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Ciclo XXVIII

Transformation of regulated and emerging mycotoxins
upon food processing:
from field to digestion

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Preface

Mycotoxins are common food and feed contaminants produced by fungi growing on edible plants, representing a potential risk for human and animal health. Depending on different factors (agricultural practices, genetic varieties and climatic influences), mycotoxins can occur during pre-harvest, but advantageous conditions for fungal growth during storage and processing steps could contribute to their evolution and modification in several commodities and foodstuffs, in particular cereals and cereal-based products.

Knowledge related to mycotoxins is continuously changing: list of contaminants is annually enriched by new detected metabolites, due to the climatic scenario and to advanced investigation concerning plants and their cellular metabolism. For these reasons, further researches are required by international authorities. Maximum legal limits have been set for the most-known and documented mycotoxins in several commodities. Many other metabolites have to be evaluated in light of their occurrence worldwide to better understand their impact and toxicological role, as required in the last decade by the European Food Safety Authority (EFSA).

The main Ph.D. project target is to provide tangible information about the potential mitigation from field to the finished product for those mycotoxins representing a well-documented risk for consumers and having a great impact in term of economic losses on the supply chain, providing also concrete outputs about toxicological role of those metabolites currently under discussion.

Strategic production chains have been investigated: in particular, common and durum wheat play a main role for the production of several cereal-based products widely consumed among populations worldwide as bread or pasta.

The Ph.D. thesis is organized in three main sections. The first section is devoted to study different durum wheat lines in order to test their reaction ability to mycotoxin contamination. The second section is mainly concerned with the investigation on the effective impact of technological processes on mycotoxin reduction and on the development of a strategy of mycotoxin mitigation, exploiting food processing as a detoxification tool, in order to obtain an acceptable and safe product for the consumers.

The last section is devoted to explore the fate of mycotoxins after ingestion along human gastrointestinal tract through dedicated digestion protocols. For each chapter a brief introduction is provided to allow an immediate contextualization of the treated subject.

Terminology

For the reader the different meaning of some terms used in this context should be clarified.

Indeed, in this work different forms of the same analyte are treated and the terms used to designate each of them can not be confused.

The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) has applied the term “modified mycotoxins” for masked, bound mycotoxins and mycotoxin metabolites. Modified mycotoxins have recently been detected when analyzing the mycotoxins in their original form; therefore, they have commonly been termed as “masked”. In this thesis, the structurally altered forms of mycotoxins covered by the terms of reference are referred to as “modified mycotoxins”[1].

Mycotoxins in their unchanged forms are referred to as “parent compounds”.

“Free fumonisins”, as an example, is the expression used to designate parent fumonisins directly extractable from a matrix through a common extraction procedure, while “total fumonisins” indicate the total amount of fumonisins contained in a sample and quantified after the application of an alkaline hydrolysis step on the matrix. Since these analytes are chemically different from parent fumonisins, their amount is expressed as fumonisin equivalent by dividing their concentration for a correction factor, represented by the calculated hydrolyzed-to-parent fumonisin molecular weights ratio.

The term “hidden fumonisins” is used to identify those forms that interact in different ways with food constituents, thus escaping routine analysis. Their amount is established through an indirect approach, by calculating the difference between total and free fumonisins.

The terminology proposed above will be used throughout this thesis.

Reference

1. EFSA Panel on Contaminants in the Food Chain (CONTAM). (2014). Scientific opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA Journal*, 12: 3916-3976.

General Introduction

Mycotoxins are naturally occurring toxic compounds produced as secondary metabolites by many filamentous fungi (mainly *Penicillium*, *Fusarium* and *Aspergillus* genera), which may colonize the various cereal grains, like many other agricultural commodities at all stage of the production chain [1,2]. The contamination of several foodstuffs by these fungi has been widely documented [3]. About 300-400 mycotoxins are known today, but many more are expected to be found in the future [4,5]. Their production in the field depends on a variety of factors, especially environmental and biological aspects as temperature/humidity, agronomical practices and other contingent factors can have a strong influence on the fungal development. As consequence, they influence mycotoxin occurrence from year to year [6], and the growth and development can proceed during postharvest (transport, processing and storage) [7]. Their presence causes tremendous economic losses to crop producers worldwide [8]: it is estimated that 10-30% of the harvested cereal grains are lost due to the fungal infection, whereas 25-50% of harvested crops have been contaminated with mycotoxins annually [9]. Among chemical hazards, the contamination of foods and feeds by mycotoxins has been identified by the WHO as a significant source of foodborne illness: they have been ranked as the most important dietary risk factor, higher than synthetic contaminants, additives or pesticides [10,11]. Mycotoxins can be found alone or can co-occur with other metabolites in cereals and cereal-based foods, especially when potentially produced by the same fungal species [12].

Several million tons of wheat, maize (about 160 and 100 million metric tons, respectively) and other cereals and raw materials are shipped around the planet annually: inevitably, mycotoxins, although related to specific regions of origin, are distributed worldwide [13,14].

Trichothecenes are the well-known mycotoxins: they are esters of sesquiterpenoid alcohols containing the trichothecene tricycling ring, which is characterized by a chemical bond at C9-C10 and an epoxide at C12-C1. Over 200 metabolites have been reported [15] and categorized into four groups; the most representative are group A and group B trichothecenes. The first one mainly consists of T-2 and HT-2 toxins, while group B is mainly represented by deoxynivalenol (DON) [16]. DON (Figure 1) is the key

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representative contaminant of wheat (it is the predominant mycotoxin detected in the bran fraction [17]), but it is found in many cereals including barley, oats, rye, and maize [16], being detected in more than 90% of all-contaminated raw materials [18]. It has been implicated in incidents of mycotoxicoses in both humans and farm animals: DON can impact on health through immunomodulation, protein synthesis inhibition, ribosome disaggregation, mitochondrial function compromise, lipid peroxidation, and cellular death [15]. Although no indication of carcinogenic or mutagenic effects has been reported, DON has been linked with human gastroenteritis [15,19].

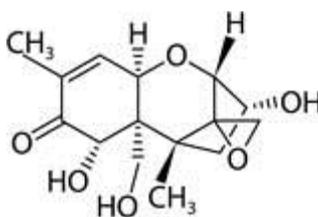


Figure 1. Chemical structure of deoxynivalenol (DON).

In addition to trichothecenes, other important mycotoxins are aflatoxins (AFs), zearalenone (ZEA), fumonisins (FBs) and ochratoxin A (OTA) [20]. In particular, Fumonisin B1 (FB1) and OTA have been evaluated as possible human carcinogenic, in the group 2B by the International Agency for the Research on Cancer (IARC) [21].

FBs are structurally characterized by an eicosane backbone, esterified with two tricarballic acid groups (Figure 2). Their natural occurrence has been reported in different foods and feeds from many countries, especially in maize. Besides FB1, other important representative of this group is Fumonisin B2 (FB2) [22]: they have been reported to be responsible for several animal diseases, e.g. equine leukoencephalomalacia and porcine pulmonary oedema, interfering on the sphingosine/sphinganine metabolism [23]; Sydenham et al. [24] reported FB1 statistically correlation with human esophageal cancer rates in South Africa.

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Due to their toxicological role and the great relevance as food and feed contaminants, regulatory maximum limits and guidelines for relevant compounds, based on scientific opinions of authoritative organizations such as the FAO/WHO Joint Expert Committee on Food Additives of the United Nations (JEFCA) or the European Food Safety Authority (EFSA), have been set in over 90 countries worldwide [28,29]. In the European Union (EU), regulatory has undergone a significant development in the last decade, resulting in a harmonization of stringent legal limits for approximately 50 toxin-food commodity combinations: current legislation regulates mycotoxin levels by means of the Regulation (EC) 1881/2006 and its subsequent amendments (Regulation (EC) 1126/2007, Regulation (EU) 165/2010, and Regulation (EU) 594/2012) [30-33]. The limits have been established for DON, the sum of FB1 and FB2, zearalenone (ZEA), aflatoxins (AFs), OTA and patulin (PAT). For T-2 and HT-2 toxins, group A trichotecenes, maximum permitted levels in cereals and derived products are currently under discussion [34]. In particular, DON maximum limit ranges between 500 and 750 µg/kg, depending on the type of cereal intended for direct human consumption; FBs (levels are regulated only for maize and maize-based products) have a maximum level set at 800 µg/kg for the sum of FB1 and FB2; OTA maximum level is established at 3 µg/kg in the finished product (Table 1).

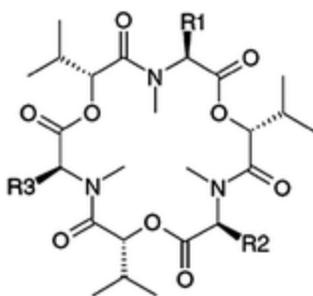
Table 1. Legal limits for ochratoxin A (OTA), deoxynivalenol (DON) and fumonisins (FBs).

2.2	Ochratoxin A	Maximum levels ($\mu\text{g}/\text{kg}$)
2.2.1	Unprocessed cereals	5,0
2.2.2	All products derived from unprocessed cereals, including processed cereal products and cereals intended for human consumption	3,0
2.4	Deoxynivalenol	
2.4.1	Unprocessed cereals other than durum wheat, oats and maize	1250
2.4.2	Unprocessed durum wheat and oats	1750
2.4.5	Pasta (dry)	750
2.4.6	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast	500
2.6	Fumonisin	Sum of FB1 and FB2
2.6.1	Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	4000
2.6.2	Maize intended for direct human consumption, maize-based foods for direct human consumption	1000
2.6.3	Maize-based breakfast cereals and maize-based snacks	800

Changing climatic conditions and innovative agricultural practices have led to modifications in the spectra of fungal agents growing in the field and, as consequence, the extent of mycotoxin contamination is constantly changing [35]. For this reason, in addition to the regulated compounds, other metabolites, defined as “emerging mycotoxins” have been detected. Over the last few years, “emerging mycotoxins” have acquired importance because of their occurrence and their potential toxic implications for human and animal health such as cardiotoxicity, cytotoxicity or teratogenicity [12]. Among these compounds, Enniatin A, A1, B and B1 (ENN A, ENN A1, ENN B and ENN B1, respectively) are cyclic hexadepsipeptides consisting of three D-2-hydroxycarboxylic and N-methylamino acid moieties (Figure 4). Studies about their incidence in food have indicated their presence in grain cereals, 50-90% in wheat, corn and barley, frequent contamination in potatoes and apples, as well as in cereal-based products from different countries in the world, especially from the Mediterranean area where contamination levels of several hundred mg/kg have been reported [36-39]. Enniatins (ENNs) are found to be toxic to cell lines of human origin such as

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hepatocellular carcinoma-line Hep G2 and fibroblast-like fetal lung cell line MRC-5 [40]. Furthermore, ENNs can also act as inhibitors on acyl-CoA:cholesterol acyltransferase (ACAT) and therefore interfere with lipid metabolism [41]. They have been showed synergistic effects and ENN B is the most bioactive ENNs in light of some recent studies [42]. Although their effective impact on human and animal health remains unclear yet, due to their late recognition and the very limited data available on *in vitro* and *in vivo* toxicity, concentration levels and occurrence, “emerging mycotoxins” represent a serious concern. Different EU authorities are pointing out the need to minimise the levels of mycotoxins, regulated and not, promoting several good agricultural practices from the cultivation to the distribution of cereals (such as crop rotation or dry storage), as well as food transformation by technological processes into the finished product.



Enniatin	R1	R2	R3
A	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃
A ₁	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃) ₂
B	CH(CH ₃) ₂	CH(CH ₃) ₂	CH(CH ₃) ₂
B ₁	CH(CH ₃) ₂	CH(CH ₃) ₂	CH(CH ₃)CH ₂ CH ₃

Figure 4. Chemical structure of enniatins (ENNs)

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SECTION I. MODIFIED MYCOTOXINS: FORMATION, OCCURRENCE AND IMPACT IN FOOD

State of the art

Cereals are the most important source of food and feed for humans and animals worldwide. In particular, wheat (common and durum) was one of the first domesticated cereals and for 8000 years has been the basic stable food of European and North African populations, feeding about 40% of human beings and providing about 20% of total food calories and proteins in human nutrition [1]. Wheat is subjected to various abiotic stresses that interfere with its normal growth and development and that cause yield losses, at all growth stages and in all natural environments. Among a series of diseases, *Fusarium* head blight (FHB) is the most relevant one and it can be caused by a complex of *Fusarium* species including the two principal species, *Fusarium graminearum* and *Fusarium culmorum* (Figure 1). FHB can cause the destruction of an entire wheat crop within a few weeks and yield losses may reach 50-60%, reducing nutritive and technological quality of the grain, as lower protein levels and semolina discolouring [2].



Figure 1. Typical *Fusarium* head blight (FHB) symptoms in wheat (A); Wheat spikelets at different contamination stages (B); Wheat kernels contaminated by *Fusarium* species.

Additionally, FHB is the principal cause by which mycotoxins develop in the grain before harvest [3]. Recently, it has been shown that plants can reduce the toxicity of mycotoxins either by chemical modification or by inclusion into plant matrix components by producing mycotoxin conjugates [4]. The so called “modified mycotoxins” can be mainly produced via enzymatic transformation (phase II metabolites) [5,6] exerted to counteract pathogen invasion [7,8]. In the last decade, several studies have been conducted with the aim to better investigate the formation and role of modified mycotoxins in cereals and food. These forms are undetectable by conventional analytical methods because of their different chemical behavior with respect to the parent compounds [9]: the occurrence of modified metabolites generally implies a possible underestimation of the mycotoxin contamination level of a certain commodity, as the structural changes induce a consequent potential changing in the suitable experimental conditions. The subsequent failure to recognize hazardous contamination on mycotoxin monitoring should lead to higher exposure of the consumer [10].

Plant metabolites have been identified so far for deoxynivalenol (DON), and, in this context, the major detoxification pathway is the conjugation of DON to a glucose moiety giving rise to deoxynivalenol-3- β -D-glucoside (DON-3-Glc) [11] (Figure 2). The occurrence of this derivative has been described in naturally infected cereals such as wheat, maize, oats, barley, malt and beer [12-14].

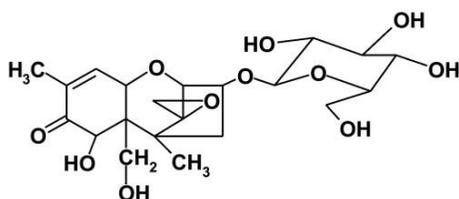


Figure 2. DON-3-Glc chemical structure.

Studies showed that the relative proportion of DON-3-Glc to DON is more or less stable (15-20%) but value up to 70% have been observed depending on year-campaign and genotype characterization [15,16]. DON-3-Glc occurrence was also proven in cereal-based finished products as bread, biscuits, pasta, and beer, and some samples resulted in DON-3-Glc concentrations even exceeding those of DON [17,18].

Indeed, chemical transformation of mycotoxins may also occur during transformation processes of the raw materials, especially when thermal treatment or fermentation steps are involved: it has been shown that DON-3-Glc levels increase after malting of barley grains and the contaminant is transferred into beer as a consequence of enzymatic degradation of polysaccharides [13]. Also during bakery processes, a variety of enzymes, principally amylases, proteases, pectinases, are involved and available data on their effect on mycotoxins conjugates are rather contradictory [19,20].

As far as the toxicological aspects are concerned, modified forms have not been included in the regulatory limits so far. Despite the frequent occurrence, toxicological data regarding DON-3-Glc are still limited. Although the toxin seems to be rather stable under conditions simulating early stages of mammalian digestion, lactic acid bacteria were able to cleave DON-3-Glc [21]. Dall'Erta et al. [22] described an extensive liberation of DON after incubation of the glucoside in human fecal slurries. The first *in vivo* reports on the fate of orally administered DON-3-Glc in rats and pigs showed a nearly complete hydrolysis in the digestive and intestinal tract [23,24]. However, more toxicological data are urgently needed to evaluate the potential contribution to DON exposure. A recent request for evaluation of modified forms have been issued by the European Commission (EC) to the European Food Safety Authority (EFSA): data of co-occurrence of these forms in food and feed and an estimation of the human dietary exposure compared with the dietary exposure to the parent compounds have been required in order to assess the final human and animal health risk. Furthermore, it has been noted that there is a need for further investigation on the fate of modified forms upon food and feed processing [25].

Starting from a genetic approach, Miller et al. [26] first speculated that formation of a less toxic DON conjugate might be responsible for partial FHB resistance of wheat. Resistant cereal lines inoculated with pathogens of the genus *Fusarium* contain lower amounts of DON in the grain compared to susceptible ones. It has been demonstrated that the ability of wheat lines to convert DON to DON-3-Glc was linked to a quantitative trait locus (QTL), which had been previously reported to be associated with FHB resistance

[7]. The presence of this gene clearly decreases FHB symptoms but raises the DON/DON-3-Glc ratio.

Considering these preliminary evidences of a link between resistance to FHB pathogen and the ability of plants to metabolize DON into DON-3-Glc, a greenhouse trial on different wheat lines has been conducted during this Ph.D. project. In the following Chapter 1, experimental approach, analytical methods and results are properly discussed.

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CHAPTER 1. DURUM WHEAT LINES SHOW DIFFERENT ABILITIES TO FORM MODIFIED MYCOTOXINS UNDER GREENHOUSE CONDITIONS*

*Cirlini, M., Generotti, S., Dall'Erta, A., Lancioni, P., Ferrazzano, G., Massi A., Galaverna, G., Dall'Asta, C. (2014). Wheat (*Triticum Durum* Desf.) lines show different abilities to form masked mycotoxins under greenhouse conditions. *Toxins*, 6: 81-95

1.1 Introduction

Fusarium head blight (FHB) is one of the most deleterious fungal diseases affecting wheat worldwide: it is related to infection by pathogenic fungi of the *Fusarium* spp. and it is widely diffused, especially in those areas with inductive climatic conditions (hot/warm temperatures and high/medium-high humidity) [1–3]. FHB causes severe yield losses (up to 70%), affecting the quality of grains which show low protein content and color defects. Moreover, fungal infection may lead to the accumulation of mycotoxins: depending on the chemotype of the fungus, the type B trichothecenes deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), and 15-acetyldeoxynivalenol(15-AcDON) often accumulate in the developing grain [4].

This contamination is especially critical for durum wheat, which is used primarily for human consumption. Although the best economic and ecological strategy for reducing FHB damage is the utilization of resistant cultivars, the attempts to define durum wheat resistant lines have been unsuccessful so far.

In soft wheat, numerous QTL related to FHB resistance have been described [5]. In particular, several studies performed using the high resistant Chinese Spring wheat line Sumai-3 showed that the two most effective QTLs related to FHB resistance are positioned on the short arm of chromosome 3B(*Fhb1*) and on chromosome 5A (*Qfhs.ifa-5A*) [6–8]. Durum wheat cultivars were generally considered to be susceptible to FHB. Indeed, no variation in resistance to FHB has been found within *T. durum* lines, even among large germplasm collections of several thousand lines [9,10].

This fact may be due to several factors: a narrow genetic base compared to hexaploid wheat might be linked to the fact that durum wheat is tetraploid and that limited breeding efforts have been undertaken on this crop [11]. Attempts to transfer resistance from hexaploid into tetraploid wheat have been met with limited success [12,13].

Several types of resistance to FHB are known in wheat, classified as type I (resistance towards initial infection of spikelets), type II (resistance against spread of pathogen within spike) [14,15] and type III (resistance to DON accumulation in grains) [16,17].

In particular, DON was proven to inhibit protein synthesis in eukaryotic cells and acts as a virulence factor during fungal pathogenesis, therefore resistance to DON is considered an important component of resistance against FHB [18]. As reported by several studies, one mechanism of resistance to DON is the conversion of DON into the less toxic metabolite deoxynivalenol-3-O-glucoside (DON-3-Glc) [19–21].

In particular, in a wheat population segregating for *Fhb1*, lines containing the *Fhb1* resistance allele efficiently conjugate DON to the less toxic DON-3-Glc [19]. In this work, the authors reported a good correlation between FHB resistance and DON conversion rate, expressed as [DON-3-Glc]/[DON] ratio. This hypothesis was recently questioned by Gunnaiah et al. [22], whose study showed that DON resistance is not a major mechanism of FHB resistance associated with Nyubai Alleles of *Fhb1*. The co-occurrence of DON and DON-3-Glc in durum wheat harvested in Northern Italy was recently reported [23], showing a diffuse contamination of most samples with both compounds at significant levels.

1.2 Aim of the work

The present work is aimed at the study of the DON-to-DON-3-Glc conversion ability of wheat lines (two durum wheat genotypes and one soft wheat genotype) under controlled greenhouse conditions.

In particular, the soft wheat line Sumai-3 was chosen as reference standard based on its well-known resistance towards FHB [24–26], while durum wheat lines (Kofa and Svevo) were chosen on account of their technological performances and because have been widely used in the most relevant durum breeding programs.

Plants have been treated with DON at anthesis under controlled growing conditions and samples have been analyzed for free and modified mycotoxins content.

1.3 Experimental section

1.3.1 Chemicals

Sodium acetate, formic acid, methanol, hexane and acetonitrile were purchased from Sigma-Aldrich (Taufkirchen, Germany). All solvents were HPLC grade. Ultra-pure water was in-house produced by using a Milli-Q System (Millipore, Bedford, MA, USA). The following mycotoxin standard solutions were purchased from RomerLabs (Tulln, Austria): 13C-Deoxynivalenol (internal standard, 25 mg/L in acetonitrile), DON-3-Glc (50 mg/L in acetonitrile), mix A + B trichothecenes containing DON, NIV, T-2 and HT-2 (10 mg/L each in acetonitrile). All standard solutions were stored at -20°C and brought to room temperature before use. A working solution (2.5 $\mu\text{g}/\text{mL}$) containing all the target mycotoxins was prepared in water/methanol (80/20, v/v) by combining and properly diluting suitable aliquots of each standard.

1.3.2 Wheat lines background information

For this study, two durum wheat lines (Kofa and Svevo) and one soft wheat genotype (Sumai-3) have been chosen.

Kofa, is a Southwestern United States cv. released by Western Plant Breeders (Arizona) obtained from a population based on multiple parents (dicoccum alpha pop-85 S-1) mainly related to the American and CIMMYT germplasm, with the inclusion of emmer accessions. Svevo, an Italian cv. released by Società Produttori Sementi, has been obtained from the cross between a CIMMYT line (pedigree rok/fg//stil/3/dur1/4/sapi/teal//hui) related to the cv. Yavaros, genetic background (Jori/Anhinga//Flamingo), and the cv. Zenit originating from a cross between Italian and American accessions (Valriccardo/Vic). Both Kofa and Svevo are well adapted to the Mediterranean climate and can be classified as early-flowering genotypes in such conditions, and are susceptible to FHB.

Lines were initially chosen based on the different susceptibility to DON accumulation and to FHB symptoms; moreover, the selected lines well represent the genetic diversity of the main ameliorated pool of durum wheat adapted to grow in the Mediterranean area.

The soft wheat cv. Sumai 3 was used as control for its known resistance ability towards FHB (type II resistance, linked to the QTL *QFhs.ndsu-3B*).

1.3.3 Design of the greenhouse experiment

The artificial inoculation experiment on durum wheat plants has been carried out according to a RCB (Randomized Complete Block) scheme within a greenhouse plant at Produttori Sementi S.p.A. (Argelato, Bologna, Italy). All the plants were singularly sown and maintained at low temperature (5 °C) for 30 days to mimic vernal conditions (vernalization), then they were transferred into the greenhouse, fixing the ambient conditions in order to mimic first the spring period and then the summer season. For the experiment, two lines of durum wheat (Svevo and Kofa) and one line of soft wheat chosen as a reference line for its resistance to FHB (Sumai 3) were selected.

For each line, two groups were considered, each formed by five plants: at the flowering stage (zadok 59–60) the first group was artificially contaminated with a DON solution, whereas the second group was considered as the control one. All the groups, as well as the negative (not contaminated) control, were replicated five times. The entire experiment was performed in triplicate in order to allow two different sampling steps at four different maturation stages. Sampling steps were fixed from the inoculation step as follows: after one day (S1d), after two days (S2d), after five days (S5d) and at harvest (SH). The total plant number was 600. The treatment was performed on four spikelets in the central part of the spike: each spikelet was inoculated with 20 µL DON standard solution (exact title: 0.828 mg/mL). Control plants were mock-treated with distilled water.

At each sampling stage, spikes were separately collected and milled, before UPLC-MS analysis.

1.3.4 Free and modified mycotoxin analysis

Samples were prepared according to Berthiller et al. [32] with slight modifications. Briefly, the whole ears were finely grounded and mixed, then 0.5 g of sample was extracted for 90 min at 200 strokes/min on a shaker with 2 mL of acetonitrile/water (80/20, v/v) acidified with 0.1% of formic acid. The extract was collected and centrifuged for 10 min at 14000 rpm at room temperature and 1 mL of the supernatant was evaporated

to dryness under nitrogen. After addition of the internal standard (13C-DON, 20 µL, obtaining a final concentration of 100 µg/kg), the residue was dissolved in 1 mL of water/methanol (80/20, v/v) and analyzed by UPLC-ESI/MS.

The UPLC-ESI/MS analyses were carried out according to Dall'Asta et al. [23], using 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 as follows: column, Acquity UPLC BEH C18 (1.7 µm, 2.1 × 50 mm); flow rate, 0.35 mL/min; column temperature, 40 °C; injection volume, 5 µL; gradient elution using 0.1 mM sodium acetate solution in water (eluent A) and methanol (eluent B), both acidified with 0.2% formic acid. Gradient conditions: initial conditions were set at 2% B for 1 min, then eluent B was increased to 20% in 1 min; after an isocratic step (6 min), eluent B was increased to 90% in 9 min; after a 3 min isocratic step, the system was re-equilibrated to initial conditions for 3 min. The total analysis time was 23 min. The ESI source was operated in positive ionization mode. 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 L/h; desolvation gas, 850 L/h. Detection was performed using single ion monitoring mode and monitoring the [M + Na]⁺ ion, as reported by Dall'Asta et al. [23].

Matrix-matched calibration curves (calibration range 100–2500 µg/kg) were used for target analyte quantification. 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/kg and 10 µg/kg, respectively.

1.4 Results

1.4.1 Set up of the trials

Trials were performed within this study, following the general scheme reported in Figure 1. The trial involved two genotypes of *Triticum durum* (Kofa and Svevo) and one genotype of *T. aestivum* (Sumai-3), selected on the basis of their resistance towards *Fusarium* head blight (FHB) under in-field conditions and their genetic background. In particular, the DON detoxification ability related to FHB resistance was largely studied

in soft wheat [27], while very few studies only reported the occurrence of DON-3-Glc in durum wheat under natural infection conditions, so far [23].

We decided to directly treat plants with DON, as already performed by Lemmens et al. [19], in order to maximize those enzymatic mechanisms carried out by the plant to limit the DON toxic action during fungal infection.

The use of this simplified system, although very far from the complicated cross-talk phenomena occurring upon in field fungal inoculation, allows distinguishing the possible differences in the transformation occurring in plants. Differently from Lemmens et al. [19], we decided to apply an amount of DON 10-times lower (total amount: 80 µg) to better resemble the common conditions experimented under in-field infection.

Plants (n = 600) were split into two groups and underwent different treatments at the flowering stage, namely treatment with DON solution and mock-treatment with distilled water (control group). Each group was sampled after one day, two days, five days and at harvest (Figure 1). Sampling times were chosen in order to highlight the DON detoxification ability in the first phases after the contamination, since reports published until now commonly focused on samples collected at harvest (full maturation stage) [19]. A recent study reported that UDP-glucosyltransferase proteins in Sumai-3, involved in the DON detoxification process, are over-expressed already 32–48 h from the fungal inoculation [29]; thus, short times after treatment were considered to better understand the DON-3-Glc formation at the early-stage. In addition, spikes at full maturation were harvested and analysed to evaluate the final content of DON and DON-3-Glc.

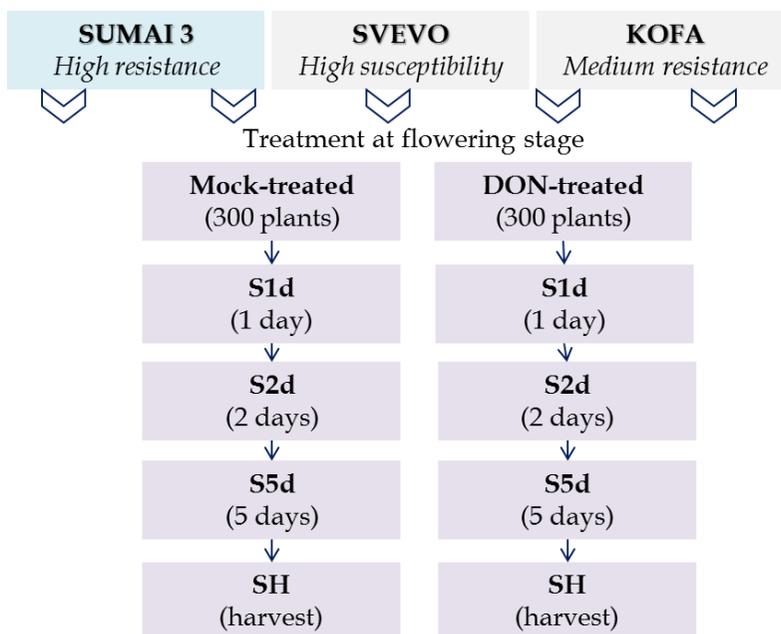


Figure 1. Experiment set-up.

1.4.2 DON-treated samples

All the DON-treated and control spikes were separately analysed for DON and DON-3-Glc content; each analysis was performed in duplicate.

No detectable DON contamination was found in the control spikes, while all DON-treated samples were found to be positive for both DON and DON-3-Glc. Data, expressed as conversion ratio of [DON-3-Glc]/[TDON] (where TDON is the sum of DON, AcDON and DON-3-Glc) found in the samples at the different sampling stages, are reported in Table 1. Conversion rate values were statistically compared by ANOVA followed by post-hoc Tuckey test. The sampling stage was found to be significant for all the considered lines ($p = 0.000$).

Table 1. Conversion rate obtained at each sampling stage for the DON-treated lines considered in the trial.

	SUMAI 3	SVEVO	KOFA
Sd1	0,246	0,056	0,046
Sd2	0,655	0,154	0,391
Sd5	0,619	0,115	0,421
SH	0,843	0,312	0,530

As a general comment, DON-3-Glc was found to be higher or comparable to DON at harvest stage in all the considered lines, but an important conversion was already noticed five days after the contamination (S5d).

DON-3-Glc was found in all the analysed samples already after 24 hours from the treatment, thus indicating that the detoxification pathway is promptly active towards DON in plant. This fact is in agreement with the expression data recently reported by Gottwald et al. [29] for FHB resistant soft wheat lines, among those Sumai-3. In particular, the authors stated that the expression profiles of genes related to detoxification processes in resistant genotypes are induced in the early stage, while susceptible lines typically show late inductions.

As reported in Table 1, the Sumai-3 line confirmed the highest conversion rate already after 24 h from the treatment and its detoxification ability is confirmed as very high at harvest, being the ratio between DON-3-Glc and total DON-related compounds (TDON) 0.843 at SH.

Concerning durum wheat lines, Kofa showed the highest conversion ability; a higher detoxification property compared to Svevo already after two days from the treatment ($p=0.003$ at Sd2) and this difference became particularly evident at harvest time. The data here presented are clearly in agreement with Gottwald et al. [29], since Svevo, which is

usually assumed to be less resistant than Kofa towards FHB, showed a delayed conversion of DON to DON-3-Glc.

1.5 Conclusions

The study presented herein showed for the first time the DON-to-DON-3-Glc conversion ability of two durum wheat lines in comparison to Sumai-3 soft wheat under greenhouse conditions. In addition, for the first time, the DON-3-Glc formation in plants already 24 h from the DON treatment was reported. Results upon DON-treatment suggest that low glycosylation ability is related to a high susceptibility towards fungal infection and toxin accumulation, at least under greenhouse conditions. In particular, Kofa cv. showed a good glycosylation activity towards DON and it is a promising candidate for further studies to better define the DON detoxification activity in durum wheat.

Data here represented support thus the hypothesis reported by Lemmens et al. [19]: the ability to convert DON to DON-3-Glc in these lines seems actually to be related to their resistance towards FHB. The role played by DON-3-Glc as DON detoxification products in durum wheat was demonstrated, thus pinpointing as well the necessity for careful monitoring of this modified mycotoxin in durum wheat.

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SECTION II. TRANSFORMATION OF MYCOTOXINS UPON FOOD PROCESSING*

*Suman, M., Generotti, S. Transformation of mycotoxins upon food processing: masking, binding and degradation phenomena. In *Masked Mycotoxins in Food: Formation, Occurrence and Toxicological Relevance*; RSC Publishing: London, UK, 2015.

State of the art

The occurrence of mycotoxins in cereal-based foods is a global issue of high concern due to their potential health for humans. Despite low consumer awareness of the problem, health risks related to mycotoxin ingestion have been quantified as exceeding risk from other food-related contaminants, such as pesticides, additives, and heavy metals. Besides mycotoxins can never be completely removed, food processing can have an impact on mycotoxin levels, but details of the effects remain mostly unclear. Due to their thermal and chemical stability, mycotoxins can only be partly removed or redistributed by food processing procedures that may involve physical and chemical steps, so preventive measures should be adopted at all stages of the production chain to prevent contamination of final food products. There is a clear health issue associated to mycotoxins ingestion and the need for regulating modified forms has been already recognized by European regulatory bodies, nevertheless, due to the lack of analytical, exposure and toxicological data, implementation remained vague until now.

The first examples of “post-harvest action” devoted to a potential reduction of the mycotoxin contamination in the grains are represented by sorting, cleaning, dehulling and debranning steps: in any case the removal efficiency of all these pre-milling processes can be significantly variable. Then, as in cleaning and debranning, the milling process may redistribute and concentrate in certain fractions (bran or outer layers of the grains) the mycotoxin concentrations. Mycotoxins have always been considered to be thermostable compounds. However, interest in knowing the effects of food processing or cooking on mycotoxin levels has increased during the last decade. The technological processes employed seem to play an important role in the phenomenon associated with “masking”: the mechanical energy of heat during the transformation process can cause significant changes, which can prompt reaction with macromolecules, such as sugars,

Section II

proteins or lipids, or release the parent compound of the toxin after decomposition of the conjugated compound.

Recent evidence suggests that some food production processes lead to the reduction of levels of parent mycotoxins in finished products compared to the corresponding raw materials and ingredient, especially when high temperature conditions are involved. Anyway, in some other circumstances processing may stimulate the release of parent compounds from the conjugated forms, especially when fermentation steps are involved: a possible explanation may be the enzymatic release of the parent compound from some bound forms occurring in the raw materials.

It is reasonable to consider that a significant effect of food processing is to reduce the overall toxicity of the finished products, as there are scientific indications that attest to a lower toxicity of the compounds obtained within the processing of foods than their precursors in raw materials. Nevertheless, there are large areas to explore: one direction regards the actual influence of the many variants of technological treatments that industry is able to carry. The other one is determining whether the transformations that generate modified-bound forms related may be defined as a real “detoxification” pathway, where was proven their effective lower combined toxicity to the release, both during food processing and, of course, in the digestive system of human beings.

During the next chapters, the impact of food processing, as technological steps and recipe formulation, has been evaluated for different production chains on the final mycotoxin content. The first three chapters are aimed to monitor mycotoxin evolution, in particular fumonisins (FBs), deoxynivalenol (DON) and its major modified form deoxynivalenol-3-glucoside (DON-3-Glc), and enniatin B (ENN B), taking into account industrial setting or pilot-plant production; starting from chapter 5, the objective is the development of a strategic mycotoxin mitigation in bakery products to minimize their content in the finished products.

The approach was explored with a particular attention of the technological requirements remaining in an acceptable range from the organoleptic point of view.

CHAPTER 2. INFLUENCE OF THE INDUSTRIAL PROCESS FROM CARYOPSIS TO CORNMEAL SEMOLINA ON LEVELS OF FUMONISINS AND THEIR MODIFIED FORMS*

*Generotti, S., Cirilini, M., Dall'Asta, C., Suman, M. (2015). Influence of the industrial process from caryopsis to cornmeal semolina of fumonisins and their masked forms. *Food Control*, 48, 170-174.

2.1 Introduction

Fumonisins are a group of secondary metabolites mainly produced by *Fusarium verticilloides* and *Fusarium proliferatum* that can occur in corn and corn-based products. These mycotoxins, especially those pertaining to B series (FBs), may cause a variety of undesirable effects in mammals, such as hepatocarcinogenic, hepatotoxic, nephrotoxic, and cytotoxic diseases. In human, the high incidence of esophageal cancer has been related with FB1 exposure [1]. The consumption of fumonisin-contaminated maize has also been associated with the occurrence of neural tube defects [2]. For these reasons FB1 has been classified as a 2B carcinogenic by the International Agency of Research on Cancer (IARC) [3]. The joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined a maximum tolerable daily intake (PMTDI) of 2 µg/kg body weight per day for FB1, FB2 and FB3 separately or in combination [4].

Contamination from group B fumonisins is a quite diffuse problem in the Southern part of Europe, especially in Italy where particular climatic condition can bring to high content of fumonisins in maize [5]. For this reason, European Union regulated the maximum limits for fumonisins (sum of FB1 and FB2) in unprocessed maize (4000 µg/kg), milling fractions of maize with particle size ≤500 micron (2000 µg/kg), milling fractions of maize with particle size >500 micron (1400 µg/kg), maize destined to human consumption (1000 µg/kg) with a particular attention to breakfast cereals and snacks (800 µg/kg) as well as toward maize destined to baby food (200 µg/kg) [6].

Fusarium contamination in maize occurs directly in field during the growing season, in particular after the dough stage, and climatic condition such as high humidity and temperature can enhance the fungi growth and the consequent production of fumonisins [7].

Food and feed processes can modify and sometimes reduce the initial content of mycotoxins in the raw materials. In particular, sorting, that is the mechanical or manual removing of contaminated units, can bring to a decrease of the total contamination [8]. Cleaning procedure removes broken kernels and those with evident and extensive mould growth but also fine materials with size < 3 mm. It was demonstrated that this step can reduce the fumonisin amount from 26 to 69% [9]. Fumonisin distribution during dry-milling process was investigated in several studies [8-13], indicating that contamination increased, with respect to unprocessed maize, in products as bran, germ, middlings (animal feed flour), while it decreased in flour destined for human consumption. Pietri et al. [14] carried out a study on the distribution of fumonisins in dry-milled maize fractions, observing that if fumonisins absolute amount presence and mass balances were considered, undersized flour that represented about 20% of final product, collected about 70% of fumonisins contained in unprocessed maize.

Fumonisin are relatively stable toward high temperatures, as significant changes occur only above 50 – 200° C, when thermal processing such as baking, frying, roasting or extruding are applied [15]. However, recent results have shown that this decrease could be ascribed to possible modifications of the mycotoxin structure by interaction with other food components leading to the formation of conjugates [16].

In particular, it is well known that fumonisin B1 can react with reducing sugars forming strong covalent bonds during thermal treatments. For example, N-(carboxymethyl)fumonisin B1 (NCM) is a reaction product deriving from FB1 and D-glucose and formed after submitting corn grits to an industrial extrusion procedure at 160-180°C [17], while N-(deoxy-D-fructos-1-yl)fumonisin B1 (NDF) represents the main derivative obtained from the condensation between FB1 and D-glucose [18]. However, the possible formation of other bound forms can be hypothesized [16]. As a consequence of the masking phenomena, it might be considered that the total concentration of fumonisins in processed corn-based food could result underestimated. So it become important to estimate both free and modified fumonisins in order to have an idea of the real potential toxicity of food and feed.

2.2 Aim of the work

In this study, free and modified fumonisins have been determined on various products (unprocessed and cleaned corn, uncooked and cooked broken corn, cornflake and cornmeal semolina) collected from a cornmeal industrial production plant, as well as on byproducts (“fumetto” flour, germ and middlings) with the aim to estimate and establish the distribution of the fumonisin contamination from raw materials to cornmeal semolina.

2.3 Experimental section

2.3.1 Chemicals

Fumonisin B1, B2 and B3 standard solutions, 50 µL/mL each, in acetonitrile/water, 1:1 v/v, was purchased from Biopure (Tulln, Austria). All of the solvents used were of LC grade. Methanol was obtained from Carlo Erba (Milan, Italy), acetonitrile was from J. T. Baker (Mallinckrodt Baker, Phillipsburgh, NJ, USA); bidistilled water was produced in our laboratory utilizing an Alpha-Qsystem (Millipore, Marlborough, MA, USA). Potassium hydroxide was obtained 100 from Carlo Erba (Milan, Italy).

2.3.2 Moisture content determination

The moisture contents of the samples were measured by heating 5 g samples in a thermostatic laboratory oven at 105 °C for over 12 h. Through this further collected information, all the results were calibrated and compared on a dry matter (d.m.) basis.

2.3.3 Preparation of hydrolyzed fumonisins standard solution

1 mL of standard solution of hydrolyzed fumonisins B1, B2 and B3 (HFB1, HFB2 and HFB3, respectively) was prepared according to the hydrolysis procedure proposed by Dall’Asta et al. [19]. Briefly, the procedure was as follows: 90 µL of the FB1 and FB2 mixed standard solution (50 µg/mL) and 90 µL of standard solution of FB3 (50 µg/mL) were evaporated to dryness. The residue was redissolved in 2M KOH (1 mL) in an ambered vial, then allowed to react for 12 hours at room temperature. After hydrolysis, the mixture was extracted twice by liquid-liquid partition using twice 1 mL of acetonitrile. The organic phases were pooled and evaporated under N₂ stream and the

residue was redissolved in 1 mL of control matrix (extract from no fumonisin-contaminated maize). The conversion yield was quantitative. Calibration curves were prepared by proper dilution of the obtained standard solution.

2.3.4 Sample collection

Ten products and byproducts (unprocessed –raw material- and cleaned corn –corn after cleaning step-, uncooked –cleaning corn after breaking stage- and cooked broken corn –broken corn thermally treated-, cornflake–cooked broken corn after roller milling- and cooled cornflake–cornflake after cooling step-, flour “fumetto”-flour obtained during processing and intended for confectionar-, cornmeal semolina –final flour for polenta production-, germ and middlings –byproducts, intended for animal feed-) were obtained from a cornmeal (dry-milling plant) industrial production plant (February 2013) in Northern Italy (Figure 1), considering different processing steps:

- cleaning step: dust, stones, different cereals and seeds, outer layer of caryopsis were removed through a system characterized by a separator with aspirator and sieve, a magnet and an optical selector. “Usable” waste was milled and intended for animal feed (middlings);
- de-germination step: cleaned corn was passed through a series of crushers (in order to break caryopsis), plansichters (able to remove bran from the other fractions) and densimetric tables (in order to separate germ and broken corn exploiting different specific weight);
- refining step: the aim of this step was to refine broken corn size and it was regulated by using a series of 5 roller mills, 2 plansichter and 4 semolina mills stages. During this stage flour “fumetto”, characterized by a fine granulometry and usually intended in confectionery and cornflake were obtained.

In order to obtain a representative sampling and a global sample (conveniently homogenized) of 1,5 kg for all fractions, an aliquot of about 100 g each 2 min for 60 min was collected from each considered processing step.

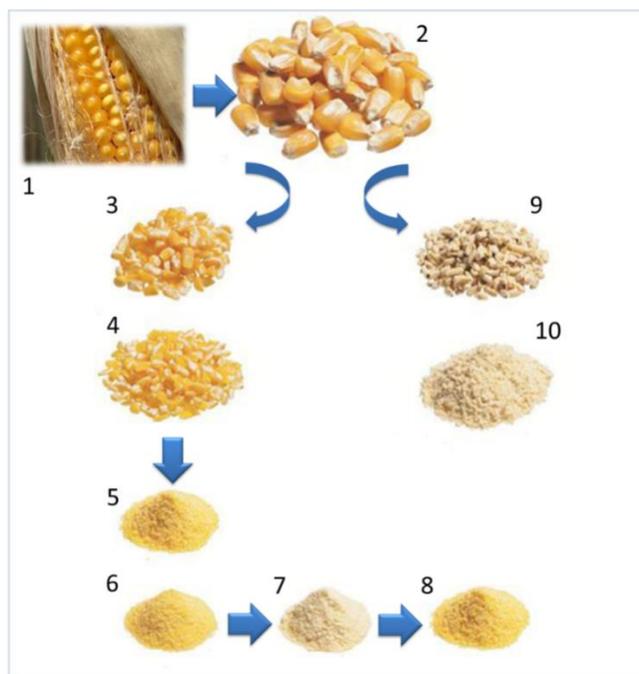


Figure 1. Sampling collection from a cornmeal industrial process: 1. unprocessed and 2. cleaned corn, 3. uncooked and 4. cooked broken corn, 5. cornflake and 6. cooled corn-flake, 7. flour «fumetto», 8. cornmeal semolina, 9. germ and 10. middlings

2.3.5 Sample preparation for free and total fumonisins analysis

Extraction and analysis of fumonisins were performed according to Dall'Asta et al. [19], with slight modifications. Briefly, for free fumonisins analysis, aliquots (5 g) of finely ground sample was extracted with 40 mL of water/methanol, 30:70 v/v, by shaking with a high speed blender (Ultraturrax T18, IKA, Stauffen, Germany) for 3 min at 14000 rpm. After filtration through Whatman no. 4 filter papers, 1 mL of extract was analyzed by LC-MS/MS.

For total fumonisins analysis, 2,5 g of finely ground sample were blended in a high speed blender (Ultraturrax T18, IKA, Stauffen, Germany) with 50 mL of 2 M KOH for 2 min at 14000 rpm and stirred for 60 min (20°C, 650 rpm). Then, 50 mL of acetonitrile was added, and after homogenization with Ultraturrax T18 (IKA, Stauffen, Germany) for 5 min at 14000 rpm, the two layers were separated by centrifugation at 3500 rpm for 15 min. The extract (4 ml) was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in 400 µL of water/methanol (30:70 v/v) and analyzed by LC-

MS/MS. Fumonisinins obtained after sample hydrolysis were measured as the sum of hydrolyzed fumonisins B1, B2 and B3. All of the results are expressed as the sum of FB1, FB2 and FB3 equivalents, considering a correction factor due to the different molecular weight of parent and hydrolyzed compounds and referred to as “total fumonisins”. Modified fumonisins were calculated as the difference between total fumonisins and free fumonisins.

2.3.6 LC-MS/MS analysis of free and total fumonisins

Free and hydrolyzed fumonisins were detected by LC-MS/MS analysis. For each sample, the entire procedure was performed in triplicate ($n=3$). Validation experiments were based on the analysis of spiked samples already measured as a blank for both free and modified fumonisins. The spiking experiments were performed at six concentration levels in the range 25-5000 $\mu\text{g}/\text{kg}$. For the total fumonisin determination, the spiked samples were previously submitted to hydrolysis, and the hydrolyzed forms were then determined. Recovery was found to be 93% for FB1 and FB2, 88% for FB3, 91% for HFB1, and 88% for HFB2 and HFB3. Repeatability (six determinations at three spiking levels) was found to be in the range of 6-9% for free fumonisins and 8-11% for hydrolyzed fumonisins. The limit of quantification (LOQ) was 25 $\mu\text{g}/\text{kg}$ for both free and hydrolyzed fumonisins.

Limits of detection (LOD) were found to be lower than 10 $\mu\text{g}/\text{kg}$ for all of the considered analytes. All of the results were corrected for recovery. Samples showing contamination levels higher than the highest calibration level were diluted to match the proper calibration range.

LC-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, U.K.) according to Falavigna et al. [16]. Chromatographic conditions were the following: the column was a Synergy Fusion RP50 x 2 mm (Phenomenex); the flow rate was 0.2 mL/min; the column temperature was set at 30°C; the injection volume was 10 μL ; gradient elution was performed using bidistilled water (eluent A) and methanol (eluent B) both acidified with 0.2% formic acid: initial condition at 70% A, 0-1 min isocratic step, 1-3 min linear gradient to 65% B, 3-

15min linear gradient to 90% B, 15-18 min isocratic step at 90% B, 18-19 min linear gradient to 70% A, and re-equilibration step at 70% A for 9 min (total analysis time: 28 min). MS parameters: ESI + (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 50 V; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow, 50 L/h and 700 L/h, respectively. Detection was performed using a multiple reaction monitoring (MRM) mode by monitoring two transitions for each analyte, as follows: 722.4→334.4 (CE 40 eV), 187 722.4→352.4 (CE 40 eV) for FB1, 706.4→336.4 and 706.4→318.4 (CE 35 eV) for FB2 and FB3, 188 406.5→370.5 and 406.5→388.5 (CE 20 eV) for HFB1, 390.5→336.4 and 390.5→372.5 (CE 20 eV) for HFB1 and HFB3. The first transition reported was used for quantification, while the second transition was chosen as qualifier.

2.4 Results

The contemporary presence of free and modified fumonisins was investigated in all the collected samples by LC-MS/MS. The method allowed to screen the co-occurrence of free fumonisins (FB1, FB2 and FB3) and their hydrolysed forms (HFB1, HFB2, HFB3), which may arise from cornmeal processing. In addition, total fumonisins, obtained upon alkaline hydrolysis of the sample and expressed as FBs equivalents, were also determined. Statistical analysis was performed using SPSS v. 17.0 (SPSS Italia, Bologna, Italy). Data were statistically compared using OneWay-ANOVA test ($\alpha=0.05$). The results obtained for free fumonisins for each sampled fraction are reported in Table 1.

Table 1. Results obtained for free fumonisins determination in the cornmeal processing samples.

Samples	FB1+FB2+FB3	Samples	FB1+FB2+FB3
	µg/kg d.m.*		µg/kg d.m.*
Unprocessed corn	981 ± 305 ^b	Cooled cornflakes	929 ± 372 ^b
Cleaned corn	926 ± 267 ^b	Flour “fumetto”	1382 ± 103 ^b
Uncooked broken corn	1006 ± 108 ^b	Cornmeal semolina	584 ± 162 ^c
Cooked broken corn	1123 ± 432 ^b	Germ	936 ± 206 ^b
Cornflakes	1107 ± 644 ^b	Middlings	6168 ± 238 ^a

*All the results were calibrated on a dry matter (d.m.) basis. Significant differences are highlighted by different letters (One-way ANOVA, Tukey’s post hoc test).

The raw material (unprocessed corn) was compliant with the EU legislation, being the FB1+FB2 amount 772 µg/Kg. In order to better allow comparison between samples collected along the production chain, all the results are reported on dry matter.

From the obtained data, the cleaning step did not affect FBs concentration, that remained almost stable passing from unprocessed corn (981 ± 301 µg/kg) to cleaned corn (926 ± 267 µg/kg). These results are in contrast to those obtained by Fandohan et al. [20], who demonstrated that cleaning of raw maize brought to a loss in fumonisin content of about 45 %. Otherwise, it should underlined that an unprocessed raw material with a relatively low contamination was used for this study, while Fandohan et al. [20] reported the cleaning effect obtained on maize kernels at higher contamination level (2890 µg/kg).

Similarly, also the other considered processing steps did not seem to significantly affect the FBs content. Among the considered fractions, the higher fumonisin content was found in middlings (6168 µg/kg). Statistical analysis showed a significant difference between middlings and unprocessed corn ($p < 0.01$), while germ was not significantly different.

A significant decrease in contamination ($p < 0.01$) was found in cornmeal semolina compared to unprocessed corn, being FBs amount in the former 584 µg/kg. To better

evaluate the percentage of FBs reduction during the pre-cooked cornmeal production, data were plotted on a percentage scale (Figure 2).

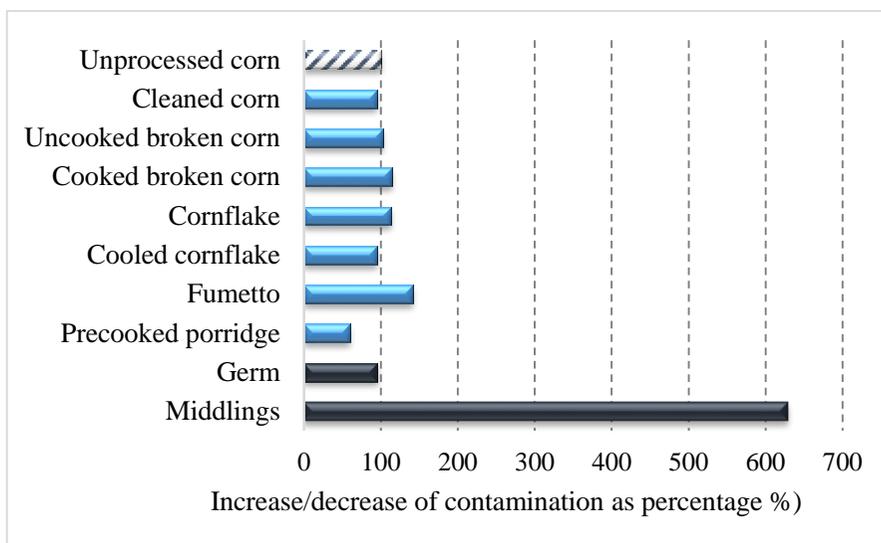


Figure 2. FBs contamination reduction/increase in samples collected from the processing chain compared to unprocessed corn. Data are expressed in percentage.

From the data, a significant decrease of 40% in FBs contamination was obtained in the final products (cornmeal semolina). On the other hand, a very significant increase in FBs contamination (about 6 fold compared to the unprocessed material) was observed in middlings, commonly intended for feed production. Similar results were already obtained by Castells et al. [21] and by Vanara et al. [13]. They demonstrated that the greatest fumonisin quantities were detected in corn flours destined to animal consumption and that this fumonisin distribution was probably due to a sort of inverse correlation between corn particle size and fumonisin contents [22]. In this study, the germ had the same fumonisin content as the unprocessed raw material according to the Italian study conducted by Vanara et al. [13]. Contamination trend found in these by-products was similar to those found in other studies [10,12,14,22], in which an increase of fumonisin concentration in bran, germ and fractions intended for animal feed and a decrease in products intended for human consumption were reported. Pietri et al. [14] noted that the highest fraction of FB1 remained in the undersized flour and conveyed into animal feed flour, concluding that contamination increased in lower particle size final products [10].

However, it should be noticed that all the analysed samples intended for human consumption showed levels of fumonisin contamination in compliance with the legal limits reported in the EC Regulation No 1126/2007.

The presence of modified fumonisins, covalently and non-covalently bound, was investigated in all considered samples. Total fumonisin fraction was obtained by an alkaline hydrolysis of the matrix and the amount of modified fumonisin was calculated as difference considering free fumonisin content. According to our results, any significant differences were found between total and free fumonisins in the samples considered within this study. Modified fumonisins in corn-based products can be due to the sum of non-covalent forms, already present in the plant [19], and covalent conjugates that might form during processing [15]. As recently reported, both the environmental and genetical factors play a significant role in the fumonisins masking rate in plants [19]. Since non-covalently modified fumonisins did not occur in the raw material used within this study and the free FBs concentration was low, it is reasonable that the any significant difference arose during the processing. Furthermore, the condition applied herein during processing were not suitable to favor any degradation or side reaction involving fumonisins, thus preventing the formation of FBs conjugates. As reported by the literature, significant FBs degradation was obtained during the production of fried tortilla chips, where hard thermal and extrusion conditions were applied [23]. Otherwise, an addition of 10% of glucose led to a decrease in FB1 content of 75-85% with a contemporary formation of N-(deoxy-D-fructos-1-yl)fumonisin B1 (NDF) levels after extrusion, also at milder conditions. Since the process applied within this study did not present neither high temperature nor the addition of ingredients such as sugars, it could be concluded that the formation of FB conjugates under the applied conditions is unlikely.

2.5 Conclusions

In conclusion, this is the first study specifically focused on the fate of fumonisins along the complete industrial cornmeal production process.

All the analyzed samples intended for human consumption showed levels of fumonisin contamination below the European legal limits and no modified forms were detected, probably due to several factors as hybrid selection, low contamination or advantageous conditions of growth. Findings showed also a significant reduction in fumonisin content from the unprocessed corn to cornmeal semolina (this one in particular was significantly lower than the other fractions achieving 40% of reduction), mainly due to the cooking treatments.

At the same time, results obtained for by-products, especially middlings, showed a very high fumonisins increase, thus pointing out the necessity to carefully manage the use of these by-products as feed for livestock.

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CHAPTER 3. THERMAL STABILITY OF DEOXYNIVALENOL AND DEOXYNIVALENOL-3-GLUCOSIDE DURING BREAD-BAKING PROCESS

3.1 Introduction

Bread is a fundamental source of carbohydrates, proteins, and vitamins. In the last decade, the fate of mycotoxins through bread-making process has been hardly studied, and a huge variability in observed results has been reported. In particular, Vidal et al. [1] studied the fate of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-Glc) during the bread making: DON increased from the unkneced mix to fermented dough and decreased during baking, depending on the initial concentration in the flour. These observations were also in agreement with other previous studies in which DON decreased up to 25% in the baked bread. Baking time seemed to have a much important effect than baking temperatures on DON stability because the maximum temperature reached in the centre of the crumb was independent of the oven temperature. DON-3-Glc content increased both during kneading and fermentation as suggested by Zachariasova et al. [2], and also during baking, most likely due to the glycosylation of DON in the initial stages of baking before enzyme inactivation, as also confirmed in a following study by Vidal et al. [1]. These results are in contrast with previous investigations conducted by Kostelanska et al. [3] and Simsek et al. [4]. Kostelanska et al. [3] noted that when so-called bread improver enzyme mixtures were employed as a dough ingredient, a distinct increase (up to 145%) of conjugated DON-3-Glc occurred in fermented dough, whereas some decrease in both DON-3-Glc and DON (10% and 13%, respectively, compared to fermented dough, and mainly in the crust) took place during baking. A Chinese study conducted by Zhang et al. [5] on steamed bread processing reported a decrease of DON-3-Glc amount and an increase of DON content after dough making and steaming, respectively, confirming these results in a further study conducted by the same authors [6]. A similar trend was observed either in the past [7] or very recently in the study of Simsek et al. [4]: DON levels detected during the fermentation stage were significantly higher in the mixed dough, and the baked bread had less DON-3-Glc detected than the dough. DON detection levels were higher after treatment with protease (16%) and

xylanase (39%) compared to the wheat composite: the significant increases in apparent xylanase activity could cause the hydrolysis of cell wall material in the dough, resulting in a release of the bound DON and an increase in the DON concentration in the baking process samples.

The same DON trend observed by Vidal et al. [1] during bread-making was already found by Bergamini et al. [8], studying how DON concentration may be influenced by modifying bread-making parameters, with a special emphasis on the fermentation and baking stages, exploiting the power of a Design of Experiments (DoE) approach to consider only those modifications that can really be applied on the industrial scale to obtain a final product that can be considered acceptable by consumers. The authors supposed that the DON increase during fermentation could be due to the enzymatic release of native DON from some bound forms occurring in the raw material. Studies at fermentation temperatures higher than 30 °C reported a reduction in DON concentrations from kneaded dough to fermented dough [9].

A similar approach was employed by Suman et al. [10] at the pilot plant/industrial scale to assess the effects of the changes in five technological parameters (fermentation time and temperature, baking time and temperature, and presence of sodium bicarbonate) during fermentation and baking on the concentration of DON in crackers. The results showed that the evolution of the toxin was significantly influenced by the baking temperature and time, while the other parameters seemed to have a smaller influence, most likely because of possible thermal degradation or rebinding with matrix constituents. The presence and the content of sodium bicarbonate suggested an opposite impact on DON concentration, due to a probable effect related to the pH conditions in the dough, which could modify the reactivity of the bound forms of the toxin.

3.2 Aim of the work

The objective of the assay presented herein was to assess the effect of dough fermentation and baking on DON and DON-3-Glc content during bread-making process. Starting from a naturally contaminated wheat flour, the process was simulated in a pilot-plant scale according to internal technological knowledge. Samples have been analyzed by UPLC-MS/MS to evaluate mycotoxin evolution throughout technological phases.

3.3 Experimental section

3.3.1 Chemicals

Sodium acetate, formic acid, methanol, hexane and acetonitrile were purchased from Sigma-Aldrich (Taufkirchen, Germany). All solvents were HPLC grade. Ultra-pure water was in-house produced by using a Milli-Q System (Millipore, Bedford, MA, USA). The following mycotoxin standard solutions were purchased from RomerLabs (Tulln, Austria): 13C-Deoxynivalenol (internal standard, 25 mg/L in acetonitrile), DON (10 mg/L in acetonitrile), DON-3-Glc (50 mg/L in acetonitrile). All standard solutions were stored at $-20\text{ }^{\circ}\text{C}$ and brought to room temperature before use. A working solution (2.5 $\mu\text{g}/\text{mL}$) containing all the target mycotoxins was prepared in water/methanol (80/20, v/v) by combining and properly diluting suitable aliquots of each standard.

3.3.2 Moisture content determination

The moisture contents of the samples were measured by taking 5 g samples and heating them in a thermostatic oven at $105\text{ }^{\circ}\text{C}$ for over 12 h. Through this further collected information, all the results were calibrated and compared on a dry matter (d.m.) basis.

3.3.3 Dough preparation and baking

For the bread experiment, a batch of wheat flour naturally infected with *Fusarium* spp. was analysed with a focus on DON and DON-3-Glc contamination levels and selected according to the technological characteristics for bread-making.

Different doughs were prepared in order to obtain a final loaf of about 120 g; the ingredients were wheat flour (62%), water (32%), yeasts (*Saccharomyces cerevisiae*), fractionated palm oil, sugar and salt (6% overall). The process consists substantially of the following steps: firstly, flour is mixed with all solid powders ingredients (2 min) then moistened with salt suspended in water and oil (3 min). Dough is mixed with a baking test kneader. The optimal amount of water for each flour as well as reference baking conditions are established on the basis of previous experiments and internal technological knowledge. The overall breadmaking process is summarized in Figure 1.

Two identical doughs were used for the experiment: mycotoxins were analysed in the starting raw material, before and after the fermentation step and after baking. Concerning the pilot-plant, the fermentation step was performed in a leaven cell (Fermalievita, AL, USA) at a relative humidity (RU, %) of 70% for 56 min at 32 °C, while the baking step was performed in a dynamic pilot-scale oven (Pollin, Verona, Italy) for 22 min at 210 °C. Before analysis, samples were stored at -20°C. Each sample was extracted in duplicate and each extract was measured in duplicate.

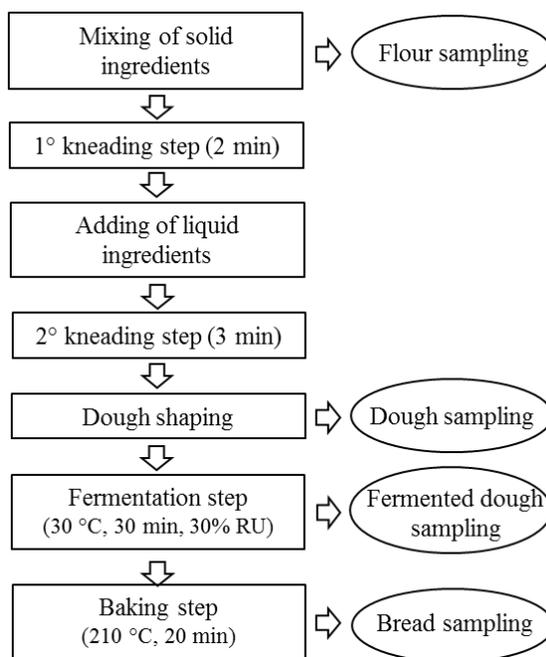


Figure 1. Scheme of the bread-making process along pilot-plant line.

3.3.4 Sample extraction and analysis

Samples were prepared according to Berthiller et al. [11] with slight modifications. Briefly, 0.5 g of ground sample was extracted for 90 min at 200 strokes/min on a shaker with 2 mL of acetonitrile/water (80/20, v/v) acidified with 0.1% of formic acid. The extract was collected and centrifuged for 10 min at 14000 rpm at room temperature and 1 mL of the supernatant was evaporated to dryness under nitrogen. After addition of the internal standard (13C-DON, 20 µL, obtaining a final concentration of 100 µg/kg), the

residue was dissolved in 1 mL of water/methanol (80/20, v/v) and analyzed by UPLC-MS/MS.

The UPLC-ESI/MS analyses were carried out according to Dall'Asta et al. [12], using a Dionex Ultimate® 3000 UHPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a triple quadrupole TSQ Vantage MS/MS system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chromatographic separation was performed at 40 °C on a Kinetex® C18-column, 100 x 2.1 mm i.d., 2.6 µm particle size (Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases were acidified with 0.2% of acetic acid and they were water 5mM ammonium acetate (eluent A) and methanol (eluent B). After an initial time of 1 min at 2% B, the proportion of B was increased linearly to 20% within 2 min. Further linear increase of B to 90% within 17 min was followed by an isocratic step of 3 min at 90% B and a linear decrease of B to 2% within 1 min. A re-equilibration step at 2% for 9 min was observed (total analysis time: 30 min). The flow rate was 0.35 mL/min. ESI-MS/MS was performed in positive ionization mode (ESI+) with the following settings: capillary temperature, 270 °C; spray voltage, 3500 kV; sheat gas pressure, 50 units; auxiliary gas pressure, 5 units. Detection was performed using selected reaction monitoring mode (SRM). The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standard (diluted in a 1:1 mixture of eluent A and B) into the MS source at a flow rate of 5 µL/min for the corresponding values.

3.3.5 Method performance

The analytical methods used were assessed for linearity, precision and recovery. Calibration curves were generated by linear regression of peak areas against concentrations ($r^2=0.99$ for DON and DON-3-Glc, respectively). Precision was established by determining DON and DON-3-Glc levels in flour, dough and bread samples in triplicate, fortified at three concentration levels (500 µg/kg, 1000 µg/kg and 2000 µg/kg) in order to calculate the recovery rates. Method performance characteristics are summarized in Table 1.

Table 1. Method performance for deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-Glc) in the analyzed matrices.

Mycotoxins	Product	n	Spiking level (µg/kg)	Recovery (%)^a
DON	Flour	3	500	101 ± 5
		3	1000	93 ± 8
		3	2000	108 ± 7
	Dough	3	500	95 ± 11
		3	1000	87 ± 3
		3	2000	106 ± 16
	Bread	3	500	120 ± 29
		3	1000	129 ± 12
		3	2000	101 ± 3
DON-3-Glc	Flour	3	500	75 ± 11
		3	1000	90 ± 5
		3	2000	100 ± 3
	Dough	3	500	87 ± 6
		3	1000	65 ± 13
		3	2000	92 ± 5
	Bread	3	500	98 ± 14
		3	1000	102 ± 6
		3	2000	101 ± 5

^aMean value ± standard deviation.

3.3.6 Statistics

Multifactorial ANOVA was applied to assess the significance of sample traits in the observed mycotoxin concentration levels as well as in the calculated percentage of increase/reduction at a $p < 0.05$. The results obtained were processed in two different ways: (a) DON and DON-3-Glc content in each step in dry matter basis (d.m.); (b) concentration in wet basis (w.b.). In the first case it was possible to assess the real impact of the processing steps in the mycotoxin reduction.

3.4 Results

3.4.1 Effect of fermentation and baking on DON and DON-3-Glc content

Average DON content in the initial starting flour and kneaded dough was different (5734 $\mu\text{g}/\text{kg}$ and 4825 $\mu\text{g}/\text{kg}$ for the overall mean, respectively), and a percentage of reduction of about 16% was calculated (Table 2). Similar results were reported in other investigations where no increase in DON content was observed with respect to the initial flour [8,13]. At the same time, DON-3-Glc content seemed to be significantly higher after kneading, recording an increase of 48%. This result is in contrast with what observed in analogous study that usually reported a decrease in content especially due to the effect of dilution of recipe formulation. Data could not be explicable by possible interferences of the matrix on the extraction capability of mycotoxin or with other components related to ingredients into the matrix. The heterogeneous contamination of the raw material could be an explanation of the increase due to a not homogeneous mycotoxin distribution and a consequent non-representative sampling during kneading step. Concerning fermentation stage, DON and DON-3-Glc were not significantly different ($p > 0.05$) with respect to data reported for the unfermented dough, according to previous studies where no changes or reduction in DON content were observed, especially when no improvers were used [3]. A small reduction was described when fermentation temperatures were higher than 30 °C [9]. By contrast, other published investigations on the effect of fermentation on DON content generally showed an increase when enzymes were used as flour improvers [1,4]. Such release might be attributed to both enzymes contained in the improver and to

the metabolism of bacteria which may be able to either transfer DON precursor into DON or release bond DON [14].

Table 2. Evolution of mycotoxins in the different step of bread-making process. Data are expressed on dry matter (d.m.) basis.

Mycotoxin	Flour ($\mu\text{g/kg}$)*	Dough ($\mu\text{g/kg}$)*	Fermented dough ($\mu\text{g/kg}$)*	Bread ($\mu\text{g/kg}$)*
DON	5734 \pm 79	4825 \pm 59	4689 \pm 40	4349 \pm 387
DON-3-Glc	258 \pm 39	385 \pm 7	343 \pm 11	295 \pm 7

*Data are expressed as mean value \pm standard deviation.

Concerning the baking step, a reduction of 8% and 14% from the fermented dough was reported for DON and DON-3-Glc (Figure 2), respectively, as observed by other researchers [3,4]. On the other hand, Vidal et al. [1] showed a significant increase in DON-3-Glc content in the finished product, highlighting the need of further investigation because of little knowledge exists in the toxicological effects of this metabolite.

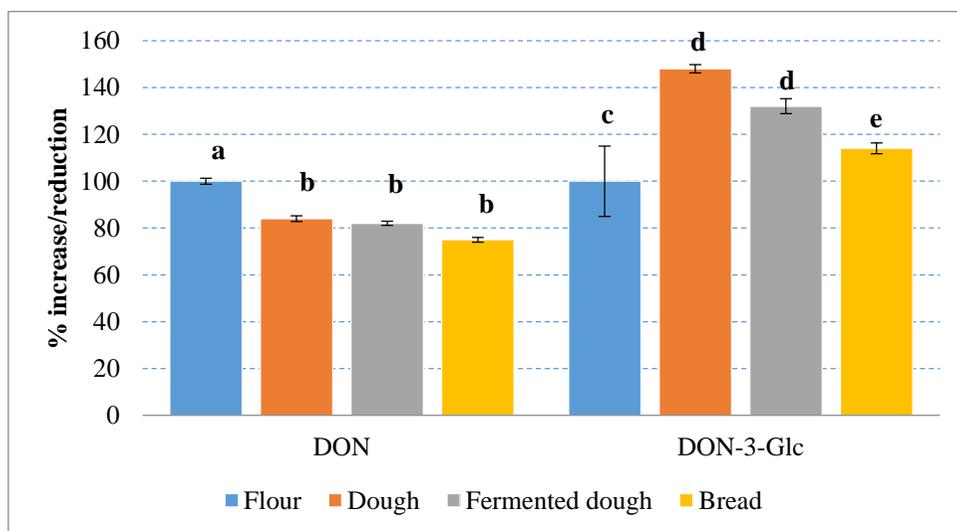


Figure 2. Percentage of increase/reduction in mean DON and DON-3-Glc content due to bread-making process on dry matter basis. If the letter on a bar is different from the letter on another bar, there is a significant difference among samples ($p < 0.05$).

Contradictory reports exist regarding the fate of DON during the baking step. Most studies described a DON reduction, while others showed an increase in DON concentration after baking [13,15,16]. The extent of reduction could be significantly different because of the great variability of factors that can affect the final mycotoxin content, leading to such contradictory outputs. Time/temperature setting and bread sized may play a fundamental role in the final reduction: higher reduction has been observed in small size loaves (40-80 g) with respect to bigger ones where temperature recorded in the inner part of the dough was usually lower than 98 °C. Moreover, contradictory results could be attributed to the use of mycotoxin-spiked products instead of naturally contaminated raw materials, with lower effects of baking in the latest ones due to effective matrix interferences.

3.4.2 DON and DON-3-Glc concentrations on wet basis

DON and DON-3-Glc concentrations in dough and baked bread depend on the “dilution factor” of the toxins with the addition of other ingredients, which depends on the recipe formulation, and finally on the stability of the toxins through the process. Concerning DON, the results suggest that from the contaminated flour it is possible to produce bread with a significantly lower contamination (42%), while the calculation on dry matter (d.m.) basis leads to 25% reduction (Figure 3). This reduction seems to be strictly correlated to the dilution effect of the recipe encountered during kneading of the ingredients. At the same time, DON-3-Glc level in the fermented dough was not significantly different to that in the flour suggesting that the dilution effect was balanced by DON-3-Glc increase during kneading due to enzymatic effect. Data showed a minor impact of the baking step from the fermented dough for DON and DON-3-Glc levels with respect to those expressed on dry matter (d.m.) basis: the lower percentages of reduction may be attributed to the evaporation of water and other dough components in the oven, with a concentration of the toxins as consequence.

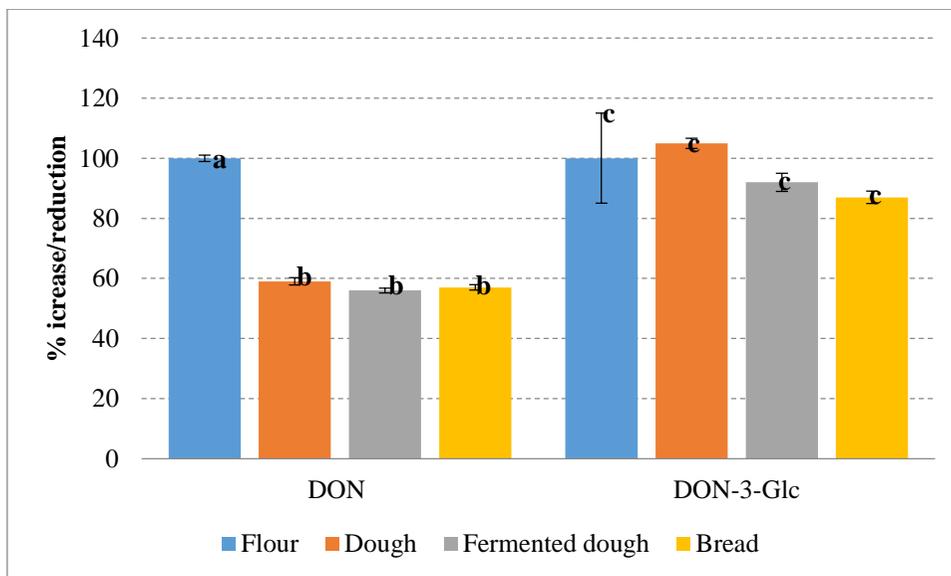


Figure 3. Percentage of increase/reduction in mean DON and DON-3-Glc content due to bread-making process and “dilution factor” of recipe formulation on wet basis. If the letter on a bar is different from the letter on another bar, there is a significant difference among samples ($p < 0.05$).

3.5 Conclusions

As conclusions, DON and DON-3-Glc levels can be controlled during bread-making process, focusing on time/temperature parameters, recipe formulation, and size of the products. DON and DON-3-Glc concentrations were affected by kneading step in different ways; in particular, the results on DON-3-Glc suggest the need of further investigations because of the marked release observed in this specific study.

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CHAPTER 4. DEGRADATION STUDY OF ENNIATIN B DURING BREAD-MAKING PROCESS

4.1 Introduction

Mycotoxin contamination represents a global issue for agriculture as for food and feed industry. Starting from the field up to the finished product, toxic compounds can affect food and feed safety, compromising human and animal health. Beside the well-known mycotoxins, regulated by European legislation [1,2], as deoxynivalenol (DON), zearalenone (ZEA), aflatoxins (AFs), fumonisins (FBs) and ochratoxin A (OTA), in the last decades a remarkable interest has been arisen upon the so called “*emerging*” mycotoxins. Different *Fusarium* species as *F. avenaceum*, *F. moniliforme*, or *F. proliferatum* [3] are able to produce these minor compounds, in particular they are responsible for enniatins (ENNs) production. The occurrence of ENNs has been widely reported in wheat, barley and maize, but they have been also found in oats and rye although less frequently at contamination level up to hundred mg/kg [4-6]. They have been also detected in processed products, such as bread, pasta, biscuits and breakfast cereals [7].

ENNs represent a group of cyclic hexadepsipeptides and the most reported are four ENNs (ENN A, ENN A1, ENN B and ENN B1): ENN B seems to be the most dominating toxin in different studies [8,9]. Basically, the contamination levels are higher in samples collected in warmer and temperate areas of central and south-eastern Europe than in central and northern regions, due to the different climatic conditions [10,11]. Despite the limited availability of *in vitro* and *in vivo* toxicological data, ENNs might pose a potential risk for animal and human health: they are found to be toxic to cell lines of human origin such as hepatocellular carcinoma-line Hep G2 and fibroblast-like fetal cell line MRC-5 [12,13]. Furthermore, *in vivo* studies with ENN B have shown that this compound is absorbed after oral and pulmonary application, showing an acute LC50 of 21 mg/ml in shrimps [14].

Although several studies have been published in relation to food processing and the most-known mycotoxins in order to evaluate their evolution, only few data are available on thermal degradation of ENNs. Because of the widespread occurrence of these metabolites

in cereals as in cereal-based foods, a better investigation about their fate along different production chains is needed, in order to assess their potential risk on the consumers. Vaclavikova et al. [15] determined ENNs levels during beer and bread production, observing that during bread-baking ENNs decreased compared to their concentration in the initial flour, whereas these mycotoxins were not detected in the final beer. A similar study upon bread making with sourdough was conducted by Hu et al. [16], by using wheat and rye as raw materials: after milling of grains, approximately 70-82% of ENNs were found in the bran fraction, while overall 25-41% of ENNs were reduced during the whole sourdough bread-making process. Serrano et al. [17] evaluated ENNs thermal degradation by simulation of pasta cooking, observing high percentages of reduction (81-100%).

4.2 Aim of the work

Because of the considerable importance of bread as a diet-based food in many regions all over the world and the lack of data related to ENNs evolution, in particular ENN B, upon thermal treatment, the aim of the current study is to evaluate its reduction during bread-making process. For this purpose, pilot-plant trials were set to simulate an industrial production.

4.3 Experimental section

4.3.1 Chemicals

Methanol and acetonitrile (both LC gradient grade) were purchased from Sigma (Stuttgart, Germany). Formic acid (99%) was obtained from Acros Organics (New Jersey, United States). Bidistilled water was in house produced using an Alpha-Q System (Millipore, Marlborough, MA, USA). ENN B was purchased from Sigma (Stuttgart, Germany). Standard solution was prepared dissolving 1 ml of analyte in 1 ml of methanol, obtaining a stock solution of 1000 mg/l. The stock solutions were then diluted with pure methanol in order to obtain the appropriate working solutions. Solutions were stored at -20 °C and were brought to room temperature before use.

4.3.2 Raw material

Inoculation experiments of a blank of commercial flour were performed with an ENNs producing strain of *Fusarium tricinctum* [18]. *Fusarium tricinctum* strain (CECT 20150) was obtained from the Spanish Type Culture (CECT Valencia, Spain). A suspension of conidia in concentration of 10⁶ conidia/mL of *Fusarium tricinctum* in PDB, was used for inoculation. Conidial concentration was measured by optical density at 600 nm. For fermentation experiments, 250 g of flour were inoculated with macronidia suspensions of *Fusarium tricinctum* and incubated during 42 days at 25°C on an orbital shaker (IKA Ks 260 basic, Stanfen, Germany). Then, the contaminated flour was autoclaved at 121°C for 15 min in a Presoclave II-75 autoclave (P Selecta, Barcelona, Spain) to promote fungi inactivation. Finally, the autoclaved semolina was dried at 100°C to reach 15% of moisture content.

In order to obtain two different levels of contaminated flours to exploit for bread-making experiments focused on ENN B content, the inoculated sample was properly homogenized with a not-contaminated wheat flour. Because the starting flour was highly contaminated with ENN B (198 mg/kg), 7,5 g and 22,5 g of inoculated flour, respectively, was added to 1000 g of blank wheat flour. ENN B was 1584 ± 95 µg/kg and 5149 ± 186 µg/kg in each flour, respectively, representing a medium-to-high range of contamination.

4.3.3 Moisture content determination

The moisture contents of samples from the bread-making process (flour, dough and bread) were measured by taking a 5 g ground sample and heating it in a thermostatic oven at 105 °C for over 6 h. All the results were calibrated and compared on a dry matter (d.m.) basis.

4.3.4 Dough preparation and bread-making

Different doughs were prepared throughout a pilot-plant line starting from the selected contaminated wheat flours for obtaining a final loaf of about 800 g; the ingredients were wheat flour (62%), water (32%), yeasts (*Saccharomyces cerevisiae*), sugar, oil, and salt (6% overall). Concerning the high-contaminated flour (5149 ± 186 µg/kg of ENN B), another dough was prepared according to the same recipe formulation but replacing

yeasts by sodium bicarbonate in order to evaluate the potential effects of the two agents and to better understand the origin of the potential changes (pH value impact and/or yeast effect) on the mycotoxin stability/degradation. The process consists substantially of the following steps: firstly, wheat flour was mixed with all solid powders ingredients (2 min) then moistened with salt suspended in water and oil (3 min). Dough was mixed using a test planetary kneader and, after that, fermented in a leavening cell (FermaLievita Alaska, Bologna, Italy) for 60 min at 30 °C at a relative humidity (RU, %) of 85%. Baking was performed in a pilot-scale dynamic oven (Tagliavini, Parma, Italy) at 210 °C for 20 min. The overall bread-making process is summarized in Figure 1. Two identical doughs were used for each single experiment: one was analysed after the fermentation step; the other one was analysed for after the baking process. Before analysis, samples were stored at -20 °C. Each sample was extracted in duplicate and each extract was measured in duplicate.

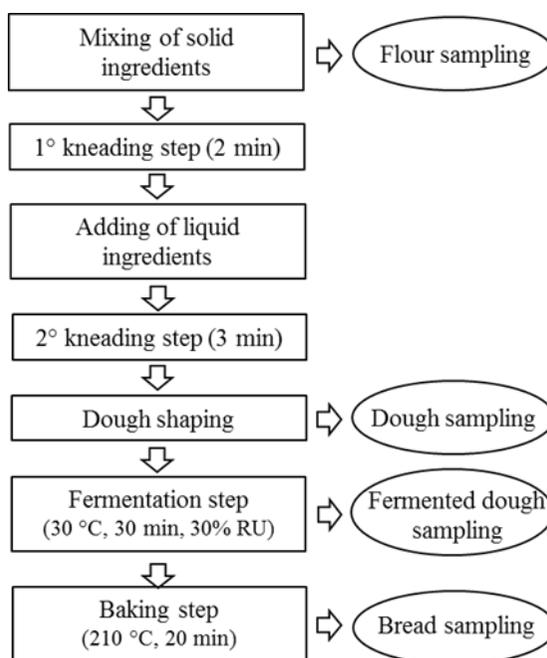


Figure 1. Scheme of the bread-making process along pilot-plant line.

4.3.5 Extraction procedure

Sample preparation was carried out according to Malachova et al. [19] with slight modifications. In brief, 5.00 ± 0.01 g of the ground samples were extracted with 20 ml acetonitrile/water/acetic acid (79/20/1, v/v/v) for 90 min using a Stuart SSL2 reciprocating shaker (Bibby Scientific Limited, Stone, United Kingdom) and subsequently, centrifuged for 15 min at 4000 rpm on a 5810R centrifuge (Eppendorf AG, Hamburg, Germany). Then, the extract was transferred into glass vials using Pasteur pipettes, and a 350 μ l aliquot was diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20/79/1, v/v/v). After appropriate mixing, 2 μ l of the diluted extract were injected into the UHPLC-MS/MS system without further pre-treatment.

4.3.6 Instrumental conditions

Detection and quantification was performed with a Dionex Ultimate® 3000 UHPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a triple quadrupole TSQ Vantage MS/MS system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chromatographic separation was performed at 40 °C on a Kinetex® C18-column, 100 x 2.1 mm i.d., 2.6 μ m particle size (Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases were acidified with 0.2% of formic acid and they were water (eluent A) and methanol (eluent B). After an initial time of 1 min at 30% B, the proportion of B was increased linearly to 65% within 8 min. Further linear increase of B to 90% within 20 min was followed by an isocratic step of 2 min at 90% B and a linear decrease of B to 30% within 23 min. A re-equilibration step at 30% for 7 min was observed (total analysis time: 30 min). The flow rate was 0.35 mL/min. ESI-MS/MS was performed in positive ionization mode (ESI+) with the following settings: spray voltage, 3000 V; capillary temperature, 270 °C; desolvation temperature, 200 °C; collision gas pressure, 80 psi. Table 1 summarizes the parameters of the optimized transitions.

Table 1. Optimized ESI-MS/MS conditions.

Compounds	Precursor ion (<i>m/z</i>)	Product ions (<i>m/z</i>)	Collision energy (V)
Enniatin B	640.6 [M+H] ⁺	86.0 ^b	49
		186.2 ^b	35
		196.2 ^a	23
		214.2 ^a	23
		314.0 ^b	31
^a quantifier ion; ^b qualifier ion		527.9 ^b	20

4.3.7 Statistical analysis

Data processing from the analysis of each sample type was performed by Excalibur software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Descriptive statistics and ANOVA analysis ($\alpha=0.05$) were performed by SPSS v.19 (IBM Italia, Milano, Italy).

4.4 Results

4.4.1 Method performance

The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standard (diluted in a 1:1 mixture of eluent A and B) into the MS source at a flow rate of 5 $\mu\text{L}/\text{min}$ for the corresponding values. Retention time for ENN B is 12.59 min. The mean limit of detection (LOD) and limit of quantification (LOQ) for the tested matrices are 13 $\mu\text{g}/\text{kg}$ and 22 $\mu\text{g}/\text{kg}$, respectively.

Performance characteristics of the method were verified for the studied matrices (flour, dough and bread) prior to analysis. Spiking experiments were carried out at three different concentration levels: 500 $\mu\text{g}/\text{kg}$, 1500 $\mu\text{g}/\text{kg}$ and 4500 $\mu\text{g}/\text{kg}$ (each in five repetitions). Concentration range of the spiked sample was chosen to cover the LOD of the toxin, the estimated linear range of calibration and the levels employed in the performed experiments. External calibration was prepared by dilution of appropriate amounts of the final working solution at levels corresponding to those employed in spiked samples. Taking into account the matrix induced signal enhancement or suppression, two points

were added to ensure that all spiking levels fall into the calibration range. Method performance characteristics for ENN B are summarized in Table 2.

Table 2. Method performance characteristics for ENN B in the analyzed matrices.

Mycotoxin	Product	<i>n</i>	Spiking level (µg/kg)	Recovery (%)
Enniatin B	Flour	3	500	104 ± 8
		3	1500	110 ± 16
		3	4500	101 ± 7
	Dough	3	500	108 ± 15
		3	1500	110 ± 3
		3	4500	99 ± 7
	Bread	3	500	115 ± 18
		3	1500	105 ± 29
		3	4500	96 ± 3

4.4.2 ENN B behavior during bread-making process

Two different levels of contamination (medium-to-high) were evaluated when dough was prepared with yeasts (*S. cerevisiae*). ENN B changes during different stages of bread-making are summarized in Table 3. A different trend was observed depending on the starting contamination level of the raw material: for the lower level, no significant ($p < 0.05$) changes of ENN B occurred during kneading, according to what reported in an analogous study where a maximum reduction of 4% has been described [16]; on the other hand, a 25% reduction was measured in the high contaminated dough (Figure 2). A similar inexplicable evolution was showed after fermentation step where an opposite behavior has been recorded: a significant decrease of about 48% has been reported in medium contaminated dough, whereas a 21% increase has been revealed in the higher one. These particular modifications have also been reported in a study on ENNs during bread-baking by Vaclavikoka et al. [15]: ENN B was first reduced significantly after fermentation of 1 h at 30 °C and then increased significantly after proofing for 1 h at the same temperature. Hu et al. [16] recorded a difference between rye sourdough breads after proofing conducted at different temperatures (30°C and 50°C, respectively): at

lower temperature ENN B was reduced by 13-19% compared to the original level in the starting raw material, whereas at 50°C proofing reduced 16,5-28% of the initial contamination level, suggesting that higher fermentation temperature was more effective in ENN B reduction.

Table 3. Changes of ENN B during bread-making process (medium vs high contamination level).

Enniatin B	Medium level		High level	
	µg/kg ^a	Recovered (%) ^b	µg/kg ^a	Recovered (%) ^b
Flour	1584± 113	100.0 a	5194 ± 377	100.0 a
Kneaded dough	1589 ± 131	100.8 a	3907 ± 325	75.2 b
Fermented dough	824 ± 73	52.0 b	4739 ± 362	91.2 c
Bread	1054 ± 95	66.4 b	4785 ± 404	92.1 c

Values of different letters in the same column are significantly different ($p < 0.05$).

^aData are expressed as mean value ± standard deviation on dry matter (d.m.) basis; ^bPercentage of recovered amount with respect to the starting wheat flour.

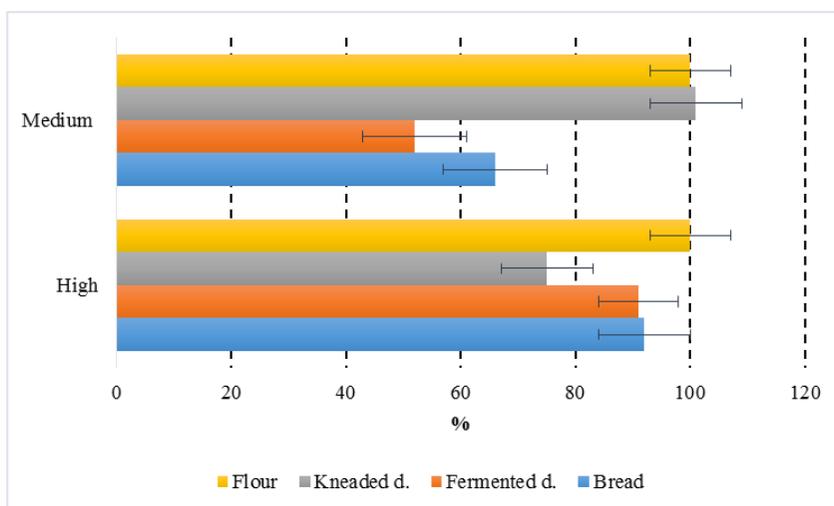


Figure 2. Reduction/increase of ENN B during bread-making (medium vs high contamination level).

Apparently, the behavior of ENN B seem to be case-dependent (starting contamination level, fermentation type, time/temperature, etc.). To better investigate this point, a supplementary test has been carried out to evaluate the potential implication of the leavening agent on ENN B evolution and trend during kneading and fermentation. Starting from the same high contaminated flour, a further bread was prepared with the substitution of yeasts with sodium bicarbonate in the recipe formulation. Data are summarized in Table 4. ENN B showed an analogous evolution with respect to *S. cerevisiae* recipe: a reduction of about 25% was observed after kneading stage, whereas an increase was described after fermentation compared to the unfermented dough (Figure 3). ENN B changes seem to be not influenced by the type of leavening agents adopted, but more related to the starting contamination level of raw material and, as consequence, to the matrix effect.

Table 4. Changes of ENN B during bread-making process (medium vs high contamination level).

Enniatin B	<i>S. cerevisiae</i>		Sodium bicarbonate	
	$\mu\text{g}/\text{kg}^{\text{a}}$	Recovered (%) ^b	$\mu\text{g}/\text{kg}^{\text{a}}$	Recovered (%) ^b
Flour	5194 \pm 377	100.0 a	5020 \pm 357	100.0 a
Kneaded dough	3907 \pm 325	75.2 b	3711 \pm 285	73.9 b
Fermented dough	4739 \pm 362	91.2 c	4174 \pm 336	83.1 c
Bread	4785 \pm 404	92.1 c	4464 \pm 255	88.9 c

Values of different letters in the same column are significantly different ($p < 0.05$).

^aData are expressed as mean value \pm standard deviation on dry matter (d.m.) basis; ^bPercentage of recovered amount with respect to the starting wheat flour.

Concerning baking, inferred results are less controversial: for both medium and high contaminate bread (Figure 2), the final thermal treatment, conducted at 210°C for 20 min, affects in a negligible way the final ENN B content, being differences not significant (Table 3).

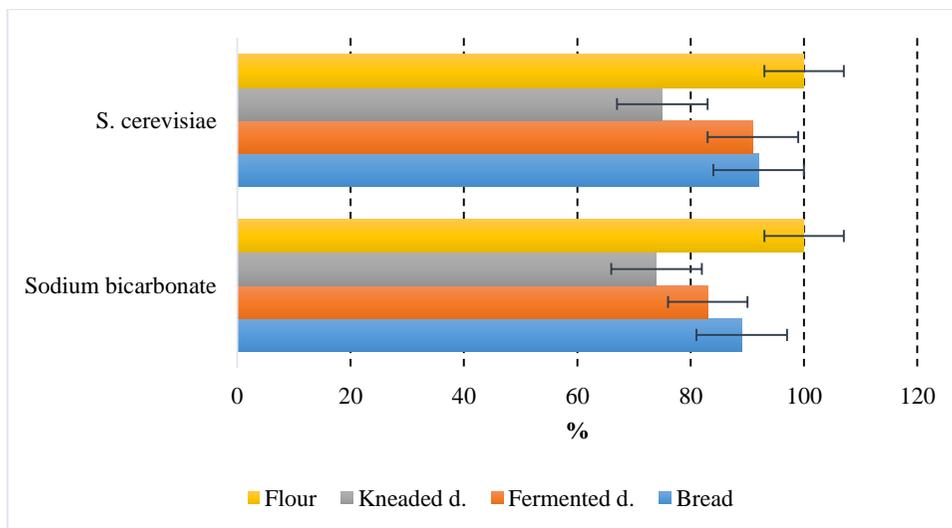


Figure 3. Reduction/increase of ENN B during bread-making (*S.cerevisiae* vs sodium bicarbonate).

Overall, the whole bread-making process reduced ENN B by 8-33% from the starting wheat flour, according to what observed in a previous study where a final reduction ranged from 25% to 41% has been reported [16]. Vaclavikova et al. [15] showed a higher reduction up to 60% of the original level in flour, but bread-making process was conducted at longer fermentation time (120 min vs 60 min) and at higher baking temperature (240°C vs 210°C), conditions that could be more effective in removing ENN B.

4.5 Conclusions

The presented work revealed that, in general, bread-making process had a slight effect (up to 30%) in removing ENN B from naturally contaminated bread. In particular, it has been shown that the effect of the bread-making process on ENN B is dependent on the initial mycotoxin concentration, while the use of yeasts or sodium bicarbonate for the fermentation step do not affect the considered compound. For these reasons, more research will be needed to explore the fluctuation of ENN B levels during bread processing, as well as, to identify the compounds generated by the possible degradation of this toxin.

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CHAPTER 5. STRATEGIC MYCOTOXIN MITIGATION WITHIN INDUSTRIAL BAKERY PROCESSES*

*Generotti, S., Cirlini, M., Malachova, A., Sulyok, M., Berthiller, F., Dall'Asta, C., Suman, M. (2015). Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology. *Toxins*, 7, 2773-2790

*Generotti, S., Cirlini, M., Šarkanj, B., Sulyok, M., Berthiller, F., Dall'Asta, C., Suman, M. Strategic mycotoxin mitigation within industrial biscuit-making technology. Under revision

5.1 Introduction

Grain quality and safety represent one of the major issue in the bakery production chain. Cereals and derived products are at the base of the human and animal diet [1]. Approximately 600 million tons are produced per year all over the world and most of them are converted to wheat flour for human consumption and destined to bakery products [2]. Exposure to toxic metabolites, which may occur on grains used for foodstuffs production, represent a food safety concern. During the last decades, there is an increasing attention to the occurrence and modification of mycotoxins during bakery production: it is fundamental to better evaluate all the aspects of mycotoxin contamination starting from their level due to the pre-harvest fungal development, up to the effect of the transformation processes and the mycotoxin occurrence in the finished products. The stability of mycotoxins during various baking process practices have been studied and documented throughout the world and the inactivation of mycotoxins during baking and the influence of raw material content are not clear yet. The extent of deoxynivalenol (DON) reduction, during various thermal processing operations along the production chain, has been the target of several studies with controversial outcomes in some circumstances depending on the conditions applied. Thermal treatment has been reported to reduce DON contamination by some authors [3,4], while others have suggested that DON is still highly stable in this process at the temperature of 170-300°C, with almost no reduction of DON concentration [5-7]. Kabak [8] reported that DON is stable in processing steps involving high temperatures, being the effect of the treatment strictly related to the technological parameters, in particular, to the setting of the temperature/time conditions [9,10].

Some studies with ochratoxin A (OTA) contaminated flour reported that this mycotoxin is stable at high temperatures, and that its concentration does not decrease during baking [11,12].

Furthermore, in the recent years, the contribution of modified forms, as deoxynivalenol-3-glucoside (DON-3-Glc), has been considered [13]: technological processes play an important role in the modifying mechanism, since reactions with other matrix components such as sugars, proteins or lipids can be induced upon treatments or parent compounds can be released from the conjugated forms. Vidal et al. [12] studied the fate of DON-3-Glc during a baking process observing an increase after the thermal treatment, hypothesizing a possible glycosylation of DON in the initial stages of baking before enzyme inactivation, in contrast to what described by other authors such as Kostelanska et al. [14] and Simsek et al. [15].

Basically, data show a clear health issue associated to mycotoxin ingestion and the need for regulating modified forms has been already recognized by European regulatory bodies. Nevertheless, due to the lack of analytical, exposure and toxicological data, legal official restrictions have still to be concretely implemented.

5.2 Aim of the work

The scope of the present work was to verify how mycotoxin levels, focusing on DON, DON-3-Glc, OTA and culmorin compounds, are influenced by modifications of technological parameters and ingredients during the production of three representative bakery products, as rusks, wholegrain and cocoa biscuits. Rusk processing offers the opportunity to investigate the effects of a series of technological processes: fermentation, baking, and toasting; on the other hand, biscuit production is characterized by a various setting of recipe formulation.

Experiments were performed starting from naturally contaminated raw materials (wheat bran and cocoa) and the model was developed using statistical Design of Experiment (DoE) schemes which allow, when multiple parameters may influence the desired outputs, to explore the relationship between the analytical responses and independent variables, conducting to an optimization of the process. The approach takes into account only those modifications that could be actually applied at industrial level in order to

minimize mycotoxin levels in the finished product that has to be appreciable by the consumers from the organoleptic point of view.

Aside from monitoring some specific mycotoxin levels, the application and comparison of two LC-MS/MS multi-mycotoxin methods, which allowed the concurrent detection and quantification of all the major mycotoxins and other secondary fungal metabolites in cereals, was performed. Additionally, to better understand the influence of the texture complexity on mycotoxin extraction capability, morphological investigation was conducted exploiting Environmental Scanning Electron Microscope (ESEM) analytical potentialities.

5.3 Experimental section

5.3.1 Chemicals

Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands) and ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Mycotoxin standards were obtained from the following commercial sources: Romer Labs®Inc. (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland) and LCGPromochem GmbH (Wesel, Germany). DON-3-Glc was isolated from wheat treated with DON [16]. All solutions were stored at -20°C and were brought to room temperature before use. OASIS® HLB 3cc (60 mg) Extraction Cartridges were purchased from Waters (Manchester, UK). Deionized water was used for all procedures. Glass vials with septum screw caps were purchased from Phenomenex (Torrance, CA, USA). Centrifugal filter units (Ultrafree MC 0.22 µm, diameter 10 mm) were obtained from Millipore (Billerica, MA, USA).

5.3.2 Raw material selection

For the baking experiments, different batches of wheat bran naturally infected with *Fusarium* spp. were analyzed with a focus on DON contamination levels. Among the analyzed batches, three bran batches were established, according to DON contamination level and technological characteristics. In particular, three different levels were selected (bran A, $600 \pm 16 \mu\text{g/kg}$; bran B, $1050 \pm 48 \mu\text{g/kg}$; bran C, $1500 \pm 92 \mu\text{g/kg}$). Industrial wholegrain flours (mix A, mix B, and mix C) ready for the experiments were obtained by mixing the contaminated bran (percentage 7-10% as maximum) with common wheat flours that present a contamination content of about 150-300 $\mu\text{g/kg}$ (depending on the lots). In addition, different batches of cocoa powders were selected for the cocoa biscuit trials, focusing on OTA level and three cocoa batches were established according to technological requirements (cocoa A, 1ppb; cocoa B, 5 ppb; cocoa C, 10 ppb). Among ingredients, cocoa content was considered for a fixed percentage (4%) according to the standard cocoa biscuit recipe.

5.3.3 Moisture content determination

The moisture contents of samples (flours, dough and baked/toasted products) were measured by taking a 5 g ground sample and heating it in a thermostatic oven at 105°C for over 6 h. All the results were calibrated and compared on dry matter basis.

5.3.4 Experimental design

The DoE method is used in many industrial sectors in the optimization of manufacturing process, making a set of experiments representative with regard to a given question. One of the main types of DoE-application is the screening and identification of important factors (the “treatments”): initial screening experimental designs are used to locate the most fruitful part of the experimental region. The main objective is to explore many factors in order to reveal whether they have an influence on the responses allowing to extract an answer with regard to the influence of an important factor. Information is also gained about how to modify the settings of the important factors, to possibly further enhance the final result [17]. In details, the present work is based on a fractional

Screening Design of Experiments (sDoE) in which the experiments varied different parameters of the transformation processes.

Among rusk-making parameters, several conditions were varied during the experiments: DON contamination level on wheat bran, levels of dextrose, levels of yeasts (*Saccharomyces cerevisiae*) and promoting agents content (% in recipe), fermentation time and temperature, baking time and temperature as well as toasting time and temperature. Concerning wholegrain and cocoa-making trials, DON and OTA contamination level on wheat bran and cocoa, dextrose, margarine, egg, milk content (% in recipe), pH value (sodium bicarbonate content) and baking time/temperature were varied during the experiments. Each selected treatment was varied within a range defined according to the technological requirements to obtain an appreciable product for consumers: the central experimental values, indicated in the Table 1 as “optimum”, represent the optimal combination of ingredients/recipe and operative conditions that permit achievement of the better finished product. Experimental data were then analyzed by a multi-variate analysis approach based on the partial least-squares (PLS) technique, using a dedicated statistical package (MODDE software, version 9.1; Umetrics, Umea, Sweden).

The statistical model required 19 single experiments per product; after analysis, MODDE software was exploited to point out the influence of a particular factor and to calculate the regression coefficient in a correspondent developed polynomial model. The statistical model efficiency was evaluated by two main parameters: fitting (R^2) and prediction (Q^2) values. R^2 measures the proportion to which the statistical model accounts for the variation of a given data set, while Q^2 refers to its ability to generate prediction. Moreover, model robustness was evaluated by ANOVA analysis. All values were collected and computationally combined in a Variable Importance Plot (VIP), illustrating the importance of a variable to the measurements of a process/phenomenon, in order to better understand how each factor influences mycotoxin response. VIP represents the most condensed way of expressing output as a weighed summary of all loadings and across all responses. On the other hand, the development of a response contour plot was able to provide suggestions concerning how to modify the setting of the factors and, potentially, to minimise mycotoxin content in the final product.

Table 1. Processing conditions during rusk and biscuit experiments in the pilot-scale plant.

Treatment	Rusk making		
	Minimum	Optimum	Maximum
DON bran level ($\mu\text{g}/\text{kg}$)	600	1050	1500
Dextrose (%)	2	4	6
Yeast (%)	2	3	4
Promoting agents (%)	0	0.5	1
Fermentation time (min)	40	50	60
Fermentation temperature ($^{\circ}\text{C}$)	26	36	46
Baking time (min)	12	21	30
Baking temperature ($^{\circ}\text{C}$)	180	195	210
Toasting time (min)	15	20	25
Toasting temperature ($^{\circ}\text{C}$)	110	130	150
Treatment	Wholegrain biscuit-making		
	Minimum	Optimum	Maximum
DON bran level ($\mu\text{g}/\text{kg}$)	600	1050	1500
Dextrose (%)	15	19	23
Eggs (%)	4	6	7
Margarine (%)	10	15	20
pH value	5	6.5	8
Baking time (min)	5	6.5	8
Baking temperature ($^{\circ}\text{C}$)	180	190	200
Treatment	Cocoa biscuit-making		
	Minimum	Optimum	Maximum
DON bran level ($\mu\text{g}/\text{kg}$)	600	1050	1500
OTA cocoa level ($\mu\text{g}/\text{kg}$)	1	5	10
Dextrose (%)	15	19	23
Milk (%)	5	6.5	8
pH value	5	6.5	8
Baking time (min)	5	6.5	8
Baking temperature ($^{\circ}\text{C}$)	180	190	200

5.3.5 Rusk and biscuit-making

Final doughs of about 800 g were prepared per each experiments. The ingredients for rusk and biscuit production were added as indicated in the three experimental design models (Table 2 and 3, respectively). The optimal amount of water to be added to each dough sample was established on the basis of previous experiments and internal technological knowledge.

The rusk process consists substantially of the following steps: firstly, all ingredients were mixed using a test planetary kneader for 3 min and dough was left to rest for 5 min at room temperature. Dough was shaped (laminating step) and fermented at different conditions in a leavening cell (FermaLievita Alaska, Bologna, Italy) at a relative humidity (RH%) of 85%. Baking was performed in a pilot-scale dynamic oven (Tagliavini, Parma, Italy). Mycotoxin content was examined in the mix flour and bran, before the fermentation step, after the baking process, and after the toasting process.

The process for wholegrain and cocoa biscuit production consisted essentially of the following steps: creaming, dough preparation and baking. Firstly, wheat flour was mixed with all solid powder ingredients using a test planetary kneader for 2 min. Dextrose and margarine were mixed separately by using another test planetary kneader for 3 min (creaming step). At a later stage, cream and powders were mixed together for 3 min. Dough was shaped and rounded pieces of about 4 cm diameter (approximately 10 g) were obtained from dough and rested for 10 min at room temperature. Baking step was performed in a pilot-scale dynamic oven (Tagliavini, Parma, Italy). Mycotoxin content was examined in mix powders, before and after baking process.

Before the mycotoxin content analysis, samples were stored at -20 °C. Each sample was extracted in duplicate and each extract was measured in duplicate.

The overall technological processes are summarized in Figure 1.

Table 2. Data set for screening variables effects on mycotoxin levels within the rusk-making process steps.

Experiment number	DON bran (µg/kg)	Dextrose (%)	Yeasts (%)	Promoting agent (%)	Fermentation stage		Baking stage		Toasting stage	
					Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)
1	600	2	2	0	40	26	12	180	25	150
2	1500	2	2	0	60	26	30	210	15	110
3	600	6	2	0	60	46	12	210	15	110
4	1500	6	2	0	40	46	30	180	25	150
5	600	2	4	0	60	46	30	180	15	150
6	1500	2	4	0	40	46	12	210	25	110
7	600	6	4	0	40	26	30	210	25	110
8	1500	6	4	0	60	26	12	180	15	150
9	600	2	2	1	40	46	30	210	15	150
10	1500	2	2	1	60	46	12	180	25	110
11	600	6	2	1	60	26	30	180	25	110
12	1500	6	2	1	40	26	12	210	15	150
13	600	2	4	1	60	26	12	210	25	150
14	1500	2	4	1	40	26	30	180	15	110
15	600	6	4	1	40	46	12	180	15	110
16	1500	6	4	1	60	46	30	210	25	150
17	1050	4	3	0.5	50	36	21	195	20	130
18	1050	4	3	0.5	50	36	21	195	20	130
19	1050	4	3	0.5	50	36	21	195	20	130

Table 3. Data set for screening variables effects on mycotoxin levels within the biscuit-making process steps.

Wholegrain test	DON bran (µg/kg)	Dextrose (%)	Margarine (%)	pH	Eggs (%)	Baking stage		Cocoa Test	DON bran (µg/kg)	OTA cocoa (µg/kg)	Dextrose (%)	Milk (%)	pH	Baking stage	
						min	°C							min	°C
1	600	15	10	5	4	5	180	1	600	1	23	5	5	5	180
2	1500	15	10	5	7	5	200	2	600	1	15	8	8	5	180
3	600	23	10	5	7	8	180	3	1500	1	15	5	5	8	180
4	1500	23	10	5	4	8	200	4	1500	1	23	8	8	8	180
5	600	15	20	5	7	8	200	5	600	1	23	5	8	5	200
6	1500	15	20	5	4	8	180	6	1500	1	23	8	5	5	200
7	600	23	20	5	4	5	200	7	1500	1	15	5	8	8	200
8	1500	23	20	5	7	5	180	8	600	1	15	8	5	8	200
9	600	15	10	8	4	8	200	9	1500	10	23	5	8	5	180
10	1500	15	10	8	7	8	180	10	600	10	23	8	5	5	180
11	600	23	10	8	7	5	200	11	600	10	15	5	8	8	180
12	1500	23	10	8	4	5	180	12	1500	10	15	8	5	8	180
13	600	15	20	8	7	5	180	13	600	10	15	5	5	5	200
14	1500	15	20	8	4	5	200	14	1500	10	15	8	8	5	200
15	600	23	20	8	4	8	180	15	1500	10	23	5	5	8	200
16	1500	23	20	8	7	8	200	16	600	10	23	8	8	8	200
17	1050	19	15	6.5	6	6.5	190	17	1050	5.5	19	6.5	6.5	6.5	190
18	1050	19	15	6.5	6	6.5	190	18	1050	5.5	19	6.5	6.5	6.5	190
19	1050	19	15	6.5	6	6.5	190	19	1050	5.5	19	6.5	6.5	6.5	190

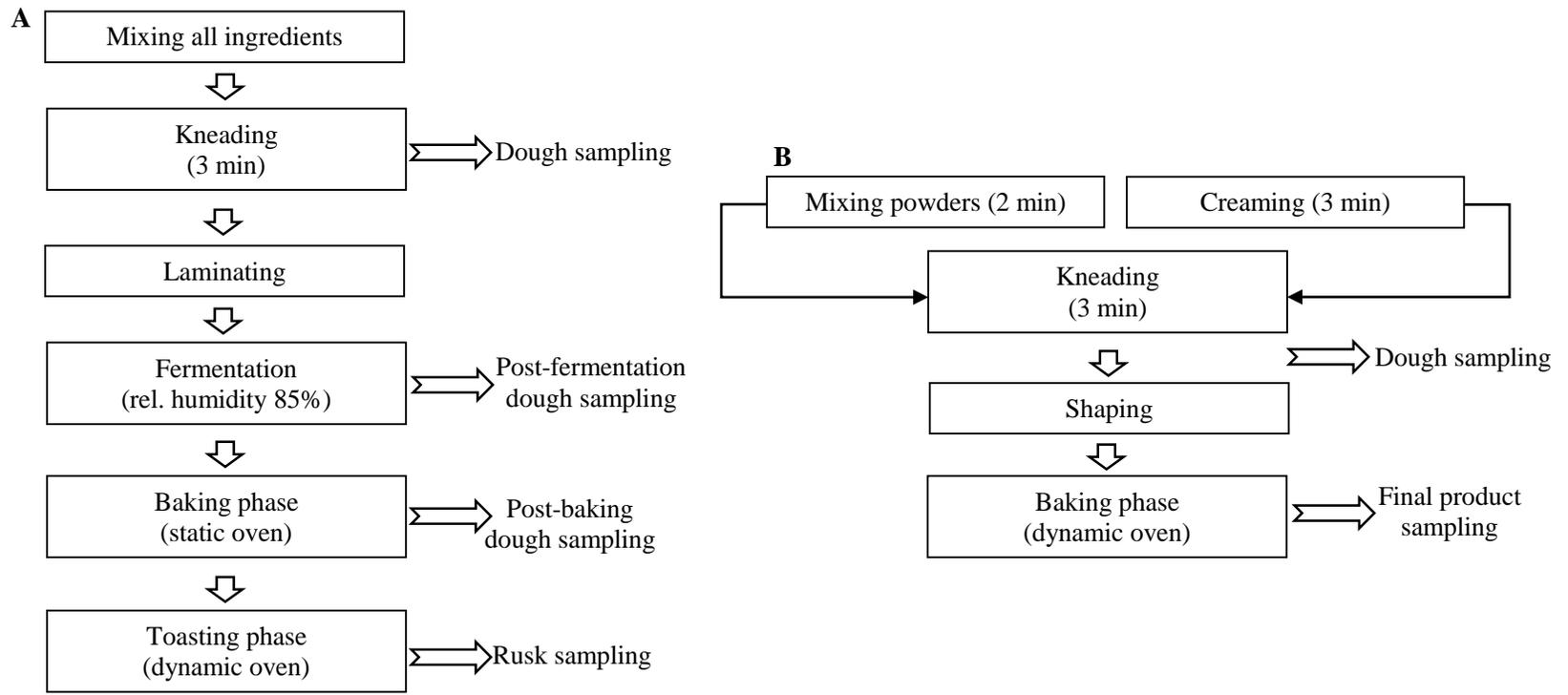


Figure 1. Scheme of the rusk-making process (A); scheme of wholegrain and cocoa biscuit-making process (B).

5.3.6 *Sample extraction and instrumental conditions – method A*

Samples were extracted according to a previously published procedure [18] with slight modifications. Briefly, a total of 10g of sample were extracted with 100 ml of a mixture acetonitrile/water (84/16, v/v) by homogenization at a medium-to-high speed for 2 min using a mixer. The extract was settled down for 15 min and 5 ml were poured into a 10 ml vial and immediately evaporated to dryness under a nitrogen stream. Then, the extract was reconstituted with 100 µl of an internal standard solution and 900 µl of water. Each extraction cartridge column was activated using 2 ml of methanol, and 2 ml of methanol/water (10/90, v/v) and sample extract was then slowly passed through the column using a vacuum chamber system. A solution of methanol:water (20:80, v/v) was used in the washing step, followed by elution with 1 ml of methanol. The eluate was evaporated under a gentle stream of nitrogen (methanol/water, 20/80 v/v, 0.5% acetic acid, and 1 mM ammonium acetate) to be used in the UHPLC-MS/MS analysis.

Ultrahigh-performance liquid chromatography (UHPLC) was performed using a Dionex Ultimate® 3000 LC systems (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a Kinetex Biphenyl column (2.6 µm; 100 x 2.10 mm; Phenomenex). The flow rate of the mobile phase was 400 µl/min, and the injection volume was 20 µl. The column oven was set to 30 °C. A linear binary gradient composed of (A) water (0.5% acetic acid, 1 mM ammonium acetate) and (B) methanol (0.5% acetic acid, 1 mM ammonium acetate) was present. The gradient elution step was performed as follows: 0-4 min to 40% B; 4-20 min to 80% B; 20-22 min, isocratic step 80% B; finally, a re-equilibration step at 10% B (the initial value) was performed for another 3 min, bringing the total analysis time to 25 min. Before the UHPLC-MS/MS analysis, all samples were filtered through centrifugal filter units for clarification.

ESI-MS/MS was carried out by a Q-Exactive (Thermo Fisher Scientific Inc., Waltham, MA, USA) mass spectrometer. Experiments were performed in full MS data scan for quantification and data-dependent scan for confirmation both in positive and negative polarities in two separate chromatographic runs per sample with the following settings: the capillary temperature was set to 300 °C; the sheath gas and auxiliary gas flow rates were set to 40 and 10 units, respectively; the spray voltage was set to 3500 kV; and the

S-lens RF level was set to 55 v. All equipment control and data processing were performed by Excalibur software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Mycotoxin measurements in all the samples were performed contextually with dedicated correspondent matrix-matched calibration curves with isotopically labeled standards and results were corrected for recovery.

5.3.7 Sample extraction and instrumental conditions – method B

Sample preparation was carried out according to Malachova et al. [19]. In brief, 5.00 ± 0.01 g of the ground samples were extracted with 20 ml acetonitrile/water/acetic acid (79/20/1, v/v/v) for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and subsequently, centrifuged for 2 min at 3000 rpm (radius 15 cm) on a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA). Then, the extract was transferred into glass vials using Pasteur pipettes, and 350 μ l aliquot was diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20/79/1, v/v/v). After appropriate mixing, 5 μ l of the diluted extract was injected into the LC-MS/MS system without further pre-treatment. Detection and quantification was performed with a QTrap 5500 MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150 x 4.6 mm i.d., 5 μ m particle size, equipped with a C18, 4 x 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases contained 5mM ammonium acetate and they were composed of methanol/water/acetic acid 10/89/1 (v/v/v; eluent A) and 97/2/1 (v/v/v; eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100% within 9 min was followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100%. The flow rate was 1 mL/min. The column effluent was transferred via a six-port valve (VICI Valco Instruments, Houston, TX, USA) either to the mass spectrometer (between 2 and 17 min; no flow splitting was used) or to the waste. ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs per

sample with the following settings: source temperature, 550 °C; curtain gas, 30 psi (69 kPa of max. 99.5% nitrogen); ion source gas 1 (sheath gas), 80 psi (345 kPa of nitrogen); ion source gas 2 (drying gas), 80 psi (345 kPa of nitrogen); ion spray voltage, -4.500V and +5.500 V, respectively; collision gas (nitrogen), medium; MRM dwell time, 100ms; pause between mass ranges, 5 ms. The sMRM detection window of each analyte was set to the respective retention time ± 27 s and ± 42 s in positive and in negative mode, respectively. The target scan time was set to 1 s. The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (diluted in a 1:1 mixture of eluent A and B) into the MS source using a 11 Plus syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 10 $\mu\text{L}/\text{min}$ for the corresponding values. Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte, which yields 4.0 identification points according to commission decision 2002/657/EC.

5.3.8 *Environmental scanning electron microscope (ESEM) analysis*

Morphological analysis was carried out by using an environmental scanning electron microscope (ESEM) QuantaTM 250 FEG (FEI Company, Oregon, USA), in order to verify and control potential matrix interferences on mycotoxin extraction capability due to texture complexity. ESEM microscopy was used to record the images of wholegrain biscuits and cocoa biscuits differentiated by the absence/presence of milk in recipe, demonstrating the negative/positive impact of considered ingredient on extraction procedure. Samples were placed on double-sided adhesive carbon tape fixed to metal sample holder, mounted in the microscope chamber. All the micrographs were acquired using an accelerating voltage of 7kV under different magnifications (114 x, 800 x, and 1600 x).

5.4 Results

Performed methods are characterized by distinguished extraction and instrument criteria and by number of detected metabolites: method A allowed to determine most of the major mycotoxins such as DON, DON-3-Glc and OTA but not other secondary compounds that was detected exploiting method B. At the end of the analytical work carried out on the samples set, MODDE software extracted an answer concerning the performed methods, comparing the expected and the found mycotoxin level in finished product. The replicate values of mycotoxin reduction (calculated as the difference between mycotoxin concentration in the initial flour and in the final product) per experiment were averaged and used in the statistical elaboration. Data were expressed on dry matter basis (d.m.), corrected according to recipe formulation. Comparing data set obtaining by the two analytical methods, method A seemed to be more efficient than method B. Referring to the MODDE output settings, method A gave a higher fitting value ($R^2 > 0.7$) and a better prediction capability ($Q^2 > 0.6$) in the three model data sets. Furthermore, model A seemed to have a greater robustness with respect to model B and this aspect was also confirmed by ANOVA plot especially for cocoa biscuit system, being standard deviation of the regression much larger than standard deviation of the residuals (data are not shown). For this reason, data from method A were chosen to perform statistical elaboration (except for results of other secondary metabolites), in order to better investigate the influence of a particular factor and to evaluate the regression coefficient in a correspondent developed polynomial model.

5.4.1 DON and DON-3-Glc levels during rusk-making

The replicated values of DON and DON-3-Glc reduction (calculated as the difference between DON and DON-3-Glc concentration in the initial flour-bran mix and in the final toasted rusks) in each experiment were averaged and used in the statistical elaboration. Data for DON and DON-3-Glc concentrations were expressed on dry matter (d.m), corrected according to the recipe formulation: they are reported in Table 4.

Table 4. Design of experiments and corresponding analytical results for DON and DON-3-Glc levels throughout rusk-making process.

Test	Bran ($\mu\text{g}/\text{kg}$ d.m.)		Dough ($\mu\text{g}/\text{kg}$ d.m.)		Fermented dough ($\mu\text{g}/\text{kg}$ d.m.)		Baked rusk ($\mu\text{g}/\text{kg}$ d.m.)		Toasted rusk ($\mu\text{g}/\text{kg}$ d.m.)	
	DON	DON3Glc	DON	DON3Glc	DON	DON3Glc	DON	DON3Glc	DON	DON3Glc
1	600 ± 16	29 ± 6	273 ± 25	29 ± 5	273 ± 29	22 ± 6	239 ± 10	22 ± 6	194 ± 16	23 ± 3
2	1500 ± 92	77 ± 10	349 ± 34	77 ± 9	340 ± 25	27 ± 5	334 ± 12	27 ± 5	329 ± 7	25 ± 4
3	600 ± 16	36 ± 3	379 ± 21	41 ± 4	333 ± 11	30 ± 3	338 ± 5	30 ± 3	322 ± 20	6 ± 1
4	1500 ± 92	46 ± 7	348 ± 30	64 ± 9	359 ± 18	31 ± 4	351 ± 23	31 ± 4	199 ± 16	12 ± 3
5	600 ± 16	23 ± 4	259 ± 15	22 ± 5	261 ± 12	18 ± 2	246 ± 12	18 ± 2	188 ± 20	21 ± 4
6	1500 ± 92	60 ± 6	392 ± 30	64 ± 7	339 ± 26	34 ± 5	360 ± 15	34 ± 5	317 ± 11	6 ± 2
7	600 ± 16	18 ± 3	257 ± 16	18 ± 4	242 ± 10	8 ± 1	199 ± 24	8 ± 1	172 ± 28	18 ± 3
8	1500 ± 92	39 ± 4	306 ± 12	45 ± 6	300 ± 5	23 ± 3	324 ± 15	23 ± 3	239 ± 15	7 ± 0
9	600 ± 16	23 ± 2	242 ± 18	29 ± 3	210 ± 22	12 ± 2	198 ± 15	12 ± 2	165 ± 26	27 ± 6
10	1500 ± 92	54 ± 5	339 ± 31	65 ± 7	333 ± 15	32 ± 4	343 ± 12	32 ± 4	315 ± 10	10 ± 2
11	600 ± 16	19 ± 3	213 ± 14	17 ± 2	220 ± 18	14 ± 3	229 ± 19	14 ± 3	200 ± 11	20 ± 5
12	1500 ± 92	41 ± 5	318 ± 27	43 ± 3	298 ± 32	30 ± 4	347 ± 36	30 ± 4	290 ± 15	8 ± 4
13	600 ± 16	26 ± 2	253 ± 12	29 ± 2	256 ± 20	19 ± 3	243 ± 20	19 ± 3	149 ± 19	20 ± 5
14	1500 ± 92	41 ± 2	339 ± 31	61 ± 5	311 ± 23	28 ± 3	330 ± 18	28 ± 3	308 ± 12	10 ± 3
15	600 ± 16	17 ± 3	211 ± 18	19 ± 2	215 ± 26	14 ± 2	253 ± 3	14 ± 2	212 ± 5	<0.1
16	1500 ± 92	30 ± 4	280 ± 14	40 ± 3	313 ± 21	9 ± 1	197 ± 32	9 ± 1	68 ± 11	9 ± 1
17	1050 ± 48	17 ± 3	219 ± 10	30 ± 5	229 ± 19	16 ± 4	225 ± 21	16 ± 4	194 ± 16	8 ± 2
18	1050 ± 48	11 ± 5	272 ± 8	19 ± 2	248 ± 30	15 ± 3	225 ± 19	15 ± 3	196 ± 12	9 ± 1
19	1050 ± 48	17 ± 3	251 ± 20	24 ± 4	213 ± 19	14 ± 5	218 ± 24	14 ± 5	186 ± 21	23 ± 3

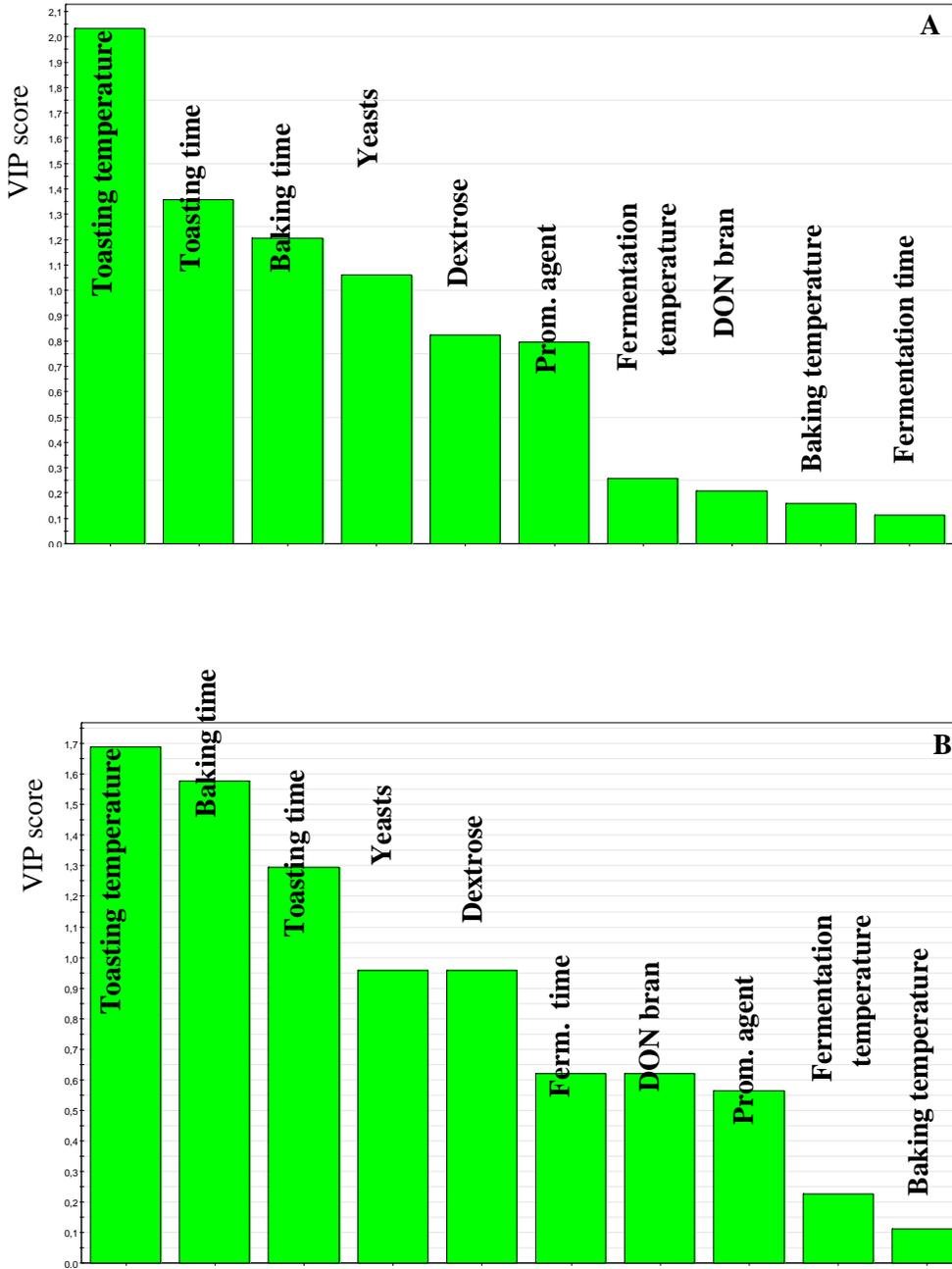


Figure 2. Variable Importance Plot (VIP) obtained from Design of Experiments rusk-making experiments: data referred to DON levels (A) and DON-3-Glc levels (B).

Figure 2(A) shows a variable trend after the fermentation step, where DON is only slightly influenced by fermentation time and temperature but more affected by initial yeast amount in the recipe; this outcome differs from results obtained in other studies [6,20] in which DON increase was observed. A hypothesis that could justify some of the different findings in fermentation effects is the use of different “yeast mixtures” (i.e., different strains of *Saccharomyces cerevisiae*, or mix with lactic acid bacteria).

Coming now to consider the thermal influence, in the present case, VIP would suggest that the evolution of DON appears to be significantly affected by the baking time, toasting time, and temperature: among these factors, the statistical analysis suggests that baking temperature has a less important effect than baking time on DON stability, according to what is reported by previous studies [12,21]. The lower effect of baking temperature compared to baking time can be explained taking into consideration the heating gradient occurring in the loaf during baking. In this case, temperature in the core of the loaf is about 100 °C, independently of the oven temperature.

The fermentation phase, as said above, together with the other involved parameters, had a smaller or negligible effect on final DON concentration. This is in agreement with another research work conducted by Suman et al. [22] on cracker-making and a reasonable explanation can be the following: taking into account the overall processes and correspondent technological parameters, the influence of heat-exchange phenomena is crucial. Rusks undergo to two subsequent heating treatments (baking the entire loaf and then toasting after having cut the loaf into several slices) and both crackers and rusks (in the baking and toasting phases, respectively) have a significantly higher heat-exchange surface with respect to bread.

One-way analysis of variance was carried out by applying Turkey’s post hoc test ($p < 0.05$) using SPSS statistics 21.0 software (SPSS Inc., Chicago, IL, USA, 2003): after baking and toasting, a decrease from 8% to 19% and from 19% to 65% in DON levels, respectively, were observed and this phenomenon indicates probable degradation, due to the thermal treatment at high temperature. A potential reduction within the total processing up to 30% may be feasible: the opportune increase of the toasting time/temperature can determine such a reduction of the free DON concentration in the

final rusks, remaining in an acceptable range of technological and organoleptic conditions.

As a naturally contaminated raw material was used for rusk production, other mycotoxins can co-occur in the final products: DON-3-Glc was observed within a range of about 5-15% of the DON concentration, similarly to what found in a previous work [23]. The experimental design reported herein was planned taking into consideration only DON contamination in bran, while DON-3-Glc co-occurrence was found later. For this reason, DON-3-Glc concentration was not significantly different in the three initial batches, and it cannot be considered as a factor for the statistical evaluation. However, although its average concentration is relatively low ($32 \pm 2 \mu\text{g/kg}$), data are still significant in term of corresponding uncertainties (Table 4) and some indication can be obtained as well. In particular, an increase up to 48% can be envisaged after the fermentation step, followed by a reduction during baking and toasting, as already reported by other studies [14,15]. As a result of the overall variability of the statistical approach, the final concentration seems to be affected only by three factors: toasting time, temperature, and baking time, as reported in Figure 2(B).

Considering the modelling of DON and DON-3-Glc evolution during rusk-making, it can be reasonably assumed that toasting and baking stages support toxin degradation.

5.4.2 DON and DON-3-Glc levels during wholegrain biscuit-making

Taking into consideration the MODDE outputs, DON levels seemed to be more influenced by recipe formulation than thermal treatment, as shown in Figure 3(A). Basically, in the present case, VIP would suggest that the pH value (expressed as sodium bicarbonate content) had the most relevant effect on the final DON concentration. A potential reduction within the total processing up to 10% may be feasible still remaining in an acceptable range of technologic and organoleptic properties: as reported by the contour plot (Figure 3(B)), pH value increase can determine a reduction of free DON concentration in the final biscuit, according to what reported by Suman et al. [22].

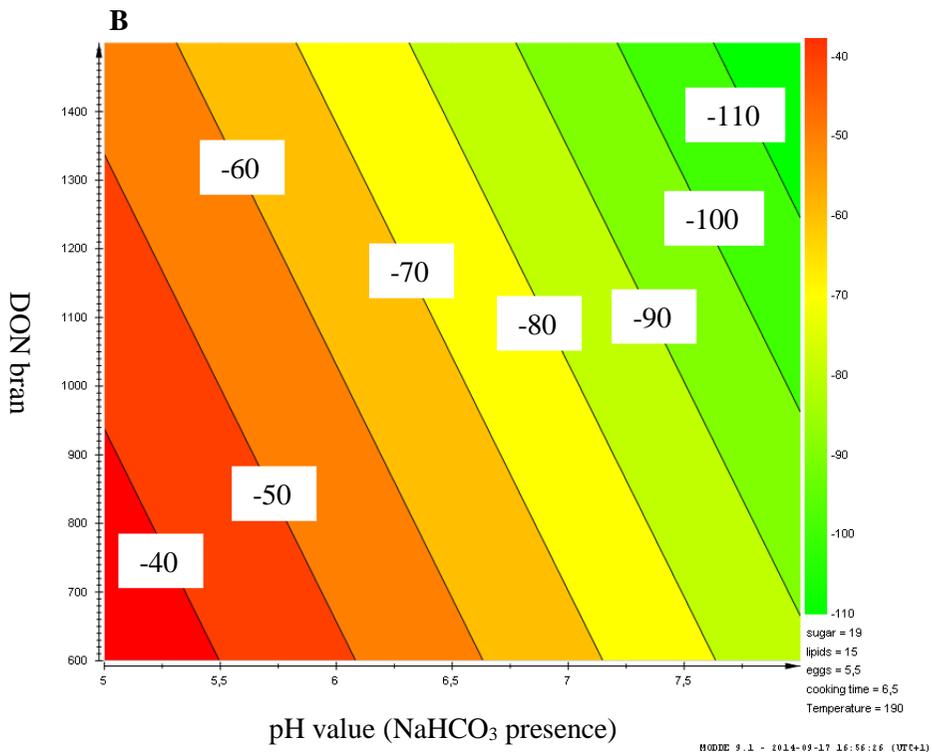
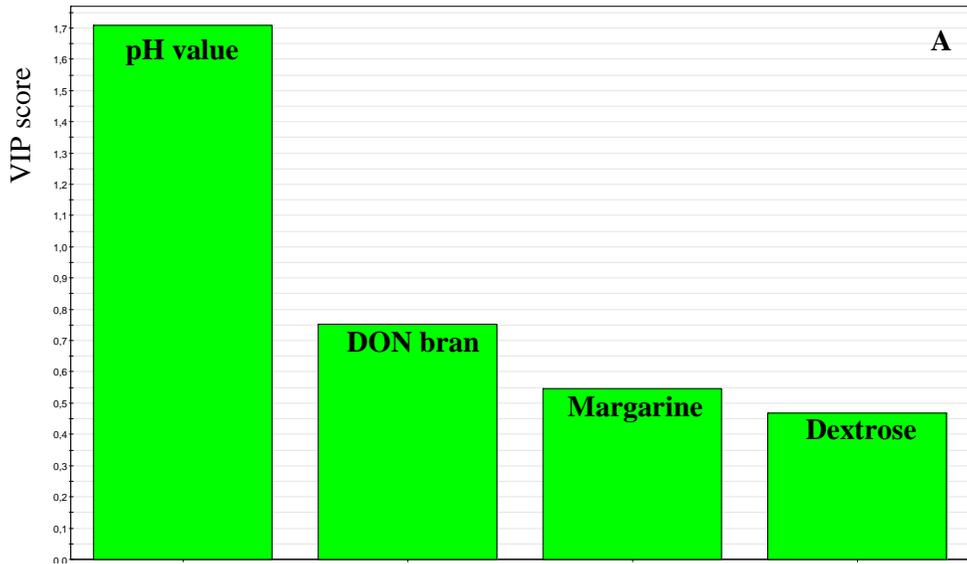


Figure 3. MODDE outputs referred to DON concentration during wholegrain biscuit-making process: Variable Importance plot (VIP) (A); Response Contour Plot -DON bran vs pH value (B).

The amount of sodium bicarbonate suggested an opposite influence on the DON levels (Table 5). This phenomenon could be connected to a possible effect related to the pH conditions in the dough that could modify the reactivity of the modified forms of the toxin, but it needs to be better investigated. The second aspect, which can be inferred from the VIP plot (Figure 3(A)), the initial DON contamination of wheat bran appeared to affect the evolution of DON in the finished product, according to an analogous study conducted by Vidal et al. [12], where reduction trend depended on the initial concentration of DON in the flour, leading to a more marked reduction for medium-to-high concentrations. By contrast, statistical analysis suggested that baking parameters (time/temperature) had a negligible effect on DON stability, in disagreement with what reported for rusk production. The limited effect of baking step can be attributed to the close range of time/temperature and to the small thermal gap among experiments, reaching similar values into the final products, with respect to the recipe formulation. On the other hand, if all the other variables are assumed as constants, baking time and temperature become more relevant as reported in Table 6. Considering experiments carried out within the most severe time/temperature baking conditions, a reduction ranged from 9 to 68% was reported. In particular, the greatest baking time/temperature combination power was observed for high initial DON contamination bran when baking step was conducted at 200°C for 8 min, remaining in an acceptable range of technological and organoleptic conditions. Extending these considerations to DON-3-Glc, a similar reduction trend was observed (Table 6) if baking step was assumed as the main parameter. Reduction ranged from 31 to 75% and decrease seemed to be affected by initial DON contamination level. Because of the relatively low DON-3-Glc values and the corresponding uncertainties in raw materials, it is difficult to identify influences of the ingredients on the DON-3-Glc levels or extract any relevant cause-effect phenomena directly related in the different production phases.

Table 5. Design of the experiments and corresponding analytical results for the effects of the screening variables on the deoxynivalenol (DON) levels throughout the wholegrain biscuit-making process.

Test number	NaHCO ₃ (g)	DON in initial wholegrain flour (µg/kg d.m.)*	Baking stage		
			Time (min)	Temperature (°C)	DON in wholegrain biscuits (µg/kg d.m.)*
1	0	219 ± 8	5	180	119 ± 13
2	0	304 ± 9	5	200	263 ± 0
3	0	219 ± 8	8	180	186 ± 3
4	0	304 ± 9	8	200	274 ± 1
5	0	219 ± 8	8	200	172 ± 13
6	0	304 ± 9	8	180	213 ± 20
7	0	219 ± 8	5	200	165 ± 0
8	0	304 ± 9	5	180	228 ± 1
9	9	219 ± 8	8	200	129 ± 7
10	9	304 ± 9	8	180	208 ± 2
11	9	219 ± 8	5	200	105 ± 3
12	9	304 ± 9	5	180	155 ± 7
13	9	219 ± 8	5	180	154 ± 1
14	9	304 ± 9	5	200	192 ± 4
15	9	219 ± 8	8	180	117 ± 3
16	9	304 ± 9	8	200	138 ± 2
17	4	237 ± 19	6.5	190	188 ± 7
18	4	237 ± 19	6.5	190	189 ± 1
19	4	237 ± 19	6.5	190	203 ± 15

*Data expressed as mean value ± standard deviation on dry matter basis (d.m.) of a final number of four replicates

Table 6. Thermal overall effect in wholegrain biscuit processing on DON and DON-3-Glc levels.

Test number	DON bran (µg/kg)	Baking stage		Reduction		Time/Temperature combination power
		Time (min)	Temperature (°C)	DON	DON3Glc	
1	600	5	180	9 %	36 %	VERY LOW
2	1500	5	200	31 %	31 %	LOW
15	600	8	180	64 %	64 %	MEDIUM
16	1500	8	200	68 %	75 %	HIGH

5.4.3 DON, DON-3-Glc and OTA levels during cocoa biscuit-making

Samples obtained from the cocoa biscuit-making experiments were analyzed for DON, DON-3-Glc and OTA content, in order to build a prediction model describing the process. Concerning DON evolution, VIP plot (Figure 4) illustrated that this mycotoxin is significantly influenced by milk content, while baking parameters seemed to have smaller influences. Taking into account the achieved results and the recipe variability, the texture of the obtained cocoa biscuits was investigated by Environmental Scanning Electron Microscopy (ESEM) under different magnifications. In order to compare different matrices, products from analogous recipes distinguished by milk presence/absence were analyzed (Figure 5). As a general comment it can be stated that milk seemed to have a not negligible impact on product texture, appearing the more milk in recipe, the more complex was the matrix. As shown in Figure 5(B), starch seemed to be embedded into the matrix with reduced area, not useful for increasing the extraction capability of mycotoxins.

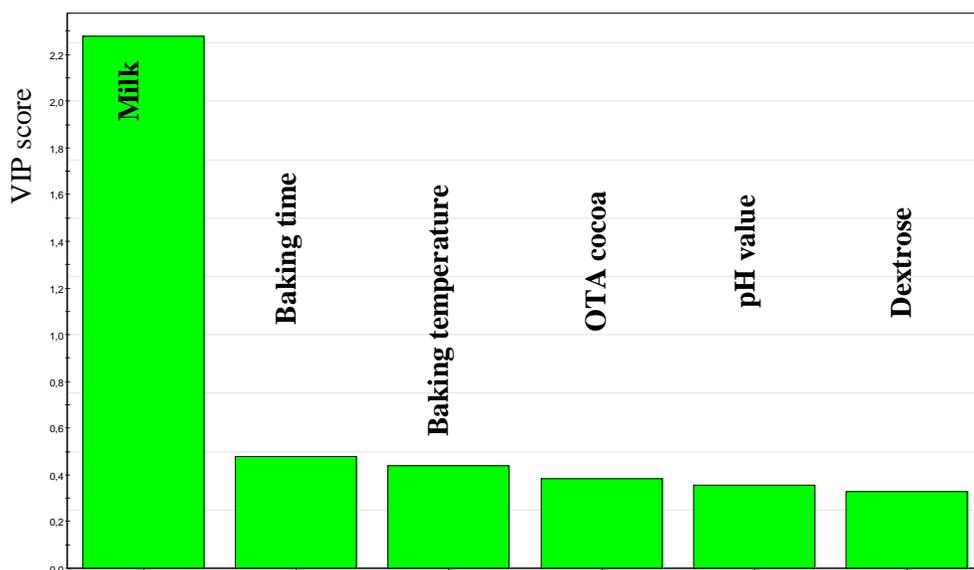


Figure 4. Variable Importance Plot (VIP) obtained for the data referred to DON concentration during cocoa biscuit-making process.

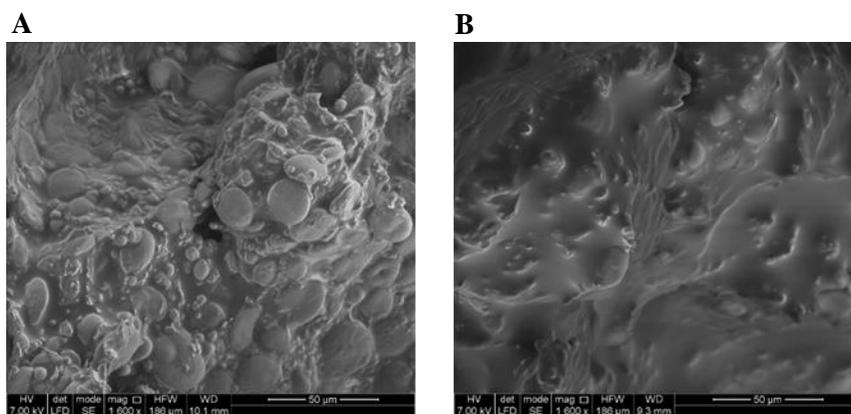


Figure 5. Scanning electron micrographs of cocoa biscuits: matrix without milk (A), matrix with milk (B) under magnification (1600 x).

Taking into account of this hypothesis, baking parameters become the most relevant and a reduction up to 27% (Table 7) was achieved, remaining in an acceptable range of technologic and organoleptic requirements for consumers, as previously indicated. The greatest reduction was reported when baking step was conducted at 200°C for 8 min, becoming baking time more relevant than temperature. Reduction percentage is not high as observed during wholegrain biscuit production, probably due to recipe formulation and matrix complexity, as reported by ESEM analysis. This effect was demonstrated by the results observed for experiment n.12 and n.15, characterized by higher milk content. For these experiments, an increasing evolution of 12 and 26%, respectively, was observed. The increase may be due to the more complex matrix and not due to thermal treatment effect. As mentioned for rusk and wholegrain biscuit production, DON-3-Glc levels were not significantly different and it is not possible to identify parameter influences phases because of the relatively low DON-3-Glc values and the corresponding uncertainties.

Regarding OTA evolution, it was not detected in biscuit samples. In order to respect the industrial pertinence, starting contamination of cocoa ingredient was set at three different levels (low/medium/high natural contamination, 1/5/10 ppb, respectively) within a reasonable range for an already high risk for consumers: in the corresponding final cocoa biscuits, OTA was estimated below limit of quantification (LOQ < 0.2), due to recipe formulation and thermal treatment effect.

Table 7. Thermal overall effect in cocoa biscuit processing on DON levels.

Test number	DON bran ($\mu\text{g}/\text{kg}$)	Baking stage		Reduction DON	Time/Temperature combination power
		Time (min)	Temperature ($^{\circ}\text{C}$)		
4	1500	8	180	25 %	MEDIUM
6	1500	5	200	17 %	LOW
7	1500	8	200	27 %	HIGH
10	600	5	180	68 %	VERY LOW

5.4.4 Other metabolites: occurrence of culmorin

Investigations showed also that culmorin is often co-occurring with type-B trichothecenes [24,25], considering that both *F. graminearum* and *F. culmorum* produce this metabolite. Culmorin is a sesquiterpene assumed to be biosynthesized from one molecule of *trans*-farnesyl pyrophosphate [26] and its effects remain vague until now. Among culmorin compounds, more than ten hydroxy-culmorins were detected and a significant difference was observed between the two *Fusarium* species: 15-hydroxy-culmorin, for example, is mainly produced by the *F. culmorum* strains [27].

In spite of rich scientific literature on mycotoxins in commodities intended for human consumption, little or no information is available with respect to culmorin compound in foods and there is a lack of data related to their thermal degradation. Also, in this study, culmorin and 15-hydroxy-culmorin were detected in all biscuit samples. Furthermore, the concentration of culmorin was positively correlated to the concentration of DON with R^2 coefficients between 0.81 and 0.99 and between 0.86 and 0.96 for wholegrain and cocoa biscuit samples, respectively, as also reported by Uhlig et al. [25]. This proportion was also inferred from VIP plot (Figure 5): DON concentration in the starting raw material seemed to have the main influence on culmorin contamination both in wholegrain and cocoa model. The concentration of culmorin in the samples were up to 245 $\mu\text{g}/\text{kg}$ and 92 $\mu\text{g}/\text{kg}$ for wholegrain biscuits in flours and baked products, respectively, while it reached 109 $\mu\text{g}/\text{kg}$ for cocoa model in baked biscuit. 15-hydroxy-culmorin occurred in all samples at concentrations ranged from 18 $\mu\text{g}/\text{kg}$ to 111 $\mu\text{g}/\text{kg}$ with higher levels in wholegrain biscuits than cocoa products. Processing, in particular thermal treatment, seemed to decrease culmorin and 15-hydroxy-culmorin content in the final products. In the

experiments carried out as system to simulate wholegrain and cocoa biscuit production, the percentage of degradation resulted variable from 25 to 80%. The greatest reduction was achieved when thermal treatment was conducted at 180°C for 8 min starting from a high DON contamination level in bran, also, confirming the more relevant role of baking time with respect to baking temperature at the aim to obtain an acceptable product for consumers.

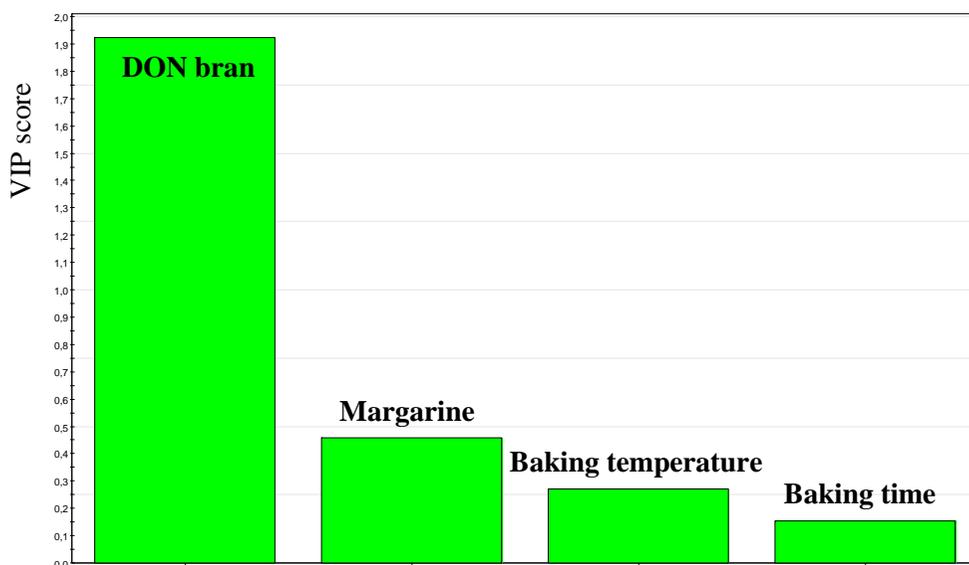


Figure 5. Variable Importance Plot (VIP) obtained for the data referred to CUL concentration during wholegrain and cocoa biscuit-making process.

5.4.5 Real-scale testing of the model prediction capability

In order to verify the prediction capability of the developed models, dedicated experiments referred to the wholegrain biscuit-making have been repeated on real-scale production. Two set of tests were carried out at pilot plant scale according to those operative conditions that resulted in significantly different rates of DON reduction. Additionally, a sampling has been conducted on an industrial line to evaluate the efficiency of the prediction at standard conditions. Data, as recipe ingredients and thermal parameters, have been processed by MODDE according to the model outputs and the predicted values of DON reduction have been compared with the achieved one, confirming the good prediction response of the model, especially for the industrial test. Data are summarized in Table 8.

Table 8. Comparison predicted vs observed DON reduction percentage during real-scale testing of wholegrain biscuit-model.

Experiment type	DON in initial							Predicted Range DON Reduction (%)		Observed DON reduction (%)
	wholegrain flour (µg/kg)	Dextrose (%)	Margarine (%)	NaHCO ₃ (g)	Eggs (%)	Baking stage		Lower	Upper	
						Min	°C			
PILOT LINE	88 ± 7	15	10	0	4	5	180	7	24	19
PILOT LINE	99 ± 15	23	20	9	7	8	200	31	58	54
INDUSTRIAL LINE	79 ± 6	19	15	4	6	6.5	190	3	34	25

5.5 Conclusions

This study represents a tangible example of how a careful control of industrial lines may conduce to mitigate mycotoxin impact, especially when their level in raw material is closed to the European legal limit (for example in the case of specific year-campaign negative circumstances), through appropriate management of cereals processing techniques and providing a powerful synergistic strategy to be combined to good agricultural practices for reducing consumers exposure.

The effect determined by modifying ingredients and industrial conditions on mycotoxin levels, with a particular focus on DON, DON-3-Glc and OTA, during bakery processes was investigated. Starting from naturally contaminated raw material, the power of dedicated DoE schemes and LC-MS/MS methods was exploited. The obtained processing models showed a good fitting, providing important information for the prediction of performances and consequent optimization of the industrial production process, suggesting the most significant parameters which can concretely contribute to the minimization of mycotoxin levels in the final food product.

The models provide the following information: (1) increasing time/temperature in the toasting phase during rusk production can reduce DON and DON-3-Glc content up to 30%; (2) increasing pH value (expressed as sodium bicarbonate content) indicates a DON decrease during wholegrain biscuit production, especially starting from high naturally contaminated flour; (3) increasing time in the baking phase, staying in an acceptable technological range, can reduce DON and DON-3-Glc content in the final product; (4) recipe formulation can contribute to matrix complexity with implications on extraction capability of mycotoxins.

Furthermore, in this study, representative culmorin compounds evolution during a baking process was investigated for the first time: concentration was positively correlated to DON concentration and a reduction up to 80% was reported, but the combined effects among fungal metabolites need to be further investigated in the future.

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CHAPTER 6. TECHNOLOGICAL APPROACH TO REDUCE ACRYLAMIDE LEVELS WITHIN BAKERY PRODUCTION

6.1 Introduction

Process contaminants are chemical substances that are produced in food during manufacturing. They are not present in raw materials but only formed when components within the food undergo to chemical changes during processing. Food industry and the European Commission have undertaken extensive efforts since 2002, when scientists from the Swedish National Food Authority and the University of Stockholm reported high levels of acrylamide in normally cooked starch-rich food compared to what had been reported earlier in other food commodities, in order to investigate pathways of formation and to reduce its levels in processed food.

Acrylamide is a low molecular weight, odorless, and colorless compound. It is typically formed in starchy food products during high temperature cooking, including frying, baking and roasting through a series of reactions, known as Maillard reactions. It forms from an amino acid, primarily asparagine, and a reducing sugar such as fructose or glucose: when the sugar and the amino acid, are heated, they combine to form substances giving new flavors, causing the browning of the food and producing acrylamide as well. Its formation begins at temperature around 120 °C and peaks at temperatures between 160 and 180°C [1,2]. Coffee, fried potato products, biscuits, crackers, breads and certain baby foods are important dietary sources of acrylamide [3-5]. Experts, including the international Joint Food and Agriculture Organization and the World Health Organization Expert Committee on Food Additives (JEFCA), have concluded that current global levels of dietary exposure to acrylamide indicate a human health concern [6]. More than one-third of the calories consumed among populations contain acrylamide and mean dietary intake in adults averages 0.5 µg/kg of body weight per day, whereas intake is higher among children [7]. Laboratory tests showed acrylamide is carcinogenic to experimental mice and rats, causing tumors at multiple organ sites in both species when given in drinking water or by other means [8,9], whereas in humans, neurotoxicity is currently the only demonstrated effect [10,11]. Primarily on the basis of the animal studies, the International Agency for Research on Cancer classified it as “a probable

human carcinogenic”, placed in group 2A [12]. Due to its toxicity, the European Commission recommends indicative values of 80 µg/kg in wheat based bread, 400 µg/kg in bran products/wholegrain cereals, and 500 µg/kg in biscuits [13].

Several studies have been published upon main mitigation strategies for bakery products [14-20]. The majority of the proposed tools are focused on the processing stage, recipes and thermal treatments. Changing of recipe formulation (for example, adding ingredients which inhibit the reaction of acrylamide formation) and baking process are regarded as a further realistic mitigation strategy. Acrylamide formation is favored by a high baking temperature and time treatment [21-23]. Decreasing of thermal input represents an effective way of mitigation. Reduction can be obtained by applying prolonged heating at lower temperatures, eventually at lower pressure than the atmospheric one [14] or by optimizing the oven profile. On the other hand, decreased thermal input has to be taken into consideration in order to achieve appropriate hygienic and sensory properties. The effects of thermal treatment is a crucial factor not only for the reduction of acrylamide [24-26] but also into controlling other food contaminants as microorganism, pathogens and mycotoxins produced by fungal agents. At this regard, we have demonstrated how increasing time and temperature during baking exert a positive impact on mycotoxin reduction in the finished product [27,28].

To date, there are no legal regulations regarding acrylamide levels in food; anyway, European and national authorities already recommend reducing acrylamide in food as much as possible and provide dietary and food preparation advice to consumers and food producers. Each strategy could present limiting factors in their applicability depending on the product type and industrial settings as feasibility and compatibility with processing, formulation, impact on sensory and nutritional characteristics, regulatory compliance and costs. In this regard, predictive models would represent a time and cost saving tool for finding the most suitable conditions.

6.2 Aim of the work

The present investigation was aimed to extend the considerations of the previous chapter in order to evaluate the impact of the developed mycotoxin mitigation strategy on acrylamide formation and its evolution in the finished products, in particular for wholegrain and cocoa biscuits, as to better understand if the mentioned strategy, mainly related to stress thermal input, could involve further implication. Starting from the same biscuits and exploiting the same approach based on dedicated Design of Experiments (DoE), the main goal of this work is to verify how acrylamide concentration is influenced by modifications of technological parameters (recipe formulation and baking time/temperature) during biscuit-making process within a potential mycotoxin mitigation range and without affecting the sensory properties.

6.3 Experimental section

6.3.1 Chemicals

Methanol and formic acid (p.a.), both HPLC gradient grade, were obtained from BDH VWR International Ltd. (Poole, UK). Standard acrylamide solution was purchased from Sigma-Aldrich (Milan, Italy). Acrylamide internal standard ($^{13}\text{C}_3$ -acrylamide, 1 mg/ml in methanol) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Deionized water was used for all procedures.

6.3.2 Moisture content determination

The moisture contents of mix flours, doughs, and baked products were measured by taking a 5 g ground sample and heating it in a thermostatic oven at 105 °C for over 6 h. All the results were calibrated and compared on a dry matter (d.m.) basis. Each sample was extracted and analyzed in duplicate.

6.3.3 Experimental design

Among the wholegrain and cocoa biscuit-making parameters, several conditions were varied during the experiments: DON and OTA contamination level on wheat bran and cocoa, respectively; dextrose, margarine, egg and milk content (as percentage in recipe); pH value (as sodium bicarbonate content) and baking time and temperature. Each

selected treatment was varied within a range defined according to the technological requirements to obtain an organoleptically appreciable product for consumers as previously explained in Chapter 5 (Table 1). Acrylamide level was measured by LC-MS/MS in the starting flours, before and after baking step. Experimental data were then analysed by a multi-variate analysis approach based on the partial least-squares (PLS) technique, using a dedicated statistical package (MODDE software, version 9.1; Umetrics, Umea, Sweden).

Table 1. Processing conditions assumed during wholegrain and cocoa biscuit experiments in the pilot-scale plant.

Treatment	Wholegrain biscuit			Cocoa biscuit		
	Minimum	Optimum	Maximum	Minimum	Optimum	Maximum
DON bran ($\mu\text{g}/\text{kg}$)	600	1050	1500	600	1050	1500
OTA cocoa ($\mu\text{g}/\text{kg}$)	-	-	-	1	5	10
Dextrose (%)	15	19	23	15	19	23
Milk (%)	-	-	-	5	6.5	8
Eggs (%)	4	6	7	-	-	-
Margarine (%)	10	15	20	-	-	-
pH value	5	6.5	8	5	6.5	8
Baking time (min)	5	6.5	8	5	6.5	8
Baking temperature ($^{\circ}\text{C}$)	180	190	200	180	190	200

6.3.4 *Sample extraction and instrumental conditions*

Sample extraction was performed according to a previously published procedure [29] with slight modifications. Briefly, samples were finely grounded in a blender to homogeneity before extraction. 1 g of sample was weighed into a polypropylene graduated conical tube and different volumes of a 300 $\mu\text{l ml}^{-1}$ internal standard solution

($^{13}\text{C}_3$ -labeled acrylamide in 0.1 % (v/v) formic acid) followed by 10 ml 0.1 % (v/v) formic acid were added on the base of the acrylamide concentrations supposed to be present in the sample. After mixing for 10 min on a vortex the extract was centrifuged at 10000 rpm for 5 min. A 3-ml portion of clarified solution was removed avoiding to collect top oil layer when present and filtered through a 0.45 μm nylon syringe filter (Phenomenex, Torrance, CA, USA) before injection into the LC-MS/MS system (injection volume 10 μL).

LC-MS/MS in positive ion mode analysis was achieved using a linear ion trap LXQ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled to a Surveyor LC quaternary pump separation system (Thermo Fisher Scientific Inc.). Chromatographic separation was performed using a Synergi Hydro-RP (150 x 2.0 mm) 4 μm analytical column (Phenomenex, Torrance, CA, USA). Elution was carried out at a flow rate of 0.2 ml/min, in isocratic conditions, at 30 $^\circ\text{C}$ using as mobile phase a mixture of 98.9 % water, 1 % methanol and 1 % formic acid (v/v/v). In these conditions, the retention time of acrylamide was about 4 min. A time programmed valve was used to discard the eluate from the column for the first 2.5 min in order to eliminate the compounds with retention times shorter than acrylamide. At 8 min the column flow was again diverted and the mobile phase changed to 100 % methanol in order to clean the column from strongly retained compounds within a total run time of 10 min. MS/MS conditions were set as follows: capillary temperature was set to 160 $^\circ\text{C}$; the sheath gas was set to 35 units; the spray voltage was kept at 4500 V; capillary voltage was kept at 9 V. All parts of the equipment and data processing were performed by the computer software Xcalibur (Thermo Fisher Scientific Inc.). MS/MS analysis was carried out by selecting the ions at m/z 72 and m/z 75 as precursor ions for acrylamide and $^{13}\text{C}_3$ -acrylamide respectively. The area of the chromatographic peaks of the extracted ion at m/z 55, due to the transition 72 > 55, and at m/z 58, due to the transition 75 > 58 were used for the quantitative analysis. The quantitative analysis was carried out with the method of the internal standard. The relative response factor of acrylamide with respect to $^{13}\text{C}_3$ -acrylamide was calculated daily by analysing a standard solution.

6.4 Results

6.4.1 Statistical elaboration of the experimental model

The statistical approach applied within the previous strategy of mycotoxin reduction throughout bakery-making technology was extended to this study in order to compare the expected and the found acrylamide level in finished product. MODDE software extracted an answer concerning robustness and prediction capability of the method, evaluating model efficiency by fitting (R^2) and prediction (Q^2) values: the two statistical models (wholegrain and cocoa biscuit model) gave high fitting ($R^2 > 0.7$) and good prediction value ($Q^2 = 0.5$). Model robustness was also confirmed by ANOVA plot (Figure 1), being standard deviation of the regression much larger than standard deviation of the residuals.

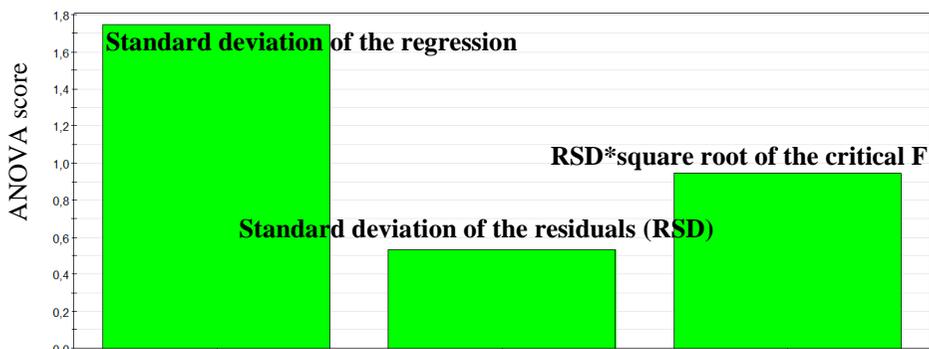


Figure 1. ANOVA plot obtained from DoE cocoa biscuit-making experiments. Data referred to acrylamide levels.

6.4.2 Acrylamide evolution within biscuit-making technology

Acrylamide concentration values were collected in a singular Variable Importance Plot (VIP, Figure 2) in order to better evaluate how each factor influences acrylamide response. In the present case, VIP would suggest that the pH value has the most relevant effect on the final acrylamide level: in fact pH increase is responsible for an acceleration of the reaction between asparagine and the reducing sugar [30], followed by baking time/temperature parameters. Basically, acrylamide content and removal seems to be affected by the food matrix, being higher for the cocoa biscuits than for the wholegrain biscuits, probably due to the additional acrylamide related to cocoa beans and roasting process where thermal energy is involved (Table 2). With regard to the sodium

bicarbonate content and the corresponding pH variation, a potential reduction in acrylamide level higher than 50% could be achieved in the finished product within an acceptable range from the sensorial point of view. Taking into account the previously mentioned mycotoxin mitigation strategy, baking time/temperature play the most important role in order to achieve a significant deoxynivalenol reduction.

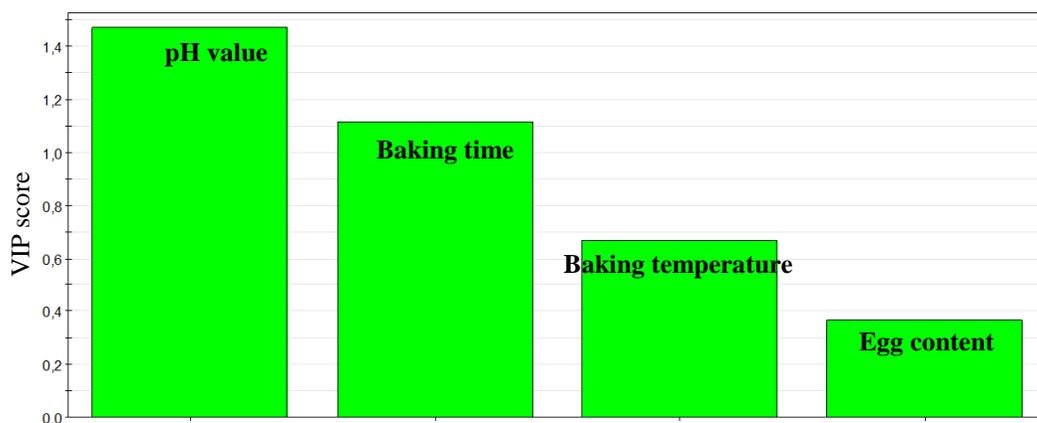


Figure 2. Variable Importance Plot (VIP) obtained for the data referred to acrylamide concentration during biscuit-making process.

As explained before, thermal inputs are strongly correlated to acrylamide formation and this aspect has been also suggested by results obtained in this study (Table 2). Baking parameters seem to have a fundamental influence on the acrylamide evolution, confirming that its formation is evidently time- and temperature-dependent. The prolongation of baking time from 5 min to 8 min resulted in the increase of acrylamide content of about 80% at 180°C, and of about 70% at 200°C, pointing out the fact that the acrylamide time-temperature (in particular time) control represents the key factor of acrylamide reduction. In our experiment, no reduction in acrylamide content was observed for longer baking time, despite many authors reported that acrylamide is subject to a partial degradation at high temperature and prolonged heating time [31,32]. Data also show that if baking was conducted at 180°C for 5 min a reduction ranged from 76 to 100% was achieved still obtaining an appreciable finished product.

Table 2. Processing conditions assumed during wholegrain and cocoa biscuit experiments in the pilot-scale plant.

Product	NaHCO ₃ (g)	Time (min)	Baking step	
			Temperature (°C)	Acrylamide (µg/kg d.m.)*
Wholegrain biscuit	8	5	180	16 ± 2
	8	8	180	125 ± 4
	8	5	200	66 ± 2
	8	8	200	306 ± 4
Cocoa biscuit	8	5	180	43 ± 3
	8	8	180	156 ± 5
	8	5	200	185 ± 5
	8	8	200	400 ± 12

*Data expressed as mean value ± standard deviation on dry matter (d.m.) basis.

Furthermore, focusing the attention on the development of a synergistic strategy in order to control different process contaminants (mycotoxin/acrylamide), suggestions about the proper setting of baking time and temperature can be inferred by the dedicated response Contour Plot (Figure 3): the increase of baking parameters within a range of mycotoxin mitigation (up to 30% of reduction) influences in a negligible way the final acrylamide content without implications on the organoleptic properties and consumer safety. With regard to the other minor ingredients, dextrose (or glucose) content seems to contribute to the overall acrylamide increase. Exploiting the effect of the combination power lower dextrose content/moderate thermal input (180°C for 8 min), a reduction up to 77% could be obtained; on the other hand, when high dextrose level and higher thermal input have been employed (200°C for 8 min) an increase up to 120% was observed (data not shown). These outcomes agree to what demonstrated by Vass et al. [33] that replacing invert sugar syrup with saccharose in wheat crackers reduced acrylamide by 60%. Similar effect were also observed by other authors [34,35], being the acrylamide formation strongly correlated to reducing sugars, as glucose is.

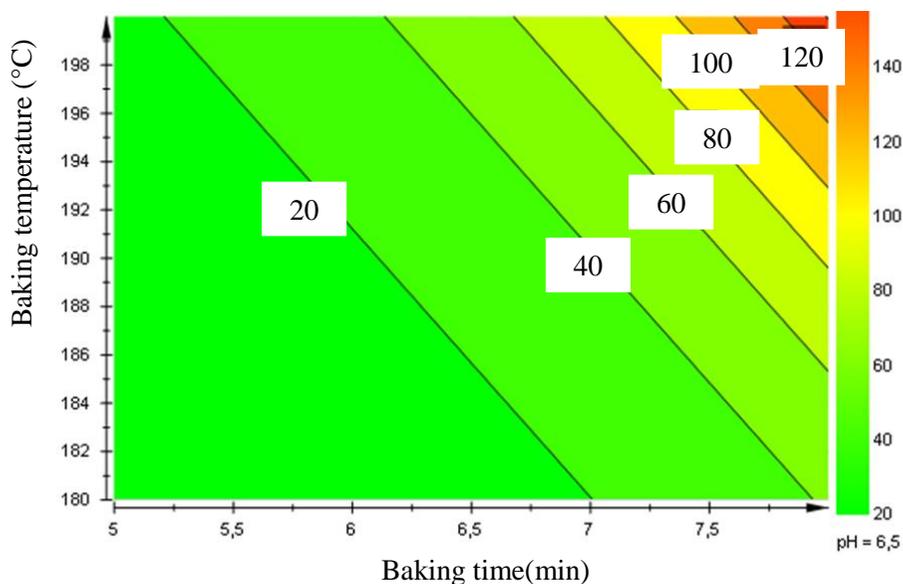


Figure 3. MODDE outputs referred to acrylamide concentration during biscuit-making process: Response Contour Plot -DON baking temperature vs baking time.

6.5 Conclusions

In the present study, the influence exerted by modifying ingredients and industrial conditions on acrylamide levels within a parallel mycotoxin mitigation strategy was investigated. Since precursors of acrylamide are present in the dough, modifications in recipe formulation and time-temperature control during baking process could be used to reduce acrylamide content in biscuits. The obtained processing models showed a good fitting, robustness and prediction capability, suggesting the most significant parameters which can concretely contribute to the reduction of acrylamide levels in the final food product. Starting from the relatively obvious premise that recipe formulation (in particular sugar content) demonstrates to have an impact on the acrylamide reduction, then the relevant highlights of the present work are the following ones: (1) acrylamide formation is evidently baking time- and temperature-dependent: prolongation of heat treatments results in higher contents of acrylamide; (2) the increasing of baking parameters within a proper range influences in a negligible way the final acrylamide content with no implications on the sensory aspects, within a parallel effective range of mycotoxin mitigation.

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**SECTION III. IMPACT ON HUMAN HEALTH OF MYCOTOXINS:
EXPLOITATION OF A STANDARDIZED STATIC IN VITRO DIGESTION
METHOD**

State of the art

In the previous sections, mycotoxin occurrence in foodstuff and their stability during food processing have been evaluated for target products widely consumed among populations. The potential presence of modified forms and their transformation upon production have been evaluated also for those compounds not regulated by legislation. Because of the lack of toxicological data, maximum limit have not been set as well as their occurrence has not been considered into the calculation of the final human intake.

It is well known that to exert toxic effects, mycotoxins have to be available from the food matrix [1]. For this reason, it is fundamental to calculate their bioaccessibility through the ingested food and the respective bioavailability. In some occasions, it could be very difficult to establish a connection between the amount of ingested mycotoxin and the expected effects. At this aim, different *in vitro* models have been developed, offering an appealing alternative to human and animal studies [2]. Results from these systems are often different to those found exploiting *in vivo* tests that usually provide most accurate information because of the complexity of the events occurring in the digestive tract. On the other hand, digestion models are usually more rapid, less expensive and labor intensive, representing a simplified strategy to perform experiment under controlled conditions with no ethical restrictions. In addition, accurately controlled conditions can reduce the high variability often encountered with *in vivo* studies, limiting the need for large numbers of replicates to obtain sufficient results.

In scientific literature, there is a huge number of digestion protocols and their variability could be related to the setting of a wide range of variables [3]:

- design of the system: procedures typically mimic the upper part of the gastrointestinal part, mouth-stomach-small intestine, as absorption mainly takes place in the small intestine; anyway, methods also include large intestinal fermentation;

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- composition of the physiological juices assayed: in some models, gastric juice is often simulated only with a strong decrease of the pH value, in other more complete systems gastric juice contain pepsin [4], and other several electrolytes [5];
- pH value: in some cases, the value is constant and usually low (1.3-2) [4], whereas for other procedures, pH decreases during simulation from higher values to lower ones in a gradual way as reported by Doll et al. [6];
- variety of food matrix: controversial results have recently been observed in the case of the bioaccessibility of DON in different types of Italian pasta [1]. Food composition, as well as contamination level, contamination type (spiked or naturally), and the ration of food bolus to digestive fluids may interfere in the way mycotoxins are absorbed through the intestinal tract;
- enzymatic activity: several studies have utilized enzymes collected from human subjects or from animal (porcine, rabbit, bovine origin) and plant sources, differing in their activity and characterization. Enzymes are usually added sequentially in order to simulate the different steps of the digestion process and they often need of more components (calcium, bile salts, etc.) to efficiently operate;
- other components: mineral type, ionic strength, phospholipids, individual enzymes, emulsifiers as well as the digestion time may alter enzyme activity and, consequently, the final results.

All these factors can impede the possibility to compare result and a compromise is needed between accuracy and easy utilization of the models. The relationship and connection among model components are crucial points in the determination of bioaccessibility: gastric pH value plays an essential role for the activity of pepsin which can contribute to the release of hidden fumonisins from the matrix [7], as an example.

In light of this, the development of a standardized digestion model is a relevant step in order to homogenize the effect of the considered parameters on the final outputs and to compare them across research-teams deducing general findings. At this aim, in the last year a consensus paper has been proposed in order to practically harmonize a static digestion method [8]. The protocol is based on physiologically relevant conditions that

can be applied for various endpoints in order to produce comparable data in the future (protocol details are provided into the following chapter).

The last section of this PhD thesis is dedicated to apply the last cited digestion protocol to investigate the stability of those mycotoxins considered in the previous studies. In particular, deoxynivalenol (DON), DON-3-glucoside (DON-3-Glc) and enniatin B (ENN B) have been selected to test the procedure by submitting standard solutions and naturally contaminated breads to the experiments in order to compare existent data from other digestion models with those obtained exploiting this international consensus.

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CHAPTER 7. STABILITY OF ENNIATIN B DURING SIMULATED GASTROINTESTINAL DIGESTION IN NATURALLY CONTAMINATED BREAD.

7.1 Introduction

The occurrence of mycotoxins, as the “emerging” mycotoxin enniatin B (ENN B), in several cereal based products leads to investigate the real impact of these compounds on human health [1,2]. Ingestion of food is the major route of exposure to mycotoxins and the total ingested amount does not always reflect the amount that is available in the body. As far as ENN B, very little is known about its bioavailability upon digestion. Some studies reported different results depending on type of applied procedure (static or dynamic digestion protocol), starting concentration level of the digested food, food composition, compound structure, and presence of natural adsorbing materials [3,4]. Indeed, data demonstrated that some prebiotic compounds are able to reduce the risk associated with the intake of *Fusarium* minor mycotoxins, through a retention mechanism similar to the phenomenon already observed for other compounds, such as minerals and polyphenols [5,6]. Other studies showed that dietary fibers are natural binding components that can capture minor mycotoxins and this affect their bioaccessibility, so it would be important to assess this effect in cereal products by using *in vitro* digestion systems [7,8].

On one hand, the pseudopeptidic structure might be somehow cleaved by human protease, thus leading to a decrease of the overall toxicity. On the other hand, the ring structure is stabilized by intramolecular H-bond, and might be thus less accessible to the enzymes.

Despite very limited data are available on its toxicity and, so far, it has not been well characterized in this respect, a potential risk from exposure to ENN B has to be assumed in light of some published data [9]. Studies have demonstrated that ENN B evidenced cytotoxic activity in different cell lines, including rodent, lepidopteran, monkey and human cells [10]. Although its bioavailability is apparently low upon oral administration, these undeniable evidences of ENN B toxicity, as well as the occurrence of high concentration of this bioactive compound in several types of food products, have persuaded the European Food Safety Authority (EFSA) to release an opinion on the risks

to human and animal health related to the presence of these “emerging” mycotoxins in food and feed, which is still in progress. In light of this, a clarification of the toxicological role of these compounds requires further investigations in order to include their occurrence in risk assessment studies.

7.2 Aim of the work

This work was carried out in order to clarify the fate of ENN B during human digestion exploiting a standardized static *in vitro* model. At this purpose, the current international consensus published by Minekus et al. [11] has been applied. Data have been evaluated on mycotoxin standard solution and naturally contamination bread at two different contamination levels, pointing out possible implication related to matrix interferences. Moreover, the effect of the gastric phase (acidic conditions) has been investigated by performing the same procedure with slight modification as the exclusion of the enzyme and by stopping the digestion after this step in order to evaluate the specific contribution of the gastric phase or the importance of the synergic effect.

7.3 Experimental section

7.3.1 Chemicals

Enniatin B (ENN B) standard was purchased from RomerLabs (Tulln, Austria). All solvents (HPLC grade) were from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was produced in our laboratory utilizing a Milli-Q system (Millipore, Marlborough, MA, USA). Salts were from Sigma-Aldrich (Taufkirchen, Germany). All chemicals for the preparation of the solutions mimicking the digestive juices (amylase from porcine pancreas, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, and bovine and ovine bile) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

7.3.2 Samples

Breads employed in the current investigation was prepared exploiting a pilot plant-line as previously described in Section II, Chapter 4. Another bread was prepared as a control sample using a mycotoxin-free wheat flour. Two loafs were prepared for each type of sample under study. With regard to ENN B, digestion has been performed upon two different levels representing a medium-to-high contamination range ($502 \pm 25 \mu\text{g}/\text{kg}$ and $1554 \pm 102 \mu\text{g}/\text{kg}$ of ENN B, respectively). In order to evaluate possible interferences on the final mycotoxin content due to matrix effect, digestion protocol was also tested on ENN B standard solution. At this purpose, a starting absolute amount of $5 \mu\text{g}$ for ENN B has been prepared into the digestion tube. Experiments were performed in triplicate.

7.3.3 *In vitro* digestion assay

All of the most important gastrointestinal digestion steps are mimed by this model, with the exception of fermentation by gut microbiota and permeation or transport across the intestinal epithelium. Since absorption takes place in the small intestine, this compartment was taken as the “end point” of the experiments. Thus, it is important to underline that the real “bioavailability” cannot be estimated by this approach. The concept “bioavailability”, in fact, comprises the availability after digestion, intestinal absorption and any metabolism of the target compound [12]. Since data concerning absorption and metabolism are not provided by the following model, is more correct use the term “bioaccessibility” to indicate the amount of considered compound that can be released from matrix after digestion and that is available for intestinal absorption.

The preparation of artificial digestive juices (Simulated Salivary Fluid, SSF, Simulated Gastric Fluid, SGF, and Simulated Intestinal Fluid, SIF) was performed according to the protocol of Minekus et al. [11] with some modifications. Table 1 resumes constituents and their respective concentrations used for the preparation of the synthetic juices. Before each experiment, all digestive juices were heated at $37 \pm 2 \text{ }^\circ\text{C}$. During the *in vitro* digestion, mixtures were stirred by a magnetic stirrer (250 rpm) to obtain a gentle but systematic mixing of the matrix with the digestive juices. The addition of enzymes, bile salts, Ca^{2+} solution etc. and water resulted in the correct electrolyte concentration in the final digestion mixture. The model has been briefly schematized in Figure 1.

Table 1. Preparation of stock solution of simulated digestion fluids. Volumes are calculated for a final volume of 500 ml (distilled water).

Constituents	Stock conc.	SSF	SGF	SIF
		pH 7	pH 3	pH7
		Vol. of stock	Vol. of stock	Vol. of stock
	g/l	ml	ml	ml
KCL	37.3	15.1	6.9	6.8
KH ₂ PO ₄	68	3.7	0.9	0.8
NaHCO ₃	84	6.8	12.5	42.5
NaCl	117	-	11.8	9.6
MgCl ₂ (H ₂ O) ₆	30.5	0.5	0.4	1.1
(NH ₄) ₂ CO ₃	48	0.06	0.5	-
For pH adjustment				
	mol/l	ml	ml	ml
NaOH	1	-	-	-
HCl	6	0.09	1.3	0.7

The digestion started by adding 3.5 ml of SSF electrolyte solution to 2.5 of ground sample and mincing them together. 0.5 ml α -amylase solution of 1500 U/ml made up in SSF electrolyte stock solution was added followed by 25 μ l of 0.3 M CaCl₂ and 975 μ l of water. The recommended time of contact with the enzyme was 2 minutes at 37°C. Then 7.5 ml of SGF were added and the mixture was incubated at 37°C for 2 hours. Porcine pepsin was calculated in order to achieve 2000 U/ml in the final digestion mixture, followed by CaCl₂. 1 M HCl was used to reduce the pH to 3.0 and the value was re-adjusted with 1 M HCl during digestion. Finally, the gastric samples-chyme was mixed with 11 ml of SIF electrolyte stock solution, 5 ml of pancreatin solution 800 U/ml made up in SIF electrolyte stock solution based on trypsin activity, 2.5 ml of bile (160 mM), 40 μ l of 0.3 M CaCl₂ and 1.31 ml of water. 0.15 ml of 1 M NaOH were required to neutralize the mixture to pH 7. A final incubation step of 2 hours at 37°C was performed. At the end of the experiment, the digestion tubes were centrifuged for 15 min at 4000 rpm, yielding the chyme (the supernatant) and the digested matrix (the pellet) in order to obtain a clear extract for the LC-MS/MS analysis.

In order to adapt the protocol to mycotoxin standard solution digestion, a scale down (14-fold) of the different amounts was calculated in order to reduce the volume of the final digested product, keeping the same balance among constituents.

Each experimental set was run in triplicate. A blank sample was prepared by mixing all the digestive fluids and enzymes in the absence of the target compounds.

7.3.4 *Step-by-step in vitro digestion experiments: gastric phase*

Another set of experiments was carried out by modifying the *in vitro* digestion protocol. The same bread and standard solution samples underwent a focused gastric phase digestion: gastric assay was run eliminating the enzymatic component in order to evaluate the effective impact of pepsin at this stage. Then, the digestion tubes were centrifuged at 400 rpm for 15 min and a proper amount of raw chyme was prepared for LC-MS/MS analysis. All the obtained results were compared with those supplied, by a complete digestion process, performed as control.

7.3.5 *Statistical analysis*

Statistical analysis were performed using SPSS v. 17.0 (SPSS Italia, Bologna, Italy) and OriginPro v. 8.0 (OriginLab, Northampton, USA). Data were statistically compared by using a OneWay ANOVA Test followed by a post-hoc Tukey Test ($p=0.05$).

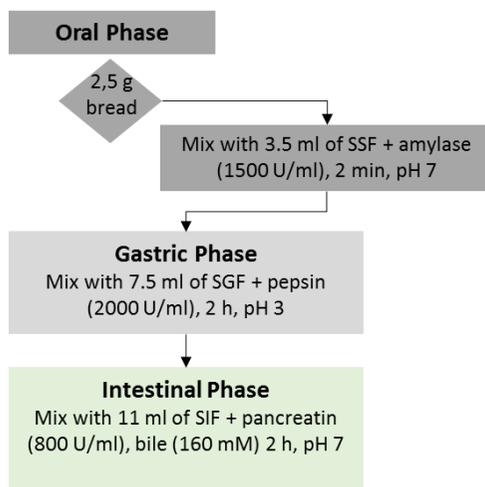


Figure 1. Overview and flow diagram of the simulated digestion method. SSF, SGF, and SIF are Simulated Salivary Fluid, Simulated Gastric Fluid and Simulated Intestinal Fluid, respectively. Enzyme activities are in units per ml of final digestion mixture at each corresponding digestion phase.

7.3.6 Instrumental conditions for ENN B analysis

Detection and quantification was performed with a Dionex Ultimate® 3000 UHPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a triple quadrupole TSQ Vantage MS/MS system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chromatographic separation was performed at 40 °C on a Kinetex® C18-column, 100 x 2.1 mm i.d., 2.6 µm particle size (Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases were acidified with 0.2% of formic acid and they were water (eluent A) and methanol (eluent B). After an initial time of 1 min at 30% B, the proportion of B was increased linearly to 65% within 8 min. Further linear increase of B to 90% within 20 min was followed by an isocratic step of 2 min at 90% B and a linear decrease of B to 30% within 23 min. A re-equilibration step at 30% for 7 min was observed (total analysis time: 30 min). The flow rate was 0.35 mL/min. ESI-MS/MS was performed in positive ionization mode (ESI+) with the following settings: spray voltage, 3000 V; capillary temperature, 270 °C; desolvation temperature, 200 °C; collision gas pressure, 80 psi. Table 2 summarizes the parameters of the optimized transitions.

Table 2. Optimized ESI-MS/MS conditions.

Compounds	Precursor ion (<i>m/z</i>)	Product ions (<i>m/z</i>)	Collisionenergy (V)
Enniatin B	640.6 [M+H] ⁺	86.0 ^b	49
		186.2 ^b	35
		196.2 ^a	23
		214.2 ^a	23
		314.0 ^b	31
		527.9 ^b	20
^a quantifier ion; ^b qualifier ion			

7.3.7 Method performance

The analytical methods used were assessed for linearity and recovery. Calibration curves were generated by linear regression of peak areas against concentrations ($r^2=0.99$ for ENN B). For the target compounds, limit of detection (LOD) and limit of quantification (LOQ) have been calculated, and they are 10 $\mu\text{g/l}$ and 30 $\mu\text{g/l}$, respectively. Recovery experiments were performed by spiking a blank chyme at a target concentration level (500 $\mu\text{g/l}$), and a mean value of 95% has been calculated. Matrix-matched calibration experiments were performed in the range 0.05-1.00 mg/l for the target analyte.

7.4 Results

Stability of ENN B toward a gastrointestinal digestion process was assessed in *in vitro* according to the last international consensus of a static *in vitro* digestion protocol [11]. Digestion was performed on ENN B standard solution and on naturally contaminated bread models. In addition, the effect of the acidic conditions of the gastric compartment has been evaluated performing a dedicated set of experiments. Sample were analysed for ENN B content by UPLC-MS/MS system. Due to the complexity of the digestive fluids, mycotoxin quantification was accomplished by interpolating data with matrix-matched calibration curves prepared in mycotoxin-free chyme. Typical extracted chromatogram for analysed mycotoxinis depicted in Figure 2 where the chromatographic peak referred to mycotoxin detection in the naturally contaminated bread is shown.

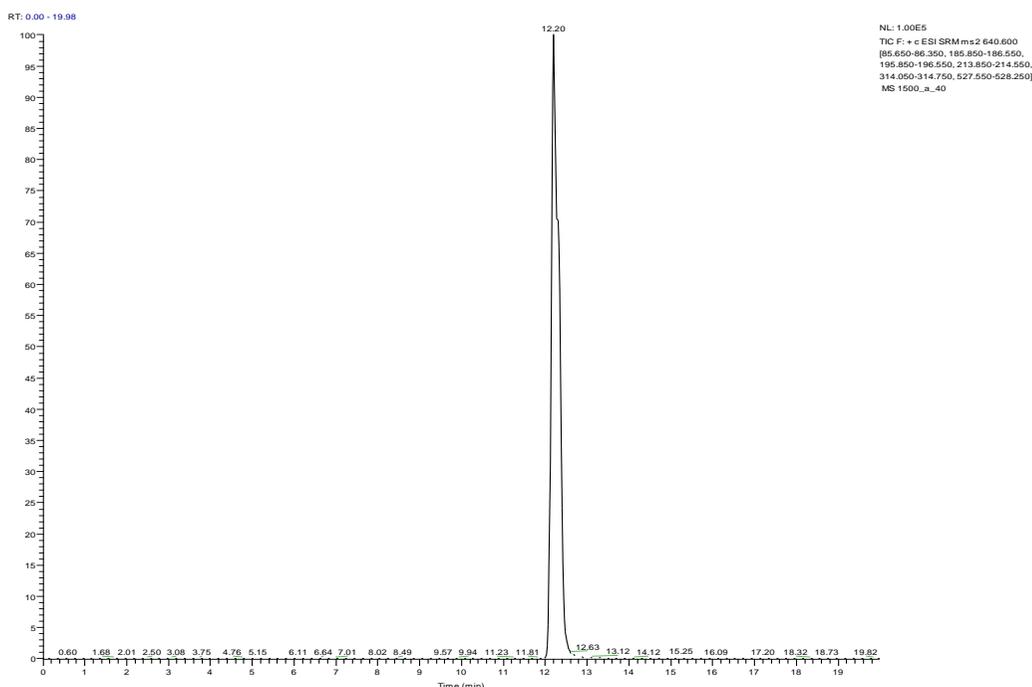


Figure 2. Extracted chromatograms referred to ENN B content: ENN B in naturally contaminated bread.

7.4.1 ENN B bioaccessibility after *in vitro* digestion

The stability of ENN B after gastrointestinal digestion of standard solution and naturally contaminated bread is shown in Figure 5. ENN B content dropped by 62% depending on the type of samples and contamination level. The lowest reduction has been recorded when 5 µg of pure mycotoxin has been digested with a mean decrease of about 57%. Concerning bread models, a significant difference has been observed depending on the mycotoxin contamination level: a 42% decrease has been calculated for the medium-contaminated bread, whereas a greater reduction has been observed when the high-contamination level has been tested (up to 62%). Moreover, results obtained after the simulation of the conditions of the gastric compartment showed that reduction took place in this tract and it seemed to be due to the acidic influence of the simulated gastric fluid, with a reduction of 38% and 60% for bread models (medium and high contamination, respectively) and a 54% decrease for ENN B standard model (Figure 5).

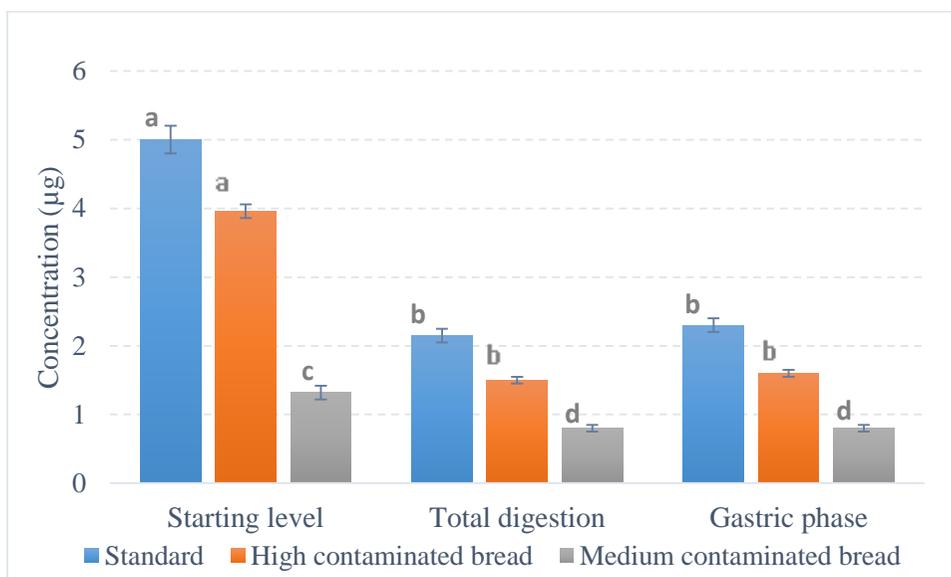


Figure 5. Trend of ENN B during digestion and after simulation of the gastric conditions of an ENN B standard solution and of naturally contaminated breads. Different letters designate statistically significant differences between data ($p < 0.05$).

The reduction in recoveries could be related to the digestion process and the use of pH values that can reduce mycotoxins levels. Moreover, according to Versanvoort et al. [13] it is possible that bioaccessibility may be underestimated because of a compound saturation that can occur in the chyme, a situation not possible *in vivo* where a compound, when it is released from the food matrix, is transported across the intestinal epithelium into the body, keeping the compound concentration low in the chyme.

7.5 Conclusions

In this study the stability of ENN B after digestion has been evaluated according to an *in vitro* standardized digestion procedure. ENN B was determined in the gastrointestinal fluid by UPLC-MS/MS and its mean bioaccessibility was about 46% depending on contamination level and matrix components. It was also demonstrated the significant role played by the acidic conditions of the gastric compartment on the final bioaccessibility value.

So far, limited studies have been developed upon ENN B gastrointestinal bioaccessibility. Meca et al. [4] calculated a reduction range from 9% to 31% after gastrointestinal digestion of wheat crispy bread depending on the contamination level of the tested material, resulting in a mean bioaccessibility of about 80%. In a following study, the same research group evaluated the effect of food composition on ENN bioaccessibility and a mean value of about 40% has been reported for wheat bran product with fibers [8]. Amount and type of fibers could be responsible as natural absorbing materials since they could combine mycotoxins, reducing their percentage of bioaccessibility. Serrano et al. [14] reported a significant difference on ENN B bioaccessibility depending on applied procedure: the duodenal bioaccessibility ranged from 0.37 to 22.41%, whereas employing the duodenal and colonic digestion, the mean bioaccessibility increased up 30%, suggesting that the macronutrient present in the matrix have the capacity to bind mycotoxins until the treatment with the colonic bacteria that hydrolyze the compounds increasing partially the bioaccessibility of the mycotoxins. On the same way, Manzini et al. [6] reported different result depending on structural protocol, observing lower bioaccessibility when static models have been applied with respect to the dynamic ones.

In conclusion, these results help to explain ENN B behavior after human ingestion, suggesting that a reduction of the risk associated to the intake of ENN B contained in foods takes place with respect to the toxin concentrations assayed in this investigation.

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**CHAPTER 8. APPLICABILITY OF A STANDARDIZED IN VITRO
DIGESTION MODEL TO ASSESS BIOACCESSIBILITY OF
DEOXYNIVALENOL & DEOXYNIVALENOL-3-GLUCOSIDE**

8.1 Introduction

In human health risk assessment, food ingestion is considered the major route of exposure to mycotoxins. Nevertheless, the total amount of the ingested contaminants (intake) does not reflect the amount that is actually available to the body. Two important concepts have to be clarified: *bioavailability* is a term used to describe the proportion of the ingested contaminant in food that reaches the systemic circulation, whereas *bioaccessibility* is the prerequisite for the following bioavailability, representing the compound released from the matrix into the digestive fluid of the gastrointestinal tract.

In the last decade, a big interest has been arisen in the use of *in vitro* methodologies to study the human bioavailability of toxin compounds as well as mycotoxins. So far, mycotoxin fate upon digestion process is not totally clear: modified mycotoxins may be degraded and their parent compounds could be partially or totally released after ingestion as well as they may exert an intrinsic toxicity, leading to an increased exposure compared to the estimated one by the conventional analytical approaches. All these aspects have to be clarified, especially considering the great variability of data due to the different approaches that researchers apply.

Concerning DON, toxicity mainly regards immune system and gastrointestinal tract [1]. Numerous studies examined its toxicokinetics, revealing two major derivative metabolites: de-epoxy deoxynivalenol (DOM-1) and deoxynivalenol-glucuronide (DON-GlcA) due to the de-epoxidation by anaerobic bacteria and conjugation of DON with a glucuronic acid molecule, respectively [2]. The presence of these characteristic metabolites in urine and feces has been used successfully as a biomarker to assess DON exposure and to get conclusions regarding its absorption and metabolism [3]. Depending on whether DON was applied in pure form or as radiolabelled compound, observed recoveries ranged from around 15 to 89% of the applied toxin dose, respectively. The

first *in vivo* case study about the human metabolism of DON suggested an average rate of DON excretion and glucuronation.

Despite, the considerable occurrence of DON-3-Glc in foodstuffs [4-6], the toxicological role in humans has not been clearly evaluated yet, due to the still unknown biological effects. Insufficient knowledge on its bioavailability may hamper an accurate risk assessment of ingested fraction in humans. DON-3-Glc is far less active as protein biosynthesis inhibitor than DON [7], but its fate after ingestion by mammals is largely unknown. So far, DON-3-Glc has not been considered in the regulatory limits established by the European Commission [8]. Some *in vitro* studies showed that in simulated gastrointestinal digestion, although the toxin seems to be rather stable under conditions simulating early stages of the mammalian digestion, certain specific intestinal bacteria are able to partially cleave DON-3-Glc to DON [9,10]. Furthermore, incubation of human fecal samples with DON-3-Glc resulted in an extensive liberation of DON [11]. These results suggest that DON may be partly bioavailable due to DON-3-Glc hydrolysis by bacterial-glucosidases in the colon and the extent cannot be predicted per each individual. Recently, two *in vivo* studies have been carried out about its metabolic fate in animals: results suggested that the majority of administered DON-3-Glc was cleaved during digestion in rats and pigs [12,13].

Considering the complex scenario, the joint FAO/WHO Expert Committee on Food Additives (JEFCA) emphasized the need of data concerning its absorption, distribution, metabolism and excretion in order to establish the potential health risk of DON-3-Glc [14].

8.2 Aim of the work

This work was carried out in order to clarify the fate of DON and DON-3-Glc during human digestion exploiting a standardized static *in vitro* model. At this purpose, the current international consensus published by Minekus et al. [15] has been applied. The main focus of the study was the comparison between data obtained by different digestion models, with a particular attention on the protocol developed by Versantvoort et al. [16], in order to evaluate which aspects can actually impact on the final outputs and

bioaccessibility data. Findings have been evaluated on mycotoxin standard solution and naturally contaminated bread, pointing out possible implications related to matrix interferences. Moreover, the effect of the gastric phase (acidic conditions) has been investigated by performing the same procedure with slight modification as the exclusion of the enzyme and by stopping the digestion after this step in order to evaluate the specific contribution of the gastric phase or the importance of the synergistic effect.

8.3 Experimental section

8.3.1 Chemicals

Mycotoxin standard deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-Glc) were purchased from RomerLabs (Tulln, Austria). All solvents (HPLC grade) were from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was produced in our laboratory utilizing a Milli-Q system (Millipore, Marlborough, MA, USA). Salts were from Sigma-Aldrich (Taufkirchen, Germany). All chemicals for the preparation of the solutions mimicking the digestive juices (amylase from porcine pancreas, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, and bovine and ovine bile) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

8.3.2 Samples

Breads employed in the current investigation were prepared exploiting a pilot plant-line as previously described in Section II, Chapter 3 for DON and DON-3-Glc. Another bread was prepared as a control sample using a mycotoxin-free wheat flour. Two loaves were prepared for each type of sample under study. Concerning DON and its modified forms, the concentration of both mycotoxins calculated on the final baked bread was found to be 4349 ± 387 $\mu\text{g}/\text{kg}$ for DON and 295 ± 7 $\mu\text{g}/\text{kg}$ for DON-3-Glc.

In order to evaluate possible interferences on the final mycotoxin content due to matrix effect, digestion protocol was also tested on DON, DON-3-Glc standard solutions. At this purpose, two starting absolute amounts of 5 μg for DON and 0.5 μg for DON-3-Glc have been prepared into the digestion tube, respectively. Experiments were performed in triplicate.

8.3.3 In vitro digestion assay

The preparation of artificial digestive juices (Simulated Salivary Fluid, SSF, Simulated Gastric Fluid, SGF, and Simulated Intestinal Fluid, SIF) was performed according to the protocol of Minekus et al. [15] with some modifications, as previously described in Chapter 7, Paragraph 7.3.3. All of the most important gastrointestinal digestion steps are mimed by this model, with the exception of fermentation by gut microbiota and permeation or transport across the intestinal epithelium.

In order to evaluate the effective impact of the acidic conditions of the gastric compartment, a dedicated set of experiments has been carried out according to the procedure reported in Chapter 7, Paragraph 7.3.4. Each experiment has been performed in triplicate.

8.3.4 Statistical analysis

Statistical analysis were performed using SPSS v. 17.0 (SPSS Italia, Bologna, Italy) and OriginPro v. 8.0 (OriginLab, Northampton, USA). Data were statistically compared by using a OneWay ANOVA Test followed by a post-hoc Tukey Test ($p=0.05$).

8.3.5 Instrumental conditions for DON and DON-3-Glc analysis

The UPLC-ESI/MS analyses were carried out according to Dall'Asta et al. [5], using a Dionex Ultimate® 3000 UHPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a triple quadrupole TSQ Vantage MS/MS system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chromatographic separation was performed at 40 °C on a Kinetex® C18-column, 100 x 2.1 mm i.d., 2.6 µm particle size (Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases were acidified with 0.2% of acetic acid and they were water 5mM ammonium acetate (eluent A) and methanol (eluent B). After an initial time of 1 min at 2% B, the proportion of B was increased linearly to 20% within 2 min. Further linear increase of B to 90% within 17 min was followed by an isocratic step of 3 min at 90% B and a linear decrease of B to 2% within 1 min. A re-equilibration step at 2% for 9 min was observed (total analysis time: 30 min). The flow rate was 0.35 mL/min. ESI-MS/MS was

performed in negative ionization mode (ESI-) with the following settings: capillary temperature, 270°C; spray voltage, 3500 kV; sheat gas pressure, 50 units; auxiliary gas pressure, 5 units. Detection was performed using selected reaction monitoring mode (SRM). The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standard (diluted in a 1:1 mixture of eluent A and B) into the MS source at a flow rate of 5 $\mu\text{L}/\text{min}$ for the corresponding values.

8.3.6 Method performance

The analytical method used was assessed for linearity and recovery. Calibration curves were generated by linear regression of peak areas against concentrations ($r^2=0.99$ for DON and DON-3-Glc, respectively). For all the target compounds, limit of detection (LOD) and limit of quantification (LOQ) have been calculated. Recovery experiments were performed by spiking a blank chyme at a target concentration level (500 $\mu\text{g}/\text{l}$). Matrix-matched calibration experiments were performed in the range 0.05-1.00 mg/l for the target analytes. Method performance characteristics are summarized in Table 1.

Table 1. Quality parameters of the analytical method.

	Deoxynivalenol	Deoxynivalenol-3-glucoside
Recovery %	95	89
LOD ^a ($\mu\text{g}/\text{l}$)	10	30
LOQ ^b ($\mu\text{g}/\text{l}$)	50	80

8.4 Results

An overview of DON content variation throughout the gastrointestinal tract referred to the standard solution and the naturally contaminated bread is shown in Figure 1. DON seemed to be not stable after gastrointestinal digestion for standard as well as for bread model. It was calculated that DON content dropped by 40% compared to the starting concentration in bread, whereas a 55% reduction has been observed for standard model probably due to a lower effect related to bread matrix interferences. Similar results have been described by De Angelis et al. [17] after digestion of a naturally contaminated bread where DON seemed to decrease of about 40%. Additionally, results inferred from the individual gastric system showed a relevant reduction (up to 45%) that was not dependent

on the pepsin action: as consequence, decrease seemed to be strictly correlated to the acidic conditions of the compartment and the lack of other substrates (Figure 1).

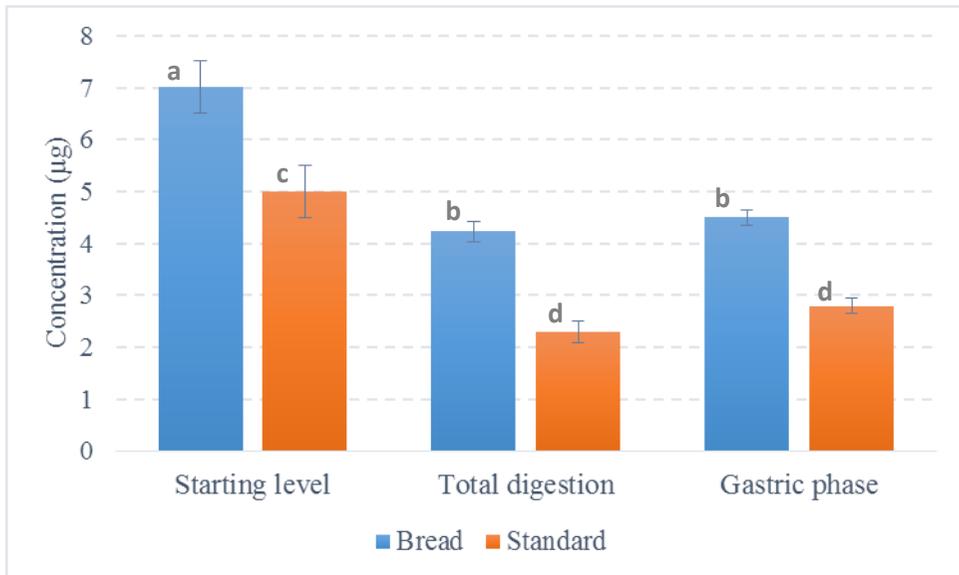


Figure 1. Trend of DON during digestion and after simulation of the gastric conditions of a DON standard solution and of a naturally contaminated bread. Different letters designate statistically significant differences between data ($p < 0.05$).

In this study, an analogous behavior has been observed for DON-3-Glc content: a considerable decrease (up to 95%) was calculated with no significant differences between standard and bread model ($p > 0.05$). Moreover, a minor impact of gastric conditions was observed, being a maximum reduction of 37% when DON-3-Glc was treated without enzyme (Figure 2). This reduction seemed to be rather inexplicable with respect to those reported by other authors where DON-3-Glc seemed to be completely fixed after digestion, suggesting that no degradation took place after the simulated digestion [11] and that less than 5% of DON-3-Glc was eventually hydrolyzed to DON [10]. In order to better explain the significant DON-3-Glc reduction observed in this investigation, the conversion of DON-3-Glc into the parent compound DON has been evaluated and DON has not detected in all analysed samples. De Angelis et al. [17] observed a final three-time higher DON-3-Glc level compared to the starting concentration, suggesting a possible conversion of the parent compound into its modified form, probably due to the

interaction of glucose with the bread matrix components. On the other hand, De Nijs et al. [10] did not detect any traces of DON-3-Glc although a DON reduction has been reported, according to what observed in this work. Taking into account the wide variety of protease activity, it could be hypothesized that it may be responsible for breaking the glycosidic linkage due to its esterase activity; for comprehensive understanding of this effect on final DON-3-Glc bioaccessibility, it is essential to study proteases in dedicated mycotoxin models, also considering the potential interference due to the occurrence of different substrates.

In light of these contradictory results, DON-3-Glc stability upon digestion conditions needs to be further investigated because of very little is known about the stability of DON-3-Glc along the gastrointestinal tract and also about the potential conversion into DON.

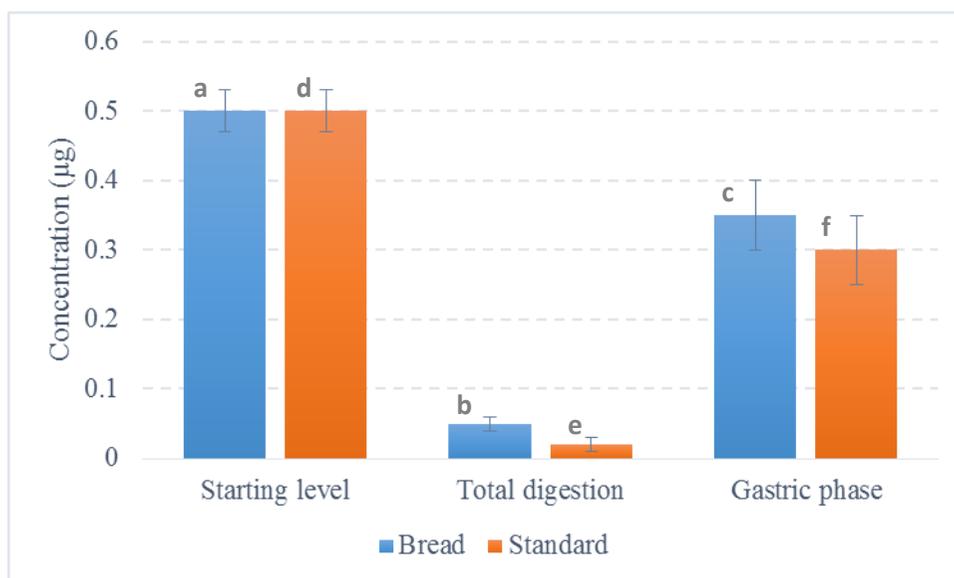


Figure 2. Trend of DON-3-Glc during digestion and after simulation of the gastric conditions of a DON-3-Glc standard solution and of a naturally contaminated bread. Different letters designate statistically significant differences between data ($p < 0.05$).

8.5 Conclusions

Considering this complex scenario, further investigations are required especially if we considered that results were obtained by different procedures and comparison was not immediate. Significant differences exist among protocols; in particular, the last international consensus proposed by Minekus et al. [15] represents a more simplified approaches compared to other digestion protocols, as that one previously proposed by Versantvoort et al. [18]. This procedure was characterized by the occurrence of more compounds and substrate into the digestive fluids as well as urea, uric acid, glucose, glucuronic acid, glucoseamine, lipase, and mucin that collaborated to better simulate the human digestive conditions. Mucin, for example, has a lubricant effect during mastication but it was not used during this standardized digestion being a minor component of saliva. Additionally, during ingestion, the pH increases depending on the buffer capacity of the food matrix. Then, pH is gradually decreasing due to hydrochloric acid secretion and the penetration of the acid in the bolus is slower with respect to what happens when hydrochloric acid is used to adjust the pH value. If Versantvoort et al. [18] took into account the amount and type of food in order to set a proper pH working range, in the last consensus pH value has been set at a fixed value for all food matrices. Another aspect is related to gastric lipase activity. Its activity is markedly lower in the gastric compartment and the addition has been omitted because of the unavailability of human gastric lipase or similar alternatives. This omission could not be adequate in order to simulate the complete digestion process because of its connection with other enzymes and interaction on their own activity. Furthermore, incubation for 2 hours at pH 3 could represent an extreme adaptation compared to the much milder *in vivo* exposure. All these factors could impact the final outputs and further investigations are needed in order to establish a final setting for different applications and to have consensus in the big scientific community.

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General Conclusions

In the present Ph.D. thesis the transformation of regulated and emerging mycotoxins has been investigated starting from the field to the finished product, considering also their potential harmful effect on human health. In the first section, a greenhouse experiment was carried out in order to better understand the conversion capability of deoxynivalenol (DON) into deoxynivalenol-3-glucoside (DON-3-Glc) as defense mechanism towards one of the most severe disease that affects wheat plants (*Fusarium* Head Blight, FHB). In this experiment, one line of common wheat (*Sumai*) and two lines of durum wheat (*Kofa* and *Svevo*) were directly contaminated with DON. Results confirmed the ability of all tested lines to convert DON to DON-3-Glc: in particular, *Sumai* (the most known resistant line) exhibited a significant conversion rate of DON to DON-3-Glc compared to durum wheat lines that displayed a different ability to express the glycosylation activity. From results, *Kofa* has been identified as a potential candidate for further investigation related to breeding program in the durum wheat production chain.

The second section of this thesis has been dedicated to the transformation of mycotoxins upon food processing along industrial and pilot plant production lines, with a particular focus on technological steps and recipe formulation. In particular, regulated mycotoxins, as DON and fumonisins (FBs), as well as mycotoxins representing an emerging issue, as DON-3-Glc and enniatin B (ENN B), have been selected in order to evaluate the potential impact of some strategic commercial production chain on their content in the final product. Concerning FBs, the conducted study was the first one specifically focused on their fate along the complete industrial cornmeal production process. Findings showed a significant reduction into the cornmeal semolina, achieving a 40% of reduction, mainly due to the cooking treatments; at the same time, results obtained for by-products showed a very high FBs increase, pointing out the need to carefully manage the use of these by-products as feed for livestock. Another fundamental production chain is that one related to bread production: bread plays a key-role in the human diet worldwide; moreover, in the last years, a big interest has been arisen towards wholegrain recipe formulation because of benefits on human health connected to adding fiber consumption. For all these

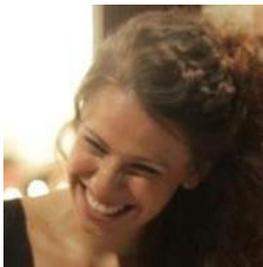
General Conclusions

reasons, wholegrain bread from naturally contaminated bran has been produced in a pilot-plant scale in order to evaluate the potential effect of bread-making process on the most known DON and DON-3-Glc mycotoxins, representing the major issue for wheat, and on ENN B, the most relevant “emerging” mycotoxin. Results for DON reported a mean reduction of about 25% with respect to the starting flour, whereas DON-3-Glc seemed to be rather stable. The study suggested their levels can be controlled during bread-making process, although DON-3-Glc needs to be further investigated because of the marked release observed during the initial production steps. In a similar way, ENN B seemed to be reduced up to 30% in the baked bread, depending on the initial mycotoxin concentration. In light of these results, food processing could be actually exploited as a mitigation strategy in order to reduce mycotoxin content in the finished product. The optimization of processing parameters, as an efficient setting of fermentation or baking time/temperature, as well as ingredients content, may lead to minimize the final mycotoxin level. At this purpose, three experimental models have been developed in order to evaluate which technological parameters could have a relevant impact on the mycotoxin mitigation. These selected parameters have been exploited to minimize mycotoxin content in the finished product, remaining in an acceptable range from a qualitative point of view. Rusks, wholegrain and cocoa biscuits, widely consumed among populations, have been selected to apply the experimental model for some mycotoxins of concern, as DON, DON-3-Glc and ochratoxin A (OTA). The approach to the model was the most innovative aspect of the study, because of the interrelation among several scientific skills: a dedicated study of the setting of the technological parameters in order to obtain an appreciable finished product for consumers, the exploitation of different pilot-plant lines to simulate the industrial production, the optimization of analytical methods for the simultaneous detection of different metabolites in more complex matrices as well as biscuits, the statistical elaboration and the interpretation of the obtained data set, finally, the evaluation of the feasibility of the performed model working on reduction direction of other process contaminants, as well as acrylamide. Results suggested potential mycotoxin reductions up to 30%, exploiting the synergy among selected parameters, in particular baking time/temperature and pH value of the dough. The study represents a tangible example of how a careful control of industrial line may

mitigate mycotoxin impact. The management of cereal processing plays an important role especially when mycotoxin levels in raw materials are closed to the European legal limits in the case of specific year-campaign negative circumstances.

In the last section, mycotoxin bioaccessibility has been studied through several *in vitro* experiments. The digestion assay allowed both to evaluate the potential risk associated to the presence of mycotoxins in a food matrix and to obtain information concerning their behavior after ingestion and the potential contribution to the overall toxicity. At this purpose, a standardized digestion model has been applied: in the scientific literature, there is a huge number of digestion protocols, impeding the possibility to compare results across research-teams. The protocol selected for this thesis has been applied in order to investigate the gastrointestinal fate of the well-known DON, as well as DON-3-Glc and ENN B. Results for DON showed a significant reduction after digestion, whereas DON-3-Glc recorded the most relevant decrease (up to 95%) and no DON has been detected into the analyzed samples. These results are rather inexplicable: a possible effect of proteases has been supposed due to the wide variability of their activity that could impact on DON-3-Glc stability but further experiments are actually needed to clarify this point. Concerning ENN B, a similar trend has been reported with reduction up to 62%, depending on the type of samples and contamination level. In light of these results, a future perspective could be the optimization of the standardized digestion protocol in order to homogenize the wide range of variables and to have consensus in the scientific community.

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Studies

Silvia Generotti got the Bachelor Degree in Food Science (University of Perugia, Italy, 110/110, Honours) in February 2009 with a particular focus on plant pathology and fungal agent characterization. In April 2012, she got the Master Degree in Food Technology and Biotechnology (University of Perugia, Italy, 110/110, Honours) with a thesis titled “Investigation about the presence of *Fusarium* species and determination of the main mycotoxins in soft and durum wheat in Umbria”. As visiting researcher, she optimized new analytical methods dedicated to mycotoxin detection (mainly, trichothecenes and “emerging” mycotoxins) at the Department of Toxicology of the University of Valencia (Spain). In June 2012, she started at Barilla in the Primary Processing Area, developing a new predictive formula related to milling yield. In January 2013, Silvia Generotti started the PhD in Food Science (University of Parma, Italy), under the supervision of Prof. Chiara Dall’Asta and Dr. Michele Suman. The PhD work has been characterized by a strict collaboration between Department of Food Science (University of Parma, Italy), Advanced Laboratory Research (Barilla, Italy) and IFA-BOKU (Vienna, Austria). Her research focus was on the fate of mycotoxins during food processing, with a particular attention on the optimization of technological parameters in order to minimize mycotoxin content in the finished products and on the investigation of the toxicological role of “emerging” contaminants in humans.

Scientific activity

Manuscripts in preparation

Degradation study of enniatin B during bread-making process

Silvia Generotti, Martina Cirlini, Ana Belen Serrano, Giuseppe Meca, Chiara Dall'Asta, Michele Suman

Technological approach to reduce acrylamide levels within bakery production

Silvia Generotti, Martina Cirlini, Dante Catellani, Chiara Dall'Asta, Michele Suman

Submitted publication

Strategic mycotoxin mitigation within industrial biscuit-making technology

Silvia Generotti, Martina Cirlini, Bojan Šarkanj, Michael Sulyok, Franz Berthiller, Chiara Dall'Asta, Michele Suman

Publications in Scientific Journal

*Wheat (*Triticum Durum* Desf.) lines show different abilities to form masked mycotoxins under greenhouse conditions*

Martina Cirlini, Silvia Generotti, Andrea Dall'Erta, Pietro Lancioni, Gianluca Ferrazzano, Andrea Massi, Gianni Galaverna
Toxins, 2014, 6, 81-95

Transformation of mycotoxins upon food processing: masking, binding and degradation phenomena

Michele Suman, Silvia Generotti

In Masked Mycotoxins in Food: Formation, Occurrence and Toxicological Relevance. RSC Publishing: London, UK, 2015

Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology

Silvia Generotti, Martina Cirlini, Alexandra Malachova, Michael Sulyok, Franz Berthiller, Chiara Dall'Asta, Michele Suman
Toxins, 2015, 7, 2773-2790

Influence of the industrial process from caryopsis to cornmeal semolina of fumonisins and their masked forms

Silvia Generotti, Martina Cirlini, Chiara Dall'Asta, Michele Suman
Food Control, 2015, 48, 170-174

Effects of orally administered fumonisin B1 (FB1), partially hydrolysed FB1, hydrolysed FB1 and N-(1-deoxy-D-fructos-1-yl) FB1 on the sphingolipid metabolism in rats

Irene Hahn, Veronika Nagl, Heidi Elisabeth Schwartz-Zimmermann, Elisabeth Varga, Christiane Schwarz, Veronika Slavik, Nicole Reisinger, Alexandra Malachová, Martina

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Biosynthesis of beauvericin and enniatins in vitro by wheat Fusarium species and natural grain contamination in an area of central Italy
Lorenzo Covarelli, Giovanni Beccari, Antonio Prodi, Silvia Generotti, Federico Etruschi, Giuseppe Meca, Cristina Juan, Jordi Mañes
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Fusarium species, chemotype characterisation and trichothecene contamination of durum and soft wheat in an area of central Italy
Lorenzo Covarelli, Giovanni Beccari, Antonio Prodi, Silvia Generotti, Federico Etruschi, Cristina Juan, Emilia Ferrer, Jordi Mañes
Journal of the Science of Food and Agriculture, 2015, 95, 540-551

Publications in proceedings

Oral communications

Fate of mycotoxins in the cornmeal processing from caryopsis to pre-cooked porridge
Silvia Generotti, Martina Cirlini, Michele Suman, Chiara Dall'Asta
Conference Abstracts of 36th Mycotoxin Workshop 2014, Göttingen, Germany, 16-18 June 2014, p. 58, L33

Strategic mycotoxin mitigation within industrial biscuit-making process
Silvia Generotti, Martina Cirlini, Bojan Šarkanj, Michael Sulyok, Franz Berthiller, Chiara Dall'Asta, Michele Suman
Conference Abstracts of Join ICC/AISTEC Conference of the World Expo "Grains for feeding the world 2015", Milan, Italy, 1-3 July 2015, Tecnica Molitoria, vol. L XVI, p.9

Poster communications

Study of the influence of the cornmeal process from caryopsis to pre-cooked porridge on levels of fumonisins and their masked forms
Silvia Generotti, Martina Cirlini, Michele Suman, Chiara Dall'Asta
Book of Abstracts of MycoRed International Conference Europe 2013, Martina Franca, Italy, 27-31 May 2013

Fate of mycotoxins in the cornmeal processing from caryopsis to pre-cooked product
Book of Abstracts of 6th International Symposium on Recent Advances in Food Analysis 2013, Prague, Czech Republic, 5-8 November 2013
Investigation of mycotoxin conversion to their masked forms in wheat
Martina Cirlini, Silvia Generotti, Chiara Dall'Asta, Michele Suman, Andrea Massi, Gianni Galaverna

Conference Abstracts of XIX Convegno Nazionale Società Italiana di Patologia Vegetale 2013, Padova, Italy, 23-25 September 2013

Fate of mycotoxins along industrial biscuit and rusk processing with deoxynivalenol and ochratoxin A as main targets

Book of Abstracts of 8th World Mycotoxin Forum Conference 2014, Vienna, Austria, 10-12 November 2014

Participation to national and international conferences

MycRed International Conference. Martina Franca, Italy, 27-31 May 2013

XIX Convegno Nazionale Società Italiana di Patologia Vegetale. Padova, Italy, 23-25 September 2013

3rd MS Food Day. Trento, Italy, 9-11 October 2013

36th Mycotoxin Workshop. Göttingen, Germany, 16-18 June 2014

8th World Mycotoxin Forum Conference. Vienna, Austria, 10-12 November 2014

Join ICC/AISTEC "Grains for feeding the world 2015". Milan, Italy, 1-3 July 2015

Participation to PhD School and Workshops

18° Corso di Spettrometria di Massa per Dottorandi di Ricerca 2014. Siena, Italy, 7-11 April 2014

Mycotoxin Summer Accademy 2014. Vienna, Austria, 30 June-11 July 2014

XX Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. Perugia, Italy, 23-25 September 2015