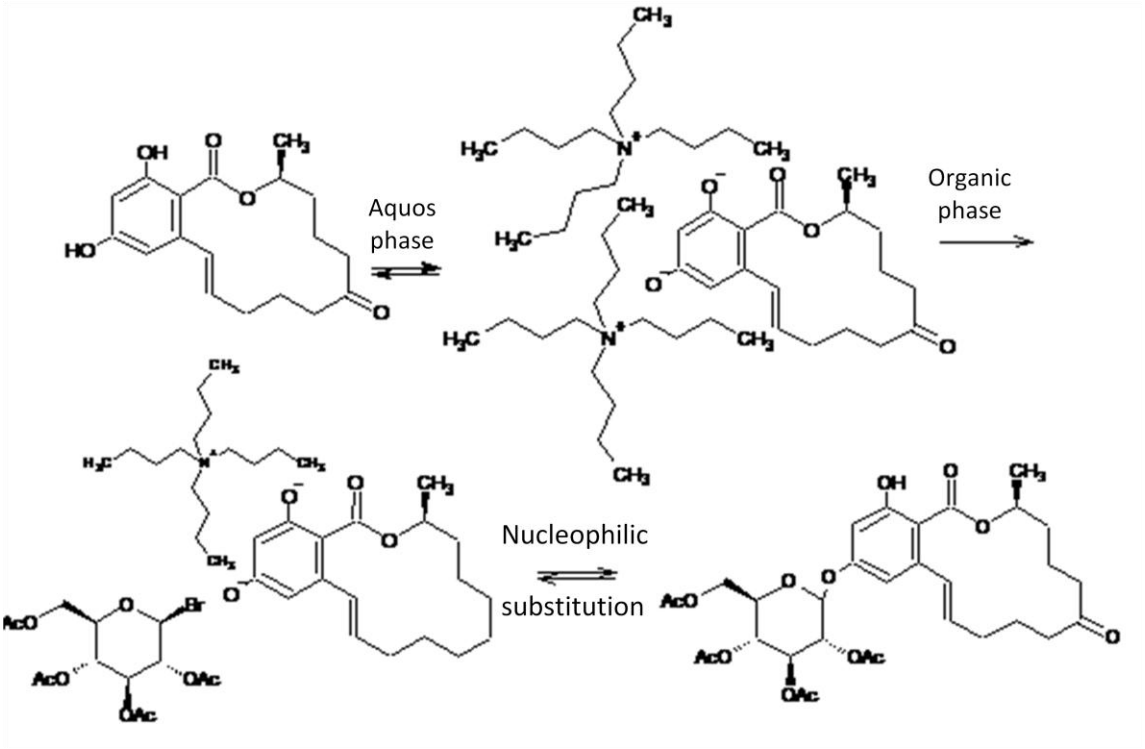
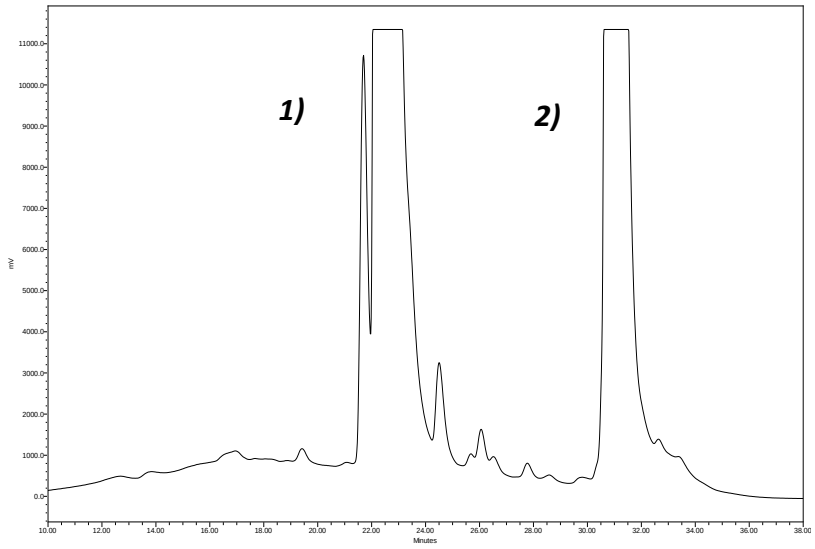


***Fig. 33*: Synthesis of ZEN-14-Glucoside.**

This first step in Z14-glucoside synthesis is carried out through the use of a phase transfer catalyst. This molecule facilitates the migration of the reactants in a heterogeneous system from one phase to another, in which the reaction can take place. In this phase the PTC (phase transfer catalyst), identified as tetrabutylammonium bromide, binds the ionic species by creating a system PTC-ion, with a hydrophilic center which contains the ion and a hydrophobic outer part, which remains in contact with the aqueous solution. Since the outer part does not have a high affinity for the aqueous environment, the ion pair formed is transferred into the organic phase, where the reaction occurs. Most of the product remains within the organic phase and the aqueous phase is washed two times with chloroform in order to transfer all the product in the organic phase[27].

Reactions were monitored with HPLC coupled with fluorescence detector and results obtained are showed in the figure below:



v

***Fig. 34*: HPLC-FLD chromatogram: 1) ZEN (t = 22 min) 2) reaction product (ZEN-14-G guarded, t = 32 min).**

Once the presence of the product was verified, it was purified through direct phase TLC, resulting as the more rapid, economic and suitable technique for the purification of small quantities of product. In the following figure results of purification step are showed: only one compound is present.

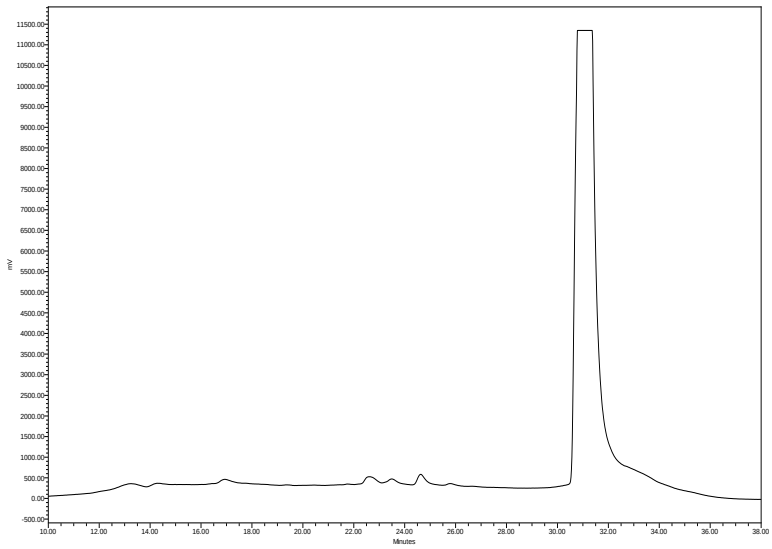


***Fig. 35*: HPLC-FLD chromatogram of purified product (ZEN-14-glucoside, t = 32 min).**

After purification, the product was deprotected to remove acetyl groups linked to glucose molecule.

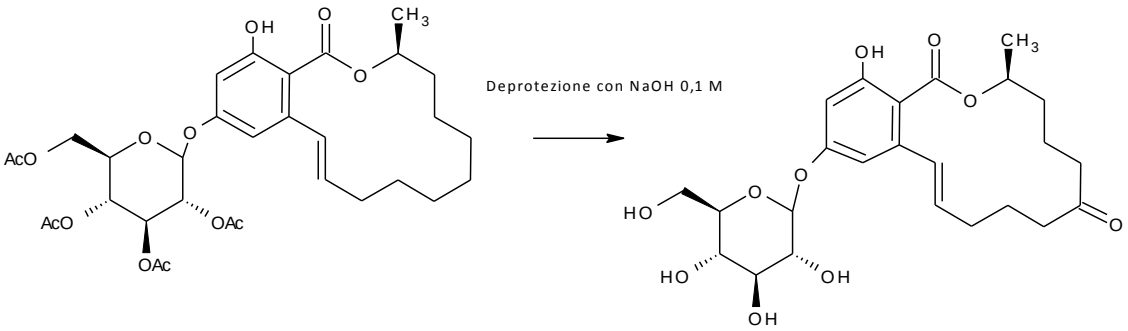


***Fig. 36*: Scheme of deprotection step.**

After this last step of the reaction protocol, the identity of the final product was confirmed by HPLC/LTQ analysis. In the figure below chromatogram of purified ZEN-14-G and the corresponding Ms spectra were showed.

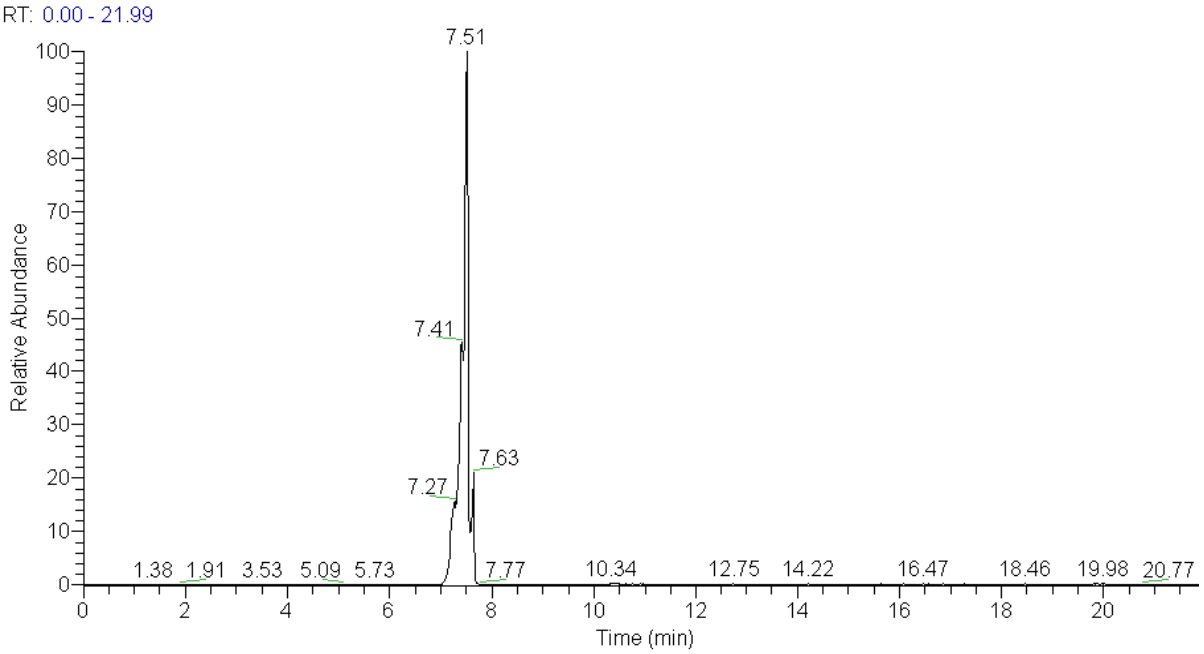


***Fig. 37*: Chromatogram of Z14G purified.**

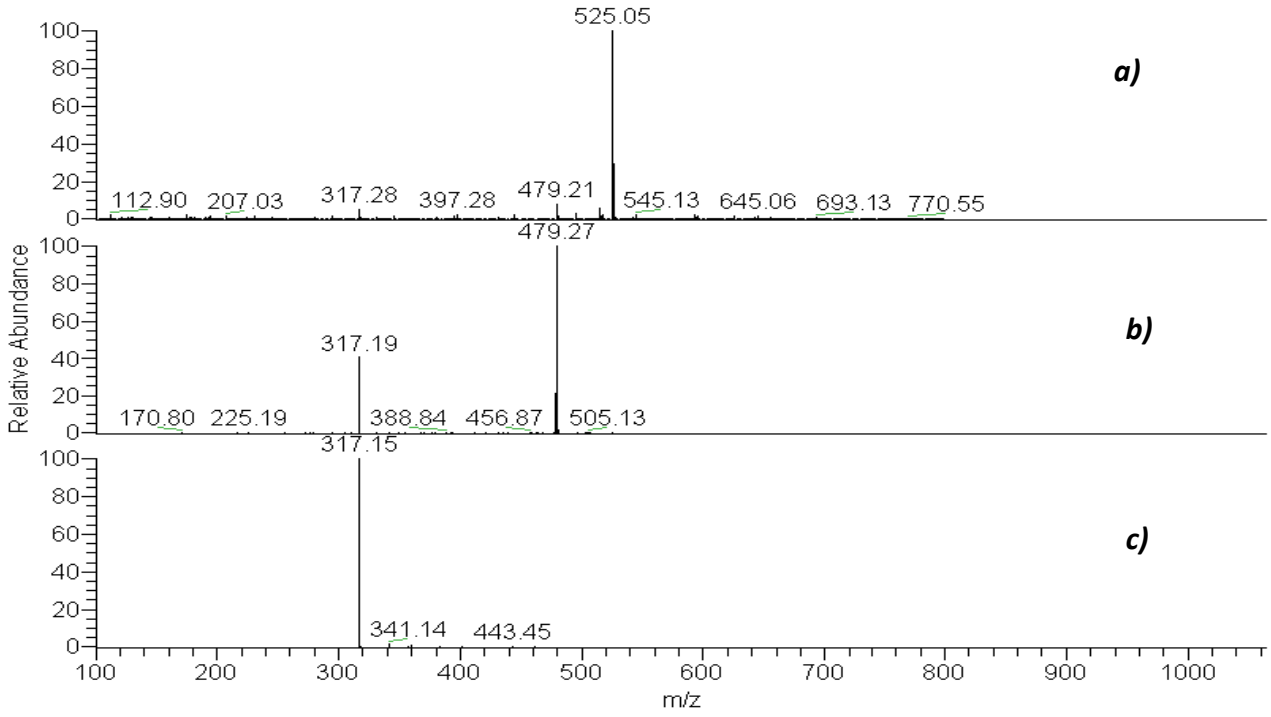


***Fig. 38*: Ms spectra of ZEN-14-glucoside a) $[M+HCOO]^-$; b) $[M-H]^-$ c) ZEN $[M-H]^-$.**

Analyzing the reaction product, a single peak with a retention time of 7.51 minutes was obtained and its corresponding Ms spectra gave specific fragments that confirmed the Z14G structure. In Fig. 6a full scan of ZEN-14-glucoside showed a fragment equal to 525 m/z, corresponding to acetate abduct of Z14G with negative charge ($[M^+HCOO]^-$); while Ms_2 fragmentation of the parent ion (Fig. 6b and c) gave two ions (479 and 317 m/z), corresponding to Z14G $[M-H]^-$ and ZEN $[M-H]^-$, thus confirming the identification of the compound.

3.14.2 Synthesis of ZEN-14-sulfate

Concentrated sulfuric acid was used for the synthesis of this second ZEN derivative. When the starting compounds reacts, the attack of acid occurs on phenolic group, that is the functional group of interest for the formation ZEN-14-sulfate. This reaction takes place in a simple and fast way and, subsequently, the product obtained was analyzed by HPLC-FLD. Result are showed in figure 7.

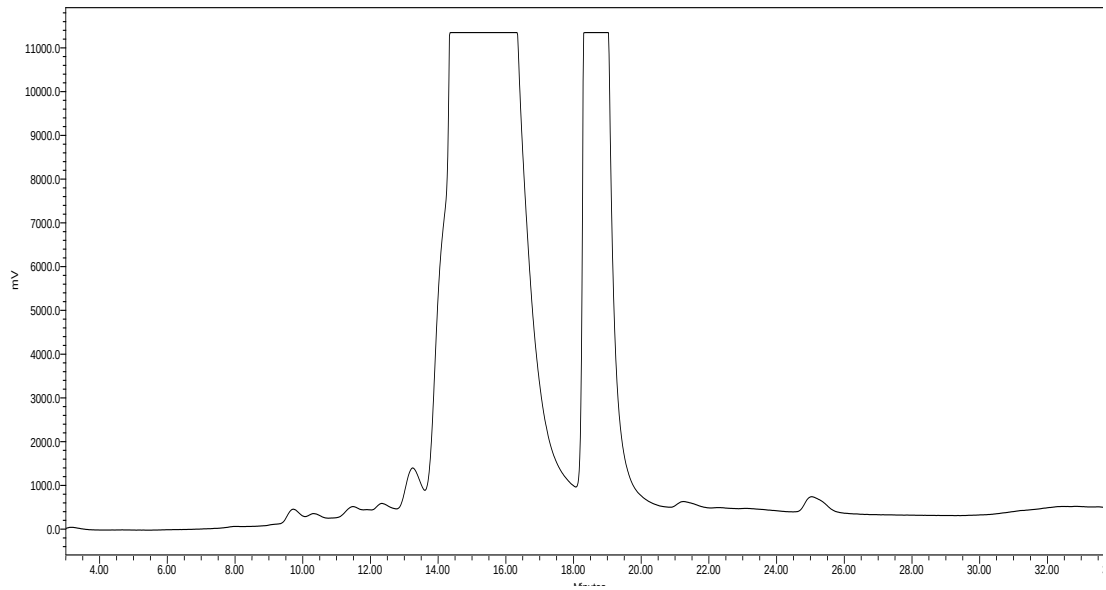


***Fig. 39*: HPLC-FLD chromatogram of reaction product.**

In this chromatogram two main peaks were considered; the first (retention time = 16 minutes) corresponds to ZEN-14-S and the second, with retention time equal to 19 minutes was identified as unreacted zearalenone. For the purification of the product, the same preparative TLC method, previously described for Z14G, was applied. Identification of purified ZEN-14-S was then confirmed by HPLC-LTQ. The characterization by LTQ analysis provided a fragmentation pattern of the molecule that exactly corresponds to ZEN-14-S. In figure 8, the chromatogram of purified Z14S and its corresponding Ms spectra were shown.

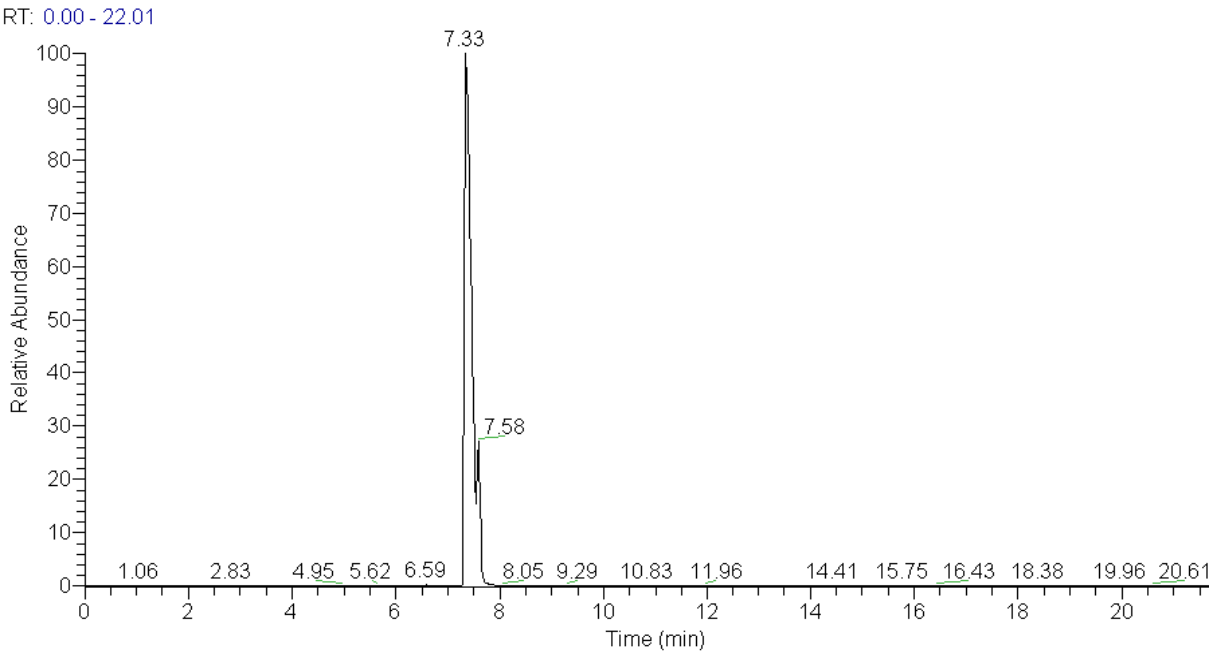


***Fig. 40*: Chromatogram of product.**

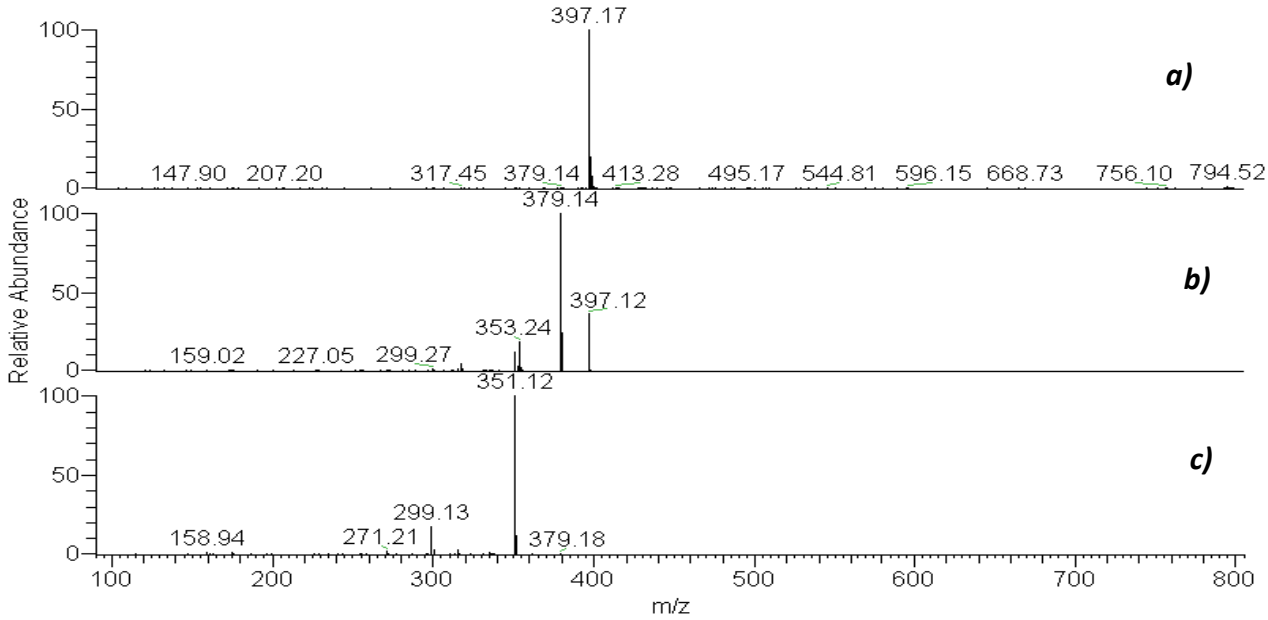


***Fig. 41*: Fig. Ms spectra of ZEN-14-sulfate a) Ms fragmentation: m/z = 397 corrisponding to ZEN-14-S [M-H]⁻; b) Ms^2 fragmentation m/z = 379 [M-H_2O]⁻ ; c) Ms^3 fragmentation m/z = 351.**

Ms analysis confirmed the identification of ZEN-14-S, since the fragmentation pattern showed a fragment equal to 397 m/z as the more abundant and corresponding to Z14S $[m-H]^-$ ion; moreover the Ms^2 fragmentation, giving a residual fragment with m/z = 397 and a major fragment with m/z = 379, corresponding to water molecule loss and Ms^3 giving a m/z = 351 fragment.

3.15 Estrogenicity test

Estrogenity in-vitro tests were carried out on both estrogen receptors (α and β) with ZEN, ZEN-14-glucoside and ZEN-14-Sulfate. Results obtained were compared with estrogen activity of 17- β-estradiol (the natural ligand of both receptors). Tests made on a strain of *S. cerevisiae* Y190 that was transformed with plasmids pGBT9-LBD-hERα and pGAD424-TIF2. The first one is an expression vector, in which was cloned the Ligand Binding Domain (LBD) of the human estrogen receptor hERα in frame with the DNA binding domain of Gal4 (Gal4-DBD). This plasmid also contains the TRP1 gene selective. In the second plasmid pGAD424 was cloned the coding sequence for the coactivator TIF2 in frame with the domain of activation of Gal4 (Gal4-AD). pGAD424 contains the gene LEU2 selective.

In vitro test on estrogen receptor were carried out by Biochemistry Department, University of Parma.

3.15.1 In-vitro test on α-receptor

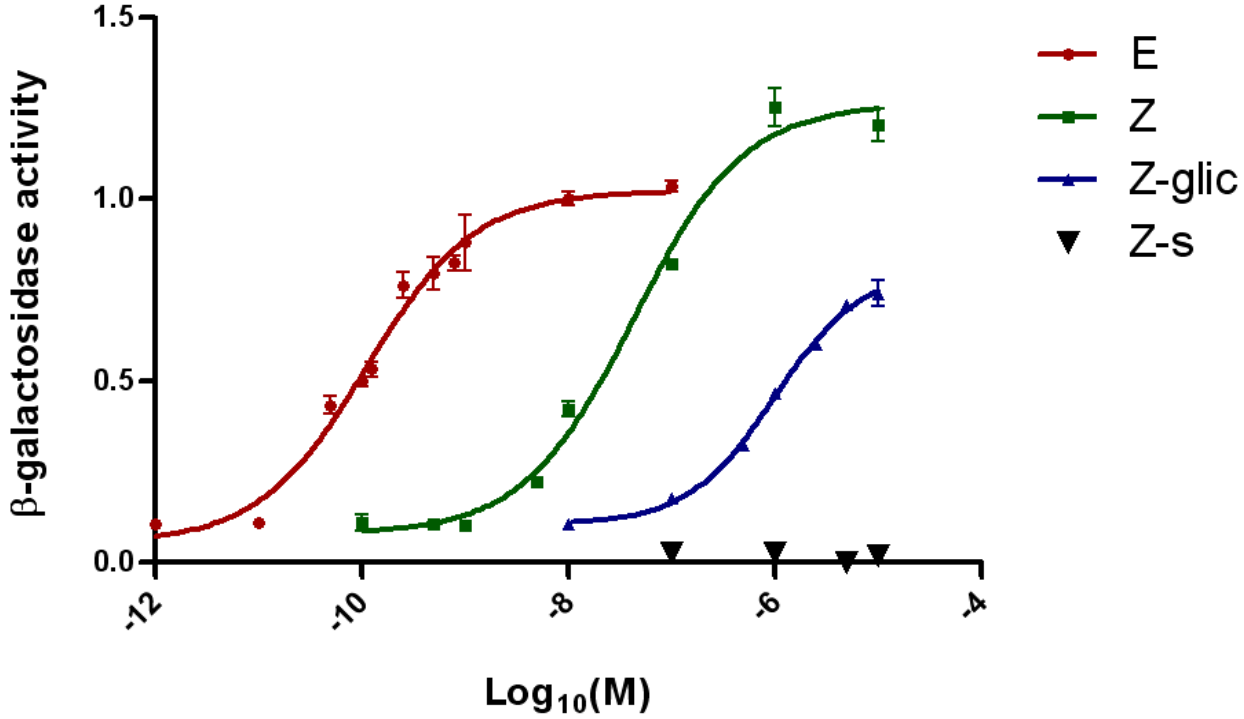


***Fig. 42*: Results of in-vitro test on α-receptor.**

In graph above results of in-vitro estrogenic tests on the agonist activity of ZEN and derivates are shown. Agonist ligands stabilize a receptor conformation that is optimal for efficient interaction with coactivators and thereby facilitates transcriptional activation. In contrast, receptor antagonists interfere with positioning of Helix 12 through a variety of mechanisms resulting in ER conformations in which the coactivator recruitment site is incomplete.[28] Red line represents the activity of 17-β-estradiol, which is the highest, since this molecule is the natural ligand of estrogen receptor. At the second position, with the EC 50 less than 2 times in logarithmic scale, there is ZEN. The term half maximal effective concentration (EC50) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure times. It is commonly used as a measure of drug potency and toxicity. The EC50 of a graded dose response curve therefore represents the concentration of a compound where 50% of its maximal effect is observed. The EC50 of a graded dose response curve represents the concentration of a compound where 50% of the population exhibit a response. This result was expected because ZEN is a well known estrogenic mycotoxin. After these molecules ZEN-14-glucoside showed the lower estrogen activity while ZEN-14-sulfate resulted devoid of such activity. This data do not agree with those of literature, that reported the absence of estrogenic activity of ZEN-14-glucoside.

Moreover this experiment showed the absence of antagonist activity of tested compounds. In table 23 the EC50 values, relatively to each considered compound, are reported.

***Table 23**: EC50 values on α -receptor for estradiol, ZEN and its derivates.*

	EC50
Estradiol	$1.11E^{-10}$
ZEN	$4.27E^{-08}$
Z14G	$9.97E^{-07}$
Z14S	n.d.

A weak EC50 value of a compound means a high activity of it on the receptor. In fact, just a reduced dose of estradiol is able to give 50% of activity, while a higher ZEN dose is necessary to obtain the same effect.

3.15.2 In-vitro test on β-receptor

The graph below shows results obtained from in-vitro test on β-receptor. Which only regarded the agonist activity of compounds, while the antagonist one of ZEN is medium/slight and absent for the other derivates. The agonist activity point out a trend similar to the other receptors. Zearalenone shows a higher activity than on α-receptor and as already evidenced in previous in-vitro test, ZEN-14-glucoside seems to have an estrogenic activity.

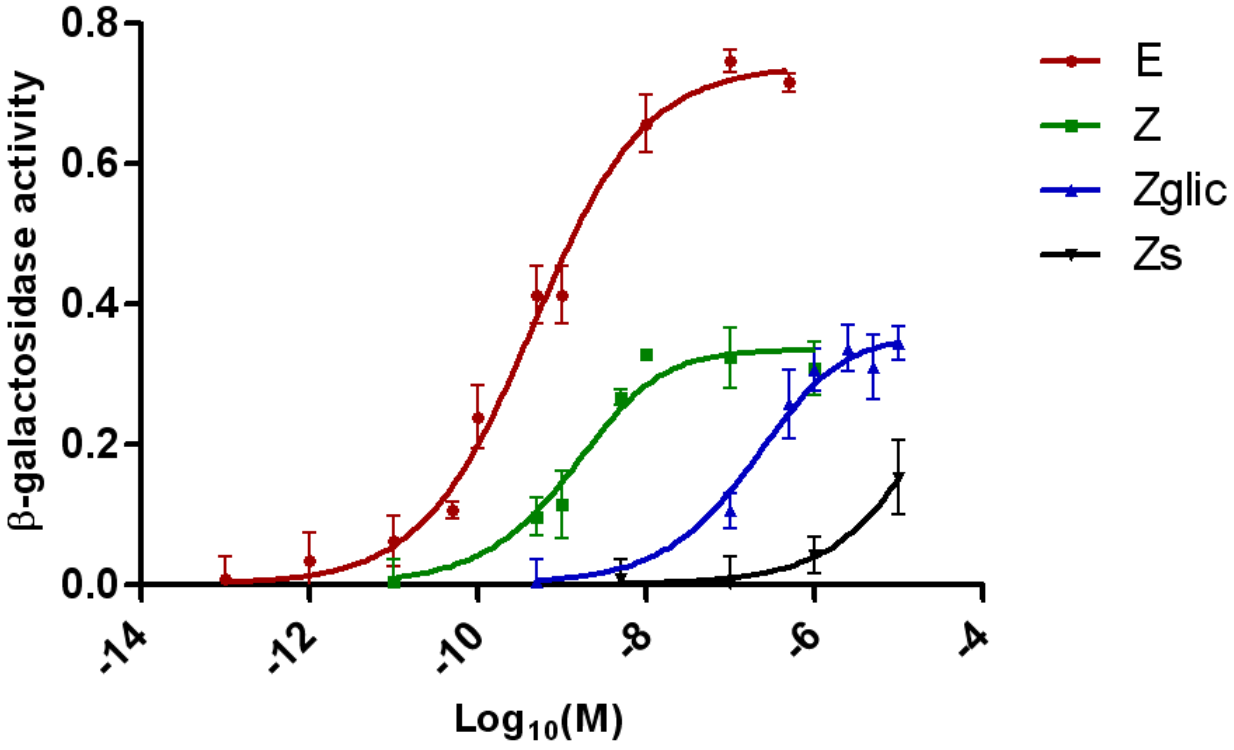


***Fig. 43**: Results of in-vitro test on β-receptor.*

In regard with experiments on β-receptor the table below shows the EC50 values about estradiol, ZEN and its derivates.

***Table 24** : EC50 values on β -receptor*

	EC50
Estradiol	4.76E-10
ZEN	1.28E-09
Z14G	1.94E-07
Z14S	n.d.

Data obtained by in-vitro estrogen tests on α and β-receptors differ from literature data. These difference could be due to a partial glycosidase activity that occurs within yeast cells and which is able to break the glucosidic bond and consequently release the native form of mycotoxin (ZEN), having an high estrogen activity.

3.15.3 In-silico test on α-receptor

In order to better understand the possibility of estrogenic activity of ZEN-14-glucoside, in-silico tests were carried out. These were performed through the use of computational studies on 3D structures of estrogen receptors and ligand. Actually this approach is widely applied in chemistry and medicinal chemistry to discover new lead compounds, to decipher mechanism of binding between protein and ligands, to predict interactions between molecules, to study structure activity relationship, to analyze biomolecule flexibility or to predict the role of water molecules in biological interactions, but it is not commonly used in food safety field yet. There are many advantages linked to this particular approach. For example it is faster and cheaper than the in-vitro tests and allows to easily explain all the possible interactions between receptor and ligand. Results obtained through these experiments are shown in figure below.

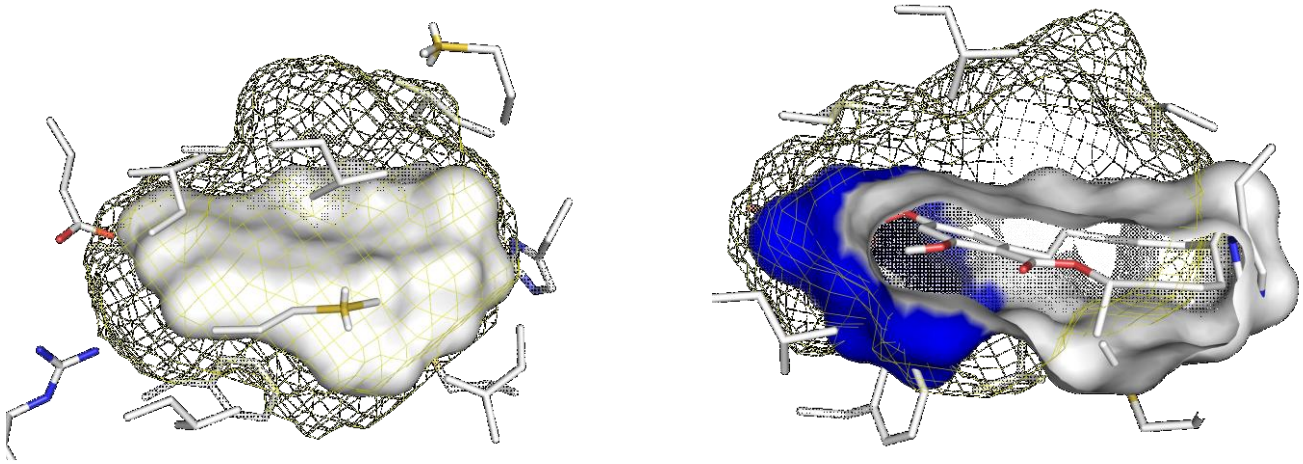


***Fig. 44*: Image of in silico test on zen and zen-14-glucoside in estrogen receptor.**

As it is possible to see in the figure zearalenone perfectly fits in the cavity of estrogen receptor (yellow grid). The program elaboration shows that polar and non-polar zones of the receptor exactly interact with those of ZEN, able to give/accept hydrogen bonds or having hydrophobic regions. Since ZEN-14-glucoside has not the same conformation, this interaction is prevented. Gluscoside moiety (blue portion) linked on zen modified the molecule conformation and does not allow compound fitting in the receptor cavity. In-silico experiments confirm the absence of estrogenic activity of ZEN-14-glucoside. This fact because if compound does not perfectly fit in the cavity of receptors, it is not able to have an estrogenic activity.

Results obtained confirm data from literature which demonstrate the absence of estrogen activity of masked form of ZEN. Another relevant aspect that will be considered is the study of masked mycotoxins resistance after oral ingestion and during the overall digestion process. In fact if these substances remain stable during digestion and fecal fermentation, it would not affect human exposure to mycotoxins. On the contrary, if aglicones are released because of the masked form degradation by intestinal microbiota, human exposure would increase, representing a potential risk for human health

3.16 Fate of masked mycotoxins

A first set of experiments was set up to simulate the gastrointestinal digestive process, according to a comprehensive protocol already in use in our laboratory13,14. In brief, the salivary step (5 min), the gastric step (120 min) and the duodenal step (120 min) were reproduced in vitro by sequential enzymatic treatments, each associated to adequate buffering and ionic strength. The digested samples were then collected and analysed by LC-ESI-MS/MS. Control experiments proved the stability of the precursor mycotoxins, DON and ZEN, during the entire digestive simulation. However, the parent mycotoxins aglycones were undetectable under the applied conditions and the conjugates D3G, Z14G and Z14S were fully recovered after digestion (99.5%, 97.3% and 98.6%, respectively), excluding any other possible degradation.

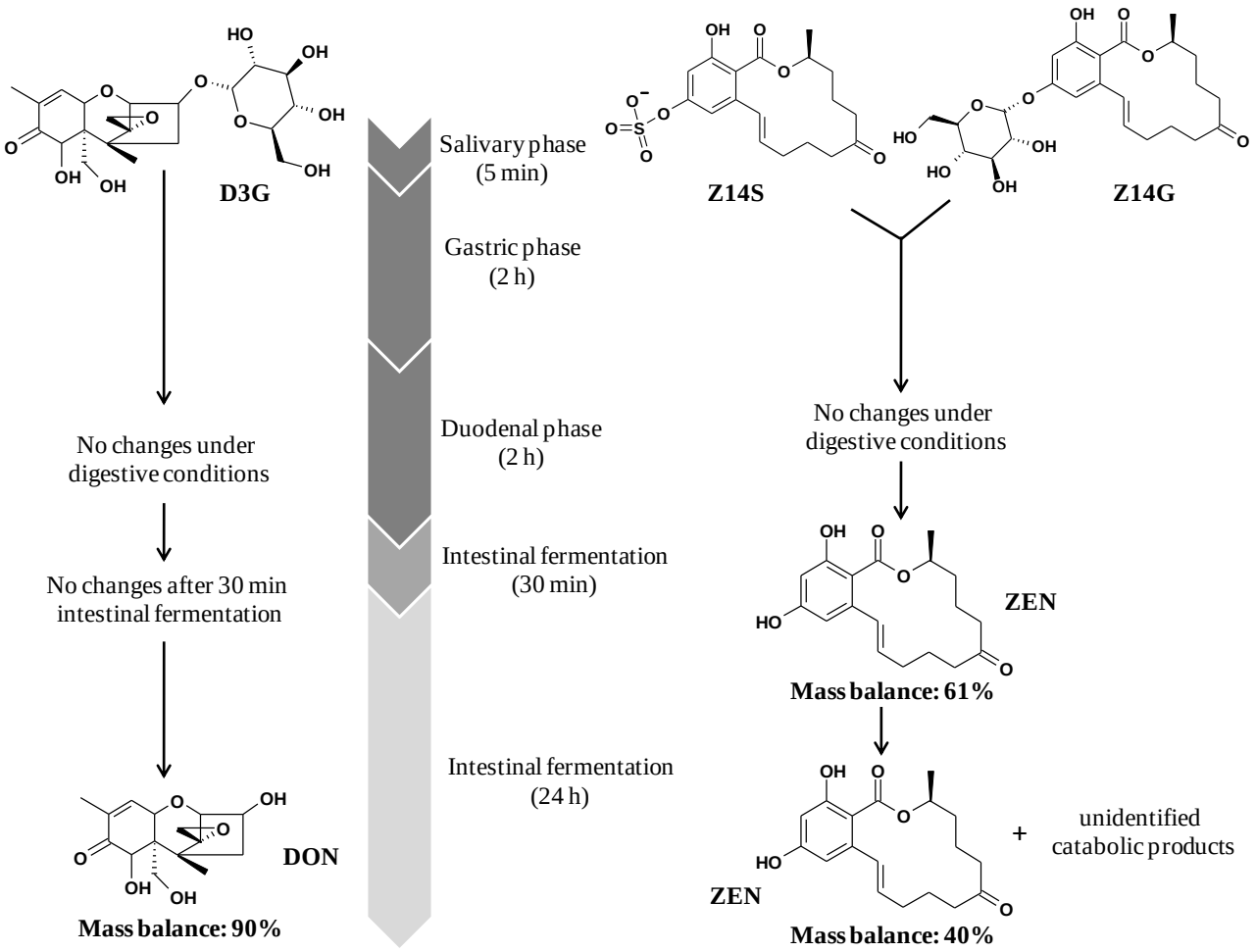


***Fig. 45*: Scheme of D3G and Z14G degradation under human gastrointestinal conditions.**

Results are in agreement with data recently reported for D3G[22,24] and fumonisin conjugation products[29]. These results confirm again that enzymes and low pH present during digestion is not able to hydrolyze masked mycotoxins to their native forms that leaving intact duodedenal tract. Has been shown that these compounds reach intact intestine and the next step regard the effect of human fecal fermentation effect of masked mycostoxins. The effect of human microbiota fermentation on D3G, Z14G and Z14S was then evaluated *in vitro,* according to the protocol developed by Dall'Asta et al [30]. Samples were collected after 30 min and 24h of fermentation and compared with control samples (t = 0 min). Negative control samples were also obtained by fermentation of D3G, Z14G and Z14S in the growth media in order to check possible chemical reactions co-occurring during the incubation period. In all the considered experiments, masked mycotoxins resulted stable after 24 hours of incubation in the absence of microbiota. Moreover, masked mycotoxins were fully recovered after 30 min and 24 h in control samples prepared by adding 10% acetonitrile to the fecal slurry before the mycotoxin addition to avoid any enzymatic activity. The analysis was performed by LC-ESI-MS/MS using a full scan mode to check the possible presence of unknown metabolites and MS^n analysis was performed for target compound confirmation. Results are reported in figure below.

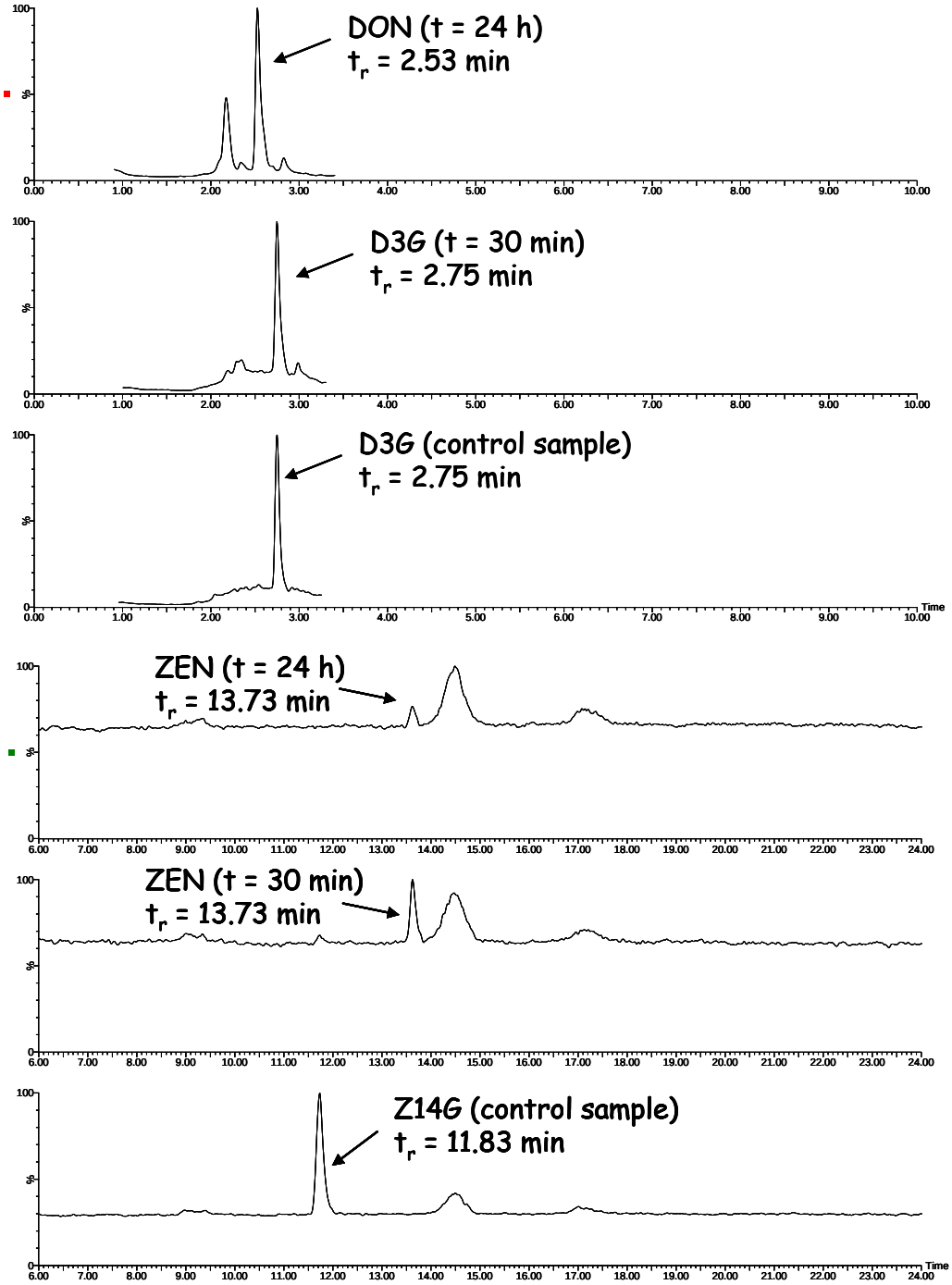


***Fig. 46:* Chromatograms obtained after fecal fermentation of D3G (upper) and Z14G (lower).**

To minimize the inter-individual variability, each set of experiments was repeated 3 times over a 6 month period, using fecal starters derived from 3 different healthy volunteers each time. The ZEN derivatives Z14G and Z14S were completely cleaved at t = 30 min; however, ZEN was only partially recovered in the fecal slurry. The ZEN degradation rate at 30 min was 39%, while after 24h only 40% of ZEN was present. The ZEN confirmation in fecal slurry was obtained by ITMS analysis, as reported in figure below.

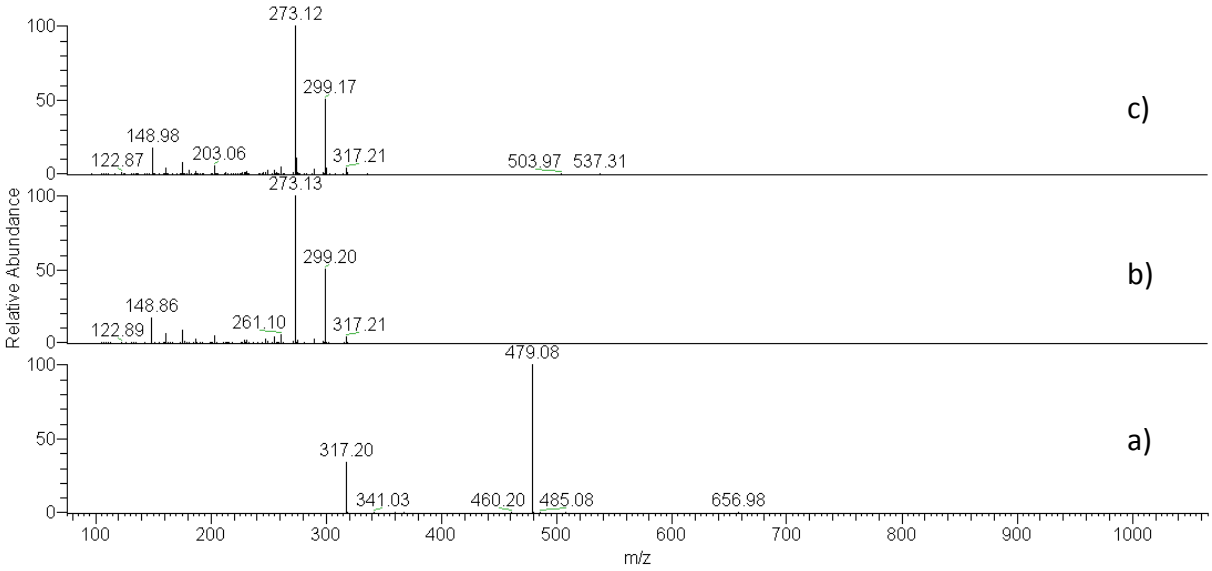


***Fig. 47*: Mass fragmentation spectra (MS2, ESI-): Z14G standard (a), ZEN standard (b) and ZEN after fecal fermentation obtained from Z14G fermentation at t = 30 min (c).**

Data dependent MS^2 spectra (ESI-) obtained for standard solutions of Z14G and ZEN were compared to that obtained for the major compound found in the fecal slurry (t = 30 min), which was univocally identified as ZEN. The same experiment was performed also for Z14S, as reported in figure below.

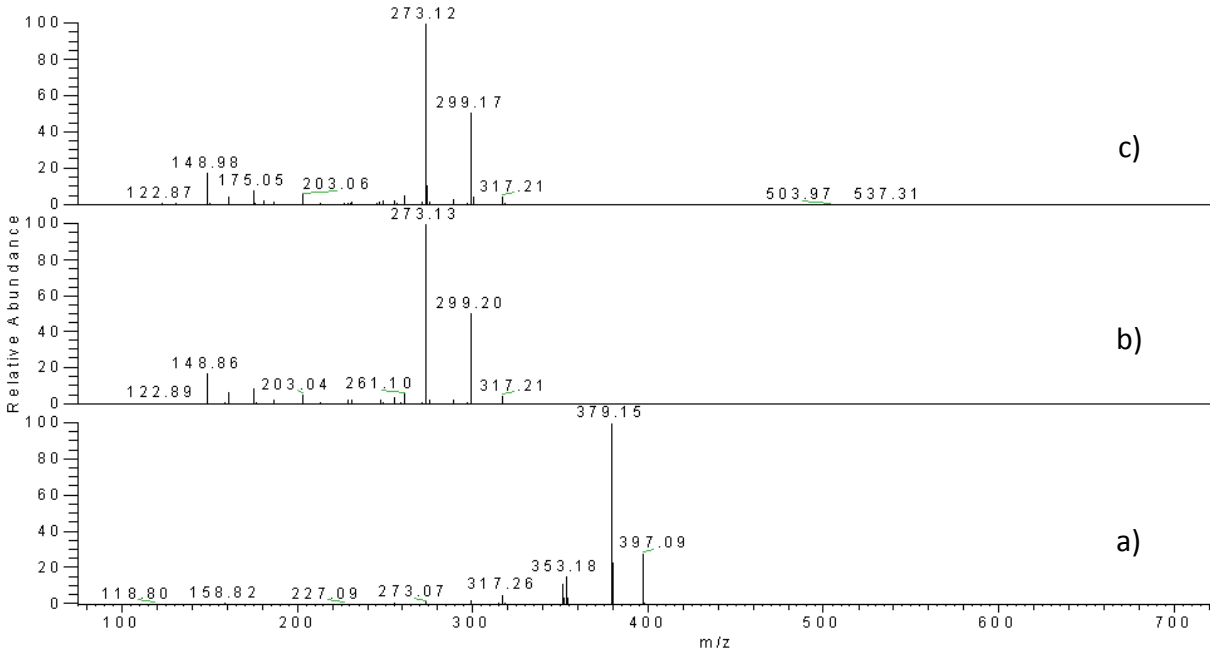


***Fig. 48*: Mass fragmentation spectra (MS2, ESI-): Z14S standard (a), ZEN standard (b) and ZEN after fecal fermentation obtained from Z14S fermentation at t = 30 min (c).**

Also in this case, ZEN was univocally identified in the fecal slurry after 30 min.

The identification and structural elucidation of at least 10 major catabolites is currently ongoing.

As far as D3G is concerned, after 30 min the deglucosilation was not significant, while the complete degradation of D3G was obtained at t = 24h. In this case, the main product found in the fecal slurry was DON (90%). The DON and D3G confirmation in fecal slurry was obtained by ITMS analysis, as reported in figure below.

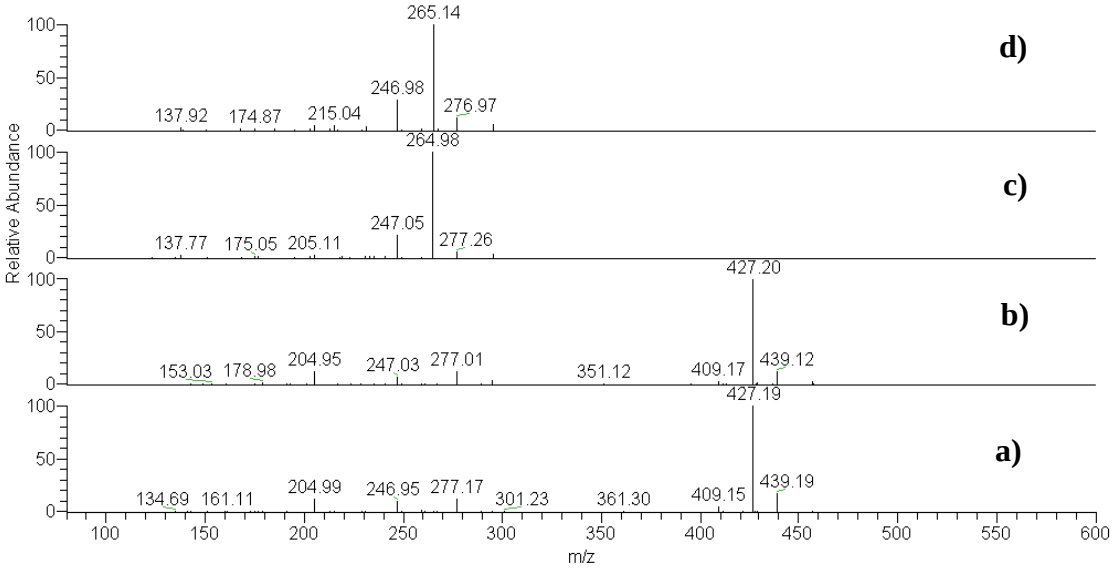


***Fig. 49*: Mass fragmentation spectra obtained for D3G standard (a), D3G after fecal fermentation at t = 30 min (b), DON standard (c) and DON after fecal fermentation obtained from D3G fermentation at t = 24 h (d).**

Data dependent MS^3 spectra (ESI-) obtained for standard solutions of D3G and DON were compared to that obtained for the major compound found in the fecal slurry at t = 30 min and at t = 24 h, which were univocally identified as D3G and DON, respectively. Only traces of the de-epoxydation product DOM-1 were detected, although this compound has been reported as the major metabolite produced by gut microbes in rat[25]. Further experiments are ongoing taking into consideration intermediate sampling points to investigate the D3G cleavage kinetic rate as well as the formation of ZEN catabolic products. To our knowledge, this is the first report describing the role played by the intestinal microbiota on the chemical modification of masked mycotoxins in humans. Results reported in this study suggest that Z14G and Z14S are easily and rapidly deconjugated by the colonic microbiota and let hypothesise that their aglyconic counterpart might therefore exert its toxic action toward local epithelial cells. Moreover, being the aglycone remarkably less polar than its conjugates, an increased absorption is obviously predictable. It should also be considered that intestinal mucosal cells may convert ZEN to their more oestrogenic phase I metabolites α- and β-ZOL[20]. Concerning D3G, we demonstrated that this masked form is efficiently deconjugated by colonic microbiota. Although it is well-known that DON absorption takes place mainly in the duodenum and in the small intestine, the release of DON from D3G in the colon cannot be neglected when neuroendocrine effects in humans are considered. As recently reviewed by Pestka[19], DON impairment of gut motility and appetite appears to involve altered neuroendocrine signalling at both the enteric and central levels. More specifically, it has been suggested that an elevation of serotonin levels in the gut could be related to DON peripherally serotonergic effects. Serotonin from the gut might also enter into the circulation, causing thus central effects[,31].

Several experiments reported that decreased feed intake and weight gain occur in pigs fed with naturally contaminated feed but not in pigs fed diet spiked with equivalent DON amounts[32]. This evidence, which is related to the neuroendocrine disruptive activity of DON, may be explained considering additional amount of D3G and other masked forms cleaved to DON by gut microflora.

In our study, DOM-1, which has been previously described as the main DON detoxification product in mammal faeces, has been found after 24 h fecal fermentation only as minor derivative. Like results have been reported in a very similar trial[33]. These authors demonstrated that 3-acetyldeoxynivalenol was metabolize to DON during the incubation period and no de-epoxidation metabolites were detected in the fecal incubates. This suggests that humans may lack the microflora for a key detoxification step for DON; further studies should be performed in order to clarify the role of the diet in human gut microflora modulation and DON detoxification. In our opinion, according to the release of parent compound due to the effect of human gut microflora and on account of occurrence data[20], a reconsideration of legal limits for Fusarium mycotoxins is needed. Since data on exposure and bioactivity are still lacking, it is currently impossible to perform a dose-response assessment (e.g. NOAEL) as well as a proper risk assessment for masked mycotoxins in food. In addition, an increasing number of masked forms of Fusarium mycotoxins are reported so far, most of them still lacking in occurrence data. All these forms clearly contribute to the toxicity of a given food and should be taken into consideration in setting future regulations. As an example, D3G usually occurs in cereals at levels up to 30% of its respective aglycone. Based on what we report here, commodities which are right below the legal limit could reasonably exceed it when the masked forms are considered. A possible approach can be the definition of proper maximum level for the sum of all relevant forms of a mycotoxin, including masked mycotoxins. In conclusion, our results clearly demonstrate for the first time that the cleavage of masked mycotoxin glucosyl- and the sulphate moieties is complete in a period of time fully compatible with the permanence of food remnants in the large intestine. This cleavage takes place through the action of microbial enzymes, whereas masked mycotoxins are untouched by proper human digestion. On the basis of this data, masked mycotoxins should be included in risk assessment studies since they are likely to contribute to the overall toxicity as they are potentially transformed into their dangerous parent compounds. In the last few years, an increasing number of reports about D3G occurrence data have been published; however, information about its stability and transformation along the manufacturing food chains are still lacking. Nonetheless, recently JECFA considered D3G, 3ADON and 15ADON as additional contributing factors for dietary exposure to DON[21], suggesting thus a reconsideration of the admitted maximum levels in food. Concerning ZEN, due to its estrogenic activity and its widespread occurrence in cereal-based products, which recently generated EFSA recommendations for further risk assessment studies in children as a potentially overexposed category[34], the new scenario should be carefully considered. The results obtained in this study definitely clarify the catabolic fate of masked mycotoxins in humans, thus depicting their relevance for risk assessment studies.

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General conclusions

The research topic of this Ph.D thesis has been the study of the occurrence and significance of masked mycotoxins in plants and their metabolic fate considering also their potential harmful effects on human health.

In particular, in the first part of the work the occurrence of deoxynivalenol-3-glucoside (D3G) in *durum* wheat naturally contaminated samples coming from different parts of Italy was reported. For the first time, evidence of the occurrence of masked mycotoxins in durum wheat was obtained, showing a very similar behaviour to that already known for other cereals, particularly for soft wheat. To perform this investigation, a new extraction protocol has been optimized and a multiresidual UPLC/MS method for mycotoxins analysis has been set up, allowing for the screening of trichothcecene mycotoxins (nivalenl, deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, T-2 toxin and HT-2 toxin) in a short time (22 min). Occurrence data have shown a widespread contamination of durum wheat samples, with a percentage of D3G reaching up to 30% of the native form (deoxynivalenol). Significant correlations were found between D3G/DON ratio and Fusarium Head Blight symptoms: analogously to what already proved in the case of soft wheat, the glycosilating activity exherted by plant seems to be one of the main defense mechanism against the infection.

In the second part of the work, a greenhouse experiment was carried out in order to better understand the masking mechanism of DON and the resistance of different durum wheat genotypes to one of the most severe disease that affects wheat plants (Fusarium Head Blight). In this experiment 4 lines of *durum* wheat and one of *aestivum* wheat were inoculated with two *Fusarium* strains or contaminated directly with the mycotoxin (DON). Results confirmed the ability of all the wheat lines to convert DON to D3G. The *aestiuvum* wheat line (Sumai, which is a known resistant line) displayed a very high conversion rate of DON to D3G compared to *durum* wheat genotypes. In any case, also interesting and different behaviours have been observed for durum wheat lines, showing a different susceptibility to the infection which is linked to the different ability to express the glycosilating activity towards DON. These results are potentially of interest for the identification of durum wheat line resistant towards FHB infection and for future breeding program in the durum wheat production chain.

In the last part of the work, investigations on the toxic effect of masked mycotoxins and their fate upon human digestion were carried out. From one side, the extrogenic activity of ZEN derivates (zearalenone-14-glucoside and zearalenone-14-sulfate) was evaluated in comparison with the native zearalenone using *in-silico* and *in-vitro* experiments. Computational studies foreseen the inability for these masked forms to interact with the estrogen receptor, in agreement with literature data. In-vitro experiments confirm these observation in the case of zearalenone-14-sulphate, whereas partially contradicting results were obtained for the glycoside and further experiments are actually needed to clarify this point. In this chapter, also the fate of masked mycotoxins (D3G, Z14G and Z14S) upon gastrointestinal digestion and fermentation was investigated, in order to evaluate their potential contribution to the overall toxicity. To perform these investigations an in vitro simulated gastrointestinal digestion and fermentation system has been set up. The results obtained shows that all these compounds are stable upon digestion. On the contrary, they are completely degraded by fecal fermentation, which induce the release of

the native forms, hydrolyzing the glycosides and the sulphate. In particular, ZEN was released immediately (30 min), while DON was recovered as free toxin after 24 h of fermentation (the mean residence time of digested food in the intestinal tract). These results demonstrate for the first time that the cleavage of masked mycotoxin is complete in a period of time fully compatible with the permanence of food remnants in the large intestine, thus potentially being readsorbed into the blood stream or potentially exerting their toxic activity towards intestinal epithelial cells. On the basis of this data, masked mycotoxins should be included in risk assessment studies since they are likely to contribute to the overall toxicity as they are potentially transformed into their dangerous parent compounds

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Author

Dall'Erta Andrea was born in Parma (Italy), October 31, 1984. He got the First Level Degree in Food Science and Technology (University of Parma) in 2007 and the Master Degree in Food Science and Technology (University of Parma) in the year 2009 with a thesis entitled "Hidden fumonisins: an emergent problem in maize".

In January 2010 he started the PhD course in Food Science and Technology at the Department of Organic and Industrial Chemistry, University of Parma, tutored by Prof. Gianni Galaverna and Dr. Chiara Dall'Asta, and he worked as research fellow on the topic "Masked mycotoxins in foods: origin and analysis". The research activity is aimed at studying the masking phenomenon of mycotoxins in cereals and cereal products, using advanced mass spectrometry techniques.

Scientific activity

Accepted papers

Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat.

C. Dall'Asta, **A. Dall'Erta**, P. Mantovani, A. Massi and G. Galaverna

World Mycotoxin Journal , 2013, in press. DOI 10.3920/WMJ2012.1463

Masked mycotoxins are efficiently hydrolysed by human colonic microbiota releasing their aglycones.

A. Dall'Erta, M. Cirlini M. Dall'Asta, D. Del Rio, G. Galaverna, C. Dall'Asta.

Chemical Research in Toxicology, 2013, accepted for pubblication.

Oral comunications

Occurence of Deoxynivalenol and Deoxynivalenol-3-glucoside in durum wheat.

A. Dall'Erta.

(XVII PhD Workshop 2012, Cesena, Italia).

Masked mycotoxins in durum wheat in durum wheat: Occurence, significance and metabolic fate. G.Galaverna, C.Dall'Asta, A.Tonelli, **A.Dall'Erta**, A.Dossena. "ChimAlSi_2012", Ischia (Na), Italy).

Occurence of Deoxynivalenol and Deoxynivalenol-3-glucoside in durum wheat.

A. Dall'Erta.

(34th Mycotoxin Workshop 2012, Braunschweig, Germania).

Poster communications

Validation of a UPLC-MS multiresidual method for the determination of trichothecenes and zearalenone in durum wheat.

A. Dall'Erta, C. Dall'Asta, G. Galaverna, A. Dossena. Poster (2nd Ms Food Day 2011, Trieste, Italia).

Simulated digestion assay for hidden fumonisin evalutation.

A. Dall'Erta, C. Falavigna, C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli.

Poster (32nd Mycotoxin Workshop 2010, Lyngby, Danimarca).

Hidden fumonisins: a study of the masking mechanism in raw maize.

Falavigna, **A. Dall'Erta**, C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli. Poster (32nd Mycotoxin Workshop 2010, Lyngby, Danimarca).

A digestion Assay for masked mycotoxin evaluation in cereals.

Falavigna, **A. Dall'Erta**, C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli.

Poster (ISM Conference 2009, Tulln, Austria).

Participation to international conferences

XVII PhD Workshop 2012. Cesena, Italy, 19-21 September 2012.

34th Mycotoxin Workshop. Braunschweig, Germany, 14-16 May 2012.

2nd MS Food Day. Trieste, Italy, 19-21 October 2011.

32nd Mycotoxin Workshop. Lyngby, Denmark, 14-16 June 2010.

Workshop: “Il triplo quadrupolo: trent’anni di successi!”. Milano, Italy, 18th December 2008.