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Free and Hidden Fumonisins in Corn (*Zea Mays* L.): Occurrence and Masking Mechanism

Coordinatore:
Chiar.mo Prof. Davide Barbanti

Tutor:
Chiar.ma Prof.ssa Chiara Dall'Asta

Dottorando: Claudia Falavigna

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PREFACE

Fumonisins are a group of toxic metabolites produced by a number of *Fusarium* species especially in maize during the pre-harvest period, leading to a risk for consumer health as well as to economical losses, in particular in the Mediterranean area. Among the several analogues that have been identified and characterized since 1988 and grouped into four series, those belonging to the B-series, comprising FB_1, FB_2 and FB_3, are the major mycotoxins produced in corn and are able to cause severe disease in animals. Since a positive correlation between FB_1-contaminated corn consumption and human oesophageal cancer was found, these metabolites were classified as potentially carcinogenic (class 2B) by IARC and, consequently, legal limits were established by the European Community concerning the maximum tolerable amount of such mycotoxins in maize intended both for human and animal use.

Thus, studies aimed to individuate the main factors that affect *Fusarium* infection as well as fumonisins production and their accumulation during the entire maize-chain is a topic of great interest in order to guarantee a good safety level for the consumer.

A new recent issue which made more intriguing the research concerning these mycotoxins is represented by the discovery of hidden fumonisins: different structurally related compounds which can be covalently or not covalently linked with various food macroconstituents.

Since these derivatives are chemically and physically different from their precursors, they are able to escape routine analysis, leading to an underestimation of the real amount of mycotoxin in a food sample, thus introducing a new serious problem concerning food safety. Indeed, these compounds may have a toxicity comparable to that shown by free forms or may be released from the matrix after food processing or digestion, exposing the consumer to a higher risk than that estimated through the common analytical procedures.

The most interesting aspect concerning this phenomenon is that masking mechanism is not yet clearly explained. To date two main hypothesis have been formulated, being the former based on the formation of covalently bound fumonisin-derivatives and the latter on the formation of associative complexations between parent forms and macromolecules. However, experimental evidences obtained are still to poor to explain such phenomenon exhaustively, therefore this topic represent an approximately unexplored search field of a great interest from a scientific point of view.

Moreover, almost all of the studies performed until now on fumonisins considered only parent forms directly detectable through routine methods of analysis, without regard to hidden derivatives. Consequently, there are only few information available concerning the occurrence of these derivatives and the conditions that affect masking phenomenon, as well as their toxicity or the fate to which hidden fumonisins are subjected during food processing and gastrointestinal digestion.

The work here presented is articulated on three levels, each of them is focused on the study of a specific aspect of fumonisins, considering especially hidden forms.

The first section is entirely dedicated to the comprehension of masking mechanism: starting from the state-of-the-art existing for this topic, a detailed research supported by several *in vitro* experiments has been performed in order to establish which type of interactions occur between the target analyte and the main maize macromolecules, such as starch and prolamin proteins.

Section II is dedicated to the research of the main factors able to promote fungal infection and fumonisin production and accumulation in growing maize, focusing the attention on the role played by the maize genotype and the macromolecular composition as well as by the plant-pathogen interaction in masking phenomenon.

In the same section, the dynamic of free and hidden fumonisins distribution and accumulation from field to flour production has been studied, evaluating also how the contamination of a maize bulk stored in a monitored silo change over time under storage conditions.

Finally, the work performed in the last section aimed to study *in vitro* fumonisins production by different *Fusarium* species, employing LC-ESI-MS/MS technique to identify and characterize also fumonisins belonging to the minor series A and C, in addition to those appertaining to the group B. Thus, the production of such metabolites by fungi under different growing conditions has been monitored with the aim to collect more information concerning the production pattern of such compounds and the conditions in which their biosynthesis takes place.

The sections of the present work are organized independently from each other: each one is provided with a proper introduction, containing both general information and detailed knowledge about the topic specifically addressed, in order to allow an immediate contextualization of the treated subject. Likewise, the literature cited throughout each section is reported at the end of the same section, in order of citation.

Moreover, each part has been divided in two chapters, organized as scientific publications and thus provided with a brief introduction focused on the specific field, with detailed experimental paragraphs and, finally, with an exhaustive description of the main results.
This particular structure has been conceived in order to allow the reader to directly approach the topic of interest, thus easily and quickly finding all the required information.

TERMINOLOGY USED

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 use to designate each of them can not be confused.

"Free extractable fumonisins" is the expression used to designate parent fumonisins directly extractable from a matrix through a common extraction procedure, based on the employ of a binary mixture of water and methanol.

"Total fumonisins after hydrolysis" or simply "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. This treatment allows the complete cleavage of the tricarballilic moieties from the central backbone of both free and hidden mycotoxins, thus releasing hydrolyzed forms. 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.

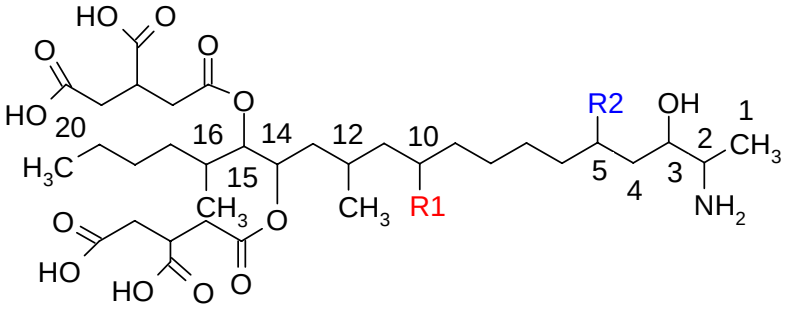
The term "Hidden fumonisins" is used to identify those forms that interact in different ways with food constituents, thus escaping routine analysis. Their amount is established through an indirect approach, by calculating the difference between total and free fumonisins. In a still more meticulous distinction, the expressions "hidden fumonisins" and "bound fumonisins" are used to indicate masked forms that interact in different manner with matrix constituents: whereas "hidden" indicates forms complexated or physically entrapped into maize macromolecules, the term "bound" designates fumonisins linked to matrix constituents through covalent interactions.

The terminology proposed above will be used throughout the entire thesis.

SECTION I. *IN VITRO* MODELS FOR STUDYING THE FUMONISIN MASKING MECHANISM IN MAIZE

FREE AND HIDDEN FUMONISINS

Fumonisins are a group of structurally-related mycotoxins firstly described and characterized in 1988 as secondary metabolites produced by a number of *Fusarium* species, notably *F. verticillioides*, *F. proliferatum* and *F. nygamai* (1, 2). These filamentous fungi are destructive pathogens on cereals crops and other commodities, in particular *F. verticillioides* is one of the most seed-borne fungi associated with corn (maize, *Zea Mays L.*) which produces fumonisins during in-field infection and also through the entire maize chain (3, 4). The fumonisins analogues can be classified into four main groups, identified as the fumonisins series A, B, C and P (5, 6). Among them, the fumonisin B analogues (FBs), comprising FB_1, FB_2 and FB_3, are the most abundant naturally occurring fumonisins in maize and maize-based products (7). Chemically, these compounds are characterized by a 20 carbon aminopolyhydroxyalkyl chain diesterified with propane-1,2,3-tricarboxylic acid (tricarballylic acid; Figure 1) (8) .



Toxin	R1	R2
FB_1	OH	OH
FB_2	H	OH
FB_3	OH	H

Figure 1. Main fumonisins structures.

Fumonisin B_1 represents, at the same time, the most common and toxic derivative, while FB_2 and FB_3 are, respectively, the 10-dehydroxy- and 5-dehydroxy-FB_1 analogues, in which the corresponding epimeric units on the backbone have the same configuration (9).

Since these compounds bear a clear structural similarity to the long-chain base backbones of sphingolipids, they can inhibit *de novo* sphingolipids biosynthesis through the inhibition of the enzyme ceramide synthase, leading to an increase of sphinganine levels and in the ratio of sphinganine to sphingosine in serum or urine in a dose-dependent manner (7, 10). Therefore, fumonisins may cause a large variety of disease in animals, affecting different target-organs depending on the species. In particular they cause equine leukoencephalomalacia (ELEM) in horses and porcine pulmonary edema (PPE) in swine, in addition to hepatocarcinogenic, hepatotoxic, nephrotoxic and cytotoxic effects in mammals (11). Moreover, recent studies have demonstrated that fumonisins are able to inhibite folate transport, thus they seem to be involved in the occurrence of neural tube defects (NTD), which are embryonic defects of the brain and spinal cord resulting from failure of the neural tube to close (12). To date, there are no data available concerning toxic effects found in human, however consumption of FB_1-contaminated corn has been associated with elevated human oesophageal cancer incidence in various part of Africa, Central America and Asia (4). For these reasons, FB_1 has been declared as a class 2B carcinogen by the International Agency for the Research on Cancer (IARC) (13) and since 2007 legal limits have been established in European Union for these contaminants both in food and in raw materials intended for human consumption. According to this Regulation, the limits for total fumonisins in unprocessed raw maize (4000 μg/Kg), maize for direct human consumption (1000 μg/Kg), maize-based breakfast cereals and snacks (800 μg/Kg) and in baby foods (200 μg/Kg) have been scheduled (14).

In the last recent years the studies regarding fumonisins have been made more attractive by the discovery of many structurally related compounds generated by plant metabolism or by food processing called “masked” or “hidden” fumonisins. Hidden or bound fumonisins are fumonisin derivatives covalently or not covalently linked with various matrix constituents that can co-exist with parent forms, both in raw maize and in maize-based products (15, 16). Since these compounds may have different chemical behaviours compared to their parent forms, they can easily escape routine analyses (17). Consequently, such compounds are detectable only after the application of an hydrolysis step on the matrix, which can cleave the tricarballylic moieties of fumonisins, that are considered the main responsible of the fumonisins-matrix interactions, thus releasing the corresponding hydrolyzed forms (16).

From a food safety point of view, the occurrence of hidden fumonisins must be considered, since they could exert toxic effects in the same way as free fumonisins or after the release of their parent forms following digestion or food processing (17). Downstream of all these consideration, it is obvious to think that the individuation of the main factors and conditions that can affect the production of fumonisins in raw maize and their accumulation during the entire maize chain is a topic of great interest in order to guarantee a good safety level for the consumers.

HIDDEN FUMONISINS: STATE-OF-THE-ART

Masked mycotoxins are mycotoxins derivatives either incorporated into macromolecules or covalently linked with more polar compounds that can co-exist with parent forms. In particular, this phenomenon is related to *Fusarium* toxins (fumonisins, trichothecenes and zearalenone). Such metabolites may be originated directly by fungi (e.g. 3-acetyl-deoxynivalenol), by plants as detoxifying mechanism in order to convert the relatively apolar mycotoxins in more polar derivatives via conjugation with sugars, amino acids or sulphate groups to compartmentalise them in vacuoles (e.g. zearalenone-4-O-glucoside and deoxynivalenol-3-O-glucoside) or during food processing after reaction with proteins, amino acids or sugars during cooking treatments (e.g. N-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (18). Among masked mycotoxins, hidden fumonisins are of particular concern, since the nature of their interaction with foods components has been not yet clearly explained. Their presence has been initially suggested in order to explain the "fumonisins paradox", according to which fumonisins can still exert toxic effect although 90% is excreted through faeces when administer orally (19). This fact could be partly explained assuming that parent fumonisins would be released from some masked forms upon digestion. In recent years the occurrence of hidden fumonisins was demonstrated by several authors both in raw maize and in maize based products though the application of an hydrolysis step on the matrix (15, 16, 20,). In fact, since these compounds have a different chemical behaviour compared with their precursors, they can easily escape conventional extractions, thus they are detectable upon alkaline hydrolysis. Figure 2 shows the conversion of fumonisin B_1 to hydrolyzed fumonisin B_1: alkaline treatment can cleave the tricarballylic moieties from the aliphatic central backbone, thus releasing hydrolyzed form.

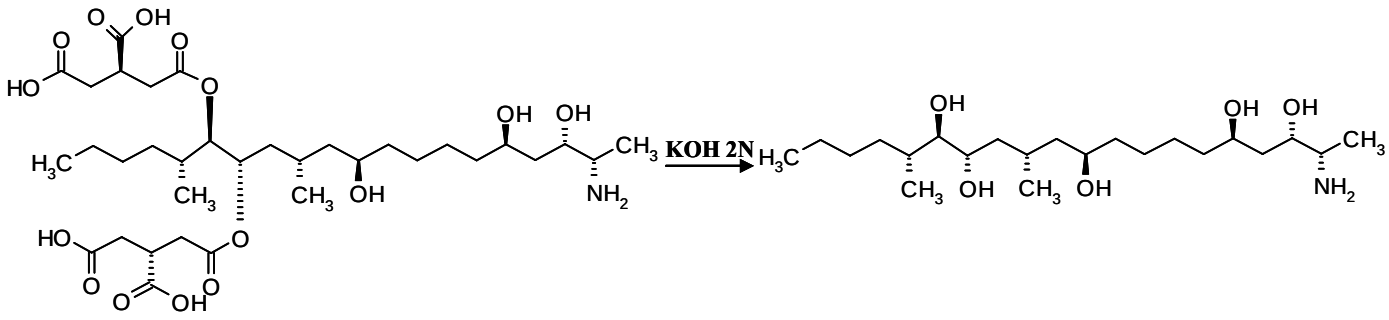


Figure 2. Conversion of fumonisin B_1 to its hydrolyzed analogue after alkaline hydrolysis.

Through this indirect approach authors have demonstrated that the amount of released hydrolyzed fumonisins is often higher than that stechiometrically derived by the conversion of the fumonisins detected by the routine analytical methods (16). As already mentioned, the nature of fumonisins-matrix interactions has not yet been clarified. To explain this phenomenon, two main hypotheses can be considered, being the former based on the formation of covalently bound FB-derivatives and the latter on the formation of associative complexations between parent forms and macromolecules. According to the first hypothesis, the nature of the masking mechanism is due to the formation of covalent bonds between the functional groups of fumonisins and the hydroxyl groups of starch or the amino or sulfidryl groups of the side chains of amino acids in proteins. Instead, according to other studies, the masking phenomenon can be due to a physical entrapment or a complexation of fumonisins by some food macromolecules including starch and proteins (16). These two theories are detailed below.

Covalent-bond hypothesis. Shier et al. (21) observed that by adding radioactively labelled FB_1 to a maize flour, only about 37% of the total radioactivity was detected through a conventional extraction after baking, while further 46% was recovered in association with the proteic fraction, after extraction using sodium dodecyl sulphate (SDS). Therefore, an activation of the molecule by the dehydration of one tricarballilic unit due to the thermal treatment and the subsequent formation of an anhydride able to react with the functional groups of amino acids or sugars was hypothesized. On the basis of this theory, Howard et al. (22) concluded that N-(Carboxymethyl) fumonisin B_1 (NCM) was the main reaction product resulting by heating FB_1 with an aqueous solution of reducing sugars. Poling et al. (23) described N-(1-Deoxy-D-fructos-1-yl) fumonisin B_1 (NDF) as the first product formed after Amadori rearrangement of the Schiff base formed by the reaction of the primary amine of

fumonisin B_1 and the aldehyde group of D-glucose. Molecular structures of these two derivatives are shown in Figure 3.

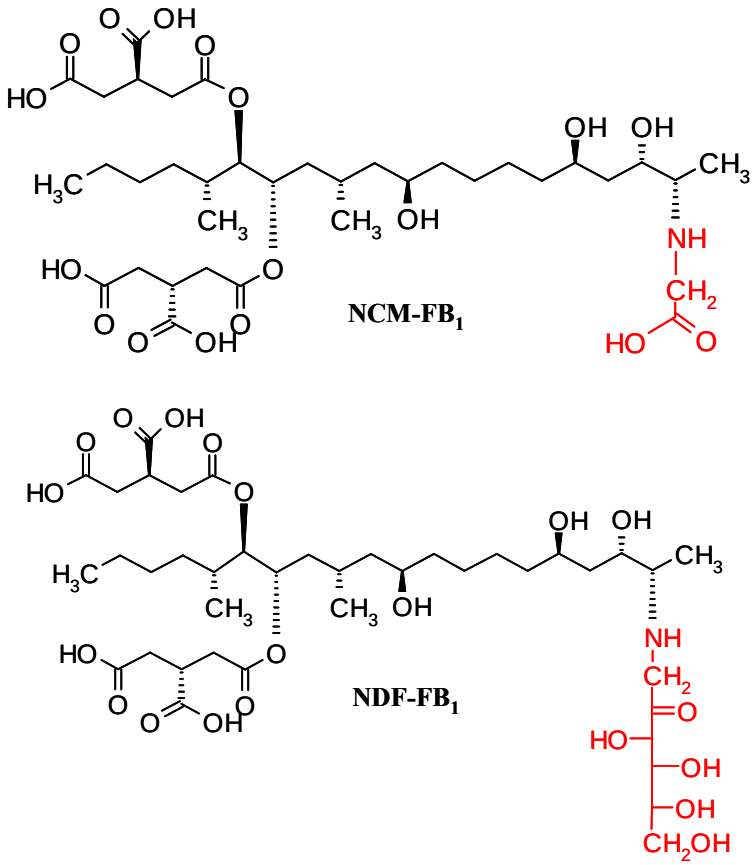


Figure 3. Molecular structure of N-(carboxymethyl) FB1 (NCM) and N-(1-Deoxy-D-fructos-1-yl) fumonisin B1 (NDF), fumonisins derivatives isolated from foods.

To study the binding of fumonisins to matrix components in thermal-treated foods, model experiments were performed by Seefelder et al.(24). In this study, fumonisin B_1 and its hydrolyzed form were incubated with α-D-glucose and sucrose (used as mono- and disaccharide models), with methyl α-D-glucopyranoside (used as starch model) and with N-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (used as protein models). The incubation of D-glucose with fumonisin B_1 or hydrolyzed fumonisin B_1 resulted in both cases in the formation of Amadori rearrangement products. Since hydrolyzed fumonisin B_1 lacks the TCA side chains, it can be concluded that the compound was formed by a Maillard-type reaction of the primary amine and the aldehyde group of glucose. Whereas sucrose, methyl α-D-glucopyranoside and amino acids derivatives reacted with fumonisin B_1, no reaction products were detected with its hydrolyzed analogue, suggesting that starch and proteins were able to bind fumonisins through their tricarballilic units, as shown in Figure 4.

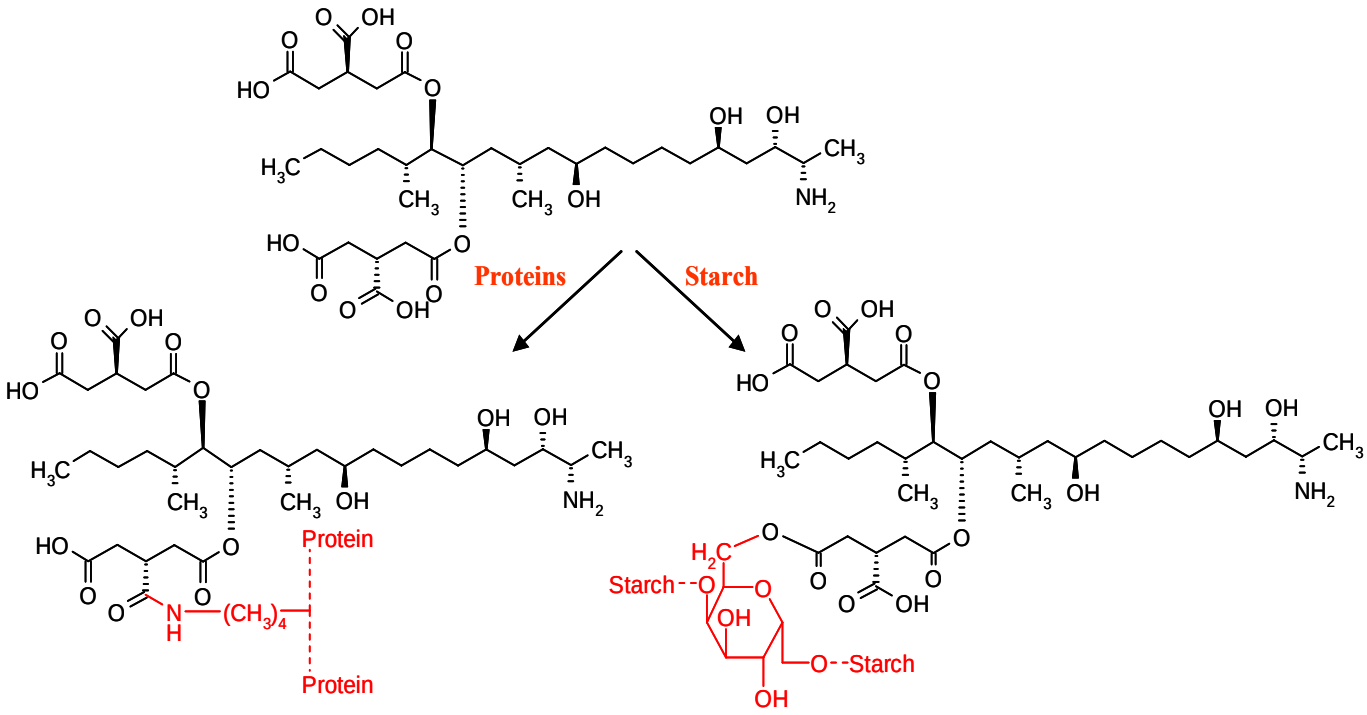


Figure 4. Proposed fumonisins derivatives by in vitro modelling.

However, only NCM and NDF were detected in corn products, while the covalent adducts postulated by *in vitro* models were never found and their presence has been not confirmed (25). Shier et al. (26) reported the occurrence of N-fatty acyl fumonisins in tortilla chips: under nixtamalization/frying conditions both parent and hydrolyzed forms were efficiently N-fatty acylated to the corresponding ceramide derivatives, probably by fatty acid anhydrides or other degradation products formed from the fat by non-oxidative thermal degradation. Very recently, Bartók et al. (27) detected and characterized from solid cultures of *F. verticillioides* three fumonisins derivatives obtained by the esterification of the target toxin with palmitic, oleic and linoleic fatty acids. The peculiarity of these covalent derivatives (called palmitoyl-oleoyl- and linoleoyl-EFBs), is that fumonisins are directly converted by fungal enzymes, thus their occurrence not depends on food processing. Further studies are ongoing in order to investigate if the toxicity of the cited derivatives is similar or significantly different from that owned by parent forms.

Complexation hypothesis. Since the covalent hypothesis previously described seems to be unsuitable to fully explain how masking phenomenon takes place in raw material, another theory was recently developed. Hidden fumonisins have been actually detected not only in thermal-treated products, but also in raw maize and in mild-treated products, as reported by Dall'Asta et al. (28). The formation of covalent bonds requires thus thermal conditions which are inconsistent with common plant growing conditions or even mild processing technologies.

The interaction between fumonisins and proteins has been studied by Kim et al. (15): hydrolyzed fumonisins were determined in corn-flakes samples after the denaturation of the proteic fraction using SDS followed by an alkaline hydrolysis. The data obtained were compared with those achieved by a common extraction procedure, showing that the amount of hydrolyzed fumonisins released from proteins was higher than the amount of free forms detected in crude extracts. Dall'Asta et al. (29) demonstrated that fumonisins were particularly bound to prolamins and glutelins: after Osborne fractionation followed by alkaline hydrolysis of each proteic fraction, significant amounts of HFBs were found among prolamins and glutelins. Figure 5 shows LC-ESI-MS/MS chromatograms obtained after the analysis of each hydrolyzed protein fraction.

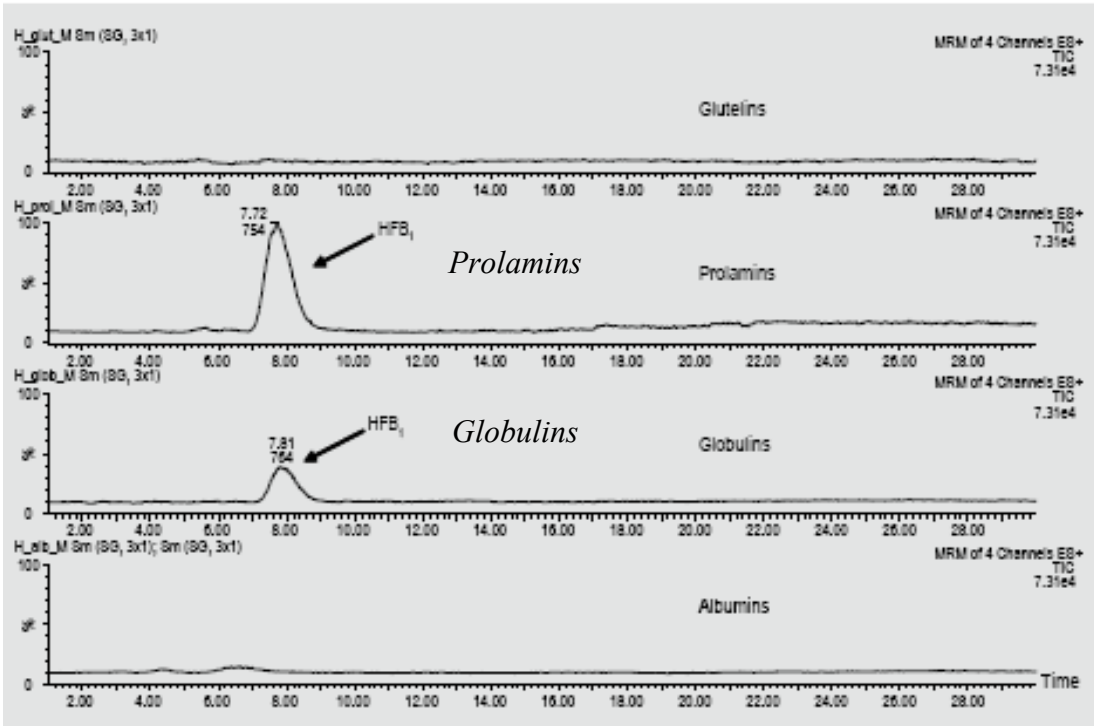


Figure 5. Distribution of bound fumonisins in the protein fraction of maize (adapted from Dall'Asta et al. [29]).

The class of corn prolamins is mainly represented by zein, an alcohol-soluble storage protein that comprises about 45-50% of the protein in corn and is deficient in essential amino acids, such as lysine and tryptophan (30). This lack of lysine leads to doubt the validity of the *in vitro* model proposed to explain the fumonisin-protein interaction: since this reaction requires the presence of amino acids having free amino groups on their side chains (such as lysine), no reactions can occur between fumonisins and zein. Moreover, zein shows a helical character, made up of nine helical segments able to host several guests such as xanthophylls in their tridimensional structure (31). On the other hand, low recoveries of fumonisin B_1 in cornstarch after spiking were observed by Kim et al. (32), suggesting a possible interaction among the

target toxin and starch. Therefore, a physical entrapment or complexation mainly due to supramolecular structures formed by zein or starch and fumonisins has been proposed by Dall'Asta et al. (16). In this study the occurrence of hidden forms in the extract obtained with the extraction solvent used for FBs determination has been observed, probably due to the solvent ability in zein dissolution from maize. During solvent extraction, in fact, prolamin bodies maintain their tertiary structure, thus potentially masking fumonisins, which cannot directly determined. Consequently, the release of hidden fumonisins from the matrix depends on physicochemical parameters able to influence both the status of zein structure and the stability of such supramolecular structure, such as pH, solvent polarity, temperature and time. This theory could explain why different extraction methods can leads to different recoveries from the same sample, even when validated procedures are used, as reported in the same work. Finally, in order to consider the occurrence of bound and hidden fumonisins in risk assessment studies, Motta and Scott (33) evaluated hidden fumonisin bioaccessibility from corn flakes after gastrointestinal digestion, by the application of an *in vitro* digestion model. The bioaccessibility of total bound fumonisins from chyme was found ranging from 37% to 64%. As suggested by the authors, masked forms released from matrix after digestion could be a substrate for the intestinal micro flora and may be hydrolyzed to give partially-hydrolyzed fumonisins (PHFBs) or HFBs, increasing the possibility of exposure to these contaminants after ingestion. Therefore, in order to guarantee good safety levels for the final consumer, the authors stated that these derivatives should be considered in the evaluation of total exposure to fumonisins.

In this section the masking mechanism has been largely study by the application of some *in vitro* experiments. As first, an *in vitro* gastrointestinal digestion model has been applied to several raw maize samples and maize-based products in order to evaluate the hidden fumonisin bioaccessibility in the human small intestine. The same protocol has been also employed to better understand the masking phenomenon by means of experiments aimed to individuate which macromolecules are mainly involved in the fumonisin-matrix interactions and, on the other hand, which type of interaction can be breakdown under digestive conditions. Subsequently, further *in vitro* experiments were performed with the aim to understand under which conditions the masking phenomenon takes place and to study the behaviour of each macroconstituent towards fumonisins.

CHAPTER 1 A DIGESTION ASSAY FOR HIDDEN FUMONISINS EVALUATION IN MAIZE AND MAIZE-BASED PRODUCTS

1.1 INTRODUCTION

In the last years hidden fumonisins have received great attention concerning food safety because they have frequently found both in maize and in maize-based products in addition to free forms (34). From a scientific point of view, the most intriguing aspect of this phenomenon is that the masking mechanism is not yet clearly explained. Although fumonisins are heat-stable up to 100°C, is known that processing induces a significant decrease of the toxin: this reduction was believed to be due not only to a chemical degradation, but also to some fumonisin modifications occurring through the interaction with food macroconstituents (35). Models supported by *in vitro* experiments using methyl α-D-glucopyranoside and protected amino acids as model compounds for starch and proteins, respectively demonstrated the possibility of covalent bond formation between the tricarballylic moiety and hydroxyl groups of carbohydrates or amino groups of amino acids (24). However, besides the fact that direct experimental evidence of the occurrence of these compounds in food was not obtained yet, the proposed reactions require high temperature which can be reached only by thermal processing, thus the occurrence of hidden forms in raw materials and in mild-treated products cannot be explained by these postulates. In this context, several authors have shown that, besides thermal effects that could give rise to covalent bond formation, other masking mechanisms such as complexation or physical entrapment of parent forms into the structure of macromolecules (mainly starch and protein) may be taken into account (15, 20, 32). This theory was strongly supported by studies demonstrating that compounds such as lipids and flavours can be associated, respectively, with starch and proteins via non-covalent interactions (31). This kind of behaviour may be also at the base of the difficulties in obtaining comparable and reproducible results using different analytical methods because such interactions could be differently broken during the extraction process, on account of different experimental parameters applied during extraction, thus leading to different recoveries of the analytes (16). To date, hidden fumonisin were only detected using an indirect approach: since

such compounds can easily escape routine analysis, they are detectable by the application of an hydrolysis step, leading to the quantification of total fumonisins present in the analyzed sample. Thus, the masked fraction can be indirectly quantified by subtracting the amount of free fumonisins extracted by a common solvent-extraction procedure to the total fumonisins amount (16). Nevertheless, alkaline hydrolysis leads to an indiscriminate breakdown of all macroconstituents and linkages existing in a treated matrix, therefore no information concerning the nature of the fumonisin-matrix interaction can be provided by this approach. Moreover, although hidden fumonisins occurrence has been demonstrated, this indirect treatment cannot give information concerning the real exposure to these forms. Very recently, the bioaccessibility of hidden forms from extruded foods (corn flakes) has been evaluated by the application of an *in vitro* digestion model, showing a low release of free FB_1 (50%) in the chyme; moreover any significant contribution from bound forms was observed.

1.2 Aim of the work

The aim of this work is to further investigate the hidden fumonisin occurrence in raw maize by the systematic application of an *in vitro* digestion model to reproduce in a simplify way the conditions occurring in the human gastrointestinal tract, giving thus an estimation of hidden fumonisin bioaccessibility in the small intestine. Moreover, the effect of the different enzyme or digestive phase have been investigated by stopping the digestion after each step or performing the process without one of the enzymes, in order to evaluate the specific contribution of each step or the importance of their synergic effect. To investigate the role of corn macromolecules in the masking phenomenon, recovery experiments were performed, by digesting blank raw maize, corn starch and corn zein spiked by target compounds. Finally, the stability under gastrointestinal conditions of four chemically synthesized covalent fumonisin adducts has been also studied, to evaluate the possible release of the free form from the covalent derivatives upon enzymatic hydrolysis.

1.3 Materials and methods

1.3.1 Reagents

Fumonisins B_1, B_2 and B_3 standard solutions (a mixture of FB_1, FB_2 and FB_3, 50 μg/ml each, in acetonitrile/water, 1:1 v/v) and Fumonisin B_1 in powder, 5 mg, were purchased from Romerlabs (Tulln, Austria). Methanol (LC grade) was obtained from Carlo Erba (Milan, Italy), acetonitrile (LC grade) was from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA). Potassium hydroxide, potassium chloride, sodium chloride, ammonium chloride, 37% hydrochloric acid, potassium dihydrogen phosphate, sodium hydrogen carbonate and dried calcium chloride were obtained from Carlo Erba (Milan, Italy), potassium thiocyanate and sodium sulphate were purchased from Riedel de Haën (Hannover, Germany), sodium dihydrogen phosphate monohydrate was from Fluka (Chemika-Biochemika, Basil, Switzerland) and magnesium chloride hexahydrate was obtained from Merck (Darmstadt, Germany). All chemicals for the preparation of the solutions mimicking the digestive juices (urea 98%, D-(+)-glucose 99.5%, D-glucuronic acid, D-(+)-glucosamine hydrochloride 99%, type III mucin from porcine stomach, uric acid, type VIII A α-amylase from barley malt, bovine serum albumin (BSA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, type III lipase from porcine pancreas and bovine and ovine bile) were purchased from Sigma (Stuttgart, Germany). The reference material was a maize flour containing fumonisins B_1 and B_2 (declared values: 2406 ± 630 and 630 ± 116 μg/kg, respectively) from Romer (Romer Labs Diagnostic GmbH, Tulln, Austria). Maize zein was from Fluka Chemika-Biochemika (Buchs, Switzerland), maize starch was a commercial product from the market (Maizena, Unilever). N-α-acetyl-L-lysine methyl ester in powder, 10 g, was from Sigma (Stuttgart, Germany). Methyl-α-D-glucopyranoside in powder, 25 mg, and sucrose, 5 Kg, were purchased from Sigma (Stuttgart, Germany).

1.3.2 Samples

Raw maize samples (n = 31) were collected in Italy over a two months period (September – October 2008) and are representative of several different maize hybrids grown under different agronomical conditions: they are indicated with the notations M1-M31. Maize-based products

were retailed from market. All maize samples were finely ground with an automatic miller (Braun GmbH, Italy). Maize flour, maize zeins and maize starch were used as purchased.

1.3.3 EXPERIMENTAL PROCEDURES

Preparation of Hydrolyzed Fumonisin Standard Solution.
90 μL of the FB_1, FB_2, and FB_3 standard solution was evaporated to dryness. The residue was redissolved in 1 mL of 2 M KOH and allowed to react for 12 hours at room temperature. After the hydrolysis, the mixture was extracted twice by liquid-liquid partition using twice 1 mL of acetonitrile. The organic phases were pooled and evaporated under N_2 stream, and the residue was redissolved in 1mL of acetonitrile/water, (1:1 v/v). Calibration curves were prepared by proper dilution of the standard solution.

Sample preparation for the analysis of fumonisins.
Extraction and analysis of FBs were performed according to Dall'Asta et al. (16, 28, 29). Briefly, 5 g of ground maize sample were blended in a high-speed blender (Ultraturrax T25, IKA, Stauffen, Germany) with 40 ml of water/methanol (30:70 v/v) for 3 min at 4,000 rpm and then filtered. After filtration on nylon filters (0.45 μm), 1 ml extract was analyzed by LC-ESI-MS/MS as described below.

Sample preparation for the analysis of hydrolyzed fumonisins.
Aliquots (5 g) of the ground maize sample were blended in a high speed blender (Ultraturrax T25, IKA, Stauffen, Germany) with 2 M KOH (50 ml) for 5 min at 4,000 rpm and then stirred for 50 min. Then, acetonitrile was added (50 ml), and after stirring for 5 min, two layers were formed which were separated by centrifugation at 3500 rpm for 15 min (Alc Centrifugette 4206, TecnoLab, Brescia, Italy). A portion of the acetonitrile rich upper layer (2 ml) was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in water/methanol (30:70 v/v), filtered through a 0.45 μm nylon filter, and analyzed by LC–MS/MS as described below. Fumonisins obtained after sample hydrolysis were measured as the sum of HFB_1, HFB_2, and HFB_3 (hydrolyzed fumonisins B_1, B_2 and B_3). All the results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of parent and hydrolyzed compounds and referred to as "total fumonisins after hydrolysis".

***In vitro* digestion assay for the evaluation oh hidden fumonisins bioaccessibility.**

The preparation of artificial digestive juices (saliva, gastric juice, duodenal juice and bile) was performed according to the original protocol of Versantvoort et al. (36). 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 ± 2°C. The digestion started by adding 3 ml saliva to 2 g of ground sample, followed by an incubation step of 5 min. Then, 6 ml gastric juice were added and the mixture was incubated for 2 hours. Finally, 6 ml duodenal juice, 3 ml bile and 1 ml bicarbonate solution (1 M) were added simultaneously to the mixture and a final incubation step of 2 hours was performed. During the *in vitro* digestion, the mixture was stirred by a magnetic stirrer (250 rpm) to obtain a gentle but systematic mixing of the matrix with the digestive juices. The pH of the chyme varied in the range 6.5 - 7. At the end of the experiment the digestion tubes were centrifuged for 15 min at 3500 rpm (Alc Centrifuge pk110, DJB Labcare Ltd, Newport Pagnell, Buckinghamshire, UK), yielding the chyme (the supernatant) and the digested matrix (the pellet). The concentration of fumonisins was determined in chyme after a desalting step through Sep-Pak C18 cartridges (Waters Co, Milford, MA, USA). Briefly, after preconditioning with 2 ml methanol followed by 2 ml bidistilled water, 2 ml chyme were loaded on the column, which was then washed again with 2 ml bidistilled water. Fumonisins were eluted using 2 ml water/acetonitrile (1:1 v/v). Then, a portion of the solution containing fumonisins (1 ml) was evaporated to dryness under a stream of nitrogen and the residue was redissolved in 1 ml water/methanol (3:7 v/v) prior to analysis. For maize-based products (generally less contaminated than raw maize) 4 ml chyme were applied to the cartridge. After elution with 4 ml water/acetonitrile (1:1), 3.5 ml filtrate were evaporated and the residue was redissolved in 1 ml water/methanol (3:7, v/v) for the analysis.

Table 1 Constituents and concentrations of the synthetic juices used for the in vitro gastrointestinal digestion.

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

The inorganic and the organic solutions must be prepared separately and augmented to 250 ml with bidistilled water. After mixing the inorganic and organic solutions, enzymes and other constituents are added to a selected volume and dissolved by heating to 37°C under stirring. If necessary, pH of each juice is adjusted to the appropriate interval using HCl 1M or NaOH 1M.

Step-by-step *in vitro* digestion experiments.

Two sets of experiments were carried out by modifying the in vitro digestion protocol. In the former, a maize sample underwent a step-by-step digestion: in the order, digestion was stopped after sample incubation with saliva, after the gastric phase and then after duodenal phase without bile addition. In all cases, the mixture was diluted to the final digestion volume (19 ml) using bidistilled water, then digestion tubes were centrifuged and 2 ml of raw chyme were prepared for LC-MS/MS analysis. In the second experiment, digestion assay was run by eliminating one component at each time: without α-amylase, without pepsin, without pancreatin, without lipase and without bile, respectively. All the obtained results were compared with those supplied by a complete digestion process, performed as control.

Recovery experiments.

FB_1 recovery experiments were performed on blank raw maize (a sample in which fumonisin contamination was lower than LOD), corn starch and corn zein, using both the routine extraction method and the digestion assay. In particular, 5 g of sample (corn flour, corn starch or corn zeins) was spiked with an appropriate amount of FB_1 solution (10 mg/L

water:acetonitrile 1:1 v/v), in order to obtain a final analyte concentration of 2 μg/g. The sample was then covered with an aluminium foil and left at room temperature for 3 days. Afterwards, the blank and the spiked samples were extracted as already reported for the determination of extractable fumonisins. Similarly, 2 g of each matrix was spiked with an appropriate amount of FB_1 solution (10 mg/L water:acetonitrile 1:1 v/v), in order to obtain a final analyte concentration of 2 μg/g. The sample was then covered with an aluminium foil and left at room temperature for 3 days as above and the blank and the spiked samples underwent the digestion assay.

All the experiments were performed in duplicate (n = 2) starting from the sample preparation and the results were statistically compared.

LC-MS/MS analysis.

LC-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a QuattroTM API triple quadrupole mass spectrometer with an electrospray source (Micromass, Waters, Manchester, UK). Chromatographic conditions were the following: column, C18 XTerra (250 mm × 2.1 mm, 5 μm); flow rate, 0.2 ml/min; column temperature, 30°C; injection volume, 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, 0-2 min isocratic step, 2-5 min linear gradient to 45% B, 5-25 min linear gradient to 90% B, 25-35 min isocratic step at 90% B, 35-36 min linear gradient to 70% A and re-equilibration step at 70% A for 15 min (total analysis time: 50 min). MS parameters: ESI+ (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 50 V for FBs and 30 V for HFBs; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow (nitrogen), 50 l/h e 700 l/h, respectively. Detection was performed using a multiple reaction monitoring (MRM) mode by monitoring two transitions for each analyte, as follow: 722.4→334.4 (CE 40 eV), 722.4→352.3 (CE 35 eV) for FB_1, 706.4→336.4 and 706.4→318.4 (CE 35 eV) for FB_2 and FB_3, 406.5→334.4 and 406.5→353.4 (CE 25 eV) for HFB_1, 390.5→336.4 and 390.5→354.4 (CE 25 eV) for HFB_2 and HFB_3. The first transition reported was used for quantification, while the second transition was chosen as qualifier. For each sample, the entire procedure (preparation, cleanup, and digestion) was performed in duplicate (n = 2). Matrix-matched calibration curves (calibration range 50-2500 μg/kg) were used for extractable fumonisins, total fumonisins after digestion, and hydrolyzed fumonisin quantification. The first transition reported was used for quantification, while the second transition was chosen as qualifier. For

each sample, the entire procedure (preparation, clean up and digestion) was performed in duplicate (n = 2). Matrix-matched calibration curves were used for extractable FBs, total FBs after digestion and HFBs quantification.

Statistical analyses.
Statistical analyses 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 ($\alpha = 0.05$).

Synthesis of fumonisin B_1-covalent derivatives.
All the reactions were performed by heating the reactants without solvent, as proposed by Seefelder et al. (24). Aliquots of stock solutions of reactants were mixed in a reaction vial and stirred for 2 min., and then the solvent was removed under a stream of nitrogen before heating.

Reaction of fumonisin B_1 with amino acid derivative. In a reaction vial a mixture of 50 µg of fumonisin B_1 (0.07 µmol) and 1 mg of N-R-acetyl-L-lysine methyl ester (4.9 µmol) was heated in a heating block for 60 min at 100°C without solvent. The reaction mixture was dissolved with 500 µl of water/acetonitrile, (1:1 v/v) and used for the digestion experiments.

Reaction of fumonisin B_1 with α-D-glucose. In a reaction vial a mixture of 40 µg of fumonisin B_1 (0.055 µmol) and 0.4 mg of D-(+)-glucose (2.2 µmol) was heated in a heating block for 60 min at 80°C without solvent. The reaction mixture was dissolved with 500 µl of water/acetonitrile, (1:1 v/v) and used for the digestion experiments.

Reaction of fumonisin B_1 with sucrose and methyl-α-D-glucopyranoside. In a reaction vial a mixture of 50 µg of fumonisin B_1 and 0.5 mg of sucrose (1.3 µmol) or 0.5 mg of methyl-α-D-glucopyranoside (2,7 µmol) was heated in a heating block for 60 min at 80°C without solvent. The reaction mixture was dissolved with 500 µl of water/acetonitrile, (1:1 v/v) and used for the digestion experiments.

***In vitro* digestion assay of fumonisin B_1 derivatives.**
The digestion started by adding 300 µL of saliva to 100 µl of the reaction product previously evaporated to dryness and resuspended with 100 µl of bidistilled water, followed by an incubation step of 5 min at 37°C. Then, 600 µl of gastric juice was added, and the mixture was incubated for 2 h. Finally, 600 µl of duodenal juice, 300 µl of bile, and 100 µl of 1M bicarbonate solution were added simultaneously to the mixture, and a final incubation step of 2 h was performed. At the end of the experiment volume was adjusted to 2 ml adding 100 µl

of bidistilled water before a desalting step through Sep-Pak C18 cartridges (Waters Co., Milford, MA, USA). Fumonisins derivatives were eluted using 2 ml of water/acetonitrile, (1:1 v/v). The whole volume was then evaporated to dryness and the residue was redissolved in 300 µl of water/acetonitrile (1:1 v/v) before LC-MS analysis. For each experiment, a control-test has been performed: 100 µl of the reaction product were evaporated to dryness under a gentle stream of nitrogen and resuspended in 100 µl of bidistilled water, and then the volume was adjusted to 2 ml prior to the desalting step performed using Sep-Pak C18 cartidges. The eluate obtained was desiccated and the residue was redissolved in 300 µl of water/acetonitrile (1:1 v/v) previous to LC-MS measurements.

LC-MS analysis.

LC-MS/MS analysis was performed by a Acquity Ultra Performance LC separation system (Waters Co., Milford, MA, USA) equipped with a Acquity SQ Detector single quadrupole mass spectrometer with an electrospray source (Waters Co., Milford, MA, USA). Chromatographic conditions were the following: the column was a 100 mm x 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH C18. The flow rate was 0.2mL/min; the column temperature was set at 35°C; the injection volume was 3 µL; gradient elution was performed using bidistilled water (eluent A) and acetonitrile (eluent B) both acidified with 0.2% formic acid: initial condition at 100% A, 0-3.5 min isocratic step, 3.50-23 min linear gradient to 40% B, 23-24 min linear gradient to 90% B, 24-27.5 min isocratic step at 90% B, 27.5-28 min linear gradient to 100% A, and reequilibration step at 100% A for 8 min (total analysis time: 36 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 (nitrogen), 50 L/h and 700 L/h, respectively. Detection was performed using a Single Ion Reaction (SIR) mode, by monitoring the molecular ion for each analyte as follow: 722.4 for parent FB_1 and 906.9, 884.5, 1047.5 and 1074.5 for the derivatives obtained by reacting the target molecule with, respectively, N-R-acetyl-L-lysine methyl ester, α-D-glucose, sucrose and methyl-α-D-glucopyranoside.

1.4 Results and discussion

1.4.1 Occurrence and bioaccessibility of hidden fumonisins in raw maize and in corn-based products: an *in vitro* digestion assay.

Hidden fumonisins were found to occur both in raw maize and maize-based products and are able to escape routine analyses (15, 16, 20), leading to a general underestimation of the total amount of mycotoxins existing in a sample and thus of the potential risk associated to these forms. The aim of this work was to study hidden fumonisins behaviour during gastrointestinal digestion and their possible release from matrix, in order to evaluate the potential consumers exposure to these derivatives. For this purpose, an *in vitro* digestion model proposed by Versantvoort et al (36) has been applied to several naturally contaminated maize samples. The digestion assay had been initially developed for food contaminants bioavailability assessment (37): in this assay the chemical composition of digestive fluids, pH and residence periods typical for each compartment (mouth, stomach, intestine) are reproduced to mimic in a simplify manner the physiological conditions in human gastrointestinal tract during the digestion process. 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 (38). 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 a target compound (38). Since data concerning absorption and metabolism are not provided by the model previously described, is more correct use the term "bioaccessibility", to indicate the amount of considered compound that can be released from matrix after digestion and is available for intestinal absorption.

At first, in order to verify if digestive processes may cause degradations of the target compound, the stability of fumonisins was checked by the application of the digestion protocol to a FB_1 standard solution. The stability of the toxin was then confirmed comparing the amount of FB_1 in the chyme after digestion with that of a not-digested standard (the control) at the same dilution. As shown in Figure 6, fumonisin B_1 was found completely stable under gastrointestinal digestion, obtaining a recovery of 100%.

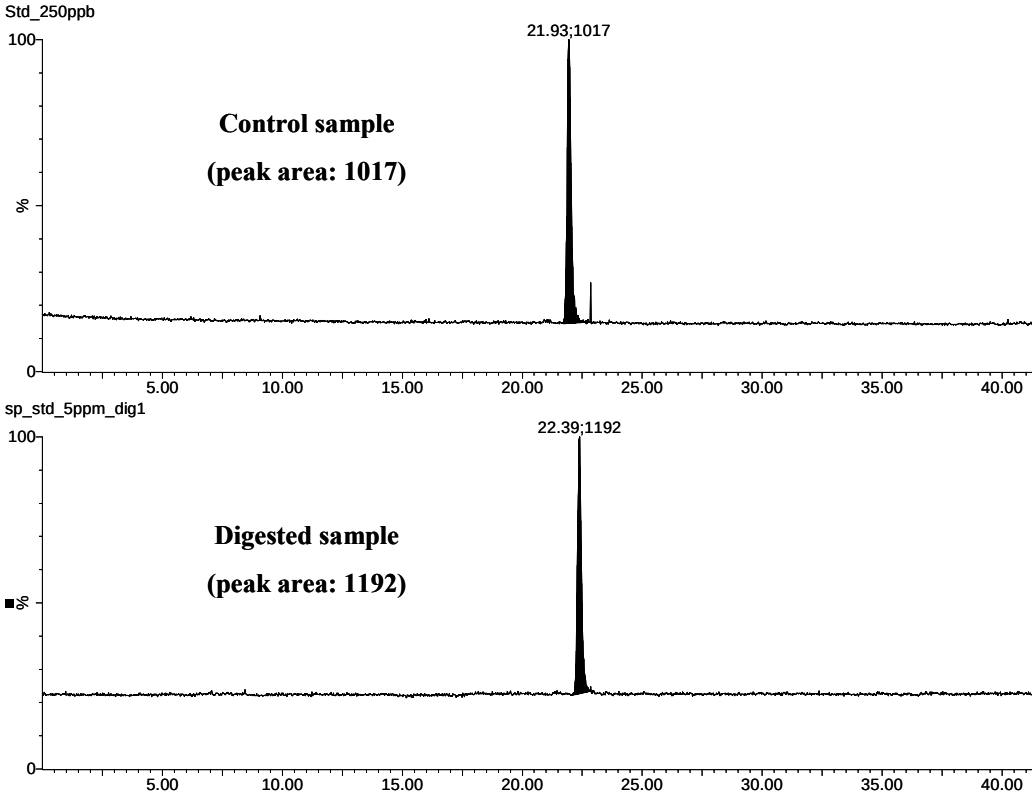


Figure 6. Comparison between control and digested sample: similar peak areas indicate that fumonisins are completely stable during digestion.

Then, three raw maize samples were analyzed for the occurrence of free fumonisins by the application of normal extraction procedures and for the occurrence of hidden fumonisins by the application of the alkaline hydrolysis and the digestion assay. The data obtained are reported in Figure 7 and expressed as the sum of FB_1, FB_2 and FB_3. All the data were statistically compared by using a OneWay-ANOVA Test ($\alpha = 0.05$).

Fumonisins were found in all the samples and, upon hydrolysis, in two out of three samples a significant increase in total fumonisins was observed. Upon digestion, no hydrolyzed or partially hydrolyzed fumonisins were found in the chyme, but only parent fumonisins were released from the matrix.

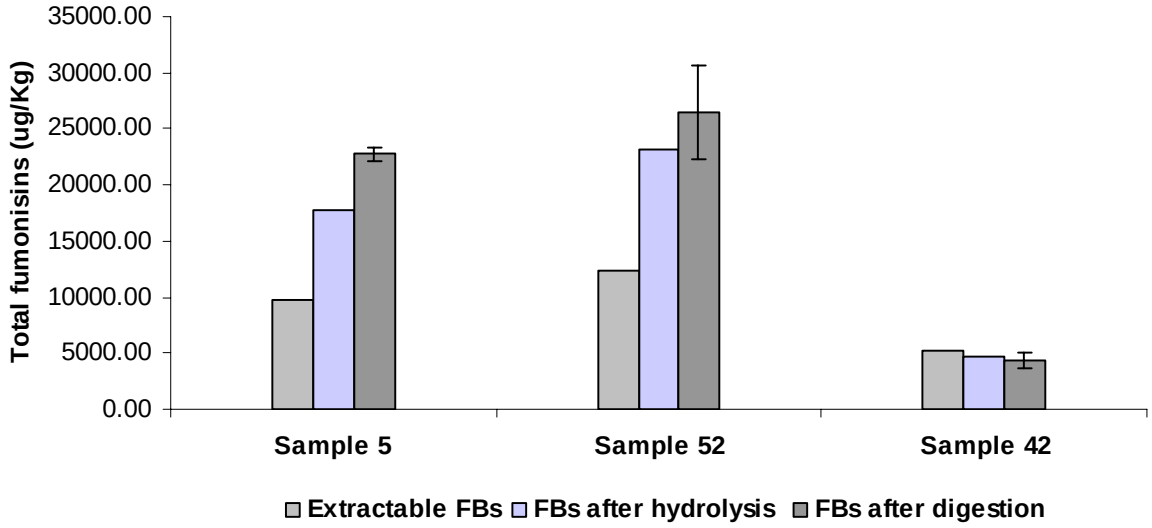


Figure 7. Comparison of the extractable fumonisins (sum of FB1, FB2, and FB3), total fumonisins found after hydrolysis (measured as hydrolyzed fumonisins and expressed as sum of FB1, FB2, and FB3 equivalents), and total fumonisins found in the samples after in vitro digestion (sum of FB1, FB2, and FB3) obtained for several raw maize samples. Different letters designate statistically significant differences between data (α = 0.05).

The analysed samples gave three different results: in comparison with the amount of detectable free fumonisins, samples M5 and M52 showed a higher content of total fumonisins after digestion, whereas sample M42 did not shown a significant difference between free and total fumonisins. Interestingly, the contamination level after digestion was always comparable with that found after hydrolysis.

These data confirmed that the gastrointestinal enzymes are able to disrupt the matrix-fumonisin interactions, thus releasing the hidden forms. Moreover, as only parent forms were detected in the chyme (and not hydrolyzed forms have found after digestion), it can be argued that fumonisins are masked by matrix constituents through the formation of non-covalent interactions which can be destroyed by enzymatic or chemical hydrolysis.

Thus, we decide to apply the digestion protocol to a larger number of maize samples (n = 31) collected in Italy over a two months period (September–October 2008). These samples were representative of several different maize hybrids grown under different agronomical conditions. For all the considered samples, the occurrence and amount of FBs was measured by the normal extraction procedure and then was compared with the amount of FBs measured in the chyme after digestion (see Table 2).

Table 2. Comparison of extractable FBs, total FBs after digestion and hidden fumonisins (calculate difference among total FBs after digestion and free extractable FBs) found in raw maize samples (n = 2; * p < 0.05; ** p < 0.01).

Sample	Extractable FBs[a] (µg/Kg)	CV%	FBs after digestion[b] (µg/Kg)	CV%	Hidden FBs[c] (µg/Kg)	Tukey test[d] (p)
M1	1145	4.6	2501	1.8	1356	**
M2	11479	20.7	22755	2.8	11276	*
M3	6318	1.6	10116	10.7	3798	*
M4	999	0.9	2729	1.3	1730	**
M5	6407	3.8	18069	17.1	11662	*
M6	1515	1.8	2785	3.1	1270	**
M7	6369	10.8	6579	7.2	210	> 0.05
M8	1997	2.8	1972	18.1	0	> 0.05
M9	1180	12.5	2696	6.0	1516	**
M10	575	0.6	1578	0.6	1003	**
M11	6934	22.9	12755	1.7	5821	*
M12	2361	3.3	3829	3.7	1468	**
M13	1287	2.6	1135	18.8	0	> 0.05
M14	4658	0.3	4677	2.4	19	> 0.05
M15	2611	3.9	10734	2.5	8123	**
M16	5057	10.2	6766	13.3	1709	> 0.05
M17	1702	2.3	2599	11.0	897	*
M18	4641	6.2	6074	5.8	1433	*
M19	3146	4.3	4427	15.4	1281	> 0.05
M20	3523	7.6	5588	0.3	2065	**
M21	2871	11.4	2935	10.8	64	> 0.05
M22	17014	5.4	40821	1.0	23807	**
M23	3258	9.7	4005	14.4	747	> 0.05
M24	1981	1.7	2626	3.8	645	*
M25	576	3.5	2112	4.4	1536	**
M26	8518	0.4	9702	0.1	1184	**
M27	15067	25.2	26503	15.6	11436	> 0.05
M28	5551	3.4	6212	9.1	661	> 0.05
M29	9689	10.8	14406	28.8	4717	> 0.05
M30	6887	2.8	12977	6.4	6090	**
M31	3749	2.7	6460	5.7	2711	**

[a] Extractable fumonisins: fumonisins obtained after routine analysis. [b] Total fumonisins: fumonisins obtained after digestion assay. [d] Hidden fumonisins: calculated difference among "total fumonisins" and "extractable fumonisins". [d] Tukey's test performed among total fumonisins and extractable fumonisins.

As a general observation, total fumonisin levels measured after digestion were higher than those measured by the routine extraction (Tukey test, $\alpha = 0.05$). Thus, from these results, it seems that the occurrence of hidden fumonisins in raw maize is a common phenomenon: this fact is of the upmost significance both for the possible consequences of the consumer health and also for the analytical implications. Indeed, consumers may be exposed to a higher level of mycotoxins in comparison with the exposure calculated on the basis of the data obtained using a standard procedure. Moreover, emerges that the currently used analytical methods are not capable to detect fumonisins hide in the matrix as intact forms.

A strong correlation among extractable forms and hidden forms has been observed after a statistical treatment of data (Pearson's test: 0.914 at $\alpha = 0.01$): samples with high extractable FBs levels showed, indeed, very high hidden fumonisin content after digestion and, on the contrary, samples showing lower free fumonisins levels, give a few release of masked forms after digestion. The correlation between extractable and total fumonisins after digestion has been shown in Figure 8. Correlation between free extractable fumonisins and total fumonisins after digestion in raw maize samples. This agreement could be used to estimate the real level of fumonisins in a maize sample when the extractable fumonisin concentration is known.

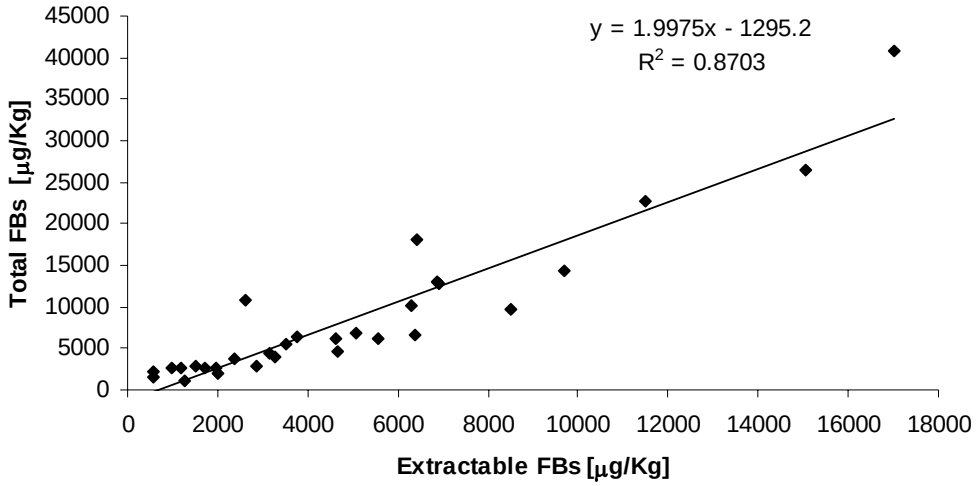


Figure 8. Correlation between free extractable fumonisins and total fumonisins after digestion in raw maize samples.

The *in vitro* digestion model has been also applied to some maize-based samples (n = 10) in order to evaluate the hidden fumonisin occurrence in processed food and their possible release from more complex matrices after gastrointestinal digestion. These samples were collected from the market and represent food categories of large consumption (snacks, cornmeal, cereals for breakfast and bread substitute). Similar to what was done for raw maize samples, fumonisins were measured by the normal extraction procedure and then the data were

compared with the amount of fumonisins measured in the chyme after digestion. Data obtained are shown in Table 3: hidden fumonisins were found in 8 of 10 samples (Tukey test, $\alpha = 0.05$), often representing the larger fraction of total fumonisin content.

Table 3. Comparison of extractable FBs, total FBs after digestion and hidden fumonisins (calculate difference among total FBs after digestion and free extractable FBs) found in maize-based samples (n = 2; * p < 0.05; ** p < 0.01).

Sample	Extractable FBs[a] (µg/Kg)	CV%	FBs after digestion[b] (µg/Kg)	CV%	Hidden FBs[c] (µg/Kg)	Tukey test[d] (p)
Biscuits	1789	55.5	8122	23.2	6333	*
Cornmeal a	1479	4.0	14027	1.9	12548	*
Cornmeal b	1479	65.5	1305	4.0	0	> 0.05
Cornmeal c	187	5.2	1972	1.1	1785	*
Corn Flakes a	55	0.7	114	5.9	59	**
Corn Flakes b	771	8.8	944	36.5	174	> 0.05
Corn Flakes c	203	2.0	651	17.6	448	*
Corn Flakes d	103	1.0	110	0.9	0	> 0.05
Crackers a	141	0.4	521	9.0	380	*
Crackers b	248	1.7	693	20.6	446	> 0.05

[a] Extractable fumonisins: fumonisins obtained after routine analysis. [b] Total fumonisins: fumonisins obtained after digestion assay. [d] Hidden fumonisins: calculated difference among "total fumonisins" and "extractable fumonisins". [d] Tukey's test performed among total fumonisins and extractable fumonisins.

Although mild-treated products (e.g. cornmeal) are the highest contaminated categories, thermally-treated and formulated products (e.g. crackers and biscuits) also showed a high contamination. These data, when confirmed by a wider survey, are of concern for the consumer health, since it is clearly shown that the bioacessible mycotoxin level after digestion may also be very high even for products which apparently do not overcome legal limits. Moreover, considering that these products are highly processed and are constituted not only by maize but also by other ingredients, these data suggested the use of high contaminated raw materials, thus highlighting a serious deficiency in controls related to food safety.

1.4.2 *IN VITRO* DIGESTION OF A CERTIFIED REFERENCE MATERIAL (CORN FLOUR).

In order to evaluate the potential impact of this problem on the accuracy of fumonisin analytical determination in food, a certified reference material (a maize flour with a declared contamination level of 2406 ± 630 µg/kg FB_1 and 630 ± 116 µg/kg FB_2 respectively) has been analyzed. Certified reference materials are widely used to validate analytical methods and are currently highly considered, as they more correctly represent the real situation as far as the interactions between sample matrix and contaminant are concerned, in comparison with

spiking experiments. Sample was analyzed applying the routine extraction methods and, in order to check for the eventual occurrence of hidden fumonisins, also applying both the hydrolysis approach and the digestion model. Analysis of the sample with the normal extraction method gave good results in accordance with the declared contamination range (z score = 0.29). Nevertheless, the amount of total fumonisins calculated upon alkaline hydrolysis and, on the other hand, after enzymatic digestion, was higher (almost double) than the amount of free fumonisins detectable by the routine extraction procedure (Figure 9).

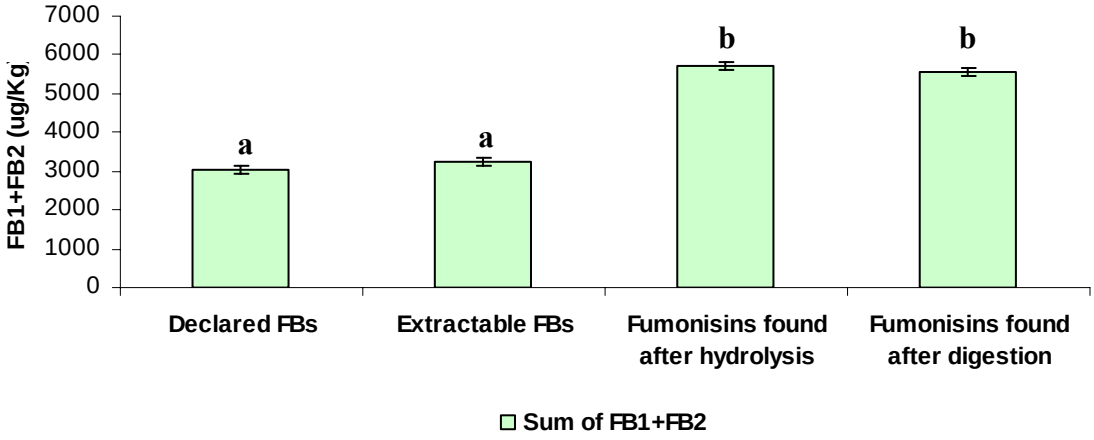


Figure 9. Comparison between extractable fumonisins (sum of FB1 and FB2) and total fumonisins (sum of FB1 and FB2) found after alkaline hydrolysis and in vitro digestion for a certified reference material (FAPAS).

Data were statistically compared by using a OneWay-ANOVA test followed by a post-hoc Tukey Test ($\alpha = 0.05$). The amount of total fumonisins after digestion and after hydrolysis were found to be statistically different from the extractable ones ($p = 0.011$ and $p = 0.019$ respectively), whereas no significant difference was found again between total fumonisins after hydrolysis and after digestion. These results showed that in the certified reference material hidden fumonisins occur which were not detected using standard approach, but can be released upon digestion as parent forms, thus potentially contributing to the overall toxicity of the contaminated product. Thus, although when setting an analytical method, recovery experiments by the spiking procedure show good performance of the method itself, a performance which can be further confirmed by using certified reference material, nevertheless naturally contaminated maize samples invariably show the scarce reliability of this approach in determining the real contamination in the case of fumonisins

1.4.3 STEP-BY-STEP DIGESTION EXPERIMENTS.

Concerning masking phenomenon, both the hydrolysis approach as well as the digestion protocol did not give us much information about the nature of fumonisin-matrix interactions. Nevertheless, whereas alkaline hydrolysis is a drastic treatment that indiscriminately break both any interaction and the toxin molecule, digestion approach could tell us much more about which macromolecules are involved in masking mechanism and the strength of these interaction, due to the use of certain enzymes that can hydrolyze only specific interactions, leaving intact fumonisins. Thus, we decided to employ the digestion model to better study the masking mechanism, by performing some *in vitro* experiments. In particular, the experiments were aimed to understand which macromolecular component (i.e. starch, proteins etc.) is primarily involved in the masking phenomenon, thus the digestion assay was accordingly modified. The first set of experiments was performed by applying a step-by-step digestion: the protocol was stopped after each digestion step (saliva incubation, gastrointestinal incubation, duodenal incubation), the total fumonisin level was determined for each fraction and compared to that obtained after a complete digestion assay of the sample (control experiment). The obtained data are reported in Figure 10.

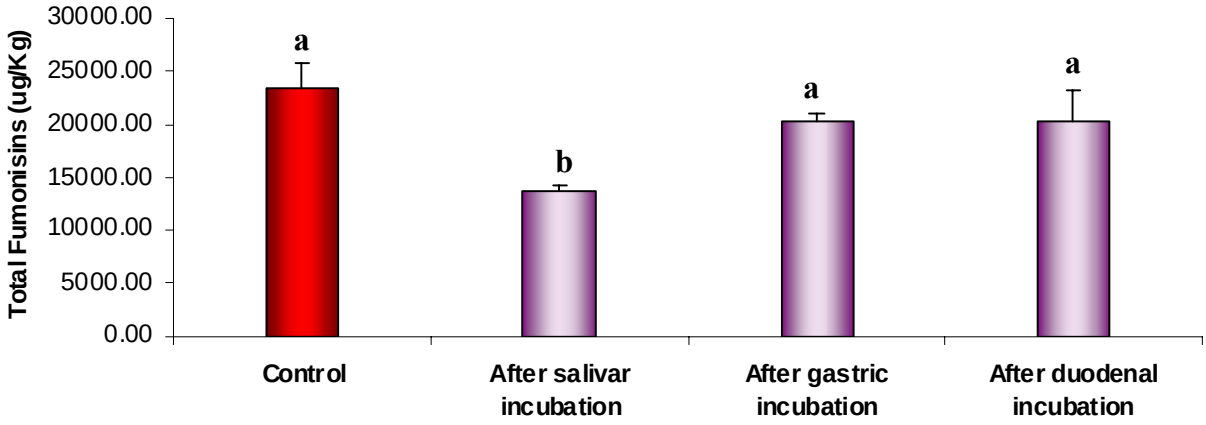


Figure 10. Total FBs (sum of FB1, FB2, and FB3) found after digestion during the step-by-step digestion experiments. "Control" (coloured in red) designates the sample subjected to the complete digestion assay.

Although even salivar incubation provides an important contribution to the release of fumonisins from the matrix, the main difference is found after the gastrointestinal incubation (Tukey test, $p = 0.025$): both salivar and gastric conditions seems to be the main responsible for the hidden fumonisin releasing, suggesting that starchy and proteic fractions are able to hide these mycotoxins.

A second set of experiments was then planned in order to better evaluate the role of each enzyme involved in the digestion assay. In particular, the protocol was applied to a naturally contaminated maize sample by eliminating a different enzyme at once. The final results in terms of total fumonisins were then compared to that obtained by applying the total digestion assay. Indeed, the latter was considered as the control sample (see Figure 11).

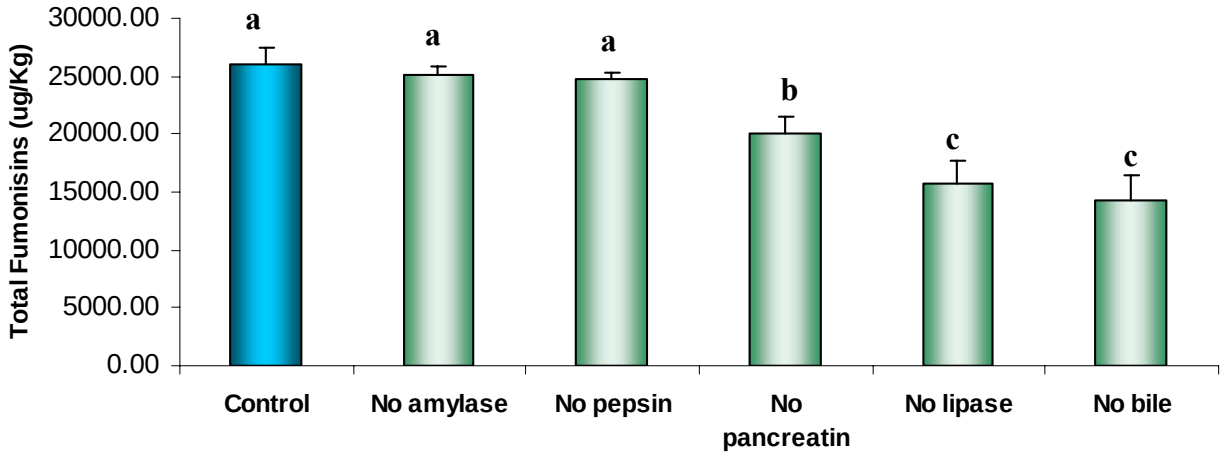


Figure 11. Total FBs (sum of FB1, FB2, and FB3) found after digestion for the experimental set obtained by removing a single enzyme at once. "Control" (coloured in blue) designates the sample subjected to the complete digestion assay.

The collected data were surprising, since neither amylase nor pepsin seemed to play a significant role on the FBs releasing process from the matrix. On the contrary, a significant decrease in FBs release was found when pancreatin ($p = 0.014$), lipase ($p < 0.001$) and bile ($p < 0.001$) were eliminated. These data seemed to support the important role played by the duodenal phase in the digestive release of fumonisins from maize. Since bile is an important emulsifying agent, its presence allows lipid emulsion and their consequent attack from lipase. These two enzymes are thus probably playing a synergistic action on the matrix disaggregation. Upon the bile or lipase removal from the digestion protocol, the emulsion of the chyme is reduced, consequently decreasing the efficiency of the whole enzymatic pool and also probably decreasing the ability to destroy the matrix-contaminant interactions. More experiments should be performed in order to better describe the role played by single enzymes during fumonisin release from food.

1.4.4 Recovery experiments from raw maize, corn starch and corn zeins.

An experiment for further investigate the masking mechanism exerted by corn macromolecules towards fumonisins was planned. Since the most representative maize components are starch and zein, the spiking experiments were performed also on blank zein and blank starch.

After spiking with FB_1 at the same contamination level (2 μg/g), the samples underwent to the routine extraction for extractable fumonisins and to the digestion assay for total fumonisins determination. Each experiment was performed in duplicate and the blank matrices were checked also by the digestion assay in order to avoid overestimation due to the occurrence of hidden fumonisins. The results are reported in Table 4.

Table 4. Recovery data obtained after spiking with FB1 a corn starch sample and a corn zein samples (all the experiments were performed in duplicate, n = 2). Different letters indicate a significant difference (T-Student Test, α = 0.05).

	Blank	Corn starch	Recovery (%)	Corn zein	Recovery (%)
Spiking level (μg/Kg)	-	2000 **a**		2000 **y**	
Extractable FBs (μg/Kg)	n.d.	952 ± 84 **b**	47.6%	693 ± 193 **z**	34.7%
FBs after digestion (μg/Kg)	n.d.	1598 ± 192 **a,b**	79.9%	595 ± 15 **z**	29.8%

As first observation, the data strongly supported the hypothesis of a complexation masking mechanism: when fumonisin B_1 is added to starch or zein and left at room temperature for some days, a poor recovery was found with the routine method. These difficulties in recovery experiments was already observed by Kim et al. (32). Then, if the same sample undergoes to the digestion assay, a significantly higher amount of analyte was found. Since the sample did not undergo to any heating process, the masking mechanism should be ascribed to physical complexation or entrapment phenomena which may unspecifically occur among the analyte and the corn macromolecules.

Moreover, a different behaviour was found for corn starch and corn zeins. Although both compounds seemed to be able to hide fumonisin B_1, zeins showed a stronger interaction with the analyte, giving a very low recovery rate for both the applied protocols (average recovery: 30 – 35%).

The obtained results suggested that the masking mechanism is ascribable to a cooperative effect exerted by the main macroconstituents of maize, being zeins responsible for a stronger masking mechanism: the recovery is difficult also after the digestion assay, thus indicating the low disaggregation of the association complexes also under gastric conditions.

1.4.5 *IN VITRO* DIGESTION OF FUMONISIN B_1-COVALENT DERIVATIVES

In order to confirm that non-covalent interactions are responsible of the masking phenomenon, and also to verify whether gastrointestinal enzymes could break down covalent linkages among fumonisins and matrix constituents, thus releasing free forms, the *in vitro* digestion protocol has been applied to four fumonisin B_1 covalent derivatives. Since the reaction between the functional groups of fumonisins and the hydroxyl groups of starch or the amino or sulfidryl groups of the side chains of amino acids in proteins at high temperature has been demonstrated by several authors under laboratory conditions (23, 24), the derivatives proposed by Seefelder et al. (24) have been chosen for our experiments. Thus, fumonisin B_1 was left to react with N-α-acetyl-L-lysine methyl ester and α-D-glucose (to simulate model reaction with, respectively, proteins and reducing sugars) and with sucrose and methyl-α-D-glucopyranoside (chosen as model for the reaction between the target toxin and starch). All the reactions were performed without solvent and at high temperature (about 80-100°C) to mimic the corn thermal processing steps. Molecular structures proposed for each fumonisin B_1 derivative are shown in Figure 12.

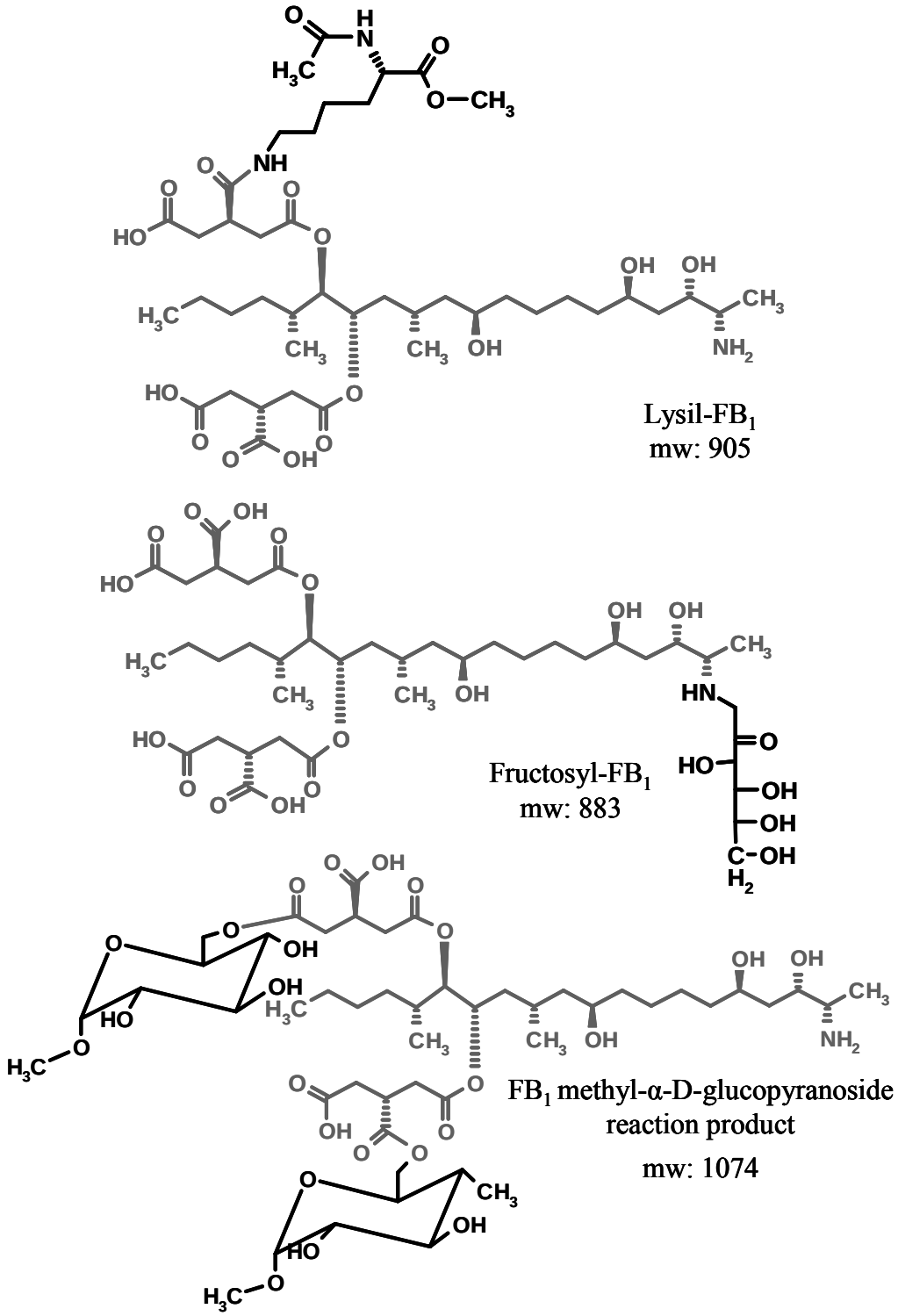


Figure 12. Molecular formulas of the reaction products obtained by letting fumonisin B1 react with N-α-acetyl-L-lysine methyl ester , α-D-glucose and methyl-α-D-glucopyranoside.

Then, the reaction products underwent the digestion assay and cleaned up on Sep-Pak C18 cartridges for desalting, before LC-MS analysis. For each digestion experiment a control-sample was prepared, by cleaning up the same amount of not-digested reaction product on Sep-Pak C18 cartridges. Volume and dilution degrees were kept constant in order to allow a

direct comparison upon analysis. To evaluate the stability of such adducts under gastrointestinal condition, the ratio between the area of the reaction product and the area of the residual fumonisin B_1 in the digested samples has been compared with the same ratio calculated in the not-digested samples.

As example, the comparison between SIR chromatograms obtained upon LC-MS analysis of both control-sample and digested sample for Fructosyl-FB_1 (the reaction product of the target toxin and glucose) is reported in Figure 13.

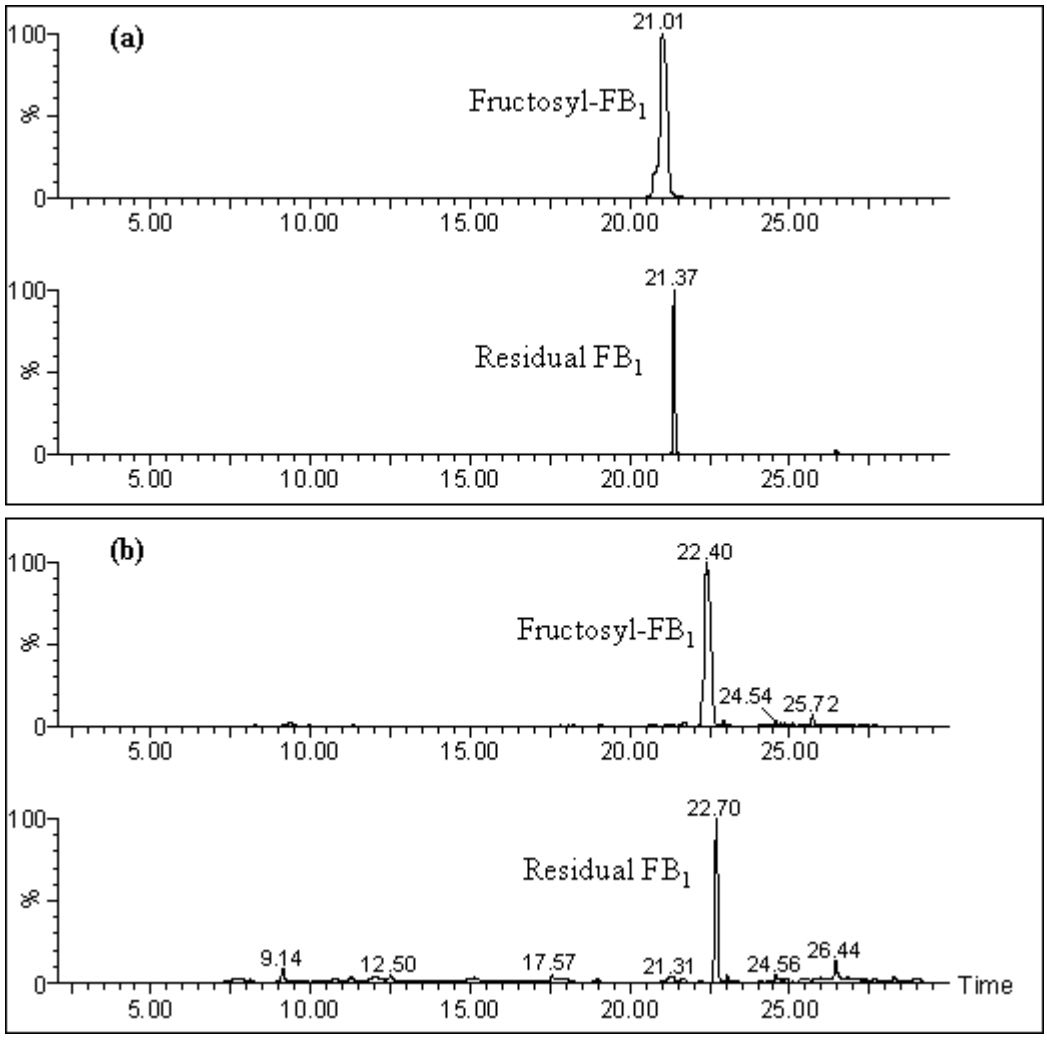


Figure 13. Comparison between SIR chromatograms obtained by LC-MS analysis of (a) not-digested Fructosyl-FB_1 reaction product passed through Sep-Pak C18 cartridges and (b) digested sample cleaned by Sep-Pak C18 cartridges.

As shown, the two chromatograms obtained are very similar, since the covalent derivative was detected also after the gastrointestinal digestion assay. This result suggests that the involved enzymes cannot break-down the covalent interaction existing among the toxin and the sugar.

Similar results have been obtained after digestion and analysis of the others FB1-derivatives, demonstrating thus that covalent bond among fumonisins and matrix constituents cannot be cleaved to release FB_1 by gastrointestinal enzymes under digestive conditions.

The reduction of the signal intensity observable upon digestion of some analytes can be due to a strong matrix-effect caused by the complex composition of the artificial digestive juices used. In particular, bile salts are difficult to remove from samples, thus they could interfere during analysis, "fouling" the chromatograms. Nevertheless, despite to the lower signal detected after digestion, a good agreement among the calculated ratios for the control and the treated samples has been found (Table 5), suggesting that this adduct cannot be cleaved in the small intestine.

Table 5. Reaction product area-to-residual FB1 area ratios: comparison among undigested and digested samples.

FB_1 adducts	Reaction product area/residual FB1 area		
	Control sample	Digested Sample	Tukey (p)
Lysil-FB_1	0.076	0.094	> 0.05
Fructosyl-FB_1	6.291	6.535	> 0.05
FB1+Sucrose reaction product	0.009	0.011	> 0.05
FB1+GLUMeOH reaction product	0.011	0.015	> 0.05

The slightly increase of the calculated ratio upon digestion can be due to a saturation of the clean-up system used to desalt samples prior to LC-MS analysis: Sep-Pak C18 cartridges may be affected by the strong matrix-effect that occurs in complex samples such those underwent to digestion, thus leading to a partial loss of the analyte.

Since the ratio values are consistent, it can be argued that the involved enzymes are not able to destroy covalent bonds such those existing in the considered derivatives. Nevertheless, as the hidden fumonisin release from the matrix upon digestion has been demonstrated, it is possible to say that only fumonisins which are complexated or physically entrapped into food macroconstituent can be released under gastrointestinal digestion conditions. Moreover, additional considerations concerning the masking mechanism emerge from these experiments: although the formation of FB_1 covalent derivatives has been demonstrated by these model reactions, the reaction yield is very low even under laboratory conditions, suggesting that their occurrence in contaminated food after cooking is reasonably very limited. Consequently, the theory based on the formation of covalent linkages among the target toxin and matrix constituents is unsuitable to explain the large amount of hidden forms often detectable in thermal-treated maize-based food.

From a food safety point of view, further studies are necessary in order to evaluate if these derivatives may be absorbed or transported through the intestinal epithelium and which is their own toxicity in comparison with their parent forms.

1.5 CONCLUSIONS

The digestion assay here applied allowed to demonstrate the release of parent fumonisins from the food matrix during gastrointestinal digestion. As shown, target compounds are stable when undergone digestive conditions. Moreover, total FBs found after digestion and FBs levels found after alkaline hydrolysis were generally comparable. From the analysis of raw maize samples, an increase of FB levels after digestion was observed as a general trend. This means that bioaccessibility of fumonisins in small intestine may be higher than that estimated by conventional techniques of analysis. For this reason, hidden fumonisins should be taken into consideration in risk assessment studies.

Moreover, an analytical issue was introduced by our experiments, since routine methods are unable to detect masked fumonisins to date, opening thus another serious problem regarding risk assessment: consumers may be, as a matter of fact, concretely exposed to a higher risk than that evaluated by routine methods.

Finally, this observation may lead to a partial explanation of the so called "fumonisin paradox", well-described by several authors: although 90% of FB_1 is excreted through faeces when administered orally, food contaminated by this toxin can still exert high toxic effects. These data can be in agreement with the occurrence of non detectable bound or hidden forms that would be released upon digestion. The experiments carried out in order to understand the masking mechanism evaluated that the most significant contribution to the matrix disaggregation was due to the duodenal digestive step and, in particular, the addition of lipase and bile seemed to be a crucial point. However, all the digestive steps seemed to play an important role in fumonisin releasing, since a complete digestion was usually necessary to obtain a total mycotoxin release from the matrix, thus supporting a non-specific masking mechanism. These observations have been also confirmed by the digestion of some FB_1-covalent derivatives, demonstrating that, among total bound fumonisins, only those complexated or physically entrapped into food macroconstituents can be released under gastrointestinal conditions.

CHAPTER 2 *IN VITRO* EXPERIMENTS FOR STUDYING FUMONISINS MASKING PHENOMENA: THE STARCH AND ZEIN BEHAVIOUR

2.1 INTRODUCTION

Among the various constituents of the maize grain, carbohydrates and proteins represent on average, respectively, the 70% and 10% of the total components. Concerning the carbohydrate fraction, despite to a low content of structural carbohydrates such as cellulose and lignin, the maize caryopsis has a high starch content, with an amylose-to-amylopectin ratio roughly equal to 26/74 but that can vary within varieties. The proteic fraction is divided into four classes: globulins (1%), albumins (3%), glutelins (35%) and prolamins (47%), defined primarily by their solubility in selected solvents, and is represented mainly by zeins (39). Zeins are alcohol-soluble storage proteins belonging to the prolamins class and comprise about 45-50% of the proteins in corn. Almost all the zeins is present in the endosperm: the function of these proteins is apparently to store nitrogen for the developing seed, however their polymeric uses are of considerable interest. Indeed, their deficiency in essential amino acids, such as lysine and tryptophan, make them poor in nutritional quality and also their insolubility in water limits their use in human food products in behalf of industrial application, for example films and fibres development (30). The zein proteins due their hydrophobic properties to their amino acid composition: they are particularly rich in glutamic acid, leucine, proline and alanine, but deficient in basic and acidic residues, thus they are insoluble in water even with low concentrations of salt and require high percentage of ethanol aqueous system to maintain their molecular conformations (40). Actually, corn zeins are classified in four types, denoted as α, β, γ and δ according to their solubility properties and α-zein represent the major fraction. Although generally found as disulfide-bridged dimers, the individual α -zeins have molecular masses of 19 and 22 kDa, the size difference being from an amino acid insertion in the C-terminal region of the 22 kDa form (30). Several analytical efforts have been made to elucidate the molecular structure of this protein. Nevertheless, whereas physicochemical and structural characterization studies of the α–zein structure

suggest that these protein has a high content of alpha-helixes (ranging between 35-60%), there are no three-dimensional structure of α–zein available but only models supported by empirical studies (41). Among the suggested models, that proposed by Momany et al. (31) may be of particular interest in the study of the fumonisin masking phenomenon, because it goes on to explain the binding of lutein, a naturally occurring xanthophyll often associated with the protein of interest. According to this model (computated for the protein solved in aqueous methanol mixture), the amino acid sequence of the 19 kDa units of α-zein (Z19) suggests that the protein has coiled-coil tendencies, resulting in nine α-helices with about four residues in the central sections. Then, the nine helical segments are arranged in three interacting superhelixes having inside a hydrophobic face formed by the non polar residue side chains. Natural carotenoid could binds into the core of each triple-helical fragments. Therefore, three lutein molecules would be include per Z19 molecule, in a position which make it difficult to remove from protein after zein extraction (Figure 14).

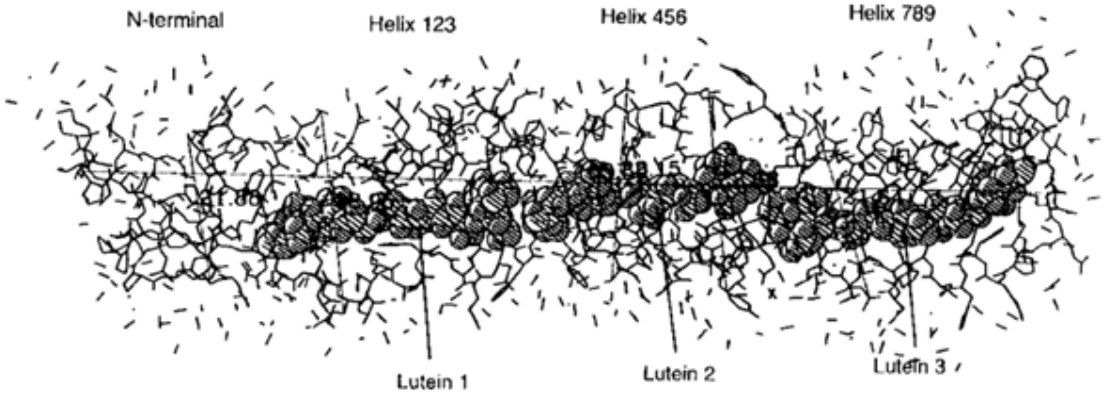


Figure 14. Three dimensional structure proposed for Z19 α-zein in aqueous methanol mixture by Momany et al (31). Methanol molecules are represented as small lines. Lutein molecules are shown in space-filling representation (reported from Momany et al., 2006 [31])

Since this model assumes that zein protein is able to interact with other molecules and also supports that these interactions are based on physical entrapment or complexation of the target molecule into coiled-coil cores, it could be taken as starting point in the study of fumonisins-zein interaction.

In addition to zein, the unbranched fraction of starch, amylose, is also know to form inclusion complexes with monoglycerides, fatty acids and surfactants (42). This type of interaction has been indirectly demonstrated by X-ray measurements of the reduction of amylose-iodine affinity: the its decaying in the presence of other molecules indicates that amylose and fatty acids form complexes similar to those which amylose forms with iodine (42, 43), with the hydrocarbon portion of the lipid located within the helical cavity of amylose (44). Moreover, factors influencing starch-lipids complexing have been investigated, demonstrating that

higher binding values correspond to lower fatty acids unsaturation degree (45). Finally, the amylose-fatty acids complex formation during food processing, such as extrusion or starch gelatinization, has been demonstrated (46, 47), suggesting that these non covalent interaction can occur not only in raw maize but also in processed foods. Since fumonisins have an aliphatic chain similar to those possessed by fatty acids and also tricarballilic moieties could be movable and fold, an analogous mechanism of interaction among fumonisins and corn starch may be supposed. Nevertheless, in literature there are only few data reporting the occurrence of hidden fumonisins associated with proteins (29), while the interaction among fumonisins and starch has never been studied. Thus, the application of the knowledge about interaction mechanisms involving macromolecules and matrix constituent as a possible explanation of fumonisins masking phenomenon is still a conjecture, but it may be taken as starting point in order to characterize zein-toxin and starch-toxin interaction.

2.2 Aim of the work

In this work several *in vitro* experiments were carried out in order to obtain more data about the nature of fumonisin interaction with zein and starch. As first, the conventional fumonisin extraction procedure from raw maize has been compared to a QuEChERS-like approach (an extraction methods based on protein salting out effect) in order to verify if hidden forms could be released after protein salting-out precipitation. Moreover, titration experiments were performed among fumonisin B_1 and zein or starch, respectively, with the aim to understand under which conditions and at which extent the masking phenomenon takes place under laboratory conditions. Additional information concerning the specific contribution of each macromolecule as well as starch fractions in fumonisin masking phenomenon were also obtained, by performing recovery experiments both on mono- and multi component model systems.

Finally, in order to assure the reliability of our results, the occurrence of analytical artefacts during hidden fumonisin evaluations has been estimated. For this purpose, an experimental set based on the determination of free and hidden fumonisins both in extracts and exhausted matrices obtained using selected solvent mixtures has been performed, giving us several information about the extraction conditions leading to supramolecular interactions involving fumonisins.

All the experiments here presented were based on *in vitro* model systems, providing a clear idea about the masking mechanism.

2.3 Materials and methods

2.3.1 Reagents

Fumonisins B_1, B_2 and B_3 standard solutions (a mixture of FB_1, FB_2 and FB_3, 50 μg/ml each, in acetonitrile/water, 1:1 v/v) and Fumonisin B_1 in powder, 5 mg, were purchased from Romerlabs (Tulln, Austria). Methanol (LC grade) and propan-1-ol were obtained from Carlo Erba (Milan, Italy), acetonitrile (LC grade) and ethanol (96%) were from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA). Formic Acid (99%) was purchased from Acros Organics (Geel, Belgium). Magnesium sulphate, sodium chloride, potassium iodide and DL-dithiothreitol (5 g) were obtained from Sigma-Aldrich (Stuttgart, Germany). Maize zein was from Fluka Chemika-Biochemika (Buchs, Switzerland), maize starch was a commercial product from the market (Maizena, Unilever). Amylose from potato and amylopectin from maize were purchased from Sigma (Stuttgart, Germany). Hydrolyzed fumonisin standard solution was prepared in our laboratory, according to the procedure described at point 1.3.3.

2.3.2 Experimental procedures

Samples preparation for the analysis of fumonisins and hydrolyzed fumonisins.
Sample preparation for the analysis of free extractable fumonisins (free FBs) and sample preparation for the analysis of total fumonisins after alkaline hydrolysis were performed as reported at point 1.3.3

QuEChERS-based extraction method.
For the determination of parent fumonisins through QuEChERS-like extraction method, the procedure reported by Zachariasova et al. (48) has been used. Briefly, 4 g of homogeneous raw maize sample were weighted into a centrifuge tube (Sterilin, ThermoFisher Scientific, Cambridge, UK) and 12.5 mL of 0.1% (v/v) aqueous formic acid and 8 mL acetonitrile were added. The suspension was shaken vigorously for 3 min (Autovortex SA6, Stuart Scientific, Keison Products, Chelmsford, UK) and then a gentle stirring was maintained for 10 min (Stuart Reciprocating Shaker SSL2, Stuart Scientific, Keison Products, Chelmsford, UK). After the simultaneous addition of 1 g NaCl and 4 g of $MgSO_4$, the mixture was shaken again

for 3 min and then centrifuged for 5 min at 3500 rpm (Alc Centrifuge pk110, DJB Labcare Ltd, Newport Pagnell, Buckinghamshire, UK) to separate the aqueous and the organic phase. Finally, the 2 mL aliquot of the upper organic phase was evaporated to dryness and the residue was redissolved in water/methanol (30:70, v/v) prior to LC-MS/MS analysis.

Zein-fumonisin B_1 titration.

A zein stock solution was obtained by dissolving 37.25 mg in 1 mL of water/ethanol (3:7, v/v) and then it was used to prepare four zein work solutions containing, respectively, 37.25, 18.75, 3.75, and 0.375 mg/mL of the protein (named as Work1, Work2, Work3, Work4). Simultaneously, three zein work solutions containing, respectively, 0.185, 0.075 and 0.04 mg/mL (named Work5, Work6 and Work7) were obtained through appropriate dilution of a second zein stock solution (5 mg/mL), prepared in the same solvent. Then, seven solution containing fumonisin B_1 and zein in different molar ratios were prepared by mixing 400 µL of a fumonisin B_1 stock solution (10 µg/mL, in water/ethanol, 3:7 v/v) and 400 µL of each zein work solution, according to the following scheme:

Table 6. Zein-FB1 titration. Experimental design: molar ratios and employed volumes of each standard solution to prepare the seven established points of the titration curve.

Zein:FB_1 (moles:moles)	Composition
100:1	400 µL stock B_1 + 400 µL zein Work1
50:1	400 µL stock B_1 + 400 µL zein Work2
10:1	400 µL stock B_1 + 400 µL zein Work3
1:1	400 µL stock B_1 + 400 µL zein Work4
1:2	400 µL stock B_1 + 400 µL zein Work5
1:5	400 µL stock B_1 + 400 µL zein Work6
1:10	400 µL stock B_1 + 400 µL zein Work7

Then, the volume of each experiment was adjusted to 2 mL by adding 1200 µL of water/ethanol (3:7, v/v) mixture. Finally, 1 mL of all solution was evaporated to dryness under a stream of nitrogen and the residue was redissolved in water/methanol (3:7, v/v) after 12 hours of incubation at room temperature and then analyzed.

Starch-fumonisin B_1 titration.

5 mg of corn starch were weighted into a glass vial and then were spiked with increasing amounts of a FB_1 standard solution (10 µg/mL, in water/methanol, 3:7 v/v) in order to obtain different weight-to-weight ratios between the target toxin and the macromolecule, as reported in the following scheme:

Table 7. Starch-B1 titration. Experimental scheme: weight-to-weight ratios and volumes of FB1 standard solutions needed to spike 5 mg of blank starch.

Starch:FB_1 (w/w)	Preparation
5000:1	5 mg starch + 100 µL stock FB_1
2000:1	5 mg starch + 250 µL stock FB_1
1000:1	5 mg starch + 500 µL stock FB_1
500:1	5 mg starch + 1000 µL stock FB_1

After spiking, solvent was removed using a gentle stream of compressed air and then samples were maintained in incubation for 12 hours in the dark at room temperature. Fumonisin B_1 was extracted using 2 mL of water/methanol (3:7, v/v) mixture and samples were filtered through a 0.45 µm nylon filter before LC-MS/MS analysis. Each experiment was performed in duplicate.

Evaluation of the effect of increasing amounts of zein on starch-FB_1 interaction degree

Four samples were prepared by mixing in 15 mL centrifuge tubes 200 mg of corn starch with, respectively, 0, 2, 20 and 40 mg of zein. Table 8 shows the amounts of starch and zein used to prepared each sample.

Table 8. Amounts of Starch and Zein used to prepare the four mixtures and ratio calculated between them.

Experiment	Starch (mg)	Zein (mg)	Starch:Zein (w/w)
1	200	0	-
2	200	2	100:1
3	200	20	10:1
4	200	40	5:1

Then, all the mixtures were spiked with 200 µL of a FB_1 standard solution (10 µg/mL, in water/methanol, 3:7 v/v). Solvent was removed by evaporation under a stream of compressed air before 12 hours of incubation in the dark at room temperature. At the end of incubation, samples were stirred with 2 mL of water/methanol (3:7 v/v) and then centrifuged (3500 rpm, 15 min) and filtered through 0.45 µm nylon filters before LC-MS/MS analysis. Each trial was run in duplicate.

Spiking experiments of individual components of starch (amylose and amylopectin)

200 µL of a FB_1 standard solution (10 µg/mL, in water/methanol, 3:7 v/v) were used to spike, respectively, 5 mg of corn starch, 5 mg of amylose, 5 mg of amylopectin and 5 mg of a mixture prepared by mixing 250 µg of amylose with 250 µg of amylopectin. For each sample, solvent was evaporated under a gentle stream of compressed air. After 12 hours of incubation

in the dark at room temperature, FB_1 was extracted with 2 mL of water/methanol (3:7 v/v). Then, samples were stirred for 2 min and filtered through a 0.45 μm nylon filters prior to LC-MS/MS analysis. Each trial was run in duplicate.

Evaluation of the effect of the extraction mixture composition on free and hidden fumonisins determination in raw maize.

Experimental design. The same batch of raw maize was split in three aliquots. Then, each ones underwent to conventional extraction using three different solvent mixtures, characterized by increasing percentages of ethanol, in order to quantify the amount of free FBs. Both the extract and the residual matrix obtained for each selected mixture were hydrolyzed to obtain data concerning hidden fumonisins. All the obtained data were compared with that achieved upon alkaline hydrolysis of the original matrix. Moreover, on the extracts a colorimetric assay to detect starch and Osborne fractionation to quantify globulins and prolamins were performed.

Sample preparation for the determination of free extractable FBs. Aliquots (5 g) of raw maize samples (belonging to the same batch) were blended in a high-speed blender (Ultraturrax T25, IKA, Stauffen, Germany) with 20 mL of water/ethanol mixture in different proportions (30:70; 20:80 and 10:90, v/v) for 3 min at 4,000 rpm and then filtered. After filtration on nylon filters (0.45 μm), 1 ml extract was analyzed by LC-ESI-MS/MS. Residual matrixes were recovered from the surface of the filters and dried to be hydrolyzed.

Sample preparation for the determination of total fumonisins in exhausted matrixes. Each residual extracted matrix, deriving from free fumonisins extraction, was recovered from the surface of the paper filter and the solvent was evaporated in a forced convection heater for 12 hours at 60°C. Then, an aliquot (2.5) underwent to alkaline hydrolysis, according to the procedure reported in 1.3.3**.**

Sample preparation for the determination of total fumonisins after hydrolysis in the extracts. Aliquots (5 mL) of each extract previously obtained were evaporated to dryness under vacuum (Rotavapor BŰCHI 461 Water Bath, BUCHI Labortechnik AG, Milan, Italy), then the residue was resuspended in 20 mL of 2N aqueous KOH and incubated for 12 hours in the dark at room temperature. At the end of this period 20 mL of acetonitrile were added and the mixture was shaken vigorously for 3 min. The aqueous and the organic phase were separated by centrifugation at 3500 rpm for 15 min (Alc Centrifugette 4206, TecnoLab, Brescia, Italy). Then, 2 mL of the organic upper layer were evaporated to dryness under a gentle stream of nitrogen and the residue was resuspended in 500 μL of the same mixture used to obtain the origin extract, prior to LC-MS/MS analysis.

Colorimetric assay for starch detection in the direct extracts. The colorimetric assay was performed using the Lugol reactive, prepared by dissolving 1 g of KI and 1 g of crystalline I_2 in 100 mL of bidistilled water. The assay was carried out by adding 2 drops of the reactive to 500 μL of each extract and the coloration obtained has been compared with that achieved by performing the same test on a little amount of pure starch.

Osborne fractionation of the direct extracts and gravimetric quantification of the protein fractions. 5 g of raw maize sample underwent to the same extraction procedure used for fumonisins determinations as previously reported, using the three selected extraction mixtures. Each extraction was performed in duplicate. Then, for each one sample, both the two extracts were evaporated under vacuum. One residue was redissolved in water/ethanol (30:70 v/v) to resuspend selectively prolamins , while the second one was redissolved in water/propan-1-ol (1:1 v/v) mixture containing 1% dithiothreitol to solve only globulins. Bothe the mixtures were transferred into previously weighted flasks and the solvent was evaporated under vacuum prior to weight again the same flask. Each measure was performed in triplicate and the amount of proteins was calculated by subtracting the weight of the flask tare to the weight of the flask containing the solid residue.

LC-MS/MS analysis

LC-MS/MS analysis for the determination of parent fumonisins and hydrolyzed fumonisins were performed as reported at point 1.3.3.

Statistical analyses

Statistical analyses 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 ($\alpha = 0.05$).

2.4 Results and discussion

2.4.1 Determination of parent fumonisins in raw maize samples by QuEChERS-like approach.

Hidden fumonisins have been found in raw maize associated with corn proteins, in particular with prolamins and globulins (29), suggesting a mechanism of interaction based on a physical entrapment of the analyte into the protein structure. This hypothesis is supported by the fact that masked forms can be released as parent fumonisins from matrix after enzymatic digestion (16, 50). Moreover, since enzymes are not able to destroy covalent interactions among the analyte and food constituents, it can be supposed that only fumonisins complexated or physically entrapped into the matrix can be released upon enzymatic hydrolysis. Finally, empirical models developed on the basis of the zein amino acidic sequence and its secondary structure suggest that this protein is able to host xanthophylls in its structure through non covalent interaction (31). This work aimed both to demonstrate the association between fumonisins and proteins and to better investigate the nature of this interaction. For this purpose, the conventional extraction procedure used for the determination of free extractable fumonisins has been compared with a QuEChERS-like approach: this is a procedure initially developed for pesticides analysis, but recently successfully proposed also for mycotoxin multiresidual determination (48, 49). This approach is based on a partitioning of acetonitrile/water mixture induced by addition of inorganic salts. While the analytes are transferred into the organic phase, several more polar matrix impurities are left in the aqueous layer. Moreover, the addition of inorganic salts cause protein coagulation and precipitation through salting-out effect. In order to evaluate if hidden fumonisins could be released after the removal of protein from the extract, several raw maize samples (n = 5) were extracted using QuEChERS-like approach, and the results were compared to those obtained by using the conventional water/methanol extraction procedure and also with the levels of total fumonisins released upon alkaline hydrolysis, as shown in Figure 15.

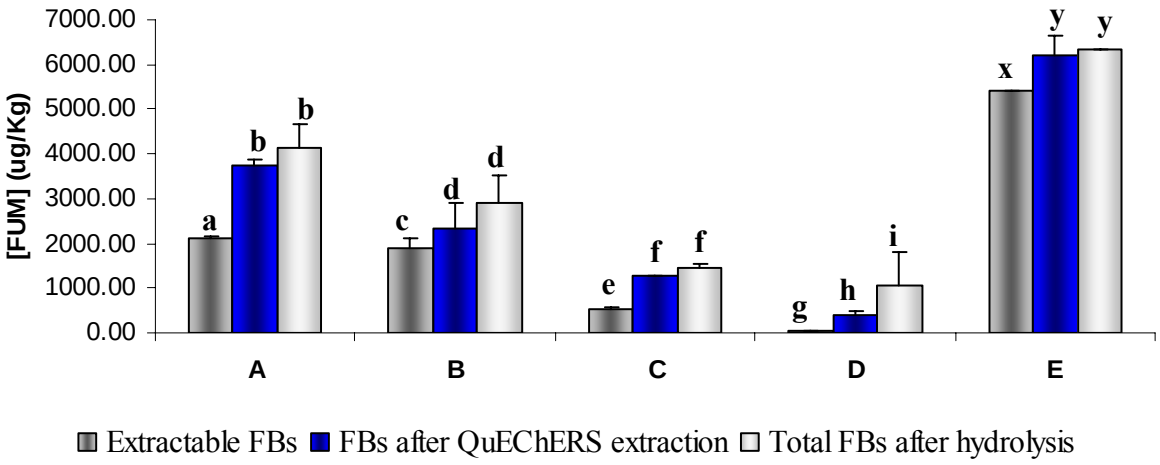


Figure 15. Comparison between the level of fumonisins found in raw maize after routine extraction, after QuEChERS extraction and after alkaline hydrolysis. Different letters designate statistically significant difference between data.

Data were statistically compared using a one-way ANOVA test followed by a post hoc Tukey test ($\alpha = 0.05$). As shown, the extractable fumonisins obtained by the QuEChERS-like method were often comparable to total fumonisins obtained after alkaline hydrolysis; moreover, even in this experiment, fumonisins and not their hydrolyzed forms were detected. Thus, the higher recovery obtained using the QuEChERS-like approach suggests again that parent fumonisins may be released from the matrix given the higher disaggregation capacity of this extraction method. This effect could be partially due to the protein removal caused by salting out induced by the inorganic salt addiction, supporting the hypothesis of a non covalent interaction between fumonisins and matrix constituents. Indeed, as a matter of fact, although total fumonisins found after alkaline hydrolysis could be ascribed either to the releasing from association complexes formed with the matrix macroconstituents or to the cleavage of covalently bound derivatives, the increased amount of extractable fumonisins found by applying the QuEChERS-like approach can be only due to the more efficient disaggregation of the matrix and to the destabilization of non covalent interactions.

2.4.2 FB_1-ZEIN AND FB_1-STARCH TITRATIONS: EXPERIMENTAL EVIDENCES OF FUMONISINS-MACROMOLECULES INTERACTIONS.

Starch and zein are the main maize macroconstituents and are both able to host several guests, such as pigments as well as fatty acids, in their tridimensional structure via non covalent interactions, as reported in literature (31, 42). Although the association of fumonisins with these macromolecules has been demonstrated in several studies (29, 50), the most conducive conditions for FB-starch and FB-zein interaction have not yet been studied. Thus, *in vitro*

titrations between zein and starch with FB_1 have been performed with the aim to better define what type of interaction exist among fumonisins and these compounds.

Concerning zein, experimental conditions were chosen on the base of the results achieved from a zein-FB_1 titration performed in a previous work (51): three solutions containing FB_1 and zein in different molar ratios (2:1, 1:1 and 1:2) were prepared in methanol and directly infused in a ESI-MS system, demonstrating that the intensity of the signal corresponding to the FB_1 molecular ion was strongly influenced just when toxin:zein molar ratio was unitary. Nevertheless, this effect can be due to an ion suppression exerted by the protein, that competes with the analyte for the protons. Therefore, in order to confirm these results and also to minimize the interference due to increasing amount of zein in solution, thus allowing the specific study of the effect due only to the masking phenomenon, the experiment was repeated in a similar way, but introducing a chromatographic separation before the MS analysis. Briefly, experiment was carried out preparing seven solution in water/ethanol (3:7 v/v) mixture containing zein and fumonisin B_1 at different molar ratios, starting from a fumonisin excess to ending in a protein excess. Then, the solvent was evaporated under a stream of nitrogen and the residue was resuspended in water/methanol (3:7 v/v) mixture after 12 hours of incubation at room temperature and analyzed through LC-MS/MS. Free fumonisins was directly detected for each solution and the results have been compared with the analysis of a FB_1 standard solution prepared without zein addition (see Figure 16). To evaluate differences, data were statistically compared using a one-way ANOVA test followed by a post hoc Tukey test ($\alpha = 0.05$).

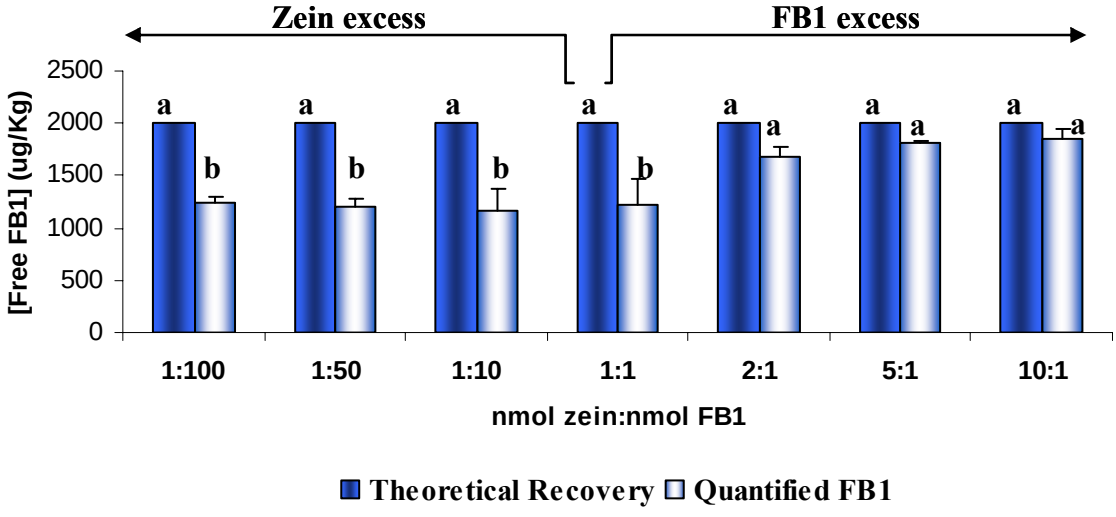


Figure 16. Zein-FB1 titration: comparison between the amount of extractable toxin detected in each sample and the expected recovery obtainable without masking effect. Different letters designate statistically significant differences between data ($\alpha = 0.05$).

The data showed that the detection of parent FB_1 was strongly influenced by the presence of the protein: only about 60% of the total FB_1 used for each experiment has been detected in four out of five experiments. In particular, this effect seems to be evident when the molar ratio among the analyte and zein was equal to 1 and remained constant also when the protein content was increased, while when FB_1 moles exceeded zein moles any effect was observed. Since in this case the detection was preceded by a chromatographic separation the effect of ion suppression has been minimized, thus it can be argued that the observed reduction of fumonisin is mainly due to a masking exerted by the proteins through physical-type interactions. Moreover, although these experiments are simple models, they still represent conditions that occur into the maize kernel, for instance the larger amount of zein compared to fumonisins. The constant masking degree that occurs since zein moles equalize FB_1 moles suggests that just under this condition the protein is saturated by fumonisins (masking rate: about 40%). Thus, it is reasonable to think that also other corn macromolecules, such as starch, are able to host fumonisins into their structure.

To evaluate the capability of starch to hide these mycotoxins, four experiments that represent the selected points of a titration curve were prepared by spiking 5 mg of blank corn starch with increasing volumes of a FB_1 standard solution. Then, after 12 hours of incubation at room temperature, parent FB_1 was extracted using water/methanol (3:7, v/v) mixture and quantified by LC-MS/MS measurement. Data obtained are reported in Figure 17: the levels of parent fumonisin detected after solvent extraction were compared with the amounts of hidden FB_1, calculated by subtracting the extractable toxin value to the initial amount used.

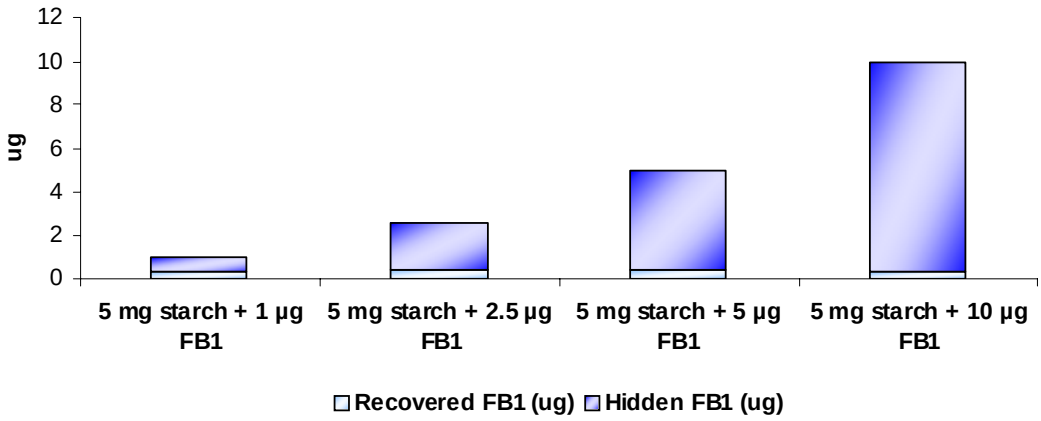


Figure 17. Starch-FB1 titration: comparison between the levels of parent FB1 detected after conventional extraction of each spiked sample and the amounts of not recovered toxin.

Likewise what found for zein experiments, starch was found to be able to hide fumonisin, although a saturation point cannot be achieved under the applied conditions.

Since starch and zein are both located in the endosperm, with a large excess of starch in comparison to proteins, a greater accessibility to this carbohydrate for fumonisin may be supposed. Thus, the masking phenomenon may involve primarily this macromolecule and later the protein fraction.

2.4.3 Contribution of single starch fractions in fumonisin masking phenomenon

Amylose and amylopectin are two macromolecular components of starch granules. Normal maize starch consist of 80% branched amylopectin; the remaining 20% is linear amylose (52). Since in the present work the capability of starch to hide FBs has been demonstrated, *in vitro* experiments on its separated fractions were performed with the aim to evaluate which of these constituents has the greatest affinity for the target toxin and also which is the specific contribution of each polysaccharide in the fumonisin masking phenomenon. Thus, both pure amylose and amylopectin were spiked with the same volume of a FB_1 standard solution and were left to incubate for 12 hours in the dark at room temperature prior to free FB_1 determination. In order to evaluate the effect of a binary system, the same experiment was also performed on a amylose/amylopectin (1:1 w/w) mixture. Moreover, two control samples were prepared: the first was corn starch spiked with FB_1 (representing the real situation in which the two polysaccharides are involved into the maize kernel), while the latter was a FB_1 standard solution a the corrected dilution rate. The results are reported in Table 9 and resumed in Figure 18.

Table 9. Recovery data obtained after spiking with FB1 a corn starch sample and a corn zein samples (all the experiments were performed in duplicate, n = 2). Different letters indicate a significant difference (Tukey test, α = 0.05).

	Spiking level (µg/Kg)	Extractable FBs (µg/Kg)	Recovery (%)
Amylose	1000	261 ± 15 **a**	26.10%
Amylopectin	1000	472 ± 22 **a,b**	47.20%
Mix (1:1)*	1000	844 ± 70 **b,c**	84.40%
Starch (Control 1)	1000	816 ± 184 **b,c**	81.60%
FB_1 1000 µg/Kg (Control 2)	1000	1069 ± 144 **c**	100

* A miscellaneous obtained by mixing 250 µg of amylose with 250 µg of amylopectin

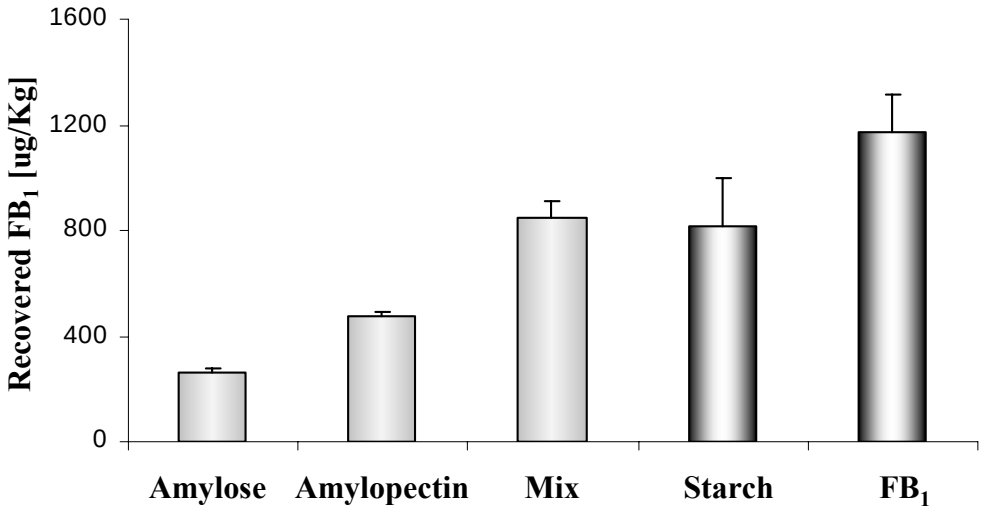


Figure 18. Recovery data obtained after spiking starch macromolecules. Spiked starch and FB_1 standard solution (named "Control 1" and "Control 2") are coloured in black.

Amylose seems to have the greatest affinity for fumonisins, as only about 26% of the total amount of initial FB_1 was recovered. Since the capability of amylose to interact with fatty acids and surfactants has been demonstrated (42, 43, 44), a similar mechanism of interaction based on the formation of inclusion complex involving this polysaccharide and the target toxin might be supposed. The unexpected data were actually those provided by amylopectin: only about 47% of the initial amount of FB_1 was recovered, demonstrating that also the branched fraction of starch is able to hide fumonisins. Nevertheless, when the two polymers occur together in the same system, a higher FB_1 recovery can be observed (about 82-84%), suggesting that the FB_1 complexation is overshadowed by the mutual interaction between amylose and amylopectin.

2.4.4 Variation of the masking degree of FB_1 measured into *in vitro* starch-zein binary systems

Until now, the FB masking phenomenon has been studied in separated binary systems consisting of FB_1-zein and FB_1-starch mixtures, and no experiments were carried out using both the macromolecules. Therefore, in order to evaluate if the behaviour of each macromolecule remains the same when also the other one occurs in the same system, the masking degree in FB_1-starch systems containing increasing amounts of zein has been evaluated. Samples were prepared by spiking several starch-zein mixtures containing increasing amounts of zein with a standard solution of fumonisin B_1. The proportion of the two constituents were chosen in order to mix them in, respectively, 100:1, 10:1 and 5:1

weight-to-weight ratios. Since starch and zein occurs into the maize kernels with a ratio of about 7:1, the last sample is the closest to a real situation.

Then, after 12 hours of incubation at room temperature, parent FB_1 was extracted using water/methanol (3:7, v/v) and determined through LC-MS/MS analysis. The results obtained are compared with a control sample, prepared by spiking the same amount of blank starch with the same volume of the FB_1 standard solution used to spike binary systems (see Figure 19). Data were statistically compared using a one-way ANOVA followed by a post-hoc Tukey test ($\alpha = 0.05$).

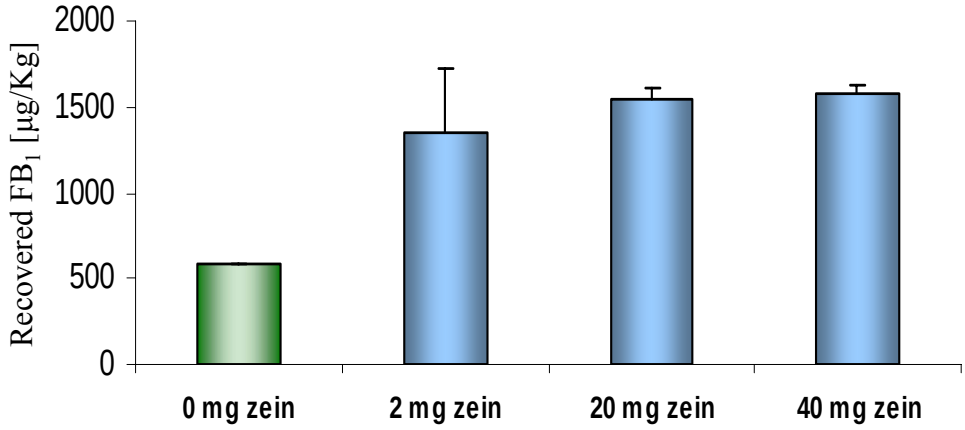


Figure 19. Comparison between the levels of parent FB_1 recovered by extracting starch-zein mixtures containing increasing amounts of zein. The control sample (starch spiked with FB_1) is coloured in green. Different letters designate statistically significant differences between data ($\alpha = 0.05$).

Whereas in a mono-component system FB_1 was poorly recovered (about 58.6%), the toxin was fully recovered when zein occurs together with starch. Surprisingly, the addition of 2 mg of protein is enough to ensure the complete recovery of the analyte, suggesting that the masking effect exerted by starch toward FB_1 is lost, showing the antagonist behaviour of the macromolecules towards fumonisins. This result was confirmed by performing a recovery experiment on blank raw maize spiked with FB_1: 5 g of sample were spiked with an appropriate amount of FB_1 solution in order to obtain a final analyte concentration of 2000 µg/Kg and the amount of free extractable fumonisin was determined with the same extraction procedure followed by LC-MS/MS analysis. The amount of FB_1 recovered through the solvent extraction was 1811±113 µg/Kg (about 90.5%), showing a nearly complete recovery of the analyte from the complex matrix. This type of experiment represent a common method used to verify the recovery of an extraction procedure and, in this particular case, the capability of the solvent used to fully recover free fumonisins has been demonstrated,

indicating also that no interaction occurs between fumonisins and maize macroconstituents under these conditions.

The lack of toxin-constituent complexes observed in a multi component system may be due to the interaction that occurs among macromolecules or to a change of their conformation in presence of other constituents. Nevertheless, since the occurrence of hidden fumonisins in naturally-contaminated raw maize has been demonstrated (16, 29, 50), some considerations concerning the difference between *in vitro* and *in vivo* systems should be made. Indeed, although zein and starch have been mixed in the same proportion occurring into the maize their organization as well as their spatial partitioning cannot be replicated into an *in vitro* multi component system. Therefore, an artificial environment represents a highly simplified system in which the constituents may interact with each other in a different way in comparison to the real system, leading to a different interaction with fumonisins.

Moreover, the fungal infection as well as the mycotoxin production occur during plant growing and ripening, involving a dynamic system subjected to continuous changes and provided to a own enzymatic activity. On the other hand, the model assay is actually a very different system: the lack of any masking effect observable in an artificially contaminated multi component system (meaning both a binary mixture of macroconstituents prepared *in vitro* or a blank raw maize sample) may be due to the absence of the complex interaction among several factors, among which the plant-pathogen cross-talk.

For that reason, although *in vitro* models provide us with several information concerning the mechanism, masking phenomenon should be study in a wider perspective, trying to understand which conditions are a prerogative in determining the masking of such mycotoxins.

2.4.5 Evaluation of analytical artefacts in hidden fumonisins determination

To date, hidden fumonisins are determined using an indirect approach, calculating the difference between the amounts of total fumonisins determined after alkaline hydrolysis and free fumonisins extractable through a solvent extraction procedure. Both these procedures are applied to solid ground matrix, thus the calculated value of hidden fumonisins has always been reported as the amount of the toxin that interacts with food constituents. Nevertheless, recently the occurrence of hidden fumonisins in the extracts has been reported (16), posing some doubts on the real occurrence of forms associated to maize macromolecules in a solid matrix, in favour of the hypothesis that hidden fumonisins may be the result of an analytical

artefact, generated during solvent extraction. Indeed, supramolecular structures formed by macromolecules such as starch and zein may be formed by the extraction solvents used for fumonisins determination thus entrapping fumonisins, which cannot be directly determined. This hypothesis is supported by the fact that the solvent used to extract fumonisins from maize samples is also able to dissolve the prolamin (zein) fraction. The behaviour of these proteins solved in a binary mixtures of water and ethanol or methanol has been studied, and the formation of globular aggregates has been demonstrated (53, 54). The dimension of such microspheres depends from the protein concentration as well as the percentage of the organic solvent in aqueous solution. These structures are of great interest in the pharmaceutical field for their ability to include small molecules, such as drugs. Thus, since the composition of the extraction solvents used for fumonisin determination is often the same that leads to the formation of the zein aggregates here described, it can be supposed that analytes may be included into these structures, thus escaping analytical determination.

In order to clarify this phenomenon and to better understand if the masking mechanism is primarily due to a real interaction that takes place into the matrix or if it is caused mainly by an analytical artefact that occurs during solvent extraction, a set of experiments has been performed. In this experimental set, three raw maize samples belonging to the same batch were extracted using three different binary mixtures of water and ethanol, characterized by increasing percentages of the organic fraction. Since the decrease of the dimension of zein aggregates at increasing levels of ethanol has been demonstrated (53), the composition of the binary mixtures was chosen based on literature reports. For each selected mixture, free extractable fumonisins were quantified in the direct extract. Then, total fumonisins after alkaline hydrolysis were determined both in the extract and in the residual matrix s. Finally, all the data obtained were compared with that achieved hydrolyzing the original matrix and the ability of each extraction mixture to generate supramolecular structures that can lead to fumonisin masking artefacts was evaluated. Figure 20 resumes the entire experimental design.

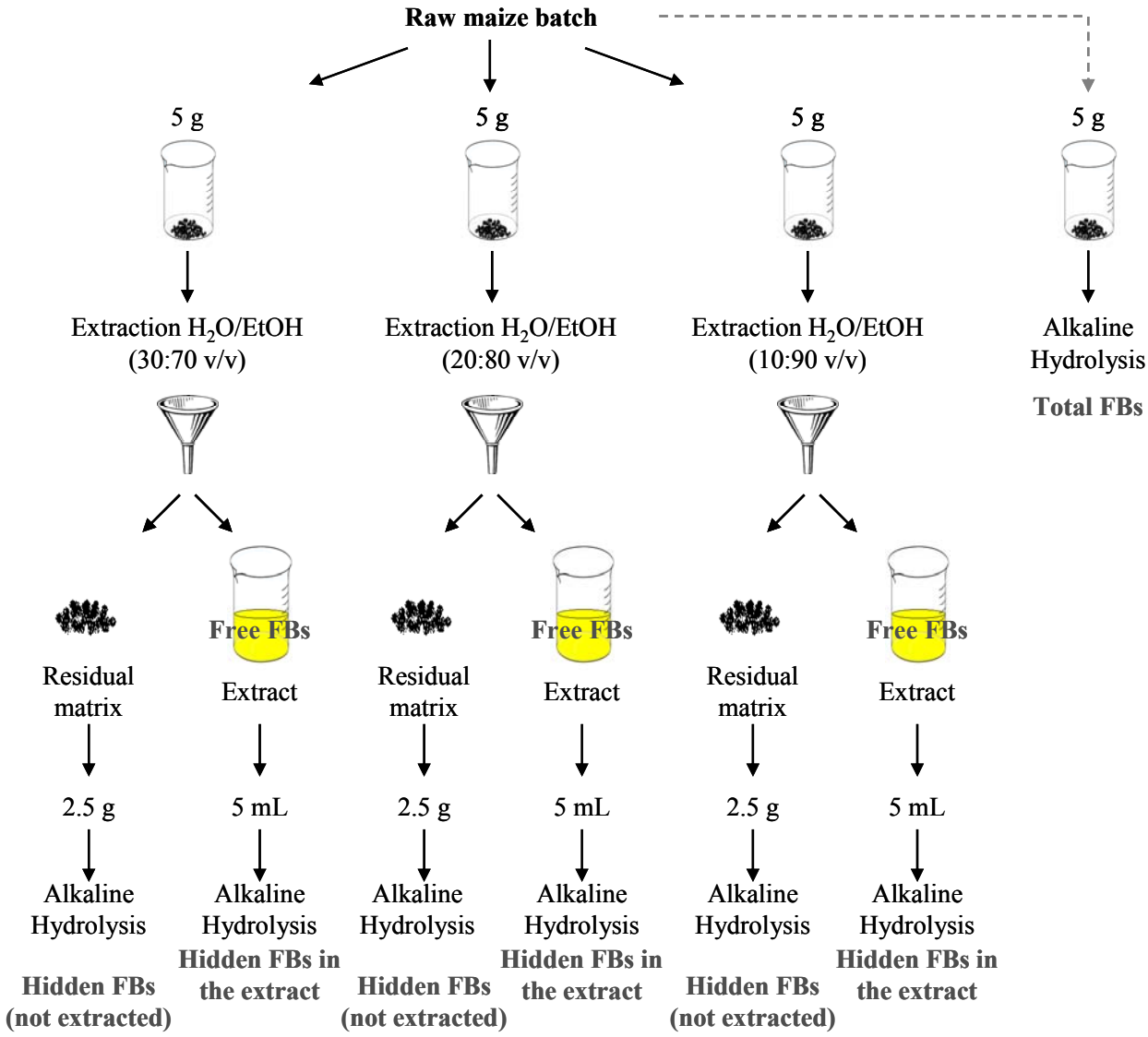


Figure 20. Schematic representation of the experimental set designed to evaluate the occurrence of analytical artefacts during hidden fumonisins evaluation.

The collected data are reported in Table 10. Extractable fumonisins, total fumonisins in each extract after hydrolysis and hidden fumonisins detected in exhausted residual matrix after hydrolysis were compared in Figure 21. Comparable results have been obtained also by using methanol instead of ethanol (data not shown).

Table 10. Amount of extractable fumonisins, hidden fumonisins found in the extract, hidden fumonisins found in residual matrix and total and hidden fumonisins found in the sample.

Samples	Extractable FBs[a] (µg/Kg)	Hidden FBs found in the extract[b] (µg/Kg)	(Total) Hidden FBs in the exhaust matrix[c] (µg/Kg)	Total FBs[d] (µg/Kg)	Hidden FBs[e] (µg/Kg)
Extr.70% EtOH	6558±304	n.s.	2982±373	8967±327	2408
Extr.80% EtOH	4149±431	1001	2698±674	8967±327	4817
Extr. 90% EtOH	3324±73	1520	3644±215	8967±327	5643

[a] Detected in the direct extract and expressed as the sum of FB_1+FB_2+FB_3

[b] Calculated as the difference between total FBs equivalents found in the extracts and extractable FBs.

"n.s." = Not Significative: the calculated difference between total FBs and extractable FBs is not significative./ The sample does not contain hidden fumonisins.

[c] Detected upon alkaline hydrolysis of the residual matrix and expressed as the sum of FB_1+FB_2+FB_3 equivalents

[d] Detected after alkaline hydrolysis of the sample and expressed as the sum of FB_1+FB_2+FB_3 equivalents

[e] Calculated as the difference between total FBs equivalents found in the sample and extractable FBs

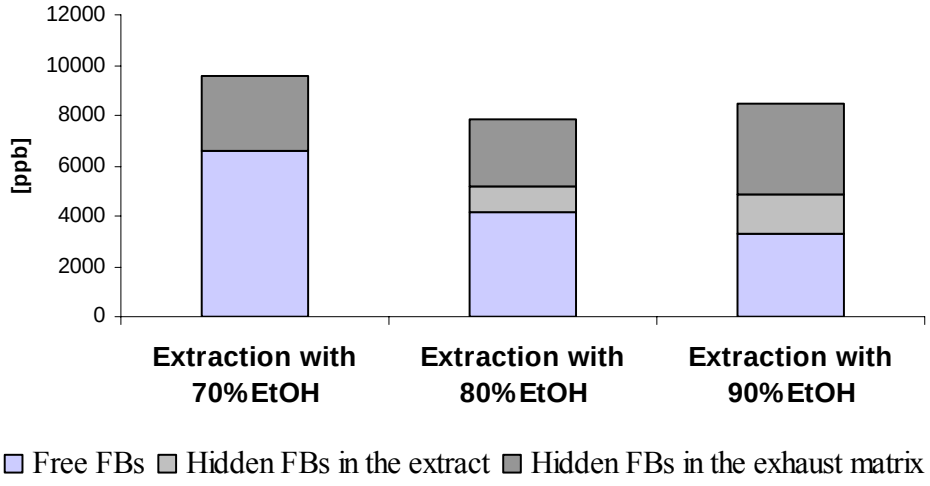


Figure 21. Comparison of extractable FBs (sum of FB_1, FB_2, and FB_3), hidden fumonisins found in the extract after hydrolysis (measured as HFBs and expressed as sum of FB_1, FB_2, and FB_3 equivalents), and hidden fumonisins found in the residual exhausted matrix after hydrolysis (measured as HFBs and expressed as sum of FB_1, FB_2, and FB_3 equivalents). For each sample, the sum of these three values represents the amount of total fumonisins contained into the matrix.

The free fumonisin amount detected in the extract decreases when the percentage of the organic solvent used in the extraction mixture is increased. In a brief analysis this result seems to be due to a different efficiency in fumonisin extraction of each binary mixture. Nevertheless, data obtained upon alkaline hydrolysis of the same extracts, shown that the levels of total fumonisins detected are very similar (respectively, 6357±145, 5150±700 and

4844±265 μg/Kg): whereas the total amount of extracted toxin does not change, the content of hidden forms in the extract increases when the amount of ethanol is increased. This means that different binary mixtures have the same efficiency in fumonisins extraction, but increasing levels of the organic constituents are able to create some conditions under which a masking artefact takes place. In order to evaluate if this is the main effect involved in masking phenomenon, exhausted matrixes after solvent extraction were hydrolyzed, showing that the quantity of total fumonisins is very similar in all three cases. Since we consider that each extraction procedure is able to extract the total amount of free extractable fumonisins existing in the sample, the levels of total fumonisins found after hydrolysis of the residual matrix can be considered as the "real" masked fraction, containing those forms that really interact with macroconstituents. The resemblance between the levels of hidden fumonisins found in the residual matrixes confirms the results obtained by the analysis of the direct extracts: all the three considered mixtures are able to extract the same amount of analyte, leaving in the solid residues similar levels of hidden mycotoxins. Finally, all the data collected for each binary water/ethanol mixture were compared with those achieved by hydrolyzing the starting material, proving that the sum of extractable fumonisins with hidden fumonisins found in the extract and hidden fumonisins remained in the exhausted matrix in all cases are consistent with the level of total fumonisins found upon the hydrolysis of the original sample.

The data collected from this experiment are very interesting and provide us with information about the masking mechanism and the occurrence of analytical artefacts during hidden fumonisins evaluation. The most interesting result regards the difference in hidden fumonisin content found for the three extracts. Indeed, the choice of the extraction mixture does not affect the amount of free fumonisins extracted, but determines the formation directly in the extract of an additional amount of hidden forms which get lost during the analysis.

Thus, increasing percentages of alcohol in the extraction mixture may aid the extraction of some constituents able to hide fumonisins in solution. In order to evaluate if starch was contained in the extracts, a colorimetric assay using the Lugol reactive has been performed on the extracts and on a control (ethanol/water solution of few grams of pure starch): for all the samples, the test gave negative results (Figure 22).

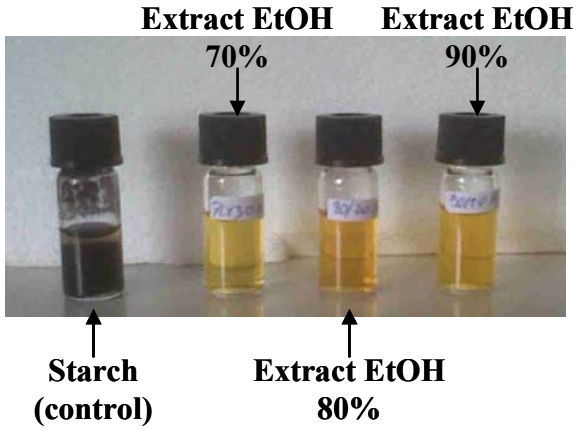


Figure 22. Colorimetric assay performed for the three extract. The blue colouring is induced by the formation of the complex starch-iodine (as obtained for the control). The lack of such colour indicates the absence of starch in the considered extracts.

Since the lack of starch has been demonstrated for all samples, the protein fraction was supposed to be the main responsible of the masking phenomenon occurring in the extract at higher ethanol percentage. The protein composition of each extract was then assessed by Osborne fractionation followed by gravimetric, mainly focusing the attention on the organic media soluble fractions (globulins and prolamins). Whereas the amount of globulins remains constant by increasing the ethanol percentage, a reduction of prolamins was observed, as reported in Figure 23.

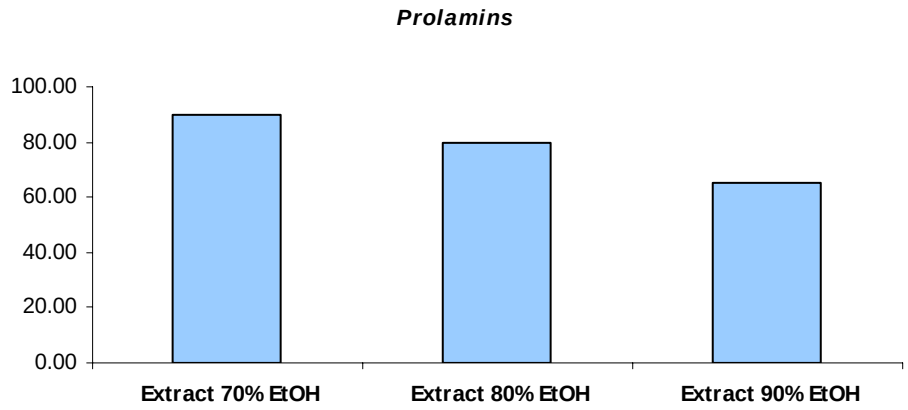


Figure 23. Prolamins content of the three extracts obtained using increasing amount of ethanol in the extraction mixture.

As prolamins (zein) are commonly extracted using a binary mixture of water/ethanol in proportion equal to 30:70 (v/v) this is not a surprisingly results, but, as a matter of fact, provides us with important information about the formation of masked FB artefacts in solution. In agreement with the literature, at the highest ethanol percentage (90%), prolamins showed indeed the smallest size of the previously described aggregates, while the largest size

is recorded at a lower ethanol percentage (about 70%) (53). The masked FB artefact formation in solution seems to be thus inversely related to the dimension of such spheres, probably on account of the distribution of zein particles around the target analyte. Since smaller aggregates can be better distributed around the target molecules than bigger particles, the occurrence of hidden forms in these conditions could be explained.

Considering that the extraction procedure utilized in fumonisins determination employ a binary mixture composed by water/methanol (30:70 v/v), it could only slightly induce the formation of supramolecular structure leading to masked FB artefacts. Therefore, the masking phenomenon observed in maize can be considered a real phenomenon, due to an authentic interaction between fumonisins and macromolecules that occurs into the matrix and does not takes place during solvent extraction.

2.5 Conclusions

The in vitro experiments here performed allowed to better study the fumonisin masking mechanism and the conditions in which it takes place. The application of a QuEChERS-like approach as well as the zein-FB1 titration trials allowed for the elucidation of the FB association with proteins. Moreover, the possible interaction among the target toxin and starch was demonstrated, perceiving a great affinity of fumonisins for this macromolecule rather than zein. Furthermore, as both the experiments were performed at room temperature, the results give further supports to the associative masking hypothesis.

The contribution of each starch fraction in the masking phenomenon has been also investigated demonstrating that, in addition to amylose, also amylopectin is able to hide fumonisins although with a lower affinity. Nevertheless, comparing the data obtained from the single-component systems with those achieved after spiking an amylose/amylopectin binary model, a reduction of masking was emerged, suggesting that the complexation of the toxin may be displaced by the mutual interaction occurring between macromolecules. Similar results were obtained after recovery experiments of spiked starch samples containing increasing amounts of zein: a minimal amount of the protein is enough to ensure the complete recovery of the analyte, by losing masking effect. Likewise, since FB_1 was fully recovered when incubated with blank raw maize, it can be argue that additional factors, such as the presence of the fungus or even the host-plant interaction, are required so that the masking occurs in a complex matrix.

The reliability of all the data concerning masking phenomenon has been demonstrated by means of the last experimental set. Indeed, the lack of analytical artefacts generated by supramolecular structure formed during solvent extraction and able to host fumonisins has been demonstrated for the extraction mixture employ in our work, showing also that the increase of fumonisin levels found after alkaline hydrolysis of a maize sample is mainly due to forms that really interact with macroconstituents into the matrix. Moreover, information concerning the interaction between fumonisins and zein aggregates in solution were obtained, suggesting that smaller particles are able to better subtract the toxins from the analytical determination rather than the larger ones.

Further studies are ongoing in order to better clarify the masking mechanism.

REFERENCES

(1) Bennet, J. W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, 16, 497–516.

(2) Sweeney, M. J.; Dobson, A. D. W. Mycotoxins production by *Asperigillus, Fusarium* and *Penicillum* species. *Int. J. Food Microbiol* .**1998**, 43, 141-158.

(3) Pitt, J.I. Toxigenic fungi and mycotoxins. *Brit. Med. Bull.* **2000**, 56, 184-192.

(4) Marasas, W.F.O. Discovery and occurrence of the fumonisins: a historical perspective. *Environ. Health Perspect.* **2001**, 109, 239-243.

(5) Rheeder, J.P.; Marasas, W.F.O. and Vismer, H.F. Production of fumonisin analogs by *Fusarium* species. *Appl. Environ. Microbiol.* **2002**, 68, 2101-2105.

(6) Sewram, V.; Mshicileli, N.; Shephard, G.S.; Vismer, H.F.; Rheeder, J.P.; Lee, Y.W.; Leslie, J.F.; Marasas, W.F.O. Production of fumonisin B and C analogues by several *Fusarium* species. *J. Agric. Food Chem.* **2005**, 53, 4861-4866.

(7) Shepard, G.S.; Thiel, P.G.; Stockenström, S.; Sydenham, E.W. Worldwide survey of fumonisin contamination of corn and corn-based products. *J. AOAC Int.* **1996**, 79, 671-687.

(8) Bezuidenhout, S.C.; Gelderblom, W.C.A.; Gorst-Allman, C.P.; Horak, R.M.; Marasas, W.F.O.; Spiteller, G.; Vleggaar, R. Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *J. Chem. Soc., Chem Commun.* **1988**, 743-745.

(9) Opinion of the Scientific Panel on contaminants in food chain on a request from the Commission related to fumonisins as undesiderable substances in animal feed. *The EFSA Journal* **2005**, 235, 1-32.

(10) Wang, E.; Norred, W.P.; Bacon, C.W.; Riley, R.T.; Merril, A.H.Jr. Inhibition of sphingolipid biosynthesis by fumonisins. *J. Biol. Chem.* **1991**, 22, 14486-14490.

(11) Stockmann-Juvala, H.; Savolainen, K.; A review of the toxic effects and mechanisms of action of fumonisin B_1. *Hum. Exp. Toxicol* .**2008**, 27, 799-809.

(12) Marasas, W.F.O.; Riley, R.T.; Hendricks, K.A.; Stevens, V.L.; Sadler, T.W.; Gelineau-van Waes, J.; Missmer, S.A.; Cabrera, J.; Torres, O.; Gelderblom, W.C.A.; Allegood, J.; Martinez, C.; Maddox, J.; Miller, J.D.; Starr, L.; Sullards, M.C.; Roman, A.; Voss, K.A.; Wang, E.; Merrill, A.H. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin contaminated maize. *J. Nutr.* **2004** 134, 711–716.

(13) International Agency for the Research on Cancer. Overall evaluation of carcinogenicity: an updationg of IARC. *Report of an IARC Expert Comm.* **1987**, volume 1-42.

(14) European Commission. Commission regulation No 1126/2006 amending regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuff as regards *Fusarium* toxins in maize and maize products. *Off. J. Eur. Union* **2007**, L. 254, 14-17.

(15) Kim, E.-K.; Scott, P. M.; Lau, B.P.-Y. Hidden fumonisins in corn flakes. *Food Addit. Contam.* **2003**, 20, 161-169.

(16) Dall'Asta, C.; Mangia, M.; Berthiller, F.; Molinelli, A.; Sulyok, M.; Schuhmacher, R.; Krska, R.; Galaverna, G.; Dossena, A.; Marchelli, R. Difficulties in fumonisin determination: the issue of hidden fumonisins. *Anal. Bioanal. Chem.* **2009**, 395, 1335-1345.

(17) Galaverna, G.; Dall'Asta, C.; Mangia, M.; Dossena, A.; Marchelli, R. Masked mycotoxins: an emerging issue for food safety. *Czech. J. Food Sci***. 2009**, 27, 89-92.

(18) Berthiller, F.; Schuhmacher, R.; Adam, G.; Krska, R. Formation, determination and significance of masked and other conjugated mycotoxins. *Anal. Bioanal. Chem.* **2009**, 395, 1243-1252.

(19) Shier, W.T. The fumonisin paradox: a review of research on oral bioavailability of fumonisin B_1, a mycotoxin produced by *Fusarium moniliforme. J. Toxicol. Toxin rev.* **2000**, 19, 161–187.

(20) Park, J.W.; Scott, P.M.; Lau, B.P.-Y.; Lewis, D.A. Analysis of heat-processed corn foods for fumonisins and bound fumonisins. *Food Addit. Contam.* **2004**, 21, 1168-1178.

(21) Shier, W.T.; Abbas, H.K.; Badria, F.A. Structure activity relationships of corn fungal toxin fumonisin B1: implications for food safety. *J. Nat. Toxins.* **1997**, 6, 225-242.

(22) Howard, P.C.; Churchwell, M.I.; Couch, L.H.; Marques, M.M.; Doerge, D.R. Formation of N-(Carboxymethyl)fumonisin B_1, following the reaction of fumonisin B_1 with reducing sugars. *J. Agric. Food Chem.* **1998**, 46, 3456-3557.

(23) Poling, S.M.; Plattner, R.D.; Weisleder, D. N-(1-Deoxy-D-fructos-1-yl) fumonisin B_1, the initial reaction product of fumonisin B_1 and D-glucose. *J. Agric. Food Chem.* **2002**, 50, 1318-1324.

(24) Seefelder, W.; Knecht, A.; Humpf, A.U. Bound Fumonisin B_1: analysis of fumonisin-B_1 glyco and amino acid conjugates by liquid chromatography-electrospray ionization-tandem mass spectrometry. *J. Agric. Food Chem.* **2003**, 51, 5567-5573.

(25) Seefelder, W.; Hartl, M.; Humpf, A.U. Determination of N-(Carboxymethyl)fumonisin B_1 in corn products by liquid chromatography/electrospray ionization-mass spectrometry. *J. Agric. Food. Chem.* **2001**, 49, 2146-2151.

(26) Shier, W.T.; Abbas, H.K,; Aboud-Karam, M.; Badria, F.A.; Resch, P.A. Fumonisins: abiogenic conversions of an environmental tumour promoter and common food contaminant. *J. Toxicol. Toxin Rev.* **2003**, 22, 591-616.

(27) Bartók, T.; Tölgyesi, L.; Mesterházy, Á.; Bartók, M.; Szécsi, Á. Identification of the first fumonisin mycotoxins with three acyl groups by ESI-ITMS and ESI-TOFMS following by RP-HPLC separation: palmitoyl, linoleoyl and oleoyl EFB_1 fumonisin isomers from solid culture of *Fusarium verticillioides*. *Food Addit.. Contam.* **2010**, 27, 1714-1723.

(28) Dall'Asta, C.; Galaverna, G.; Mangia, M.; Sforza, S.; Dossena, A.; Marchelli, R. Free and bound fumonisins in gluten-free food products. *Mol. Nutr. Food Res.* **2009**, 53, 492-499.

(29) Dall'Asta, C.; Galaverna, G.; Aureli, G.; Dossena, A.; Marchelli, R. A LC/MS/MS method for the simultaneous quantification of free and masked fumonisins in maize and maize-based products. *World Mycotoxin J.* **2008**, 1, 237-246.

(30) Shukla, R.; Cheryan, M. Zein: the industrial protein from corn. *Ind. Crop Prod.* **2001**, 13, 171-192.

(31) Momany, F.A.; Sessa, D.J.; Lawton, J.H.; Selling, G.W.; Hamaker, S.A.H.; Willett, J.L.; Structural characterization of α-zein. *J. Agric. Food Chem.* **2006**, 54, 543-547.

(32) Kim, E.K.; Scott, P.M.; Lau. B.P.; Lewis, D.A. Extraction of fumonisins B_1 and B_2 from white rice flour and their stability in white rice flour, cornstarch, cornmeal, and glucose. *J. Agric. Food Chem.* **2002**, 50, 3614-3620.

(33) Motta, E.L.; Scott, P.M. Bioaccessibility of total bound fumonisins from corn flakes. *Mycotox. Res.* **2009**, 25, 229-232.

(34) Galaverna, G.; Dall'Asta, C.; Mangia, M; Dossena, A.; Marchelli, R. Masked mycotoxins: an emerging issue for food safety. *Czech. J. Food Sci.***2009**, 27, S89-S91.

(35) Humpf, H. U.; Voss, K.A. Effects of food processing on the chemical structure and toxicity of fumonisin mycotoxins. *Mol. Nutr. Food Res.* **2004**, 48, 255-269.

(36) Versantvoort, C.H.M.; Oomen, A.G.; Van deKamp, E.; Rompelberg, C. J. M.; Sips, A. J. Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food Chem. Toxicol.* **2005**, 43, 31-40.

(37) Brandon, E.F.A.; Oomen, A.G.; Rompelberg, C.J.M.; Versantvoort, C.H.M.; van Engelen, J.G.M.; Sips, A.J.A.M. Consumer product *in vitro* digestion model: bioaccessibility of contaminants and its application in risk assessment. *Regul. Toxicol. Pharmacol.* **2006**, 44, 161-171.

(38) Oomen, A.G.; Rompelberg, C.J.M.; Bruil, A.; Dobbe, C.J.G.; Pereboom, D.P.K.H.; Sips, A.J.A.M. Develop of an *in vitro* digestion model for estimating the bioaccessibility of soil contaminants. *Arch. Environ. Contam. Toxicol.* **2003**, 44, 281-287.

(39) Nasi, F.; Lazzarotto, R.; Ghisi, R. Mais (Zea Mais L.) In *Coltivazioni erbacee*, Edition no. II; Nasi, F.; Lazzarotto, R.; Ghisi, R.; Liviana Ed.; Padova, Italy, **1999**; 1, 97-148.

(40) Argos, P.; Pedersen, K.; Marks, M.D.; Larkins, B.A. A structural model for maize zein proteins. *J. Biol. Chem.* **1982**, 257, 9984-9990.

(41) Cabra, V.; Arreguin, R.; Vazquez-Duhalt, R.; Farres, A. Effect of temperature and pH on the secondary structure and processed of oligomerization of 19 kDa alpha-zein. *Biochim. Biophys. Acta* **2006**, 1764, 1110-1118.

(42) Osman, E.M.; Leith, S.J.; Fles, M. Complexes of amylose with surfactants. *Cereal Chem.* **1961**, 38, 449-463.

(43) Mikus, F.F.; Hixon, R.M.; Rundle, R.E. The complexes of fatty acids with amylose. *J. Am. Chem. Soc.* **1946**, 68, 1115- 1123.

(44) Banks, W.; Greenwood, C.T.; On hydrogen bonding in amylose. *Biopolymers.* **1972**, 11, 315-318.

(45) Hahn, D.E.; Hood, L.F. Factors influencing corn starch-lipids complexing. *Cereal Chem.* **1987**, 64, 81-85.

(46) Bhatnagar, S.; Hanna, M.A. Amylose-lipid complex formation during single-screw extrusion of various corn starches. *Cereal Chem.* **1994**, 71, 582-587.

(47) Kawai, K.; Takato, S.; Sasaki, T.; Kajiwara, K. Complex formation, thermal properities, and *in vitro* digestibility of gelatinized potato starch-fatty acid mixtures. *Foods Hydrocolloids* **2011**, *Article in Press.*

(48) Zachariasova, M.; Lacina, O.; Malachova, A.; Kostelanska, M.; Poustka, J.; Godula, M.; Hajslova, J. Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. *Anal. Chim. Acta* **2001**, 662, 51-61.

(49) Cunha, S.C.; Fernandes, J.O. Development and validation of a method based on a QuEChERS procedure and heart-cutting GC-MS for determination of five mycotoxins in cereal products. *J. Sep. Sci.* **2010**, 33, 600-609.

(50) Dall'Asta, C.; Falavigna, C.; Galaverna, G.; Dossena, A.; Marchelli, R. In vitro Digestion Assay for determination of hidden fumonisins in maize. *J. Agric. Food Chem.* **2010**, 58, 12042-12047.

(51) Mangia, M.; Free and hidden fumonisins in maize and gluten-free products. Ph.D. Dissertation Thesis in Food Science and Technology. Faculty of Agriculture, University of Parma (Italy). Cycle XXII, 2007-2009, pages 125-127.

(52) Sandhu, K.S.; Singh, N.; Kaur, M. Characteristics of the different corn types and their grain fractions: physicochemical, thermal, morphological, and rheological properties of starches. *J. Food Eng.* **2004**, 64, 119-127.

(53) Kim, S.; Xu, J. Aggregate formation of zein and its structural inversion in aqueous ethanol. *J. Cereal Sci.* **2008**, 47, 1-5.

(54) Wang, Y.; Padua, G.W. Formation of zein microphases in ethanol-water. *Langmuir* **2010**, 26, 12897-12901.

SECTION II. FREE AND HIDDEN FUMONISINS OCCURRENCE IN RAW MAIZE (*ZEA MAYS* L.): STUDY OF THE ROLE OF MAIZE GENOTYPE AND MACROMOLECULAR COMPOSITION

FACTORS THAT INFLUENCE FUMONISINS PRODUCTION IN RAW MAIZE

Fumonisins, like other mycotoxins, are secondary metabolites, thus their biosynthesis is governed entirely by the existence of conditions that, in a first time, favour the growth of the fungus and, subsequently, lead to a situation of stress for the microorganism concerned. The genes required for fumonisin biosynthesis are organized in a cluster designated as *FUM* gene cluster, composed by 17 genes 10 of which act directly on the biosynthetic pathway (1) through a mechanism which has not been totally clarified. As often happens for other cluster genes involved in secondary metabolites production, the expression of *FUM* genes is subject to a multilevel regulation, due both to pathway-specific factors and environmental signals (2, 3). In this case, the pathway specific transcription factors are represented by a protein encoded by a gene appertaining to the same cluster and by a not yet identified transcription factor not located into the *FUM* cluster (3).

Environmental factors that may influence the fungal infection and fumonisins production in raw maize can be divided into several groups: nutritional factors associated to the substrate composition, climatic conditions, plants natural defence strategies connected to the plant-pathogen interaction, agronomical practices and genetic factors related to maize genotypes.

The effect of physicochemical and nutritional factors associated to the substrate composition such as pH, water availability and nutrients sources has been largely studied both on *in vitro Fusarium* cultures as well as under field conditions, concluding that they strongly influence the fumonisins biosynthesis at the level of genes transcription (4). As general observation, on *in vitro* cultures of several *Fusarium* strains the maximum amount of fumonisins is produced between 20 and 25°C, with high water availability (0.97-0.98) and at high C:N ratio, corresponding to high sugar concentration and little amino acids amounts (5). Whereas low pH values (4.0-5.0) are needed to ensure fumonisins biosynthesis, its strong inhibition as well as the lower stability of the target toxin in an alkaline environment have been demonstrated (6). These experimental evidences have reflected also after *in vivo* studies: a strong influence of a_w dynamic during ripening has been recently reported (7), as well as amylopectin content in kernels (8).

Concerning climatic conditions, infection from *F. verticillioides* is mainly associated with warm, dry years and insect damage. The contribution of drought-stress on fumonisin production and, at the same time, an inversely proportional trend between fumonisin concentration and rainfall have been demonstrated (9). These results were strongly supported by the studies about the influence of the growing area on the fumonisins accumulation, since an inverse correlation between latitude and fumonisins occurrence has been observed (10): poor fumonisins contamination levels detected in maize grown in continental climates, demonstrate that mediterranean scarcely rainy climate leads to high levels of contamination. Moreover, is intuitive that insect damage (the main collateral factor induced by the moisture stress) allows easier and deeper access to the fungus that can reach internal and senescent tissue where mycotoxin biosynthesis takes place (6). Likewise, other fungal diseases caused by ear-damaging pathogens may predispose corn to *F. verticillioides* infection and fumonisins accumulation (6).

Nevertheless, plants are organisms that do not remain inert during fungal infection: a complex system of communication between the fungus and its host takes place, leading to physiological and molecular perturbation in plant cells, that triggers plant defence mechanisms, which may influence both fungal growth and mycotoxin production (5). One of the first biochemical events is the production of reactive oxygen species (ROS), with a consequent perturbation of the oxidative state of the plants which may interferes with the fungus metabolism. Because the fumonisin biosynthetic pathway contains many oxidation steps, it is tempting to speculate that fumonisin biosynthesis may also be enhanced by increasing the levels of ROS, but it still has to be demonstrated (5). The increasing of the

oxidative level leads to the oxidation of polyunsaturated fatty acids, with the formation of oxylipins, key signalling molecules that regulate the expression of certain defence-related genes, thus modulating fungal sporulation and mycotoxin biosynthesis (5).

During maize kernel ripening, the expression of genes involved in fumonisin biosynthesis is modulated by the factors early mentioned. Nevertheless, the regulation degree in fumonisins production obtained is the results of their reciprocal interaction, rather than not connected actions. Figure 24 shows the schematic representation of some factors enhancing fumonisin production during maize kernel ripening. For the duration of this periods the main events occurring are related to the maize kernel physiological changes and to the fungal-host interactions. Each kernel substrate modification, as well as the increasing of the oxidative stress, induces other variations that can affect directly the transcription of the *FUM* genes.

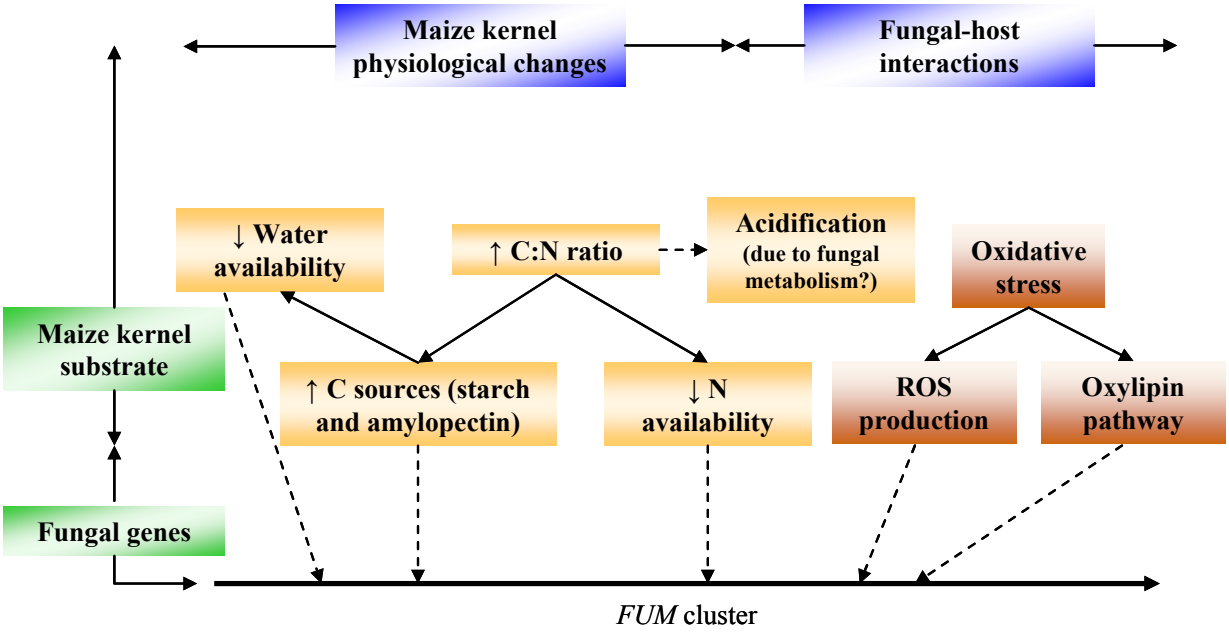


Figure 24. Schematic representation of the factors enhancing fumonisin production during maize kernel ripening. Dotted arrows indicate the direct effect on *FUM* genes expression; continuous arrows indicate the interaction occurring by various factors (adapted from Picot et al. [5]).

Agronomical practices, comprising sowing and harvest period, sowing distance, landfill waste, fertilization, crop rotation and use of fungicides may be of paramount importance in determining *Fusarium* infection and fumonisins accumulation, together with the features of the hybrids chosen. The latter include FAO class (the measure of the level of earliness), productiveness, endosperm composition and resistance to infection.

The influence of maize genotype on fumonisins levels have been studied by several authors: a general trend for higher contamination levels was observed in maize genotypes with higher FAO maturity class or dent-type endosperm (11). The contribution of the growing area on the

resistance to infection of different maize hybrids has been also investigated, suggesting that low-fumonisins hybrids exist which could be adapted to a specific region (10). However, since there are no experimental evidences of a correlation among specific genetic traits and *Fusarium* resistance on certain hybrids rather than others, this explanation remains just a speculation.

Finally, higher resistance to *Fusarium* growth was demonstrated for transgenic corn: hybrids genetically engineered with genes from the bacterium *Bacillus thuringiensis* (Bt), which produce a protein toxic for insects, reveal a percentage of infected kernels lower than traditional corn (12), suggesting that this may be the better way to reduce fumonisins contamination of corn intended for human or animal use.

So far, the studies carried out in order to identify which are the main factors that could affect the fumonisins occurrence in raw maize were focused on the fraction directly detectable through routine analysis. Indeed, in literature there are no data available or studies reported about factors that could influence the hidden fumonisins occurrence in corn as raw material. Since hidden fumonisins represent a substantial fraction of the total fumonisins detectable in raw maize, the individuation of main factors that affect their occurrence and the role played by the plant in masking mechanism are issues of great attention to ensure a good safety level both for animals and human users.

This section is dedicated to the investigation the role of maize hybrids in fumonisins accumulation as well as their effect on masking phenomena, focusing the attention on the chemical composition in terms of macroconstituents. From this purpose, a two-years experimental plan has been performed, by working both years on the same selected genotypes. The first-year activity aimed to establish the role played exclusively by the maize genotype, while during the second year the interaction between the genotype and the growing area has been investigated. Throughout the entire work, special consideration was devoted to fatty acids composition, as marker of the host-pathogen interaction.

Moreover, the growth of filamentous fungi and fumonisins accumulation during maize storage has been investigated by sampling corn stored in a vertical silos monitored for the entire storage period, in order to evaluate the role of conservation parameters on mould growth and fumonisins production, considering in particular hidden forms and possible effects on masking.

CHAPTER 3 ROLE OF MAIZE HYBRIDS AND THEIR COMPOSITION ON *FUSARIUM* INFECTION, FUMONISINS PRODUCTION AND MASKING PHENOMENA.

3.1 INTRODUCTION

Fusarium v*erticillioides* Sacc. (Nirenberg), belonging to *F.* section *Liseola*, is the most common toxigenic fungus in maize worldwide, causing *Fusarium* ear rot, a very important disease affecting maize production (13) and producing secondary toxic metabolites, known as fumonisins. Among the several analogues that have been identified and classified into the four series A, B, C, and P-series (14, 15), those appertaining to the B-series (FBs), comprising FB_1, FB_2 and FB_3 are the most naturally occurring fumonisins and are usually found at the highest levels in maize and in maize-derived foodstuff and feedstuff (16). FB_1 is by far the most prevalent in the human diet and was categorized as a Group 2B carcinogen by International Agency for Research on Cancer (17), on account of its toxic effect in human and animals (18).

Recently, besides FBs detectable through common analytical methods, several studies reported the occurrence in foods of FBs-derivatives covalently linked or strongly associated with macromolecules such as starch or protein, which can easily escape routine analyses and can be determined only after the application of a hydrolysis step on the matrix (19, 20, 21, 22). Indeed, alkaline hydrolysis allows the complete degradation of all matrix constituents and, at the same time, the separation of the tricarballilic moieties from the central backbone of the toxin, thus releasing hydrolyzed forms (HFBs) (23). In particular, it has been observed that performing alkaline hydrolysis of contaminated corn products, the amount of released HFBs is often higher than that stechiometrically derived by the conversion of the fumonisins detectable by the routine analytical methods.

The presence of these derivatives may be taken into consideration from a food safety point of view, since they can contribute to the overall toxicity after releasing upon gastrointestinal digestion (23, 24).

Lately, the occurrence of hidden fumonisins in raw maize has been reported by Dall'Asta et al. (23, 24), demonstrating that hidden forms are originated in field during infection rather than processing. Thus, although this evidence suggests that hidden fumonisins are forms simply entrapped or complexated by maize macroconstituents, the nature of masking phenomenon in the plant as well as the role played by the plant-pathogen interaction have not been yet clarified.

Fumonisins production and accumulation during maize growth is the results of a complex process governed by several factors that interact with each other, such as mould-host interaction and the ecological factors involved in the modulation of fungal secondary metabolites production.

The FBs biosynthesis is regulated by the FUM gene cluster, therefore it is known that a number of environmental factors including water activity (a_w) and temperature (25) can be pointed out as key factors regulating their expression and thus FBs production and possibly its partitioning between FB_3 and FB_4, respectively precursors of FB_1 and FB_2 (3).

Although several studies concerning the influence of physicochemical and nutritional factors such as pH, C:N ratio and amylopectin content in kernels on FBs biosynthesis have been reported (5, 6, 8, 26, 27), specific studies to investigate a possible correlation between maize composition in terms of other macroconstituents and fumonisins contamination have not been performed yet.

The influence of grain hardness on fumonisins accumulation has been also investigated, suggesting that soft grains are more susceptible than hard (28). Thus, a role of hybrids season length was supposed, but only partially confirmed by experimental results (28, 29, 30). Finally, a strong influence of a_w dynamic during ripening has been recently described by Battilani et al. (31).

The role played by the hybrid in *Fusarium* infection and fumonisins contamination is of a great interest: even if is state as important by many authors (6, 9, 11, 28, 29, 32, 33), the reasons for host resistance to *Fusaria* are not explained and have not been attributed to specific genetic traits (34). Indeed, genetic resistance has been studied (35, 36, 37), but experimental data do not support definitive conclusions as no genetically resistant hybrids are available on the market. To date, the most reliable hypothesis is that the resistance to infection of certain hybrids is mainly due to their own ability to adapt to the growing environment (29).

3.2 AIM OF THE WORK

The individuation of the main factors that affect the infection by *Fusarium* species and their accumulation during the maize kernel maturation is a topic which has not yet found a clear explanation, as well as the role of the plant-pathogen interaction. Moreover, the studies performed until now are focused only on the fraction directly detectable by application of the common extraction methods (the so-called "free fumonisins"), while the masked portion has never been considered.

The aim of this research was to study in commercial fields the role of maize hybrids in FBs production by *F*. section *Liseola* and the eventual effect on masking phenomena, by investigating a possible correlation between contamination levels and hybrid chemical composition, devoting a special consideration to the fatty acids composition, as precursors of the molecules involved in the plant-pathogen system cross-talk.

More in details, different hybrids were collected in a small geographic areas in the first year, to focus on the role of hybrids, and a wider area was sampled in the second year to evaluate the possible hybrid interaction with the growing area.

This work was carried out in collaboration with the Institute of Entomology and Plant Pathology (Università Cattolica del Sacro Cuore, Piacenza, Italy) and with the Consorzio Agrario Provinciale-CAP Parma.

Sampling was performed by the technicians of the Consorzio Agrario Provinciale (Parma).

Microbiological data regarding the percentage of infected kernels and the identification of mycotoxin-producers fungi were achieved from the Institute of Entomology and Plant Pathology (University of Piacenza, Italy).

Chemical data concerning the levels of contamination from free and hidden fumonisins and the fatty acids profile were obtained in our laboratory at the Department of Organic and Industrial chemistry (University of Parma, Italy).

Field and meteorological data, as well as the proximate composition of maize samples were purchased from the Consorzio Agrario Provinciale (Parma).

3.3 Materials and methods

3.3.1 Maize samples collection

In 2009, 60 maize fields were selected in Parma (Emilia Romagna Region-North Italy) and sampled at harvest with the aim of collecting around 30 samples of the most seeded hybrids (9 hybrids were sampled), with several replicated fields. One hundred sub-samples (around 100 g each) were collected from the kernels flux during the harvest combine machine discharge in each field; the final sample, around 10 Kg, was sent to the laboratory for mycological analysis.

In 2010, 7 hybrids were selected, based on results from 2009 and taking into account the 3 main commercial brands of maize seeds in the Italian market, and they were sampled at harvest in 5 districts of Emilia Romagna (Piacenza, Parma, Modena, Bologna and Ferrara) following the same protocol previously described.

Hourly data on air temperature, relative humidity and rain have been collected from a meteorological station placed close to the maize growing areas and data on the cropping system applied were collected for each field.

In both years, the whole production of each selected field was delivered to a store house where forced ventilation (air heated at 100°C) allowed to reduce maize humidity under 14% in around hours; grain temperature during drying was around 35-40°C. Sampling took place at the end of each drying turn, during the grain discharge, with sub-samples taken from the kernels flux, similarly to the approach followed at harvest described before. Dried samples were finely ground (0.25 mm particle size) with a laboratory mill (IKA MF 10, OptpLab, Modena, Italy) and stored at -18°C before FBs content and chemical composition determination.

3.3.2 Chemicals

Fumonisin B_1, B_2, and B_3 mixed standard solution, 50 μg/mL each, in acetonitrile/water, 1:1 v/v, were purchased from Romerlabs (Tulln, Austria). All solvents used were of LC grade. Methanol was obtained from Carlo Erba (Milan, Italy), acetonitrile and ethanol 96% were from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA).

Potassium hydroxide was purchased from Carlo Erba (Milan, Italy). Fatty acid standards were purchased by Sigma-Aldrich (Stuttgart, Germany).

3.3.3 EXPERIMENTAL PROCEDURES

Incidence of kernels infected by fungi.
Fifty kernels of raw maize were randomly selected from each 10 Kg sample collected from the harvesting combine after an accurate mixing. They were surface disinfected with a solution of 1% sodium hypochlorite and 90% ethyl alcohol for 2 minutes and washed with sterile distilled water. Kernels were plated in Petri dishes (9 cm diameter) with Potato Dextrose Agar (PDA, Oxoid®) added with streptomycin (Sigma-Aldrich®) as medium and incubated at 25°C for 7 days. White mould colonies, looking like *Fusaria*, were transferred on Petri dishes with PDA and identified at section level according to Summerell et al. (38). Black and green colonies, looking like *Aspergilli* or *Penicilli*, were observed for their macro and microscopic characters and identified at section or genus level, respectively according to Raper and Fennell (39) and Pitt (40). The result was expressed as incidence of infected kernels (%).

Preparation of Hydrolyzed Fumonisin Standard Solution.
90 µL of the FB1, FB2, and FB3 standard solution were evaporated to dryness. The residue was redissolved in 1 mL of 2 M KOH and allowed to react for 12 hours at room temperature. After the hydrolysis, the mixture was extracted by liquid-liquid partition using 1 mL of acetonitrile (twice). The organic phases were pooled and evaporated under N_2 stream, and the residue was redissolved in 1 mL of acetonitrile/water, 1:1 v/v. Calibration curves were prepared by proper dilution of the standard solution.

Sample preparation for the analysis of free fumonisins.
5 g of ground maize sample were blended in a high-speed blender (Ultraturrax T25; IKA, Stauffen, Germany) with 40 mL of water/methanol, 30:70 v/v, for 3 min at 4000 rpm and then filtered through a paper filter. After filtration of the extract obtained on 0.45 µm nylon filters, 1 mL of extract was analyzed by LC-ESI-MS/MS.

Sample preparation for the analysis of total fumonisins
Aliquots (2.5 g) of the ground maize sample were blended in a high-speed blender (Ultraturrax T25; IKA, Stauffen, Germany) with 50 mL of 2 M KOH for 5 min at 4000 rpm and then stirred for 60 min. Then, 50 mL of acetonitrile was added, and after stirring for 10

min, two layers were formed which were separated by centrifugation at 3500 rpm for 15 min. A 4 mL portion of the acetonitrile-rich upper layer was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in 400 μL of water/methanol, 30:70 v/v, filtered through a 0.45 μm nylon, and analyzed by LC-MS/MS. Fumonisins obtained after sample hydrolysis were measured as the sum of hydrolyzed FB_1, FB_2, and FB_3. Results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of parent and hydrolyzed compounds and referred to as "total FBs after hydrolysis". All the results have been weighted by dry matter since maize kernels showed different moisture content at harvesting.

Analysis of free and total fumonisins by LC-MS/MS.

LC-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, 145 MA, USA) equipped with a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass, Waters, Manchester, U.K.) according to Dall'Asta et al. (24). Chromatographic conditions were the following: the column was a 250 mm x 2.1 mm i.d., 5 μm, XTerra C18; the flow rate was 0.2 mL/min; the column temperature was set at 30°C; the injection volume was10 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, 0-2 min isocratic step, 2-5 min linear gradient to 45% B, 5-25 min linear gradient to 90% B, 25-35 min isocratic step at 90% B, 35-36 min linear gradient to 70% A, and a reequilibration step at 70% A for 15min (total analysis time: 50 min). MS parameters: ESI+ (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 50 V for FBs and 30 V for HFBs; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow (nitrogen), 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), 722.4→352.3 (CE 35 eV) for FB_1, 706.4→336.4 and 706.4→318.4 (CE 35 eV) for FB_2 and FB_3, 406.5→334.4 and 406.5→353.4 (CE 25 eV) for HFB_1, 390.5→336.4 and 390.5→354.4 (CE 25 eV) for HFB_2 and HFB3. The first transition reported was used for quantification, while the second transition was chosen as qualifier.

For each sample, the entire procedure was performed in duplicate (n = 2). Validation experiments (matrix-matched calibration, recovery, repeatability and limit of detection) were based on the analysis of spiked corn samples already measured as a blank for both free and hidden fumonisins. The spiking experiments were performed at six concentration levels in the

range 25 – 5000 μg/Kg. For the total fumonisins determination, the spiked samples were previously submitted to hydrolysis and the hydrolysed forms were then determined. Recovery was found to be 93% for FB_1 and FB_2, 89% for FB_3, 91% for HFB_1 and 88% for HFB_2 and HFB_3. Repeatability (six determinations at three spiking levels) was found to be in the range 6 – 9% for FBs and 8 – 11% for HFBs. Matrix-matched calibration curves (calibration range 25-5000 μg/Kg) were used for extractable FBs and hydrolyzed FBs quantification. Limit of quantification (LOQ) was 25 μg/Kg for both FBs and HFBs. Limits of detection (LOD) were found to be lower than 10 μg/Kg for all the considered analytes. All the results were corrected for recovery. Samples showing a contamination levels higher than the highest calibration level (5000 μg/Kg) were diluted to match the proper calibration range

Proximate composition of maize samples.

The chemical composition of maize samples, in terms of macro components (moisture, starch, fat and protein percentages), was determined by means of NIR spectroscopy.

Dispersive near infrared reflectance (NIR/VIS) data (including the visual region) were collected using a 5000 spectrophotometer model from FOSS NIR Systems, Inc, Silver Spring, MD, USA. Model 20910-2441. The spectrophotometer uses a split detector system with a Silicon (Si) detector between 1100 and 2500 nm and a tungsten halogen lamp and has an internal ceramic standard. All spectral data were recorded in duplicate as log R-1 172 , where R is the reflectance, in the wavelength range 1100-2500 nm every 2 nm, to give a total of 346 data points per sample. 4 g of ground maize were sampled for each entry. The software for scanning, mathematical processing and statistical analysis was supplied with the spectrophotometer by Infrasoft International (ISI Port 176 Matilda, PA, USA).

Sample preparation for fatty acid analysis.

5 g of ground maize sample were extracted with 60 mL diethyl ether through Soxhlet extraction. At the end of the process, the fatty residue was weighted, added with heptanoic acid internal standard and redissolved using 9 mL hexane and 3 mL of KOH solution, 5% in methanol. After 1 min of stirring, 1 μL of the upper organic phase was injected in GC-MS.

Fatty acids profile by GC/MS analysis.

GC-MS analysis was performed by a Hewlett Packard 5890 separation system (GMI Inc., Minneapolis, USA), equipped with a Hewlett Packard 5971 single quadrupole mass spectrometer with a electronic impact source (GMI Inc., Minneapolis, USA). Chromatographic conditions were the following: the column was a Carbowax 250 mm x 2.5

mm i.d., 250 nm f.t. (Supelco, Bellefonte, PA, USA); the injection volume was 1 μL; gradient elution was performed using helium as carrier gas: initial conditions at 80°C, 0-3 min isothermal step at 80°C, 3-16 min linear gradient to 210°C, 16-21 min isothermal step at 210°C (total analysis time: 21 min); injector temperature, 220°C, source block temperature, 230°C. MS detection was performed using a full scan mode from 50 to 500 m/z. Peak identification was obtained by both database matching (WILEY275, NBS75K) and comparison with standard retention time.

Statistical Analyses.

Statistical analyses were performed using SPSS v.19.0 (SPSS Italia, Bologna, Italy). Arcsin and logarithm transformation were applied respectively to data on the percentage of infected kernels and on FBs contamination before applying the ANOVA analysis (41). Hybrid, hybrid and sampling location, hybrid and year were respectively considered as factors in 2009, 2010 and in the joint data analysis. Mean data were statistically compared by a post hoc Tukey test ($\alpha = 0.05$). Data correlation were evaluated by Spearman's correlation test ($\alpha = 0.05$). Contamination data were compared by a Student's t-test ($\alpha = 0.05$). Regression parameters were statistically evaluated by Linear Regression Model.

3.4 Results and discussion

3.4.1 Data collection: comparison between 2009 and 2010.

Field data and kernel contamination

During 2009, nine maize hybrids, commercialized by different brands, were sampled from 27 fields near Parma (Emilia Romagna, Italy). The selection criteria were their season length (measured as FAO class), the resistance to infection, their productiveness and commerciability. Table 11 shows the list of selected hybrids (identified with a code), their respective FAO class and the number of fields from which they were sampled.

Table 11. Codes of the selected hybrids, number of fields from which were sampled and respective FAO class (identifying the season length).

Hybrid code	Replicates (number of fields)	FAO class (season length)
H1	3	300
H2	10	600
H3*	1	600
H4	3	500
H5*	1	600
H6	3	600
H7	3	500
H8	3	600
H9	3	500

* Not considered in data analysis

All the maize fields were seeded between early and mid April and harvested between late August and mid September; silk emergence was observed between early and mid July.

The preceding crops were variable, mainly cereals or arable crops; all fields have been plowed, around 30 cm depth, during winter and regularly fertilised. Sixty five percent of maize crops were irrigated and 18% were sprayed with pesticides for European Corn Borer (ECB; *Ostrinia nubilalis* Hübner) control. Kernels humidity at harvest ranged between 12 and 20%.

Concerning the fungal infection data, the collected samples had a mean percentage of kernels infected by fungi around 50%, ranging between 16 and 92%; most fungi isolated have been identified as *Fusaria* section *Liseola*, whose more frequent species in South Europe is *F. verticillioides*, confirmed by morphological identification in this study (42% and 74% of kernels being mean and maximum, respectively). The incidence of all the others potential

mycotoxin producers, intended as *Aspergillus* or *Penicillium* spp., was below 1% of infected kernels each.

With regard to the occurrence of FBs, all the considered samples were found to be contaminated by the main FBs (FB_1, FB_2, and FB_3). Free FBs (expressed as the sum of FB_1, FB_2 and FB_3) have been found in all the considered samples at a concentration ranging from LOQ to 8020 μg/Kg. Total FBs obtained after alkaline hydrolysis (expressed as the equivalent sum of FB_1, FB_2 and FB_3) were in the range 270-9975 μg/Kg d.m, being significantly higher than free FBs in 22 out of 28 samples (t-Student test, $\alpha = 0.05$). Hidden forms, calculated as the difference between total and free FBs, were found in all the considered samples, ranging between 11 and 96% of free FBs.

In 2010, sixty seven maize samples were collected over 5 Emilia Romagna districts, representing seven maize hybrids commercialized by the 3 main maize-seed brands and selected on the basis of the results obtained during the first year activity. Table 12 shows the list of selected hybrids (identified with a code), their respective FAO class and the number of fields from which they were sampled. The distribution of samples between districts was as follows: 2 from Piacenza, 19 from Parma, 9 from Modena, 21 from Bologna and 16 from Ferrara. The number of samples of each hybrid collected in each district varied between 1 and 5.

Table 12. Hybrids selected in 2010, number of fields from which were sampled and respective FAO class (identifying the season length).

Hybrid code	Replicates (number of fields)	FAO class (season length)
H1	2	300
H2	11	600
H3	11	600
H4	8	500
H5	15	600
H6	14	600
H10	6	500

All the maize fields were seeded between late March and April and harvested in September, with few exceptions; silk emergence was observed between early and mid July. The preceding crops were variable, mainly cereals or arable crops, all fields have been tilled and regularly fertilised. Forty percent of maize crops were irrigated and 46% were sprayed for ECB control. Kernels humidity at harvest ranged between 11 and 36%.

Concerning fungal infection, the collected samples had a mean incidence of kernels infected by fungi around 95%, ranging between 48% and 100%; most fungi isolated have been identified as *F.* section *Liseola* (mean and maximum 46% and 94% respectively); *F.*

verticillioides (identification based on morphological characters) was largely dominant, similarly to 2009. Furthermore, the mean incidence of kernel infected by *A.* section *Flavi* was around 5% with a maximum of 80%, for *A.* section *Nigri* around 1% with a maximum of 30% and for *Penicillium* spp. around 2%, with a maximum of 44% (the maximum was detected in only 1 sample for each group.

As far as FBs contamination is concerned, also in 2010 all the considered samples were found positive to FBs (Table 13) at a level higher than that recorded for the first year of sampling. The free FBs concentration ranged from LOQ to 42015 µg/Kg d.m., while the total FBs level after alkaline hydrolysis was found to be in the range LOQ-68692 µg/Kg d.m, being significantly higher than free FBs in 60 out of 67 samples (t-Student test, $\alpha = 0.05$). Hidden FBs were ranging between 388 and 26677 µg/Kg d.m.. Also for this dataset, the calculated FB_2/FB_1 and FB_3/FB_1 ratios were not significantly different for both free and total FBs. Data concerning free and total fumonisins contamination and the calculated ratios between the free and hidden fractions or analogues over two years are summarized in Table 13.

Table 13. Free and total fumonisin contamination (mean, range in µg/Kg) determined in maize samples collected in 2009 and 2010 in Emilia Romagna district (North Italy).

	2009			2010		
Toxins	**Free**	**Total**	**Rate F/T**	**Free**	**Total**	**Rate F/T**
FB_1	1266 70-5782	1983 135-6996	0.64	5203 LOQ-30075	7591 LOQ-44274	0.68
FB_2	386 LOQ-1459	634 75-1841	0.61	1643 LOQ-9342	2486 LOQ-16471	0.66
FB_3	212 LOQ-779	391 60-1138	0.54	580 LOQ-2598	1180 LOQ-7947	0.50
FB_2/FB_1	0.31 0.12-0.55	0.36 0.23-0.54		0.32 0.01-0.71	0.28 0.01-0.59	
FB_3/FB_1	0.14 0.01-0.33	0.26 0.05-0.54		0.11 0.02-0.24	0.13 0.02-0.77	
LOQ: limit of quantification (25 µg/kg)						

The FB_2 to FB_1 and FB_3 to FB_1 ratios calculated for both the free and the total forms were not significantly different for both years, thus indicating that all the target compounds were involved in the masking mechanism with the same extent.

In 2009 as well as in 2010 any relations between cropping system applied and incidence of infected kernels or their FBs content have not found.

Meteorological data

In addition to field data and kernel contamination, also meteorological data of the months comprises from sowing to harvest of the target cultures have been collected (see Table 14),

showing a very similar temperature values and limited variations in RH and rainfall. Relative humidity was generally higher in 2010, so as rainfall; the amount of rain fallen in May, June and July almost tripled in 2010 compared to 2009 and it remained abundant also in August and September.

Table 14. Mean monthly temperature (T; °C), relative humidity (RH; %) and rainfall (mm, first line, and number of rainy days, second line, in bold) registered in Parma in 2009 and 2010 during the maize growing period.

	March	April	May	June	July	August	Sept
T							
2009	8.9	13.5	19.7	22.3	24.8	25.5	20.1
2010	7.2	12.4	17.1	22.0	25.5	22.9	18.0
RH							
2009	68.8	80.2	63.6	62.8	62.7	62.6	69.2
2010	78.9	74.1	69.7	67.2	62.4	69.8	75.7
Rainfall							
2009	111	138	39	40	9	69	56
	10	**15**	**4**	**6**	**3**	**4**	**5**
2010	92	82	119	108	25	83	136
	13	**12**	**15**	**7**	**8**	**6**	**11**

Chemical composition of kernels

The chemical composition of kernels, measured in 2009 and in 2010, is reported in Table 15.

In 2009 mean starch content in kernels was about 70g/100g dry matter (d.m.), with a maximum of 73g/100g d.m., while in 2010 the mean starch content was about 71.5g/100g d.m. (ranging from 66.4 to 75.2g/100g d.m.).

Whereas in the first year fat and protein percentage were, respectively, 3.7g/100g d.m. with a maximum of 4.7g/100g d.m. and 8.0g/100g d.m with s maximum of 9.1g/100g d.m., in the second year fat mean percentage was around 3g/100g d.m. (ranging from 2.1 to 4.2g/100g d.m.) and protein mean percentage was about 7.6g/100g d.m. (ranging from 6.1 to 9.6g/100g d.m.). For both years, the obtained data concerning the mean chemical composition of kernels were in agreement with the compositional data usually reported for raw maize.

In both years the main fatty acids were found to be palmitic acid (C16:0), oleic acid (C18:1) and linoleic acids (C18:2). Palmitic acid showed a mean and maximum value of 0.45 and 0.53g/100g d.m. in 2009 and of 0.41 and 0.58g/100g d.m. in 2010; oleic acid a mean and maximum value of 0.64 and 1.44g/100g d.m in 2009 and 0.89 and 1.65g/100g d.m. in 2010; linoleic acid a mean and maximum value of 2.16 and 2.54g/100g d.m. in 2009 and 1.60 and 2.27g/100g d.m. in 2010. The oleic to linoleic ratio was calculated in the range 0.37-0.67 in the first year and ranging from 0.42 to 0.76 in the second year, with a mean, respectively, of 0.50 and 0.60.

Traces of stearic (C18:0) and linolenic (C18:3) were found in both years, but they were not quantified.

Table 15. Chemical composition (mean ± SE) of the maize hybrids sampled in 2009 and in 2010.

2009							
Hybrid	**FAO class**	**Starch (g/100g dm)**	**Fat (g/100g dm)**	**Nitrogen (g/100g dm)**	**C16:0 (g/100g dm)**	**C18:1 (g/100g dm)**	**C18:2 (g/100g dm)**
H1	300	71.21±1.55	2.80±0.33	7.95±0.57	0.43±0.08	0.78±0.07	1.58±0.08
H2	600	69.51±0.13	3.89±0.08	8.16±0.06	0.47±0.04	1.09±0.05	2.45±0.07
H3	600	68.45	3.67	9.08	0.47	1.02	2.34
H4	500	70.40±0.39	3.63±0.15	7.78±0.12	0.49±0.02	1.16±0.02	1.58±0.03
H5	600	70.20±0.77	3.74±0.18	7.81±0.39	0.49±0.03	1.23±0.02	2.28±0.02
H6	600	69.10	3.94	8.32	0.45	1.17	2.17
H7	500	70.90±0.59	3.33±0.21	7.53±0.22	0.49±0.02	1.10±0.04	2.11±0.02
H8	600	69.62±0.77	3.63±0.15	7.98±0.65	0.45±0.01	1.20±0.01	2.43±0.01
H9	500	69.24±0.45	3.93±0.23	8.05±0.03	0.45±0.01	1.00±0.04	2.02±0.04
H10#	500	-	-	-	-	-	-
2010							
Hybrid	**FAO class**	**Starch (g/100g dm)**	**Fat (g/100g dm)**	**Nitrogen (g/100g dm)**	**C16:0 (g/100g dm)**	**C18:1 (g/100g dm)**	**C18:2 (g/100g dm)**
H1	300	72.03±0.35	3.00±0.37	7.23±0.37	0.46±0.03	0.97±0.02	1.49±0.02
H2	600	71.24±0.59	3.18±0.12	8.29±0.22	0.43±0.07	0.93±0.05	1.74±0.04
H3	600	71.04±0.74	3.01±0.18	7.96±0.15	0.43±0.03	0.92±0.09	1.60±0.05
H4	500	71.39±0.66	2.98±0.16	7.60±0.14	0.42±0.03	0.96±0.11	1.39±0.11
H5	600	71.04±0.44	3.27±0.12	7.43±0.09	0.44±0.03	0.99±0.03	1.79±0.03
H6	600	72.59±0.36	2.58±0.08	7.36±0.15	0.37±0.02	0.71±0.06	1.45±0.06
H7#	500	-	-	-	-	-	-
H8#	600	-	-	-	-	-	-
H9#	500	-	-	-	-	-	-
H10	500	71.89±0.67	2.84±0.14	6.81±0.19	0.43±0.02	0.92±0.07	1.96±0.04

* Mean is reported when only 1 sample of the hybrid was collected
\# Not sampled

3.4.2 Data elaboration: statistical analysis

Evaluation of the role of the hybrid, growing area and years.

All the chemical and infection data underwent to ANOVA, in order to point out significant differences between hybrids.

Data analysis showed a not significant effect of the considered hybrids on the incidence of kernels infected by *F.* section *Liseola* over the two years of observation; the same result was obtained for starch content.

Concerning data obtained for samples collected in 2010, the statistical analysis was performed considering both the maize genotype and the harvesting district as factors. The main source of variation between samples was found to be the genotype; the harvesting district never resulted significant, as well as the interaction of both factors .

Significance for the other parameters are shown in Table 16.

Table 16. Significance obtained by ANOVA test for 2009 and 2010 datasets.

Variable	Total incidence	Free FBs	Total FBs	Fat	Protein	C16:0	C18:1	C18:2
Dataset obtained in 2009								
Hybrid	0.048	0.010	0.007	0.009	n.s	<0.001	0.002	0.012
Dataset obtained in 2010								
Hybrid	n.s.†	n.s.	n.s.	0.010	<0.001	n.s.	<0.001	<0.001
Dataset obtained for common hybrids harvested in both 2009 and 2010								
Hybrid	n.s.	n.s.	n.s.	0.022	0.001	0.011	0.001	<0.001
Year	<0.001	n.s.	n.s.	0.041	0.01	<0.001	n.s.	<0.001
Hybrid x year	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* Only significant variables are given
n.s.†: not significant (p>0.05)

In 2009, whereas hybrids does not affect the incidence of kernels infected by fungi, as well as the incidence of *F*. section *Liseola*, *A*. section *Flavi, A*. section *Nigri* and *Penicillium* spp. considered separately, they can strongly influence both total and free FBs levels (p = 0.007 and p = 0.010, respectively). In particular, concerning both total and free FBs, the hybrid H2 was found to be significantly more contaminated than hybrid H9. Nevertheless, although in 2010 the incidence of *F.* section *Liseola*, *A*. section *Flavi*, *A*. section *Nigri* and *Penicillium* spp., were not significantly different between hybrids accordingly to what observed in the first season, also the contamination for both free and hidden FBs were not affected by genotypes, contrarily to the statistical results achieved from 2009.

Considering the FB_2/FB_1 and FB_3/FB_1 ratios, in 2009 season the collected data showed a high variability between hybrids (see Figure 25), being the calculated values in the range 0.28-0.67 and 0.31-0.68 for free FB_2/FB_1 and for total FB_2/FB_1 ratio, respectively, and in the range 0.14-0.62 and 0.21-0.64 for free FB_3/FB_1 and for total FB_3/FB_1 ratio, respectively. A similar trend for FB_2/FB_1 and FB_3/FB_1 ratios was observed in 2010, being these values more similar between hybrids than those observed in the 2009 season, as shown in Figure 25. In particular, free FB_2/FB_1 and total FB_2/FB_1 ratios were both calculated in the range 0.26-0.32, while free FB_3/FB_1 and total FB_3/FB_1 ratios were calculated in the range 0.08-0.13 and 0.10-0.16, respectively.

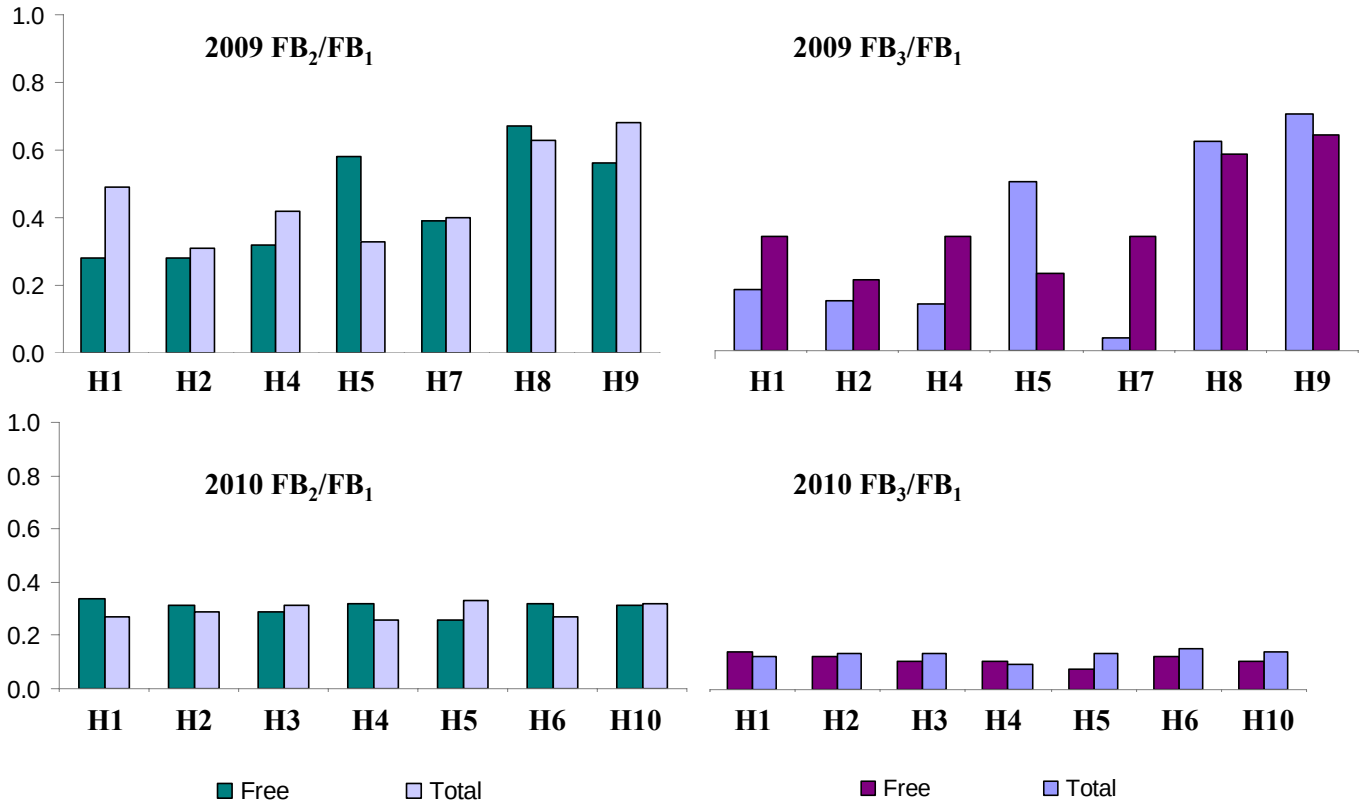


Figure 25. Free and total FB_2/FB_1 (left) and FB_3/FB_1 (right) ratios in the hybrids sampled in 2009 and 2010.

As far as the hybrid chemical composition has been concerned, the lipid fraction related variables were found to be a key parameter for both years, since the main significant differences between hybrids were observed in fat content and fatty acid profiles, as reported in Table 16. In particular, on the basis of post-hoc Tuckey's test ($\alpha = 0.05$), the fat content measured in 2009 was lower in H1 than in H2, H5 and H9. Accordingly, also changes in the fatty acid profile recorded for maize genotypes were found to be significant. In particular, H1 showed the highest and H7 and H8 the lowest C16:0 content; moreover, H1 and H2 showed a lower C18:1 amount than H8, while a higher C18:2 amount occurred in H2 and H8 in comparison to H1, as shown in Figure 26.

Regarding the data obtained in 2010, the fat content was lower in H6 in comparison to H5 and H2. Accordingly, also changes in fatty acid profile were found to be significant, as reported in Figure 26. In particular, H10 had a higher C16:0 amount than H2 and H5; it also had the highest C18:1 amount. Moreover, H6 had a lower C18:1 level than H3, H4 and H5. Hybrid H2 and H5, then, showed the highest C18:2 amount.

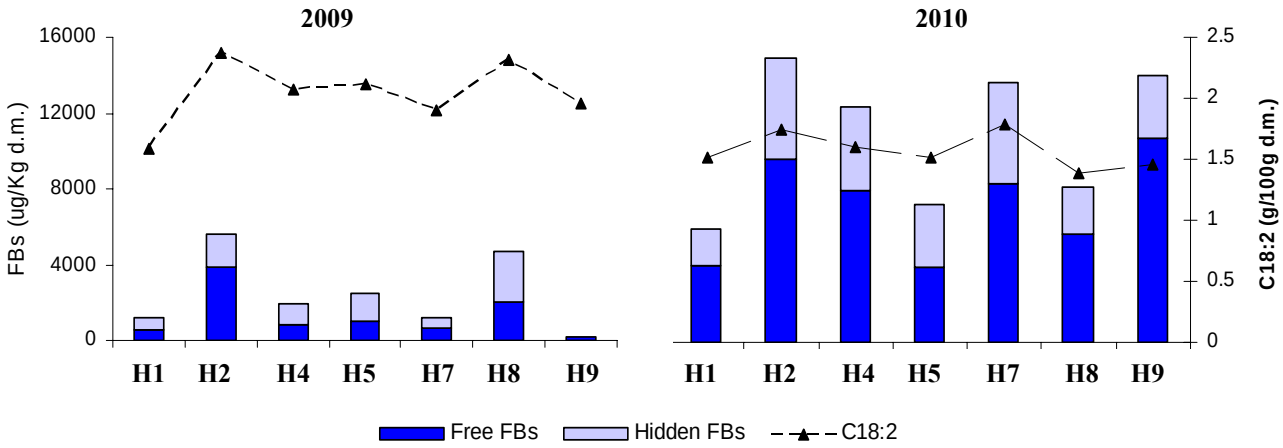


Figure 26. Free and total FBs levels and linoleic acid content in the considered maize hybrids.

Moreover, in the harvest year 2010, significant differences between hybrids were observed for protein content: besides H10, which showed the lowest protein content, H6 showed a lower value than H2, H3 and H4.

Comparison of the common hybrids

In order to better understand the role played by the considered maize hybrids towards *Fusarium* infection and FBs contamination, the data collected in 2009 and in 2010 harvest seasons were merged and statistically analysed including the 6 common hybrids; this approach is justified by the insignificant role played by the sampling place. The contamination levels were normalized on the maximum recorded amount for each year to make data comparable.

The dataset was elaborated by ANOVA considering as factors both the hybrid (H1-H6) and the year (2009, 2010). Both hybrid and year showed a statistical significance, whereas the interaction between the two factors was found to be negligible. The total infection level, expressed as percentage of infected kernels by fungi, showed significant differences over the two years of observation as well as between hybrids. On the other hand, any significant difference was found between hybrids in relation to the incidence of kernels infected by *F.* section *Liseola* and both free and total FBs contamination. The free and masked FBs levels, as well as the linoleic acid content, were reported in Figure 27: the highest the fat and the linoleic acid amount in the considered maize genotypes the highest the FBs contamination results.

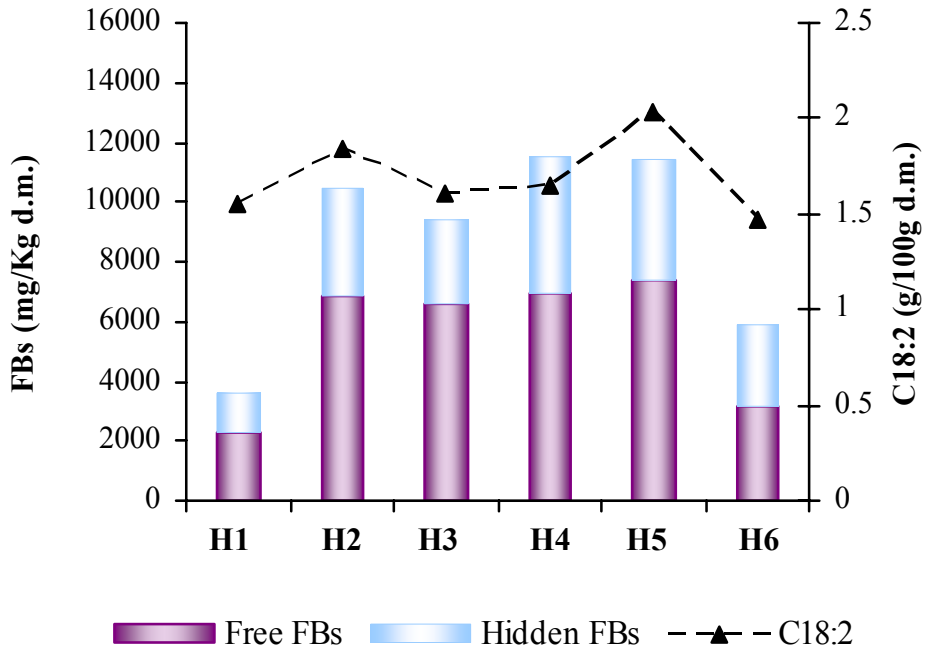


Figure 27. Free and masked FBs levels, and linoleic acid content in the common maize hybrids for year 2009 and 2010.

According to what observed also for the separated datasets, the highest variability between hybrids was due to the lipid fraction related variables. On the contrary, the oleic acid percentage was found to be related only to the genotype, being the year negligible as source of variability. More precisely, H2 showed a higher protein content, a higher fat content and a lower level of oleic acid when compared to other hybrids.

In order to better define the changes in composition occurring over two years, the mean values and the variation range were determined for the six common hybrids. Only those factors which have been found as significant by statistical analysis were considered. Regarding fatty acids, the profile composition was given in terms of relative percentages (Table 17).

Table 17. Mean value and variation range (in brackets) of selected parameters recorded for the 6 common hybrids considered in both 2009 and 2010.

	2009	2010
Fat (g/100g d.m.)	3.6 (2.8-3.9)	3.0 (2.6-3.3)
C18:1 (%)	27.7 (23.5-31.1)	30.2 (27.6-32.3)
C18:2 (%)	59.4 (56.6-64.2)	53.5 (47.9-56.3)
C18:1/C18:2	0.47 (0.37-0.55)	0.57 (0.49-0.65)
$(FB_2/FB_1)_{FREE}$	0.37 (0.28-0.58)	0.31 (0.26-0.34)
$(FB_2/FB_1)_{TOT}$	0.39 (0.31-0.49)	0.29 (0.26-0.33)
$(FB_3/FB_1)_{FREE}$	0.24 (0.36-0.50)	0.12 (0.08-0.15)
$(FB_3/FB_1)_{TOT}$	0.28 (0.21-0.34)	0.14 (0.10-0.16)
Free FBs/Tot FBs	0.58 (0.40-0.91)	0.63 (0.55-0.70)

A wide overlapping can be noticed in fat and C18:1 content in the 2 years, while C18:2 was lower in 2010, with a minimum overlapping in the range of variation also noticed in the rate C18:1/C18:2 and FB_2/FB_1, both free and total. The rate FB_3/FB_1, both free and total, had a different range of variation in 2009 and 2010 and no overlapping exist between the 2 years, being FB_3 lower in 2010 compared to 2009. The range of values variation was larger in 2009, with the exception of C18:1 and C18:1/C18:2 that showed very similar ranges in both years.

Effect of chemical composition on FBs levels

Table 18 reports significant correlations between maize kernels chemical composition and FBs contamination, according to Spearman's correlation test.

Table 18. Significant Spearman's correlations found for 2009, 2010 and common hybrids datasets. Spearman's Rho and p- (in brackets) values are given.

Dataset	**2009**			**2010**			**2009-2010**		
Variables	**% fat**	**C18:1**	**C18:2**	**% fat**	**C18:1**	**C18:2**	**% fat**	**C18:1**	**C18:2**
Free FBs	0.518 (0.008)	n.s.	0.565 (0.003)	n.s.	n.s.	n.s.	0.409 (<0.001)	n.s.	0.423 (<0.001)
Total FBs	0.466 (0.019)	0.474 (0.017)	n.s.	n.s.	n.s.	n.s.	0.387 (0.001)	n.s.	0.404 (<0.001)

Positive correlation was found between free and total FBs for samples collected in 2009, as well as for those collected in 2010 ($p<0.001$), and for the common hybrids analysed together ($p<0.001$).

Whereas starch and total N were never found correlated to contamination parameters, significant correlations were found for the lipid fraction. Indeed, the fatty acid composition was found to be positively correlated to both free and total FBs, oleic acid with total FBs and linoleic acid with free FBs in 2009. Although, no significant correlations were found in 2010, when common hybrids studied over two years were considered, the fat content was found again positively related to the free and total fumonisins levels as well as linoleic acid was correlated with both free and total FBs.

Moreover, when the masking rate (expressed as free-to-total FBs ratio) is regressed with the oleic-to-linoleic ratio of the six considered hybrids, a very good linearity was obtained (r^2 = 0.89), as reported in Figure 28.

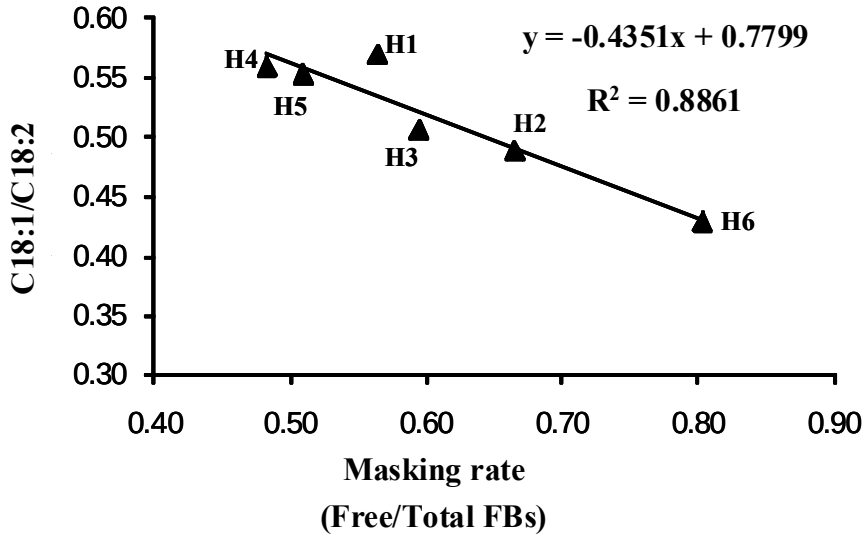


Figure 28. Linear correlation between the Free-to-Total FBs ratio and C18:1 to C18:2 ratio, calculated as mean values for common hybrids over two years.

3.4.3 CONSIDERATIONS

To date, this is the first work in which the role of the maize genotypes as well as their chemical composition in fumonisins accumulation have been evaluated, starting from data collected under field conditions and over two years of observation, also considering both the free and hidden forms.

The collected data showed a significant difference in *F.* section *Liseola* incidence as well as in FBs contamination over the two years, also when the same maize hybrid is considered. Indeed, the samples collected in 2010 showed a higher *Fusarium* incidence and higher free and hidden FBs levels than those observed for maize sampled in the first season. The severe contamination registered in the second year can be explained through the observation of the

meteorological data collected for 2009 and 2010 in Emilia Romagna (reported in Table 14): during the 2010 growing season, the average temperature in July was higher than that recorded during 2009 and more rainfall and rainy days were monitored in the second season during the maize ripening period (August-September). This resulted in a longer in fields maturation period united to favourable kernels and environmental conditions for fungal growth and FBs accumulation (31, 42).

As early mentioned, in addition to FBs extractable through routine extraction procedures (known as "free FBs"), the occurrence of hidden FBs has been determined, in order to investigate possible changes in masking rate between the considered maize genotypes.

Such extensive survey in raw maize has never been performed before, providing solid data to definitely prove the presence of a masking phenomenon in raw maize at relevant levels. In particular, hidden FBs were found in 80 and 90% of samples in 2009 and 2010 respectively, with a mean masking rate, calculated as free-to-total FBs ratio, over two years of about 0.6. All the main FBs (FB_1, FB_2 and FB_3) underwent to the masking phenomenon, with a comparable average masking rate. When the masking rate between different maize genotypes is considered, the free-to-total FBs ratio values obtained for the samples collected in 2010 was narrower than in 2009, as reported in Table 17. These data suggest that maize genotypes may support the masking phenomenon at a different extent, particularly with poorly conducive conditions for FBs accumulation.

Concerning FBs production, some interesting considerations can be done on the base of the calculated FB_2/FB_1 and FB_3/FB_1 ratios: whereas the first is quietly constant over two years, the latter was lower in 2010, suggesting that FB_1 is more efficiently obtained from its precursor FB_3 (3), in conducive conditions for the production of the final metabolite. Moreover, when hybrids are considered separately, significant difference can be highlighted (see Figure 25): the ratios calculated for the 2009 dataset showed an appreciable variability among hybrids, but it is strongly reduced in 2010. As suggested to explain the variation in masking rate, also in this case we supposed that the role played by the genotype in the FBs biosynthesis modulation is significant with low contamination, but it seems mitigated with high contamination.

By observation of the data collected over two years, fumonisins accumulation seems to be strongly related to the genotype. In particular, results obtained from the same hybrid grown in different areas during the same season showed a similar behaviour for all the considered hybrids, indicating an infection response mainly due to the genotype features.

So far, although several studied have been carried out in order to clarify the role of hybrid-related characteristic in *Fusarium* infection and FBs production in corn, the composition of the substrate in terms of macro constituents, comprising the lipid fraction, was never considered.

Whereas the amount of starch is very similar both among different hybrids and over two years, protein content was found change significantly over the two years but not between hybrids. Instead, the lipid fraction was found to vary significantly between maize genotypes, irrespective of the growing year.

In 2010, when a stronger incidence of fungi is experienced in maize, a decrease in oleic acid and a corresponding increase of linoleic acid amounts within each genotype have been recorded. Moreover, the total fat percentage, the C18:1 and the C18:2 amount ranges within the 6 common hybrids were nearer in 2010 than in 2009, when the *Fusarium* infection and the FBs contamination levels are higher. These results are coherent with those concerning the FBs accumulation and the masking rate and strongly support the hypothesis that the response to fungal attack is firmly dependent to the maize genotype at lower infection degrees, while hybrid-related response ability decreases when more conducive conditions for fungal growth occurs.

As reported in Figure 26, when the free and hidden FBs and the linoleic acid content in the considered genotypes are compared, a similar trend is recorded. This situation is observable for samples collected both in 2009 and in 2010 and also when average values for hybrids 1 to 6 are considered over two years, as shown in Figure 27, clearly pointing out a correlation between FBs accumulation and fatty acids content in maize.

Significant positive correlations were obtained between free and total FBs levels and the main lipid related factors (total fat percentage, C18:2 amounts); therefore, the highest the fat and the linoleic acid amount in the considered maize genotypes the highest the FBs contamination result, in agreement with results reported by Ebrahimi et al. (43) in peanuts and by Aziz (44) in sunflower infected by *A. flavus*.

Such relation between unsaturated fatty acids and fungal secondary metabolites may be explained by considering the role play by these macro constituents as modulators of plants resistance pathway during pathogen infection (45).

Indeed, polyunsaturated fatty acids are enzymatically or non-enzymatically oxydized to produce oxylipins, a class of compounds produced in mammals, microbes and plants which have different signalling properties (46).

Whereas fungal oxylipins are know to function in regulating developmental processes including cell growth, sexual and asexual spore differentiation, apoptosis and pathogenicity, in plant they work as molecular signals to regulate growth and development, senescence, sex determination and reproductive organs and, above all, the defence against biotic and abiotic stress as well as programmed cell death (47, 48, 49).

Although the substrates are the same, fungal oxylipins are structurally different from those produced in plants, as reported in Figure 29.

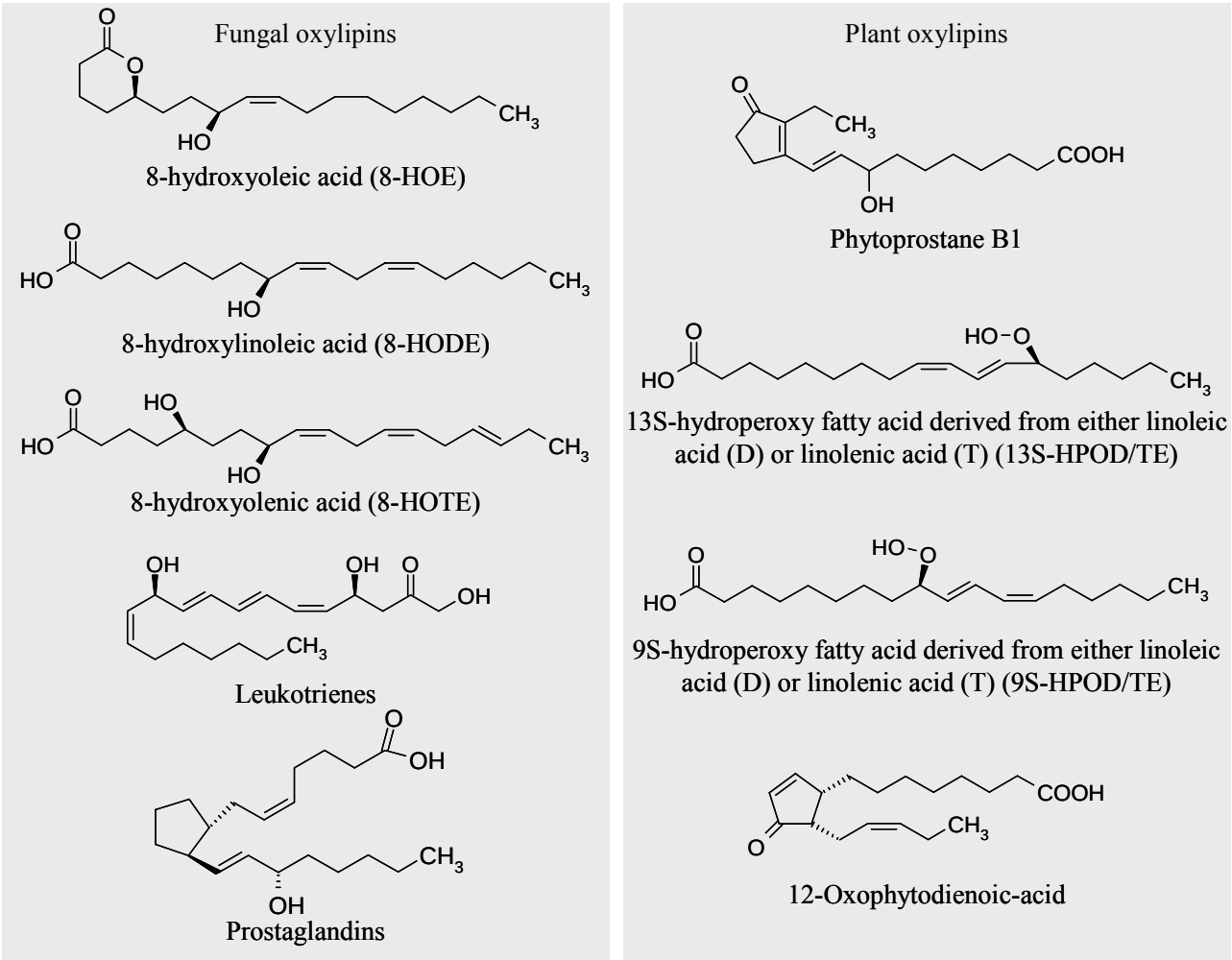


Figure 29. Structures of fungal and plants oxylipins (adapted from Christensen et al., 50).

Plants oxylipins are able to regulate the reproductive development of fungi and their secondary metabolism, thus influencing the production of mycotoxins (50). Concerning *Fusarium* mycotoxins, the ability of host oxylipins to enhance fumonisins production has been demonstrated (51).

In addition to their function as substrates for the biosynthesis of oxylipins, recent discoveries demonstrated more direct roles for fatty acids in inducing various modes in plant defence. Specifically, C16 and C18 fatty acids are the main constituents of plant cuticle, the

hydrophobic layer that covers the aerial surfaces of the plants and represent their first line of contact with the environment (52). In addition to limiting nonstomatal loss of water, gasses, and solutes, cutin influences both plant-insect and plant-microbe interactions, working as the indirect primary line of defence against pathogens by providing a physical barrier to their admittance (46).

Moreover, fatty acids are also involved in the systemic defence response, generating cascade signals able to activate mechanism belonging to the systemic acquire resistance (SAR) (46).

Fatty acids profile is strictly related to environmental and climatic conditions occurring during the flowering/growing period of plants, especially with temperatures. Indeed, since temperatures increase is related to higher oleic acid content (53), the higher relative percentage of oleic acid recorded in 2010 (the season characterized by slightly higher temperatures in July) can be explained.

Since the same statistical trend was found for free and total FBs, an involvement of fatty acids in masking phenomenon may be suggested. Very recently, Bartok et al. found several fatty acid esters of fumonisins in fungal mycelia (54). Although their occurrence has never been proven in food and/or in plants, these derivatives should be actually considered as masked fumonisins. Moreover, these compounds may be cleaved under alkaline conditions similar to those applied in this study, releasing thus the hydrolyzed forms. Although the formation of FB derivatives esterified with fatty acids could offer a possible explanation of the masking phenomenon, according to Bartok et al. these derivatives are produced by fungi in very low amount and are difficultly excreted in the media due to their low polarity. Since the masking rate reported in this paper is very high, it is very unlikely that hidden forms may be due to the formation of fatty acid FBs. More likely, the masking phenomenon is ascribable to the supramolecular interactions already hypothesised by Dall'Asta et al (22, 23, 24), while fatty acids may be actively involved in the plant-pathogen cross-talk regulating the FB accumulation and the hidden FB formation *in planta*.

The significant correlation between the average masking rate (reported as free-to-total FBs ratio) and the average linoleic-to-oleic ratio suggests that hybrids containing a higher amount of oleic acid show a higher masking rate.

The data presented in this paper suggested thus a hybrid-dependent implication of fatty acids in the plant-pathogen cross-talk inducing a modulation of FBs-related mechanisms in maize.

3.5 Conclusions

The two-years study here presented allows to obtain several new additional information concerning the role of maize hybrids and as well as their chemical composition in *F.* section *Liseola* infection and related FBs synthesis, accumulation and masking.

Fumonisin B_1, B_2 and B_3 were always detected in maize samples belonging to different hybrids, with FB_3/FB_1 ratio lower in conducive conditions for FBs synthesis; similarly, FBs masking was confirmed in raw maize with a lower rate detected in the year characterized by high FBs contamination.

So far as kernel composition is concerned, the fat content resulted correlated both to free and hidden fumonisins. A main role of fatty acids has been suggested, with higher contamination in hybrids showing a higher linoleic content. An interesting negative correlation was found between oleic/linoleic ratio and free/total FBs ratio in different hybrids. Even if the content of oleic and linoleic acid is yearly dependent, the behaviour of hybrids was confirmed in the 2-year data analysis.

Although these data were obtained in a limitedly wide geographic area and need to be confirmed in more variable conditions, they represent a basis to explain maize hybrid susceptibility to fungal infection, FBs contamination and masking not related to a specific hybrid or commercial brand, but extendable to all hybrids, a crucial result being maize hybrids characterized by a very frequent turnover on the market.

Downstream of these considerations, it can be argue that if this results were confirmed they may be very useful in breeding and hybrids selection.

Obviously, further studies are needed to investigate the possible involvement of other factors in the hybrid-related modulation of fumonisins occurrence and their masking rate.

CHAPTER 4 DYNAMIC OF FREE AND HIDDEN FUMONISINS ACCUMULATION DURING MAIZE STORAGE

4.1 INTRODUCTION

Filamentous fungi in seeds may be rather arbitrarily divided in two groups, designated field fungi and storage fungi. Whereas the fields fungi are those that invade the development or mature seed while it is on the plant, the storage fungi are those which develop on and within seeds at moisture contents often encountered in storage (55). Although *Fusarium* spp. invades maize kernel during plant growth, are anyway able to continue to grow also in storage condition, thus they may be considered as possible contributors to the storage deterioration of grains.

Besides mycotoxin production, fungi growing in stored seeds can cause other severe deteriorations, such as a reduction in germination, darkening, increasing of the fatty acids content and moisture and heating (55).

Since grain entering store carries a wide range of microorganisms including bacteria, yeasts and moulds who colonized crop during growth (56), it can be considered as a complex ecosystem in which abiotic and biotic factors interact with each other, leading to repercussions on the quality of the stored material (57). Indeed, exchanges of moisture, O_2 and CO_2 occur between the stored grain and the external environment, influencing the micro flora development and mycotoxin production as well as insect attack (Figure 30).

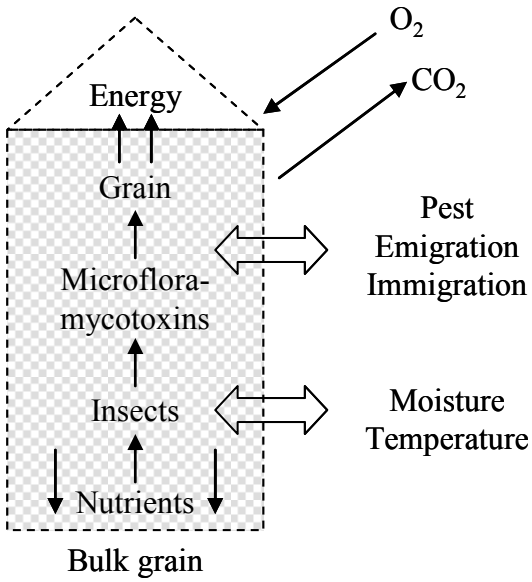


Figure 30. Diagrammatic representation of the interaction between abiotic and biotic factors in stored grain ecosystem (adapted from Magan et al. [56]).

The key interactions that influence fungal growth and mycotoxins biosynthesis are three: interaction between spoilage fungi, interaction between abiotic factors such as temperature, moisture, activity water, etc., and mycotoxin-producing fungi and relationship occurring between insects and moulds.

Spoilage fungi interact with each other through competition, antagonism and niche overlap. In this context, environmental factors may exert a selective pressure influencing the dominance of individual species, especially as concern mycotoxigenic species (56).

It must also be remembered that insects can produce metabolic heat which generates water via condensation on colder surfaces, thus creating micro-climates favourable to microorganism growth (58). Insect infestation and fungal development are together in close relation: whereas some storage insects are disseminator of fungi and other are exterminator, similarly some fungi attract insects as food source and some produce metabolites which repel them (59). Surprisingly, no studies have been conducted with regard the interaction between insect pests and fumonisin-producing fungi (56).

Although in recent years several post-harvest control strategies have been developed in order to minimize mycotoxin accumulation in stored grain (60), matter losses due to fungal proliferation and mycotoxins production still represent a real issue to be addressed in order to guarantee a good safety level for the final users. Moreover, studies conducted to date have taken into account only the free mycotoxins (the portion direct detectable through common analytical methods), without regard to eventual dynamics that may affect the occurrence and

the accumulation of masked mycotoxins (the fraction complexated with maize macroconstituents). Thus, no data concerning the effect of storage condition on hidden fumonisins occurrence in a maize stored within a silo are available.

4.2 Aim of the work

This work aimed to study the dynamic of mycotoxin producing micro flora into a maize bulk stored in a vertical silo. The accumulation of both free and hidden fumonisins has been also evaluated in order to individuate if storage conditions had some effect on masking phenomenon.

The dynamic of fungi and mycotoxins in the post-harvest period were studied using a true-scale experiment: two sampling methods were applied to evaluate how microbial population and fumonisin contamination change along both the longitudinal ad the radial section of the chosen storage system.

In addition, to evaluate any effects of the industrial milling on the occurrence of filamentous fungi as well as on the variation in masking rate, the flour obtained after kernel processing was sampled and analyzed. A complete overview of the changes occurring along the maize production chain was thus obtained.

This work was carried out in collaboration with the Institute of Entomology and Plant Pathology (Università Cattolica del Sacro Cuore, Piacenza, Italy) and with the Consorzio Agrario Provinciale-CAP Parma.

Sampling was performed by the technicians of the Consorzio Agrario Provinciale (Parma).

Microbiological data regarding the colony forming unit (CFU) numbers and the identification of mycotoxin-producers fungi were achieved from the Institute of Entomology and Plant Pathology (University of Piacenza, Italy).

Chemical data concerning the levels of contamination from free and hidden fumonisins were obtained in our laboratory at the Department of Organic and Industrial chemistry (University of Parma, Italy).

4.3 Material and methods

4.3.1 Maize samples collection

In 2009 twenty seven maize fields near Parma (Emilia Romagna Region-North Italy) sowed with different hybrids were harvested between late August and mid September. The whole production (about 450 tons) was delivered to a store house where forced ventilation (air heated at 100°C) allowed to reduce maize humidity under 14% in around 6 hours; grain temperature during drying was around 35-40°C.

The conditioned maize was cooled to 15°C and used to fill a flat-bottom vertical silos equipped with a discharge opening at the base and also with a central cochlea used to force the maize flux to the exit.

In February 2010 (after 6 month of storage) the core cylinder was emptied and 9 samples (around 3 Kg each) were collected from the kernel flux: 3 were collected immediately, 3 after 15 minutes and 3 after 30 minutes of discharge, representing, respectively, the base, the core and the top of the silo central longitudinal section (see Table 19).

Then, during 2010, the silo was completely emptied in three times (on March, April and June). At each time, the silo was deprived of 1300 quintals of kernels, which were loaded on 4 trucks equipped with trails. Sampling took place on both tracks and trails (3 portions for each one), thus obtaining 8 samples (around 5 Kg each) for each discharge date (a total of 24 samples).

Samples were finely ground (0.25 mm particle size) with a laboratory mill (IKA MF 10, OptpLab, Modena, Italy) and stored at -18°C before chemical and microbiological analysis.

Since the kernel flux exiting from the silo was intended for flour production, the flour obtained from the industrial milling was sampled to obtain 4 flour samples (about 1 Kg each) for each time (a total of 12 samples).

The sampling plan of the silo is reported in Table 19.

Table 19. Sampling calendar and sample codification.

Sampling	Date	Samples number	Code
Core cylinder	February, 10	9	CS1-9
1st empting	March, 8	12	Truck 1-4; Trail 1-4; Flour 1-4
2nd empting	April, 14	12	Truck 5-8; Trail 5-8; Flour 5-8
3rd empting	June, 8	12	Truck 9-12; Trail 9-12; Flour 9-12

4.3.2 EXPERIMENTAL PROCEDURES

Colony forming units (CFU) count

Aliquot (10 g) of ground maize sample was diluted with 90 mL of sterile peptonated water (1%) and stirred using an homogenizer (Bagmixer® 400, Interscience, Paris, France) for 3 minutes. Then, the mixture underwent to serial dilutions from 10^{-1} to 10^{-7}. From each solution 1 mL was transferred on Potato Dextrose Agar (PDA, Oxoid, Cambridge, UK) added with cloramphenicol and incubate at 25°C for 6 days. For each dilution the trial was run in triplicate.

At the end of incubation, strains belonging to *Fusarium*, *Aspergillus* and *Penicillium* species were counted. Results were expressed as colony forming units for sample gram (CFU/g).

Chemical analysis

The procedures used in sample preparation for the determination of free extractable fumonisins or total fumonisins after hydrolysis as well as the instrumental analytical methods are just reported in paragraphs 3.3.2 and 3.3.3.

Statistical analysis

Statistical analyses were performed using SPSS v.17.0 (SPSS Italia, Bologna, Italy). Data were statistically compared by using a OneWay-ANOVA Test followed by a post-hoc Tukey Test ($\alpha = 0.05$).

4.4 Results and discussion

4.4.1 Evaluation of free and hidden fumonisins occurrence in the silo core cylinder.

Between late August and mid September 2009, maize harvested in 27 fields near Parma (Emilia Romagna Region) was used to fill a vertical silo equipped with an opening at the base used for its discharge. After six month of storage (in February 2010), the core cylinder of such silo (the central longitudinal section) was emptied and sampling took place during the kernel flux through the exit in three different times. Thus, three samples were collected at the beginning of the discharge, representing the base; three after 15 minutes, representing the core and three after 30 minutes, representing the top of the longitudinal section here studied (see Figure 31).

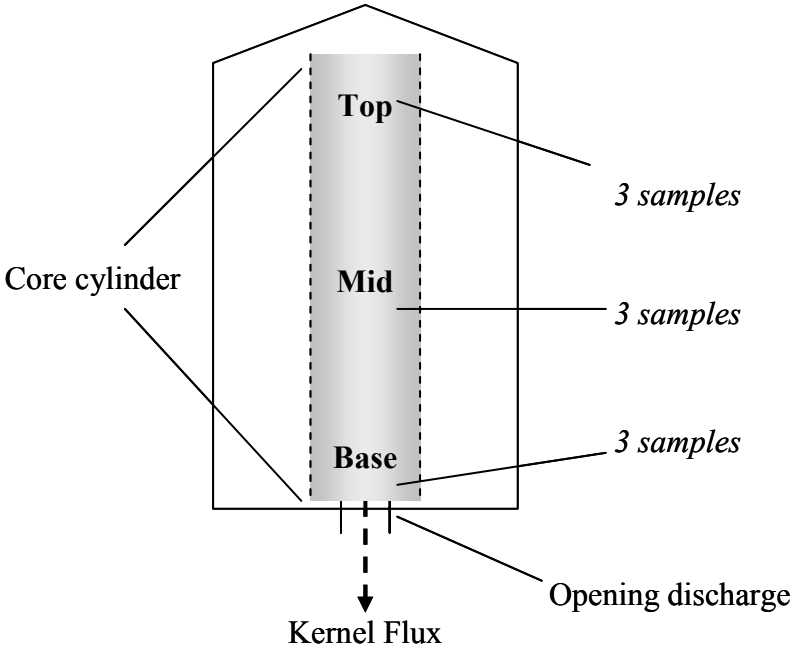


Figure 31. Schematic representation of the vertical silo used in this work and sampling of the core cylinder (the section underlined in grey).

Samples underwent to microbiological and chemical analyses, in order to collect data concerning the number of colony forming units of mycotoxin-producing fungi, as well as the amounts of free and total fumonisins occurring in the three considered portions.

The colony forming units count (carried out for gram of maize flour and expressed as CFU/g) showed a very similar occurrence of moulds among the three levels, as reported in Figure 32.

Fusarium verticillioides was the prevalent mycotoxin producing fungus, with a concentration of about 10^6 CFU/g. *Aspergilli* section *Flavi*, and *Penicillium* spp. were observed in all samples, with a similar concentration of about 10^4 CFU/g, while *Aspergilli* section *Nigri* was found only in the basal portion, at the appreciable concentration of 10^5 CFU/g.

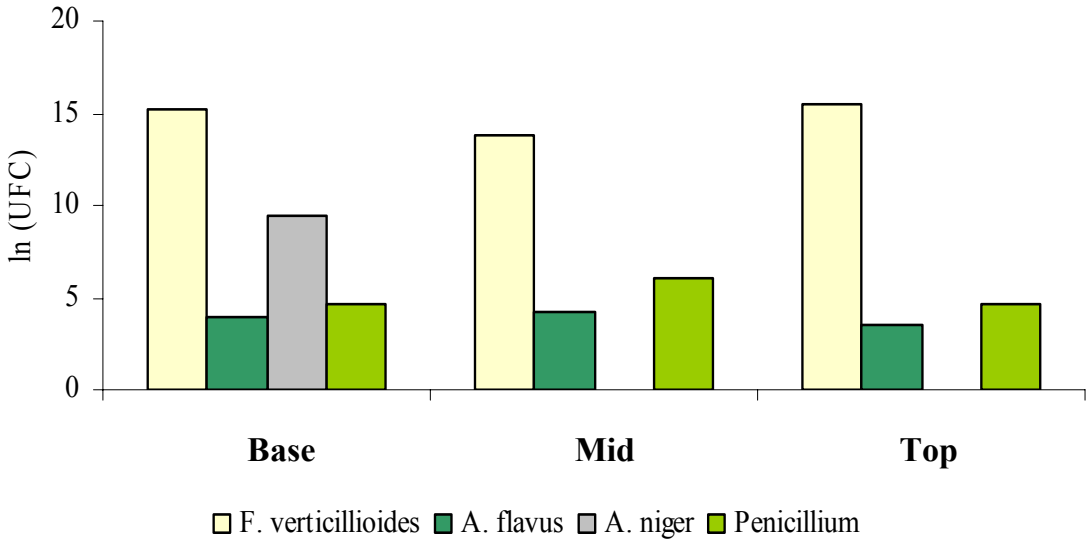


Figure 32. CFU/g count for the main mycotoxin-producing fungi occurring in the core cylinder at the base, mid and top. Results are expressed as the natural logarithm (ln) of the data obtained.

To evaluate possible differences occurring in the contamination of the three levels, data were statistically compared using a One-Way ANOVA test followed by a post-hoc Tukey test (α = 0.05): no differences were found in the fungal population found in the three considered levels, except for *A. flavus* which was found only in the first fraction of discharge.

As far as fumonisin contamination is concerned, all the considered samples were found contaminated from FBs (see Table 20). The free FBs concentration ranged from 97 to 2198 µg/Kg, while the total FBs level after alkaline hydrolysis was found to be in the range 245-4344 µg/Kg. Hidden FBs were ranging between 0 and 2496 µg/Kg. Data concerning free and total fumonisins contamination and the calculated ratios between the free and hidden fractions are summarized in Table 20.

Table 20. Free and total fumonisin contamination (mean, range in µg/Kg) determined in maize samples collected from the bottom, the middle and the top of the silo core cylinder.

	Base			Middle			Top		
	Free	**Total**	**Rate F/T**	**Free**	**Total**	**Rate F/T**	**Free**	**Total**	**Rate F/T**
FB_1	1048 653-1233	1728 985-2742	0.61	416 97-974	612 162-1282	0.68	673 225-1379	1064 511-2068	0.63
FB_2	378 262-483	582 278-1135	0.65	122 LOQ-303	195 45-317	0.63	192 LOQ-512	299 129-638	0.64
FB_3	219 146-274	290 152-467	0.76	77 LOQ-195	111 42-208	0.69	128 LOQ-308	181 81-384	0.71
FBs	1645 1061-1961	2600 1415-4344	0.63	615 97-1472	920 246-1807	0.67	994 225-2198	1544 721-3090	0.64

The free-to-total ratios calculated were not significantly different among the three levels both for the separated analogues as well as for their sum.

As reported in Figure 33, although a trend is visible from the base to the top of the core, statistical evaluation does not point our significant differences indicating that the contamination is uniform when the core cylinder is considered.

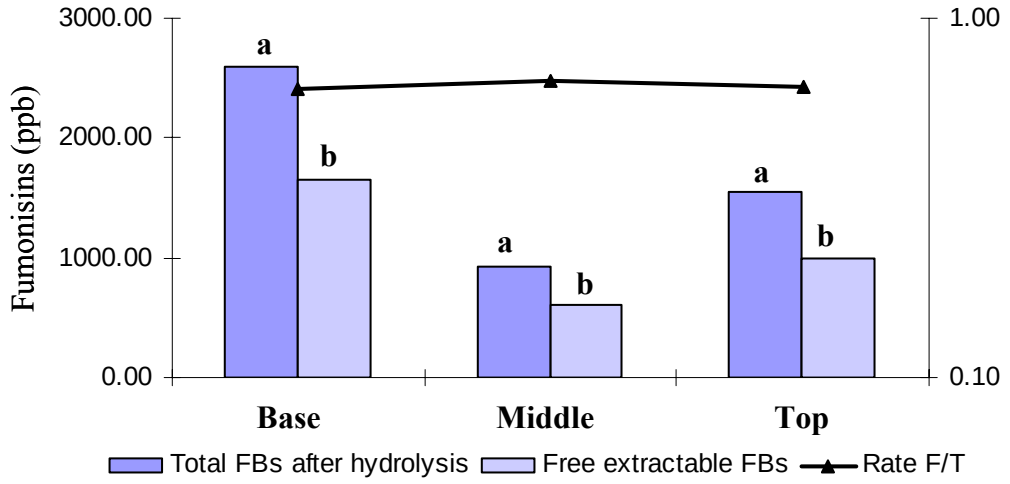


Figure 33. Free and total fumonisins levels and their calculated ratio in maize samples obtained from the discharge of the core cylinder. The same letters signify that there are no statistically difference between data (Tukey test, α = 0.05).

4.4.2 Silo Emptying and fractionation: evaluation of the radial contamination

Among March and June 2010, the vertical silo was completely emptied through three emptying steps. At each time about 1300 quintals of maize kernels were discharged from the opening at the bottom and loaded on four truck equipped with trail. Then, both tracks and trails were sampled to obtain a total of 8 samples for sampling date.

The silo was equipped of a central cochlea employed to force the kernel flux through the opening discharge, allowing to discharge firstly the inner and lower portions to continue with outer and higher fractions, as shown in Figure 34.

Moreover, at each sampling date the total amount of raw maize discharged from silo was transformed in maize flour intended for cattle feed production. Sampling was performed also at the end of this step, in order to have an overall view on the entire maize chain. Thus, for each fraction discharged and processed, 4 flour samples were obtained.

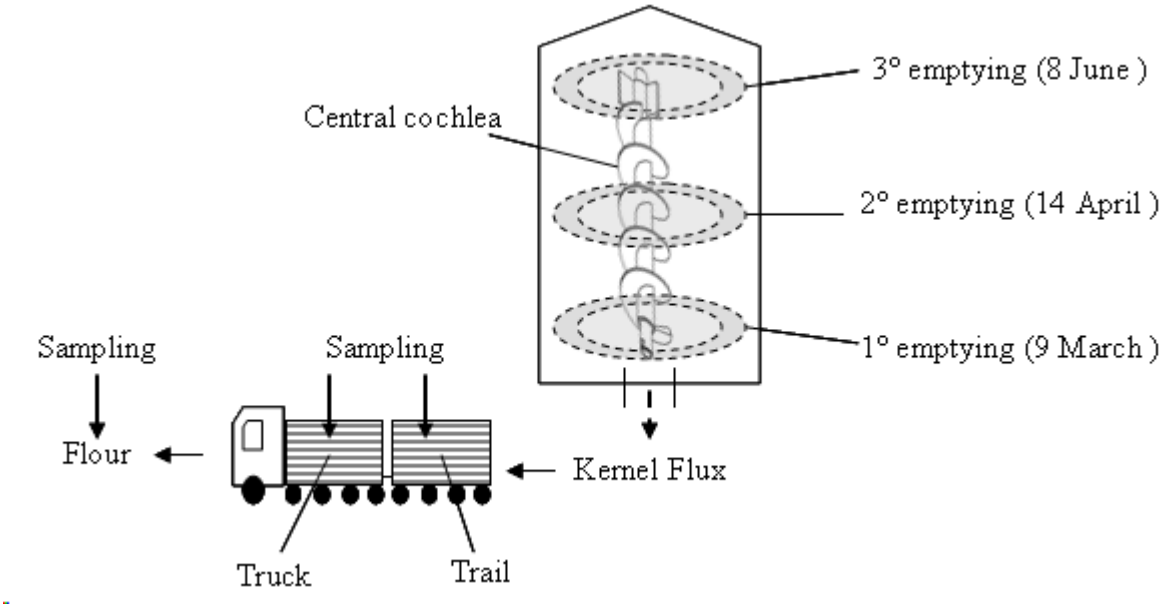


Figure 34. Schematic representation of the programmed emptying of the vertical silo.

Temperature was monitored during the entire storage period by means of temperature probes and abnormal values were not registered.

Similarly to what performed for samples obtained from the central core, also for these samples the number of colony forming units of the main mycotoxin producing fungi and the levels of free and total fumonisins have been determined. All the data concerning raw maize were grouped for trucks and trails, thus considering only temporal differences.

The *F. verticillioides* CFU was very similar to that observed for the core cylinder (about 10^6 CFU/g) and remained almost constant over the time as well as after flour production. A very similar situation was found also for *Aspergillus* and *Penicillum* species (see Figure 35). On the contrary, a significant increase of *A.* section *flavi* was registered in flour, probably due to a previously plant contamination. Also *A.* section *nigri* showed a significant growth over the three sampling dates.

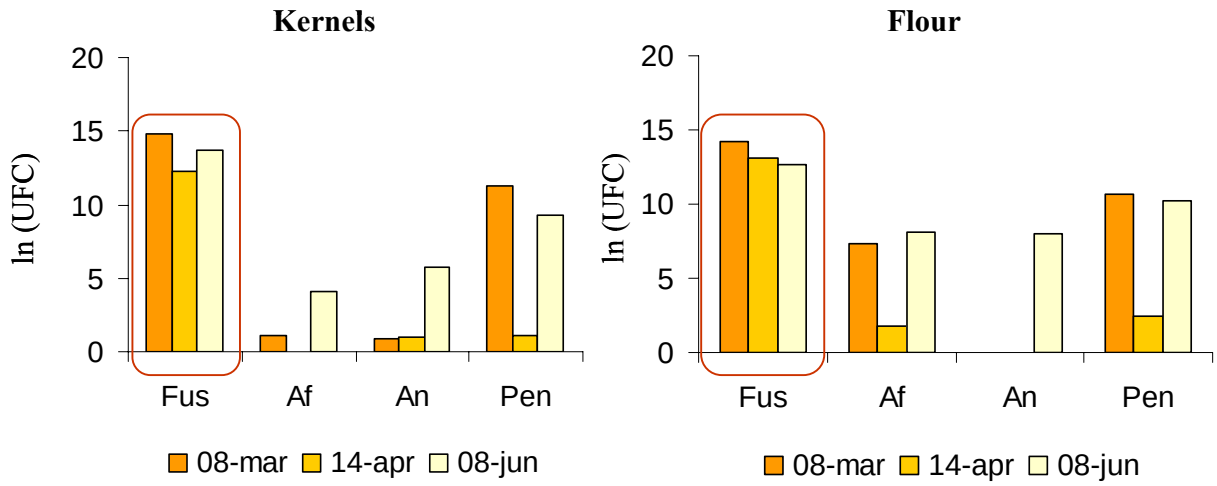


Figure 35. CFU/g count for the main mycotoxin-producing fungi (*Fusarium verticillioides, Aspergillus flavus, Aspergillus nigri* and *Penicillium* spp.) in maize discharged from silo ("kernels") and its corresponding half-processed product ("flour"). *F. verticillioides* CFU remained constant over time and after the flour production.

Concerning mycotoxin contamination, free and hidden fumonisins were found in nearly every samples. Although the free fumonisin concentration ranged from LOQ and 3232 µg/Kg in kernels, there was a slight decrease in flour, being the contamination between 215 and 3228 µg/Kg. Similarly, the total fumonisin concentration ranged from 59 to 4767 µg/Kg in the raw maize versus a concentration between 447 and 3229 µg/Kg in flour.

Data concerning free and total fumonisins contamination and the calculated ratios between the free and hidden fractions are summarized in Table 21.

Table 21. Free and total fumonisin contamination (mean, range in µg/Kg) determined in maize collected during three discharge dates (9th March, 14th April and 8th June) and in its corresponding flour.

Kernels									
	9-March			**14-April**			**8-June**		
	Free	**Total**	**Rate**	**Free**	**Total**	**Rate**	**Free**	**Total**	**Rate**
FB_1	1296	1942	0.67	687	1214	0.57	363	502	0.72
	LOQ-2752	832-2869		160-1995	73-2820		60-1026	95-1885	
FB_2	430	628	0.68	282	350	0.81	76	86	0.88
	LOQ-944	311-1009		LOQ-983	LOQ-999		LOQ-282	LOQ-680	
FB_3	289	852	0.34	92	428	0.21	6	102	0.06
	LOQ-596	152-1386		LOQ-254	LOQ-1636		LOQ-81	LOQ-1023	
FBs	2014	3422	0.59	1061	1992	0.53	455	679	0.67
	LOQ-3805	1295-4767		160-3232	88-4573		72-1390	59-3003	
Flour									
	9-March			**14-April**			**8-June**		
	Free	**Total**	**Rate**	**Free**	**Total**	**Rate**	**Free**	**Total**	**Rate**
FB_1	1250	1431	0.87	611	1231	0.50	585	810	0.72
	656-1803	765-2122		215-1253	314-2193		421-718	406-1168	
FB_2	458	504	0.91	188	380	0.50	172	112	1.54
	373-645	384-537		LOQ-399	252-608		120-228	16-314	
FB_3	370	558	0.66	75	294	0.25	LOQ	13	-
	273-461	362-954		LOQ-138	88-447		LOD-LOQ	LOQ-48	
FBs	2304	2266	1.02	875	1905	0.46	757	936	0.81
	1411-3228	1430-3229		215-1715	872-3088		640-947	447-1483	

The probable differences in fumonisin contaminations were sought over the three periods both in maize kernels and in flour samples. Moreover, such differences were evaluated also between the raw maize discharged and the corresponding flour obtained after its milling.

To confirm such variations, data were statistically compared using a One-Way ANOVA test followed by a post-hoc Tukey test ($\alpha = 0.05$). Both free and total FBs were compared.

Concerning raw maize, a strong decrease in free and total fumonisin contamination over the sampling dates has been observed, as reported in Figure 36. A similar trend, although less marked, was found for the fumonisin levels detected in flour samples. Indeed, whereas strong falling of contamination can be observed in maize kernels moving from a sampling date to the next one, such reduction is more gradual for the flour.

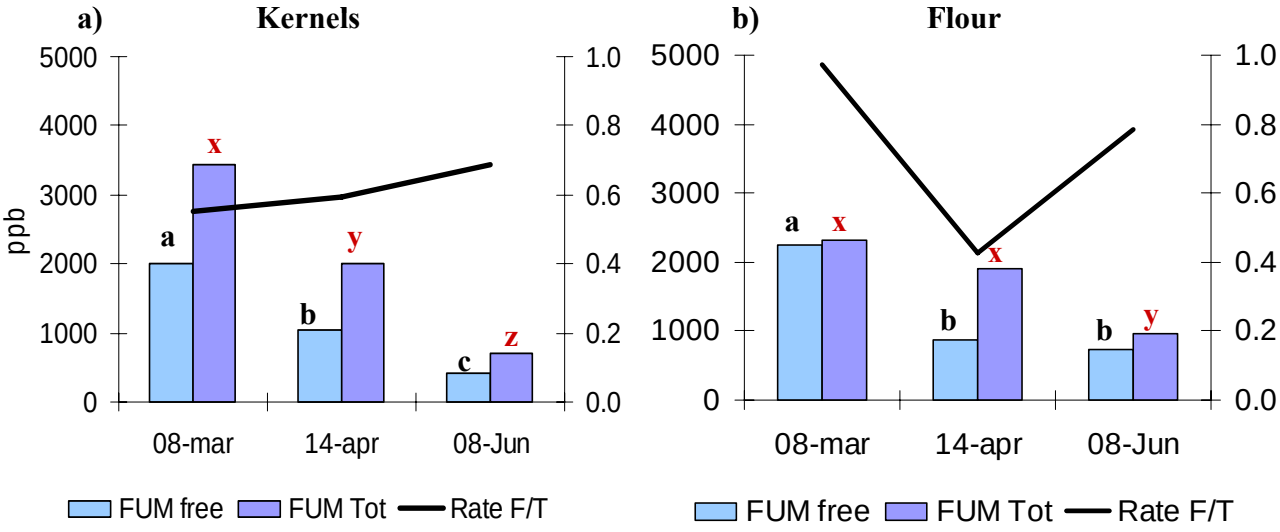


Figure 36. Free and total fumonisins levels and their calculated ratio in a) raw maize samples collected during the three dates of silo discharge and b) flour samples obtained by milling kernels exiting from the silo. Different letters designate statistically difference between data (Tukey test, α = 0.05).

As described above, the raw maize discharged from silo was forced through the exit at the bottom by means of the rotary motion of a central cochlea that pushes to the discharge first the lower and inner layers and finally the upper and external fractions. Thus, the data concerning the amounts of free and hidden forms collected during the three emptying step reflect the radial pattern of contamination of such silo. Since the maize discharged in March was found more contaminated than that sampled in April and June, it can be argued that mycotoxin contamination decreases moving from the inside of the silo towards outer and upper layers.

Although it has not been highlighted any difference between both free and total FBs levels registered in raw maize and flour ($p > 0.05$), a significant increase of the free-to-total fumonisins ratio was observed moving from kernels to flour, suggesting that flour production leads to a reduction of the masked fraction. However, such phenomenon is most likely ascribable to the mechanical disruption of cellular structures and macromolecules that allows the release of hidden forms.

The comparison between the mean values of free and total fumonisins levels detected in the core cylinder with those calculated for the maize discharged during 2010, showed no substantial differences among the longitudinal internal section and the surrounding bulk (see Figure 37). Nevertheless, whereas the core cylinder is characterized by a uniform contamination, a gradient of pollutant is realized in the more external maize, as demonstrated above.

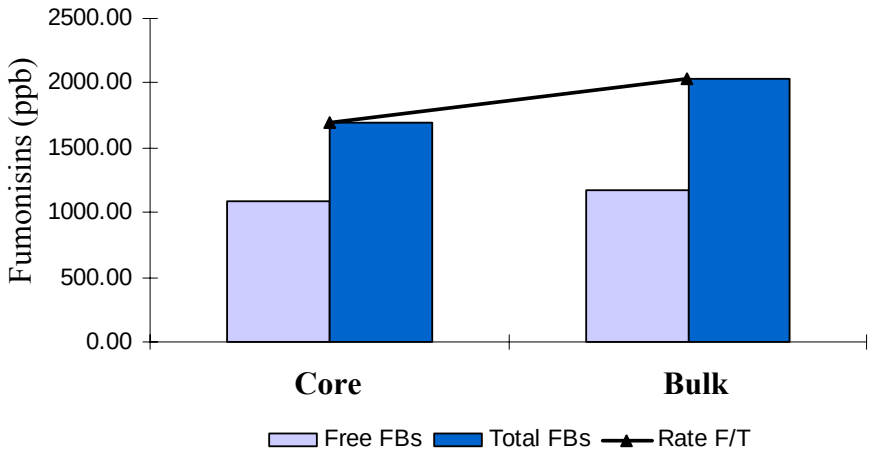


Figure 37. Free and total fumonisin contamination (mean, in µg/Kg) detected in the core cylinder and in maize discharged from March to June 2010.

4.5 CONCLUSIONS

The work here described allowed to obtain several information about the dynamic of contamination occurring in storage conditions into a vertical silo.

Concerning microbiological data, it has been demonstrated that the storage conditions do not influence the CFU counted, thus is it can be argue that the microbial contamination is slightly uniform in the whole mass stored, with a clear predominance of *Fusarium verticillioides* strains against other mycotoxins-producing fungi. Since very similar values were found also in flours, an effect of moulds decontamination determined by milling may be excluded.

On the contrary, whereas the core cylinder shows a homogeneous contamination from free and hidden fumonisins through its longitudinal section, a gradient of mycotoxin concentration seems to takes place moving from lower parts towards outer layers.

The analysis performed on flour obtained by milling the raw maize discharged from silo showed an increase of the free-to-total fumonisins ratio, probably due to the release of a certain amount of hidden forms after the mechanical disaggregation of the matrix particles.

From a food safety point of view, these data may be useful in order to forecast the contamination degree of a stored material exiting from a silo and thus to decide about its utilization.

REFERENCES

(1) Desjardins, A.E.; Proctor, R.H. Molecular biology of *Fusarium* mycotoxins. *Int. J. Food Microb.* **2007**,*119*, 47-50.

(2) Yu, J-H.; Keller, N.; Regulation of secondary metabolism in filamentous fungi. *Annu. Rev. Phytopathol.* **2005**, 43, 437–58.

(3) Gerber, R.; Lou, L.; Huffman, J.; Zhu, X.; Lin, T.; Li, L.Q.; Arreguin, I.; Butchko, R.A.E.; Proctor, R.H.; Du, L. Advances in understanding the biosynthesis of fumonisins. In: *Mycotoxin prevention and control in agriculture*, Ed. 1, Appell, M.; Kendra, D.F.; Trucksess, M.W.; ACS Symposium Series; American Chemical Society; Washington, DC, **2010**, 1031, 167-182.

(4) Reverberi, M.; Ricelli, A.; Zjialic, S.; Fabbri, A.A.; Fanelli, C. Natural function of mycotoxins and control of their biosynthesis in fungi. *Appl. Microbiol. Biotechnol.* **2010**, 87, 899-911.

(5) Picot, A.; Barreau, C.; Pinson-Gadais, L.; Caron, D. ; Lannou, C. ; Richard-Forget, F. Factors of the *Fusarium verticillioides*-maize environment modulating fumonisin production. *Crit. Rev. Microbiol.* **2010**, 36, 221-231.

(6) Miller, D.J. Factors that affect the occurrence of fumonisins. *Environ. Health Perspect.* **2001**, 109, 321-324.

(7) Battilani, P.; Formenti, S.; Rossi, V.; Ramponi, C. Maize hybrids and fumonisin contamination in kernels. *J. Cer. Sci.*, **2011**, published on line, DOI: j.jcs.2011.08.014.

(8) Bluhm, B.H.; Woloshuk, C.P. Amylopectin induces fumonisin B_1 production by *Fusarium verticillioides* during colonization of maize kernels. *Mol. Plant-Microbe Inter.*, **2005**, 18, 1333-1339.

(9) Shelby, R.A.; White, D.G.; Burke, E.M. Differential fumonisin production in maize hybrids. *Plant Dis.* **1994**, 78, 582-584.

(10) Soriano, J.M.; Dragacci, S. Occurrence of fumonisins in food. *Food Res. Int.* **2004**, 31, 985-1000.

(11) Doko, M.B.; Rapior, S.; Visconti., A.; Schjøth, J.E. Incidence and levels of fumonisin contamination in maize genotypes grown in Europe and Africa. *J. Agric. Food Chem.* **1995**, 43, 429-434.

(12) Bakan, B.; Melcion, D.; Richard-Molard, D.; Cahagnier, B. Fungal growth and *Fusarium* mycotoxin content in isogenic traditional maize and genetically modified maize grown in France and Spain. *J. Agric Food Chem.* **2002**, 50, 728-731.

(13) Miller, J. D. Epidemiology of Fusarium diseases of cereals. In *Mycotoxins in Grain: Compounds Other Than Aflatoxin*; Miller, J. D., Trenholm, H. L., Eds.; American Association of Cereal Chemists: St. Paul, MN, **1994**; pp 19-36

(14) Rheeder, J.P.; Marasas, W.F.O. and Vismer, H.F. Production of fumonisin analogs by *Fusarium* species. *Appl. Environ. Microbiol.* **2002**, 68, 2101-2105.

(15) Sewram, V.; Mshicileli, N.; Shephard, G.S.; Vismer, H.F.; Rheeder, J.P.; Lee, Y.W.; Leslie, J.F.; Marasas, W.F.O. Production of fumonisin B and C analogues by several *Fusarium* species. *J. Agric. Food Chem.* **2005**, 53, 4861-4866.

(16) Shepard, G.S.; Thiel, P.G.; Stockenström, S.; Sydenham, E.W. Worldwide survey of fumonisin contamination of corn and corn-based products. *J. AOAC Int.* **1996**, 79, 671-687.

(17) IARC. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene*. International Agency for Research on Cancer, Lyon, **2002**, 301–366.

(18) Wild, C.P.; Gong, Y.Y. Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis.*, **2010**, 31, 71-82.

(19) Kim, E.-K.; Scott, P. M.; Lau, B.P.-Y. Hidden fumonisins in corn flakes. *Food Addit. Contam.* **2003**, 20, 161-169.

(20) Galaverna, G.; Dall'Asta, C.; Mangia, M.; Dossena, A.; Marchelli, R. Masked mycotoxins: an emerging issue for food safety. *Czech. J. Food Sci***. 2009**, 27, 89-92.

(21) Park, J. W.; Scott, P. M.; Lau, B.P.-Y.; Lewis, D. A. Analysis of heat processed corn foods for fumonisins and bound fumonisins. *Food. Add. Contam.*, 2004, 21, 1168-1178

(22) Dall'Asta, C.; Galaverna, G.; Mangia, M.; Sforza, S.; Dossena, A.; Marchelli, R. Free and bound fumonisins in gluten-free food products. *Mol. Nutr. Food. Res.*, 2009, 53, 492-536 499.

(23) Dall'Asta, C.; Mangia, M.; Berthiller, F.; Molinelli, A.; Sulyok, M.; Schuhmacher, R.; Krska, R.; Galaverna, G.; Dossena, A.; Marchelli, R. Difficulties in fumonisin determination: the issue of hidden fumonisins. *Anal. Bioanal. Chem.* **2009**, 395, 1335-1345.

(24) Dall'Asta, C.; Falavigna, C.; Galaverna, G.; Dossena, A.; Marchelli, R. *In vitro* digestion assay for determination of hidden fumonisins in maize. *J Agric Food Chem*, **2010**, 58, 12042-12047.

(25) Mogensen, J.M.; Nielsen, K.F.; Samson, R.A.; Frisvad, J.C.; Thrane, U. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. *BMC Microbiology*. **2009,** 9, 281.

(26) Kim, H.; Woloshuk, C.P. Role of AREA, a regulator of nitrogen metabolism, during colonization of maize kernels and fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal. Gen. Biol.*, **2008**, 45, 947-953.

(27) Picot, A.; Barreau, C.; Pinson-Gadais, L.; Piraux, F.; Caron, D.; Lannou, C.; Richard-Forget, F. The dent stage of maize kernels is the most conducive for fumonisin biosynthesis under field condition. *Appl. Environ. Microbiol.,* 2011, published on line, DOI:10.1128/AEM.05216-11.

(28) Blandino, M.; Reyneri, A. Effect of maize hybrid maturity and grain hardness on fumonisin and zearalenone contamination. *Ital. J. Agron.*, **2008**, 3, 107-117.

(29) Battilani, P.; Pietri, A.; Barbano, C.; Scandolara, A.; Bertuzzi, T.; Marocco A. Logistic regression modelling of cropping systems to predict fumonisin contamination in maize. *J. Agric. Food Chem.*, **2008**, 56, 10433-10438.

(30) Loffler, M.; Kessel, B.; Ouzunova, M.; Miedaner, T. Population parameters for resistance to *Fusarium graminearum* and *Fusarium verticillioides* ear rot among large sets of early, mid-late and late maturing European maize (*Zea mays L.*) inbred lines. *TAG Theor. Appl. Gen.*, **2010**, 120, 1053-1062.

(31) Battilani, P.; Formenti, S.; Rossi, V.; Ramponi, C. Maize hybrids and fumonisin contamination in kernels. *J. Cer. Sci.*, **2011**, published on line, DOI: j.jcs.2011.08.014

(32) King, S.B.; Scott, G.E. Genotypic differences in maize to kernel infection by *Fusarium moniliforme. Phytopathology.* **1981**, 71, 1245-1247.

(33) Presello, D. A.; Botta, G.; Iglesias, J.; Eyherabide, G. H. Effect of disease severity on yield and grain fumonisin concentration of maize hybrids inoculated with *Fusarium verticillioides*. *Crop. Prot.*, **2008**, 27, 572-576

(34) Lanubile, A.; Pasini, L.; Lo Pinto, M.; Battilani, P.; Marocco, A.. Evaluation of broad spectrum sources of resistance to Fusarium verticillioides and advanced maize breeding lines. *W. Mycotox. J.*, **2011**, 4, 43-51.

(35) Butron, A.; Santiago, R.; Mansilla, P.; Pintos-Varela, C.; 571 Ordas, A.; Malvar, R. A. Maize (*Zea mays L.*) genetic factors for preventing fumonisin contamination. *J. Agric. Food. Chem.*, **2006**, 54, 6113-6117.

(36) Loffler, M.; Miedaner, T.; Kessel, B.; Ouzunova, M. Mycotoxin accumulation and corresponding ear rot rating in three maturity groups of European maize inoculated by two *Fusarium* species. *Euphytica*, **2010**, 174, 153-164.

(37) Miedaner, T.; Bolduan, C.; Melchinger, A. E. Aggressiveness and mycotoxin production of eight isolates each of *Fusarium graminearum* and *Fusarium verticillioides* for ear rot on susceptible and resistant early maize inbred lines. *Eur. J. Plant. Pathol.*, **2010**, 127, 113-123.

(38) Summerell, B.A.; Salleh, B.; Leslie, J.F. A Utilitarian approach to *Fusarium* identification. P*lant Dis.*, **2003**, 87, 117-128.

(39) Raper, K.B.; Fennell, D.I. 1965. The Genus *Aspergillus*. United States of America, Robert E. Krieger publishing company Inc.

(40) Pitt, J.I. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press, London, **1979**.

(41) Fowler, J.; Cohen, L. 1990. *Practical statistics for field biology*. Open University Press, Milton Keynes.

(42) Schaafsma, A.W.; Hooker D.C. Climatic models to predict occurrence of *Fusarium* toxins in wheat and maize. *Int. J. Food Microbiol.*, **2007**, 119, 116-125.

(43) Ebrahimi, T.; Khomeiri, M.; Maghsoudlou, Y.; Ahmadi, G.M. Relation between chemical compositions of peanut with its resistance to *Aspergillus flavus*. (Special issue: Flora, life form and plant chronology in Damghan rangelands.) *J. Agric. Sci. Nat. Res.*, **2009**, 16, 1-A, unpaginated.

(44) Aziz, S. Y. Evaluation of some sunflower seed hybrids for oil properties and fungal infection. *Egypt. J. Agric. Res.*, **2001**, 79, 1075-1084.

(45) Upchurch, R. G. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol. Lett.*, **2008**, 30, 967-977.

(46) Kachroo, A.; Kachroo, P. Fatty acids-derived signals in plant defence. *Annu. Rev. Phytopatol.* **2009**, 47, 153-176.

(47) Noverr, M.C.; Erb-Downward, J.R.; Huffnagle, G.B. Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbe. *Clin. Microbiol. Rev.* **2003**, 16, 517.533.

(48) Shah, J. Lipids, lipases and lipid-modifying enzymes in plant disease resistance. *Annu. Rev. Phytopatol.,* **2005**, 43, 229-260.

(49) Howe, G.A.; Jander, G.; Plant immunity to insect herbivores. *Annu. Rev. Plant. Biol.,***2008**, 59, 41-66.

(50) Christensen, S.A.; Kolomiets, M.V. The lipid language of plant-fungal interaction. *Fungal Gen. Biol.,* **2011**, 48, 4-14.

(51) Gao, X.; Shim, W.-B.; Göbel, C.; Kunze, S.; Feussner, I.; Meeley, R.; Balint-Kurti, P.; Kolomiets, M.; Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. *Mol. Plant-Microbe Interact,* **2007**, 20, 922-933.

(52) Jeffree, C.E. Structure and ontogeny of plant cuticles. In: *Plant Cuticles: An Integrated Functional Approach*. Ed. **1996**, Kersteins G, Oxford, UK: BIOS Scientific, 1996, pp 33-82.

(53) Wilson, R.A.; Calvo, A.M.; Chang, P.K.; Keller, N.P. Characterization of the *Aspergillus parasiticus* delta12-desaturase gene: a role for lipid metabolism in the *Aspergillus*-seed interaction. *Microbiol.,* **2004**, 150, 2881-2888.

(54) Bartok, T.; Tolgyesi, L.; Mesterhazy, A.; Bartok, M.; Szecsi, A. Identification of the first fumonisin mycotoxins with three acyl groups by ESI-ITMS and ESI-TOFMS following RP-HPLC separation: palmitoyl, linoleoyl and oleoyl EFB1 fumonisin isomers from a solid culture of *Fusarium verticillioides*. *Food Add Contam - Part A*, **2010**, 27, 1714-1723.

([55]) Christensen, C.M.; Kaufmann, H.H. Deterioration of stored grains by fungi. *Annu. Rev Phytopathol.*, **1965**, 3, 69-84.

(56) Magan, N.; Hope, R.; Cairns, V.; Aldred, D. Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. *Eur. J. Plant Pathol.,* **2003**, 109, 723-730.

(57) Wallace, H.A.H. and Sinha, R.N. Causal factors operative in distributional patterns and abundance of fungi: A multivariate study; In: *The Fungal Community–Its Organisation and Role in Ecosystems*, Wicklow, D.T. and Carroll, G.C. (eds) Marcell Dekker Inc., New York, USA, **1981**, pp 233-247.

(58) Sauer, D.B.; Storey, C.L. and Walker, D.E. Fungal populations in US farming-stored grain and their relationship to moisture, storage time, regions and insect infestation. *Phytopathology*, **1984**, 74, 1050-1053.

(59) Sinha, R.N. Fungus as food for some stored product insects. *J. Econ. Entomol.* **1971**, 64, 3-6

(60) Magan, N.; Aldred, D. Post-harvest control strategies: minimizing mycotoxins in the food chain. *Int. J. Food Microbiol.*, **2007**, 119, 131-139.

SECTION III. STUDY OF FUMONISIN B, A AND C PRODUCTION BY TWO *FUSARIUM* SPECIES GROWN UNDER DIFFERENT CONDITIONS THROUGH LC-ESI-MS/MS

PRODUCTION OF FUMONISINS B, A AND C BY *FUSARIUM* SPECIES: BIOSYNTHESIS AND REGULATORY FACTORS

Fumonisins are structurally related toxic metabolites produced primarily by *Fusarium* species, in particular *F. verticillioides* and *F. proliferatum* (1), that are fungal pathogens known to cause pink ear rot on maize worldwide (2). The fumonisins analogs have been characterized since 1988 and were classified into four groups, identified as fumonisins series A, B, C and P (1, 3, 4). While *F. verticillioides* seems to be able to produce several metabolites, as fumonisins B (FBs), fumonisins A (FAs), fumonisins C (FCs) and fumonisins P (FPs), *F. proliferatum* is known to produce only fumonisins belonging to the A- and B-series (1). The latter includes toxicologically important FB_1, FB_2 and FB_3, that are the most abundant naturally occurring fumonisins, with FB_1 predominant, and are usually found at high levels both in raw maize and in maize-derived foods- and feed-stuff (1). Fumonisins B are mycotoxins widely studied because they represent a serious risk for animal and human health, for their toxicity and potential carcinogenicity (IARC, 1993). Although many ecological, molecular and chemical studies have been carried on in the past (5, 6, 7, 8, 9), many issues are not yet clarified and researches are still ongoing, in particular regarding their biosynthetic pathway.

Fumonisins B are sphinganine-analog mycotoxins (SAMs): poliketide-derived natural products structurally similar to sphinganine that execute their toxicity through the competitive inhibition of sphinganine N-acetyltransferase (ceramide sinthase). This group comprises also AAL-toxins, which are characterized by the same central aliphatic backbone typical of fumonisins that define the structural similarity with sphinganine (10). Since there was limited direct evidence linking ceramide synthesis to SAMs production, polyketide-biosynthetic pathways have been suggested as possible routes for the biosynthesis of such metabolites and this hypothesis was ascertained by using isotope-labelled acetate in liquid cultures of *F. verticillioides* (11). Labelling studies remain the best approach to resolving the biosynthetic pathway of a target compound and allowed to know the biosynthetic origin of FBs (Figure 38): while the carbon backbone from C-3 to C-20 are derived from acetate, C-1 and C-2 as well as amino C-2 are derived from alanine and the two methyl groups at C-12 and C-16 are derived from methionine (12, 13, 14). Among multiple hydroxyls present on the aliphatic backbone, the one at C-3 is the only derived from acetate, whereas hydroxyl groups at C-5, C-10, C-14 and C-15 are derived from molecular oxygen, suggesting that the initial carbon chain is a highly reduced poliketide (10). The origin of the two tricarballylic units is not yet clearly explained, but they probably are derived from the citric acid cycle (15).

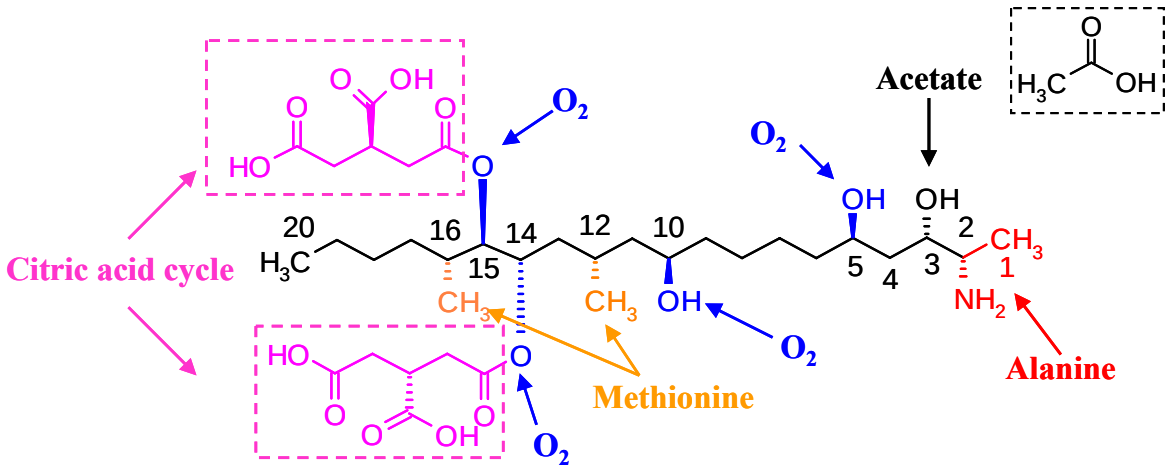


Figure 38. The biosynthetic origins of FB_1, resolved by means of labelling studies (adapted from Du et al., 2008 [12]).

A cluster of 17 genes (named *FUM* cluster), extensively studied using gene disruption, domain swapping and heterologous expression approaches, are needed for fumonisins biosynthesis (7).

The biosynthesis starts with the poliketide chain assembly, controlled by the enzyme complex poliketide synthase (PKS), encoded by *FUM1*. This complex contains seven domains: domains, β-ketoacylsynthase (KS), acyltransferase (AT), dehydratase (DH), methyltransferase

(MT), β-ketoacyl reductase (KR), enoylreductase (ER), and acyl carrier protein (ACP), that allow to synthetize polyketides in which β-carbonyl functions are fully or almost fully reduced (12). The two methyl groups at C-12 and C-16 are incorporated during, rather and after poliketide chain assembly. Then, the carbon chain is extended and an amino group is introduced through the formation of a new carbon-carbon bond between the poliketide chain and alanine (16) Within the *FUM* cluster, *FUM8* encodes for a 2-oxoaminosynthase, a group of PLP-dependent enzymes that catalyze the condensation of amino acids and acyl-CoA thioester substrates (17).

After the poliketide chain release, the 3-ketointermediate obtained undergoes to several modification during fumonisins biosynthesis, that include oxidoreductions and esterifications. The first oxidoreduction involves the terminal ketone group. The gene responsible is *FUM13* that encodes for a short chain NADPH-dependent dehydrogenase/reductase, which catalyzes the dehydrogenation or reduction of various substrates like alcohols and aromatic compounds. The two vicinal hydroxyl groups at C-14 and C-15 are introduced by one or a combination of P450 monooxygenase encoded by *FUM6* (18), thus obtaining the 3, 14, 15-trihydroxy intermediate, as known as "hydrolyzed FB_4", that is fumonisins B_4 lacking of both the tricarballylic moieties.

During the esterification of the vicinal diol at C-14 and C-15 with the two tricarballylic functions four genes (*FUM7*, *FUM10*, *FUM11*and *FUM14*) are involved. Whereas *FUM7* and *FUM11* are not essential but important for a complete esterification, both *FUM10* and *FUM14* are directly involved into the reaction (6). At the subsequent step, a monooxygenase NADPH-dependent encoded by *FUM12* that catalyzes fumonisin C-10 hydroxylation to obtain the first fumonisin analog, FB_3, that is the direct precursor of FB_1, to which is converted after the hydroxylation at C-5 by mean of a dioxygenase 3-ketoglutarate dependent encoded by *FUM3* gene. The enzyme is able to convert FB_3 in FB_1 in the presence of α-ketoglutarate, Fe^{2+}, ascorbic acid, and catalase (19).

Another way to obtain FB_1 involving the same enzymes encoded by *FUM3* and *FUM12* has been suggested: FB_4 is converted in FB_2 via hydroxylation at position C-5 and this one is in turn converted in FB_1 through a subsequent hydroxylation at position C-10 (16). Nevertheless, this way has not been established. Figure 39 shows the biosynthetic pathway proposed for fumonisins belonging to the B-series.

The biosynthesis of minor analogs has been poorly studies. Nevertheless, like B fumonisins, also FAs, FCs and FPs are characterized by a linear carbon backbone decorated with 3-5 hydroxyls, two methyl groups, one amino group, and two tricarballylic esters. Therefore, their

biosynthetic pathways may be not substantially different from that proposed for FBs. Their structural difference from the main analogs (e.g. shorter chain or a substituent linked to the amino group) may be due to the activity of enzymes with specificity for a particular substrate rather than other or encoded by genes activated only in specific conditions still unknown.

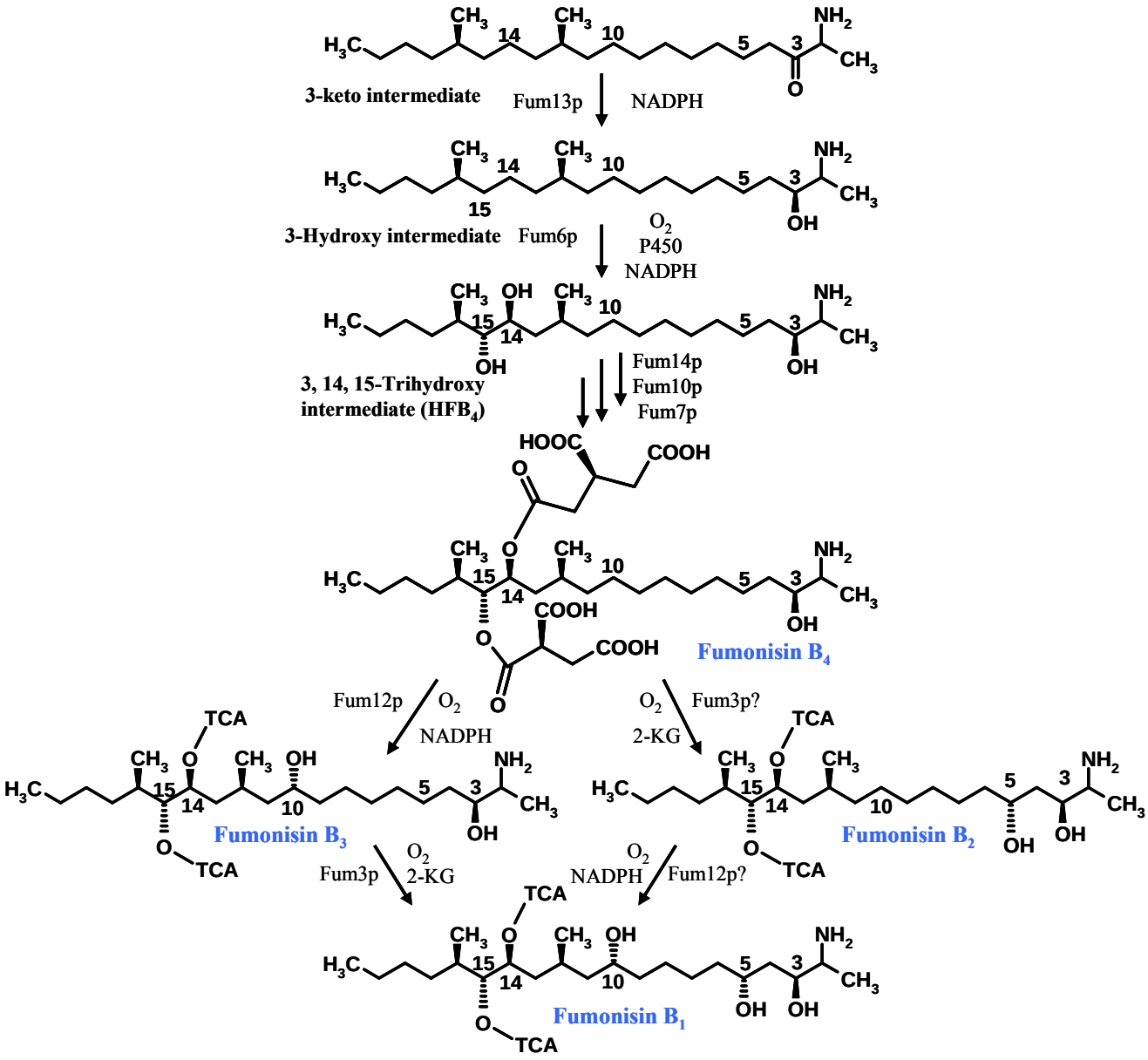


Figure 39. Proposed biosynthetic pathway for the modification of fumonisin backbone to obtain FB_1, FB_2 and FB_3. The conversion from FB_4 to FB_2 and thus FB_1 is still not ascertained (16). TCA, tricarballylic acid; 2-KG, 2-ketoglutarate.

The production of fumonisins is regulated by the synthesis of the involved enzymes, and thus by the expression of *FUM* genes. As occur for other cluster genes implicated in secondary metabolites synthesis, also the expression of *FUM* genes is regulated by transcription factors. Regulatory factors are divided in two groups: one includes factors encoded by genes appertaining to the cluster and are pathway-specific since they regulate the expression of other

genes, while the latter includes environmental signals such as pH, carbon, and nitrogen sources. This multilevel regulation by both specific and broad-domain transcription factors ensures that secondary metabolite pathways can respond to the demands of general cellular metabolism and the presence of specific pathway inducers (20). Concerning fumonisins, the pathway-specific transcription factors is represented by the protein encoded by *FUM21* (16) and also by a transcription factor not located into the fumonisin cluster (20).

Other environmental factors, such as temperature, water activity and pH, strongly influence fumonisins biosynthesis at the level of genes transcription: under moderate conditions of growth (e.g. a $_w$=0,95, 20°C, pH 5.0), the expression of the toxin biosynthesis genes and mycotoxin production were low, whereas a coordinated activation of mycotoxin gene clusters was apparent under mild-stress conditions (21).

Fusarium species have the maximal production of FB_1 and FB_2 among 20 and 25°C, with a radical decline at 30°C. However, fumonisin production by *F. verticillioides* was less inhibited than *F. proliferatum* by the higher temperature (22).

Higher water availability generally results in higher fumonisin production and higher fungal growth: optimal conditions for fumonisin production were described with a_w=0.97–0.98 (23). However, at temperatures that are not optimal for fungal biomass accumulation, fumonisin production relative to fungal growth was greater at lower a_w values, indicating that a_w stress may enhance fumonisin production (24).

Also the effect of the incubation period on fumonisins production is strongly related to the temperature: the overall maximal yield in FB_1 production was obtained after 13 weeks when *F. verticillioides* is incubated at 20°C, and after 11 weeks if the same strain is incubated at 25°C (25).

F. verticillioides and *F. proliferatum* produce the maximal amount of FB_1 when the pH is between 3.0 and 4.0 (26), while the repression of fumonisins production at alkaline pH has been demonstrated (27).

The substrate from which *Fusarium* species draw their carbon and nitrogen sources also plays a critical role in fumonisins regulation. A positive relationship was obtained between fumonisin production and sugar concentration, independently of the carbohydrates used. On the contrary, decreasing amino acid concentration led to a significant increase in fumonisin production and to a decrease in mycelial mass. These experimental evidences suggest that fumonisin production is positively influenced by an increase in the C:N ratio, to the detriment of fungal growth (28).

Since the moulds of interest have an aerobic metabolism, aeration has a profound effect both on the growth of mycelia and the production of fumonisins: reduced aeration results in slower grow rate and limited fumonisins production (26).
Finally, the effect of fungicides on *Fusarium* growth and toxin production has been evaluated. As general observation, fungicides are able to inhibit fungal growth and stimulate mycotoxin production, probably as the consequence of the direct stress effect on toxigenic species that increases the activity of enzymes involved in toxin biosynthesis. Therefore, the fungicides can affect the gene expression of toxin biosynthesis (29).
Very recently, the antifungal property of resveratrol and its ability to reduce mycotoxin accumulation were studied: although the accumulation of other mycotoxins such as zearalenone was strongly inhibit by this antioxidant, no inhibition of fumonisins accumulation has been observed (30). Thus, *FUM* gene expression seems to be not influenced by such environmental factor.
To date, studies performed in order to evaluate the effect of pathway-specific transcription factors and environmental dynamics both on the *in vitro* and *in vivo* production of fumonisins by *Fusarium* species focused the attention on the naturally occurring B analogues, in particular on FB_1 and FB_2. Although a strong similarity among the regulatory factors that affect FBs biosynthesis and those involved in the regulation of the production of minor analogs may be supposed, in literature there are no data published on the influence of the cited factors on FAs and FCs biosynthesis.

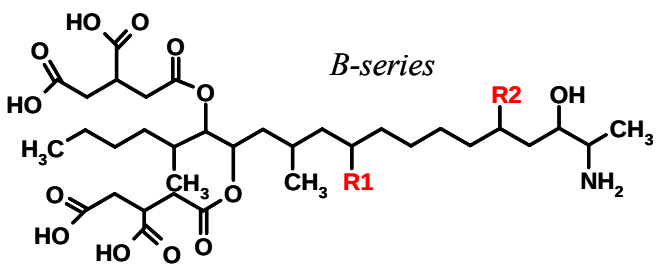
The studies performed in this section are aimed to collect more information about the production of fumonisins belonging not only to the B-series, but also to the minor series (A- and C-series) by *F. verticillioides* and *F. proliferatum*.
The work has been divided over two levels. The first part is dedicated to the detection and characterization of several fumonisins analogs produced in *F. verticillioides* broth cultures using MS/MS techniques, developing also a LC-MS/MS method to allow their simultaneous analysis. The second one aimed to better investigate the biosynthetic pathways of FBs, FAs and FCs and the influence of a_w and incubation period on their production pattern, also looking for any links between the production of minor analogues and main fumonisin biosynthesis.

CHAPTER 5 DETECTION AND CHARACTERIZATION OF TWELVE FUMONISINS ANALOGUES IN *FUSARIUM* BROTH CULTURES BY LC-ESI-MS/MS

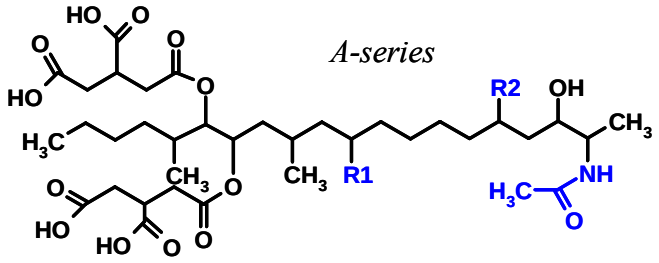
5.1 INTRODUCTION

The fumonisins, a family of food-borne carcinogenic mycotoxins, were first isolated in 1988 from cultures of *Fusarium verticillioides* (Sacc.) Nirenberg , previously known as *Fusarium moniliforme* Sheldon (31, 32). The 28 fumonisins analogs that have been characterized since 1988 can be separated into four main groups, identified as fumonisin A, B, C and P series (1), of which the toxicologically most important fumonisins are the FB analogues. Among these, FB_1 is usually found at high levels most frequently in maize and in maize-based food- and feed-stuff (1). Chemically, fumonisins belonging to the B-series are characterized by a 20 carbon aminopolyhydroxyalkyl chain diesterified with propane-1,2,3-tricarboxylic acid (tricarballylic unit, Figure 40) (33).

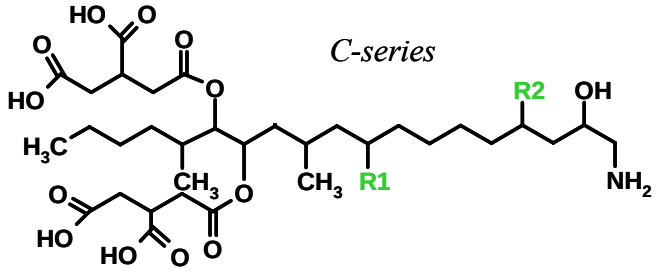
Apart from the FB series, several of the other analogs can be produced by *F. verticillioides*. Nevertheless, since the production of minor compounds is often maintained at relatively low levels (<5% of the total fumonisin present), they have been poorly studied (1, 4). Their chemical structure differs from that of the B-series because the central backbone in the C-series lacks a terminal methyl group, while FA analogues contain the acetylated derivatives of amine (33, 34). Fumonisin P has been recently isolated from *F. verticillioides* cultures. In their chemical structure, the amine found in C-2 position of the B-series in replaced with an N-linked 3-hydroxypiridinium moiety. Whereas many previously identified fumonisins occur generally at very low levels respect to FB (<5%), FPs can occur at levels up to 30% of FB_1 when grown on solid cultures (35). Figure 40 shows the chemical structures of the four series.



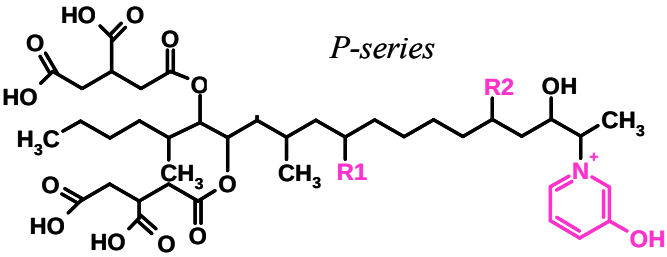
	R1	R2
FB1	OH	OH
FB2	H	OH
FB3	OH	H
FB4	H	H
FB5	OH	H



	R1	R2
FA1	OH	OH
FA2	OH	H
FA3	H	OH



	R1	R2
FC1	OH	OH
FC2	H	OH
FC3	OH	H
FC4	H	H



	R1	R2
FP1	OH	OH
FP2	OH	H
FP3	H	OH

Figure 40. Main fumonisins structures.

Among these minor compounds, only the occurrence of the C-series has been demonstrated in mouldy maize (36, 37), while there are no data available concerning FAs and FPs incidence in fumonisins-contaminated samples.

Since the lesser know analogs occur in traces, they are difficult to detect with most analytical techniques but they can easily analyzed by liquid chromatography/mass spectrometry with electrospray ion source or ESI with tandem mass spectrometry (34). Indeed, LC-ESI-MS and ESI-MS/MS techniques with different types or different combinations of mass analyzer have become very popular in mycotoxins analysis (38, 39, 40, 41, 42). In spite of their considerable costs, hyphenated techniques such as LC-ESI-MS, LC-ESI-MS/MS and LC-ESI-MS^n, are very suitable for mycotoxins monitoring, thanks to their unique sensitivity and selectivity and,

in contrast with florescence or UV detection, there is no need for derivatization (41). Moreover, in addition to mycotoxins monitoring, these techniques are also appropriate for the detection and characterization of minute amounts of compounds with unknown structures, without isolation. Indeed, the employment of LC-MS and MS^n techniques for the characterization of unknown compounds has been reported in several papers. In a recent work the detection and univocal characterization of new fumonisins mycotoxins and fumonisins-like compounds by using a multistage mass spectrometry technique has been reported by Bartók et al. (34). The same research group employ LC-MS^n techniques in structural studies for the identification of six new, higher molecular weight FB analogues having the hydroxyl groups of the TCA units esterified with long-chain fatty acids, also distinguishing among various isomers (43). Unambiguous identification and characterization of traces compounds can be obtained through MS/MS experiments and LC-ESI-MS/MS analysis, as demonstrated by Seefelder et al. (44): fumonisin B_1 glyco and amino acid conjugates were analyzed by means of LC-ESI-MS/MS and their structure were univocally determined through product ion spectra interpretation.

5.2 AIM OF THE WORK

The aim of this work was to detect and identify several fumonisins analogues produced in *Fusarium verticillioides* broth cultures, as well as their corresponding partially hydrolyzed forms, by LC-ESI-MS/MS. After finding twelve fumonisin analogues belonging to A-, B- and C-series, MS/MS experiments were performed in order to confirm their identification. Finally, two LC-ESI-MS/MS methods have been developed to allow, respectively, the multiresidual analysis of FBs, FAs and FCs and their partially hydrolyzed derivatives.

5.3 Materials and methods

5.3.1 Chemicals

Methanol (LC grade) was obtained from Carlo Erba (Milan, Italy) and acetonitrile (LC grade) was from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA).

5.3.2 Fungal isolates and media

The fungal isolate used in this study is a FB-producer belonging to the species *F. verticillioides* and collected in the fungal collection of the Institute of Entomology and Plant Pathology-UCSC, Piacenza (294 MPVP), and of the Institute of Sciences of Food Production-CNR, Bari (10027 ITEM; http://server.ispa.cnr.it/ITEM/Collection) and preserved by crio-conservation in water and glycerol (18%) at –80°C and liquid nitrogen. The strain has been isolated on maize crop from South Tuscany, Italy.

Mould was grown on Potato Dextrose Agar (PDA, Oxoid, Cambridge , UK) at 25°C for 7 days in the dark, then 10 ml of sterile distilled water was added to each plate and the mycelium was gently scraped to collect fungal conidia. The suspension was adjusted to 106 conidia ml-1 and 100 µl of conidial suspension were used as inoculum for liquid cultures.

A Ø 2 mm tassel, was collected from the whole PDA plate and used as inoculum for solid cultures.

5.3.3 Experimental procedures

Fumonisins production on synthetic media.

The conidial suspension of *F. verticillioides* strain was inoculated on static liquid culture of Malt Extract Agar (MEA), known to be a FB-inducing medium (45, 46), and incubated for 30 days at 25°C in the dark. The trial was run in triplicate.

At the end of incubation, the fresh mycelium was separated from the liquid medium, dried under vacuum (Whatman #4 filter, Ø 24 cm, Dassel, Germany) and frozen in liquid nitrogen. The medium was stored at -20°C and then used for FB analysis.

Extraction of fumonisins. Sample preparation.

An aliquot (4 mL) of liquid medium (previously separated from fungal conidia) was subjected to a clean-up through Sep-Pak C18 cartridges before LC-ESI-MS/MS analysis. Briefly, after preconditioning with 2 mL of methanol followed by 2 mL of bidistilled water, 4 mL of raw sample were loaded on the column, which was washed again with 4 mL of bidistilled water. Fumonisins were eluted using 4 mL of water/acetonitrile, 1:1 v/v, then 3 mL were evaporated to dryness under a gentle stream of compressed air and the residue was redissolved in 3 mL or 300 µL of water/methanol, 30:70 v/v, prior to LC-MS/MS analysis.

HPLC separation of fumonisins.

RP-LC analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA). Chromatographic conditions were the following: the column was a 250 mm x 2.1 mm i.d., 5 µm, XTerra C18; 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 acetonitrile (eluent B) both acidified with 0.2% formic acid: initial condition at 100% A, 0-5 min isocratic step, 5-30 min linear gradient to 100% B, 30-35 min isocratic step, 35-36 min linear gradient to 100% A and reequilibration step at 100% A for 14 min (total analysis time: 50 min).

MS spectrometric detection and characterization of fumonisins.

MS measurements were performed using a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, U.K.). MS parameters were the following: ESI+ (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 30 V; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow (nitrogen), 50 L/h and 700 L/h, respectively.

Total Ion Current (TIC) chromatograms were obtained by operating in Full Scan mode, detecting positive ions. The mass range was set between *m/z* 300-1000, with a total scan duration of 2.0 s and an inter-scan delay of 0.10 s.

The MS/MS experiments were performed at a collision energy ranging from 20 to 40 eV, acquiring in Daughter Scan mode. The mass range was set between *m/z* 300-800, with a scan duration of 0.5 s and an inter-scan delay of 0.05.

The detection of fumonisins analogues in synthetic media was achieved using a Multiple Reaction Monitoring (MRM) mode, by monitoring two transitions for each analyte, as reported in Table 22.

Table 22. MRM conditions for LC-ESI-MS/MS analysis of fumonisins (relative abundances of mass ions in brackets).

	Compound-specific ions (m/z)					
Analyte	**Precursor ion $[M+H]^+$**	**Main transition**	**Identification**	**CE (eV)**	**Qualifier ion**	**CE (eV)**
FB_1	722.4	334.4 (100)	$[M+H-2TCA-2H_2O]+$	35	352.4 (97)	35
FB_2	706.4	336.4 (100)	$[M+H-2TCA-H_2O]+$	35	318.4 (40)	35
FB_3	706.4	336.4 (100)	$[M+H-2TCA-H_2O]+$	35	318.4 (40)	35
FB_4	690.1	320.1 (97)	$[M+H-2TCA-H_2O]+$	35	338.2 (86)	35
FB_5	738.4	368.1 (98)	$[M+H-2TCA-H_2O]+$	35	350.1 (29)	35
FA_1	764.1	334.1 (73)	$[M+H-2TCA-AcOH-2H_2O]+$	35	394.1 (68)	35
FA_2	748.1	336.1 (91)	$[M+H-2TCA-AcOH]+$	35	378.1 (59)	35
FA_3	748.1	336.1 (91)	$[M+H-2TCA-AcOH]+$	35	378.1 (59)	35
FC_1	707.8	337.8 (100)	$[M+H-2TCA-H_2O]+$	35	319.1 (47)	35
FC_2	692.2	322.1 (100)	$[M+H-2TCA-H_2O]+$	35	340.2 (52)	35
FC_3	692.2	322.1 (88)	$[M+H-2TCA-H_2O]+$	35	340.2 (57)	35
FC_4	676.1	324.1 (100)	$[M+H-2TCA]+$	35	306.1 (78)	35

TCA = Tricarballylic acid moiety; CE = Collisional Energy.

HPLC separation of partially-hydrolyzed fumonisins.

RP-LC analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA). Chromatographic conditions were the following: the column was a 250 mm x 2.1 mm i.d., 5 μm, XTerra C18; 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 acetonitrile (eluent B) both acidified with 0.2% formic acid: initial condition at 100% A, 0-2 min isocratic step, 2-5 min linear gradient to 50% B, 5-20 min linear gradient to 100% B, 20-25 min isocratic step, 25-27 min linear gradient to 100% A and reequilibration step at 100% A for 13 min (total analysis time: 40 min).

MS spectrometric detection and characterization of partially-hydrolyzed fumonisins.

MS measurements were performed using a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, U.K.). MS parameters were the following: ESI+ (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 30 V; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow (nitrogen), 50 L/h and 700 L/h, respectively.

Total Ion Current (TIC) chromatograms were obtained by operating in Full Scan mode, detecting positive ions. The mass range was set between *m/z* 300-1000, with a total scan duration of 2.0 s and an inter-scan delay of 0.10 s.

The MS/MS experiments were performed at a collision energy ranging from 20 to 40 eV, acquiring in Daughter Scan mode. The mass range was set between *m/z* 100-600, with a scan duration of 0.5 s and an inter-scan delay of 0.05.

The detection of partially-hydrolyzed in synthetic media was achieved using a Multiple Reaction Monitoring (MRM) mode, by monitoring two transition for each analyte, as reported in Table 23.

Table 23. MRM conditions for LC-ESI-MS/MS analysis of partially-hydrolyzed fumonisins (relative abundances of mass ions in brackets).

	Compound-specific ions (m/z)					
Analyte	**Precursor ion $[M+H]^+$**	**Main transition**	**Identification**	**CE (eV)**	**Qualifier ion**	**CE (eV)**
$PHFB_1$	564.4	334.4 (80)	$[M+H-TCA-3H_2O]+$	30	352.4 (49)	30
$PHFB_2$	548.2	336.4 (100)	$[M+H-TCA-2H_2O]+$	30	354.2 (84)	25
$PHFB_3$	548.2	336.4 (100)	$[M+H-TCA-2H_2O]+$	30	354.2 (84)	25
$PHFB_4$	532.2	338.2 (100)	$[M+H-TCA-H_2O]+$	25	320.2 (96)	30

TCA = Tricarballylic acid moiety; CE = Collisional Energy.

Complete system control and data evaluation were performed with MassLynx 4.0 software (Micromass; Waters, Manchester, U.K.).

All the analytes were quantified using FB_1-calibration curves (calibration range 250-5000 μg/kg) since any analytical standard was commercially available.

5.4 Results and discussion

5.4.1 Detection and identification of several fumonisins analogues in *Fusarium* broth cultures by RP-HPLC-ESI-MS/MS

Although group B fumonisins are the best-know mycotoxins produced by *F. verticillioides* and *F. proliferatum*, several analogs belonging to the minor series (know as A- , C- and P-series) may be produced by these *Fusarium* species. Very recently, the detection of a large number of fumonisins analogues and fumonisins-like compounds produced by *F. verticillioides* cultures grown on long-grain rice has been reported (34). In this study, the fumonisin analogues were extracted from mycelia. The aim of the present work, on the contrary, is to identify and characterize the fumonisins analogues excreted into the synthetic media.

Broths were directly analyzed by RP-HPLC-ESI-MS/MS after sample clean up through Sep-Pak C18 cartridges, as described in the Experimental section, by operating in Full Scan mode to obtain a typical chromatographic profile, as reported in Figure 41.

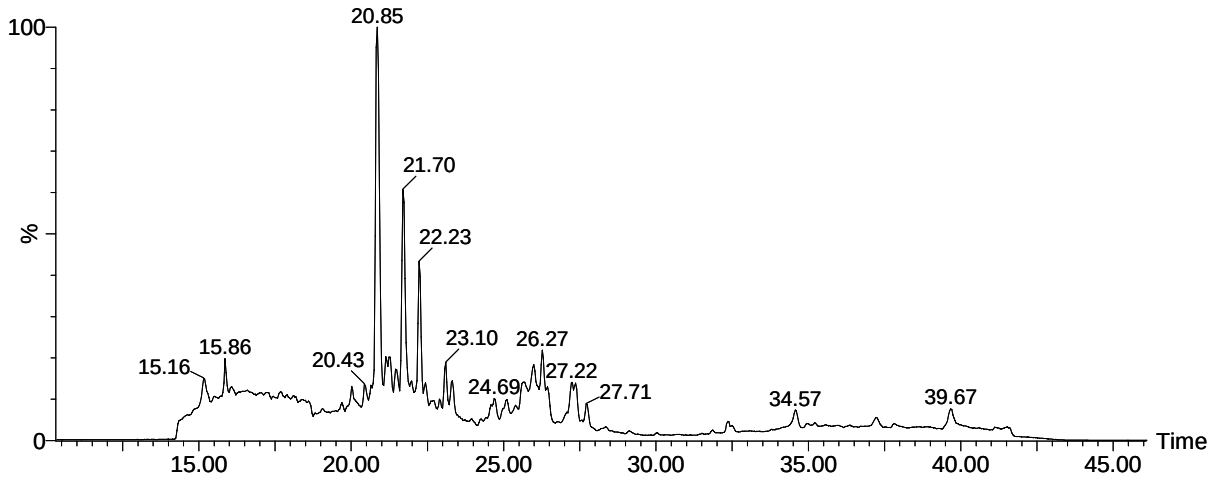


Figure 41. HPLC-ESI-MS/MS profile (Total Ion Current, TIC, chromatogram) obtained through Full Scan analysis of a contaminated culture broth. The three main chromatographic peaks (RT: 20.85, 21.70 and 22.23) correspond, in order, to FB_1, FB_3 and FB_2.

Thus, fumonisin analogs were detected by extracting for each one the *m/z* value corresponding to the molecular weight of the protonated molecule. Figure 42 shows the eXtracted Ion Current (XIC) chromatograms of the main fumonisin analogues.

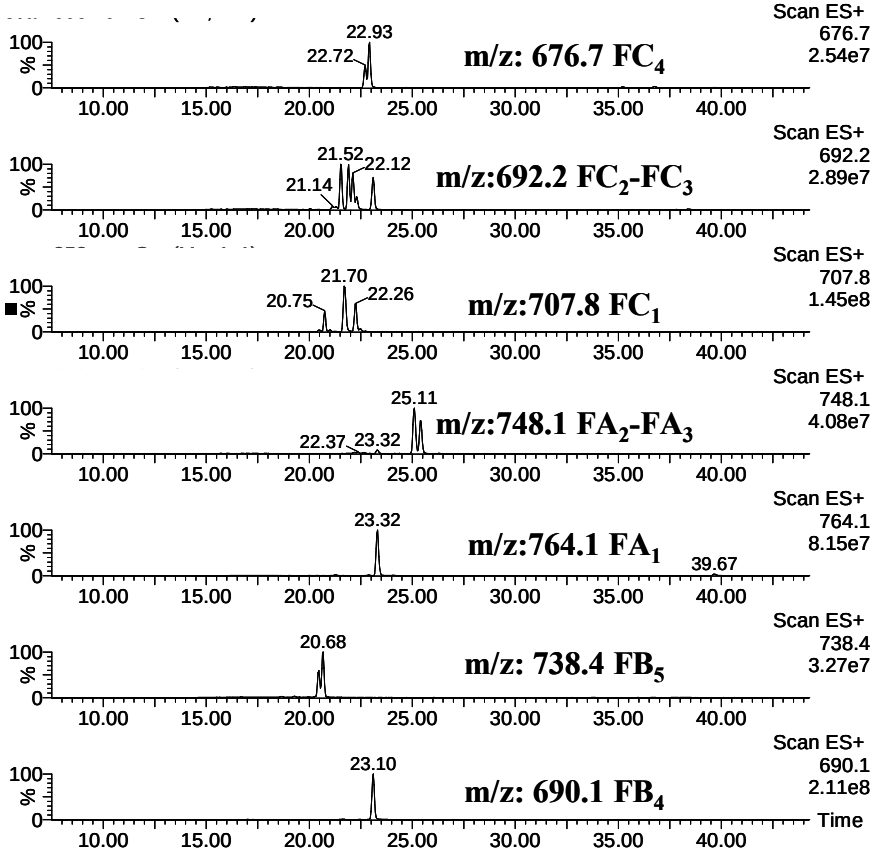


Figure 42. EXtracted Ion Current (XIC) chromatograms of nine fumonisins analogs.

As shown, FAs and FCs were detected in addition to FBs, while FPs do not occur in the examined broth.

Due to their chemical similarity, FBs, FAs and FCs lean to elute under same chromatographic conditions. Then, in order to obtain the finest separation of the analytes, thus avoiding interference during MS/MS experiments and method development, LC parameters were adjusted; the best conditions were found using a binary gradient of water and acetonitrile, both acidified with 0.2% formic acid, as described in "Materials and method". In this way, the separation of most of the toxins as well as the main isomers (e.g. $FA_{2\text{-}3}$, $FB_{2\text{-}3}$ and $FC_{2\text{-}3}$) has been obtained.

So as to univocally characterize each analyte, MS/MS experiments were performed in order to obtain product ions spectra due to the fragmentation of the selected molecular ions (see Figure 43 and Figure 44). The fragmentation patterns achieved were compared with those proposed by Bartók et al. (34), thus confirming the identity of each toxin.

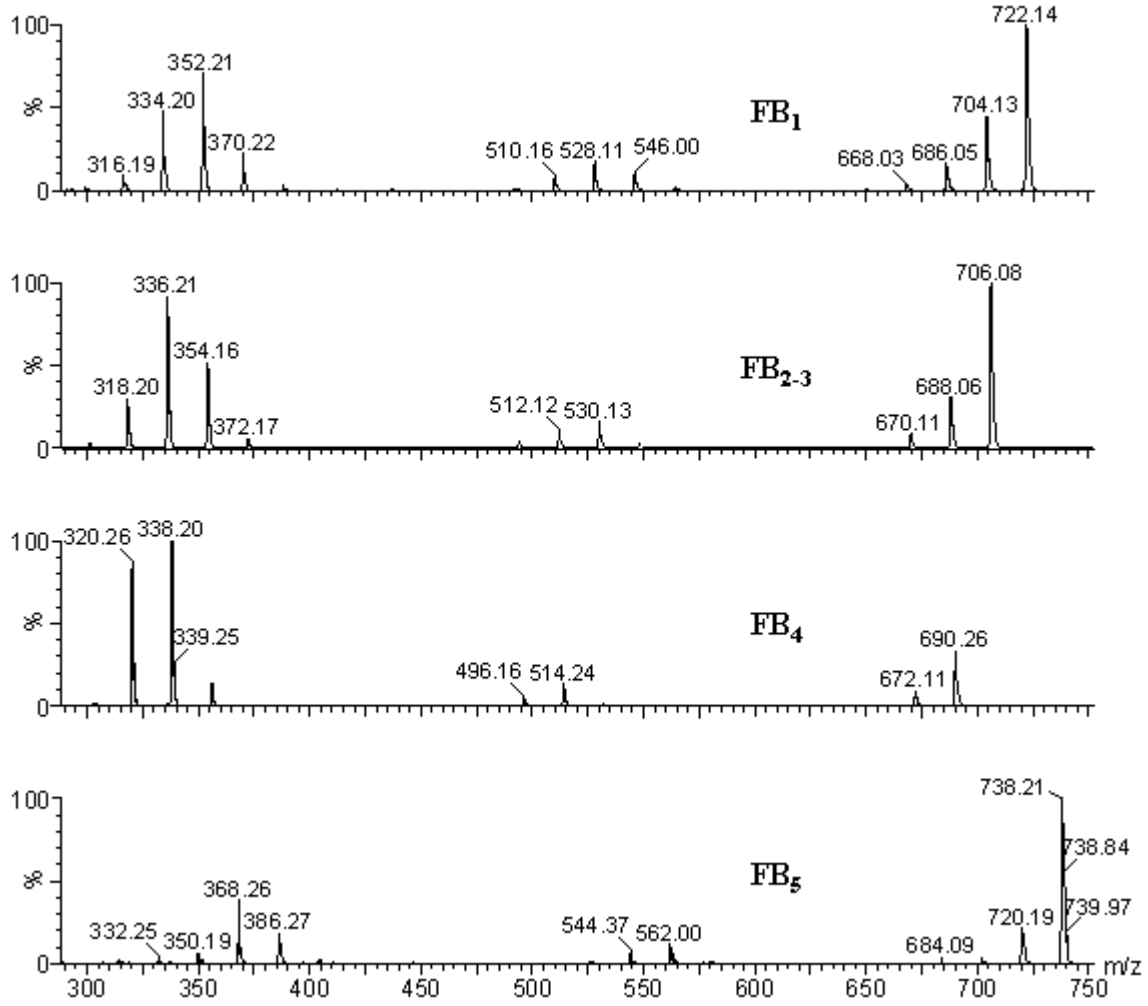


Figure 43. Product Ion spectra of group B fumonisins. Each experiment was performed using a collision energy value equal to 35 eV. Data were acquired in Daughter Scan mode.

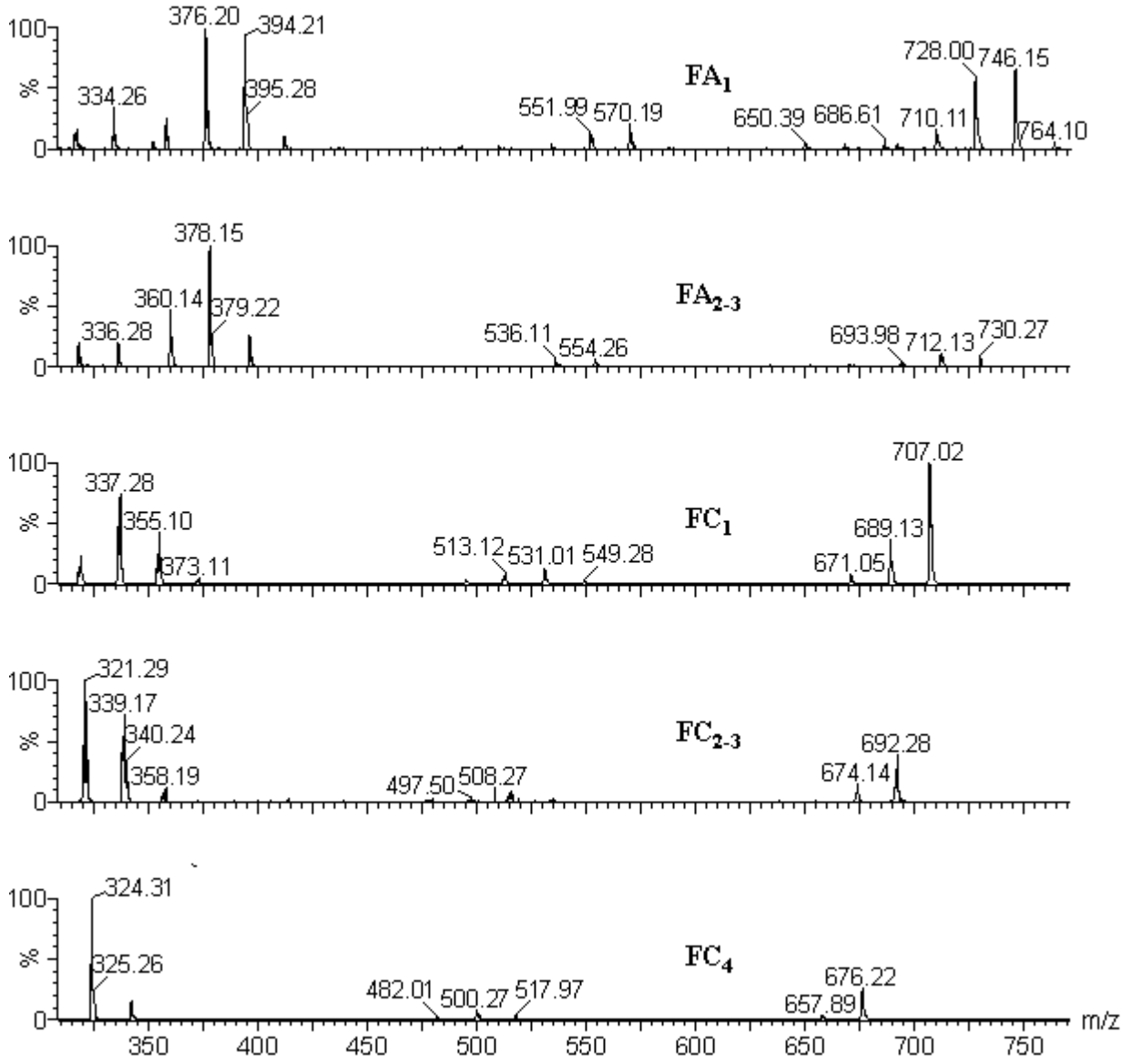


Figure 44. Product Ion spectra of A- and C-series fumonisins. Each experiment was performed using a collision energy value equal to 35 eV. Data were acquired in Daughter Scan mode.

Figure 43 and Figure 44 show the product ion spectra acquired after the fragmentation of the molecular ions of each mycotoxin. Characteristic ions of product ions spectra and their corresponding losses are reported in Table 24, Table 25 and Table 26.

Table 24. Characteristic ions of product ions spectra of fumonisins B series (relative abundances of mass ions in brackets, asterisks indicate the selected product ions).

Analyte	**Parent Ion**	**Analyte**	**Parent Ion**
FB_1	722.4	FB_2-FB_3	706.4
Fragment ions	**Corresponding losses**	**Fragment ions**	**Corresponding losses**
704.1 (47)	[M+H-H_2O]+	688.2 (30)	[M+H-H_2O]+
686.1 (17)	[M+H-2H_2O]+	670.3 (11)	[M+H-2H_2O]+
667.8 (5)	[M+H-3H_2O]+	548.2 (5)	[M+H-TCAK]+
546.0 (14)	[M+H-TCA]+	530.0 (17)	[M+H-TCA]+
528.1 (21)	[M+H-TCA-H_2O]+	511.9 (12)	[M+H-TCA-H_2O]+
510.1 (10)	[M+H-TCA-2H_2O]+	493.9 (5)	[M+H-TCA-2H_2O]+
370.2 (25)	[M+H-2TCA]+	372.3 (8)	[M+H-TCA-TCAK]+
352.4 (76)**	[M+H-2TCA-H_2O]+	354.4 (53)**	[M+H-2TCA]+
334.4 (53)**	[M+H-2TCA-2H_2O]+	336.4 (90)**	[M+H-2TCA-H_2O]+
		318.4 (31)	[M+H-2TCA-2H_2O]+
Analyte	**Parent Ion**	**Analyte**	**Parent Ion**
FB_4	690.1	FB_5	738.4
Fragment ions	**Corresponding losses**	**Fragment ions**	**Corresponding losses**
672.1 (10)	[M+H-H_2O]+	720.1 (30)	[M+H-H_2O]+
532.1 (4)	[M+H-TCAK]+	580.8 (6)	[M+H-TCAK]+
514.1 (14)	[M+H-TCA]+	562.9 (20)	[M+H-TCA]+
496.1 (8)	[M+H-TCA-H_2O]+	543.8 (9)	[M+H-TCA-H_2O]+
356.0 (15)	[M+H-TCA-TCAK]+	526.8 (5)	[M+H-TCA-2H_2O]
338.1 (100)**	[M+H-2TCA]+	386.2 (29)	[M+H-2TCA]+
320.2 (90)**	[M+H-2TCA-H_2O]+	368.1(27)**	[M+H-2TCA-H_2O]+

** Selected product ion for the subsequent LC-MS/MS method development

Table 25. Characteristic ions of product ions spectra of fumonisins A series. (relative abundances of mass ions in brackets, asterisks indicate the selected product ions).

Analyte	**Parent Ion**	**Analyte**	**Parent Ion**
FA_1	764.1	**FA_2, FA_3**	748.1
Fragment ions	**Corresponding losses**	**Fragment ions**	**Corresponding losses**
746.4 (77)	[M+H-H_2O]+	730.2 (16)	[M+H-H_2O]+
727.8 (52)	[M+H-2H_2O]+	712.2 (9)	[M+H-2H_2O]+
709.9 (14)	[M+H-3H_2O]+	670.1 (7)	[M+H-AcOH-H_2O]+
650.2 (13)	[M+H-AcOH-3H_2O]+	634.3 (3)	[M+H-AcOH-3H_2O]+
570.3 (20)	[M+H-TCA-H_2O]+	396.3 (42)	[M+H-2TCA]+
551.8 (18)	[M+H-TCA-2H_2O]+	378.1 (98)	[M+H-2TCA-H_2O]+
412.2 (25)	[M+H-2TCA]+	336.1 (100)**	[M+H-2TCA-AcOH]+
394.1 (100)**	[M+H-2TCA-H_2O]+	318.1 (41)**	[M+H-2TCA-AcOH-H_2O]+
334.1(45)**	[M+H-2TCA-AcOH-H_2O]+		
316.1 (18)	[M+H-2TCA-AcOH-H_2O]+		

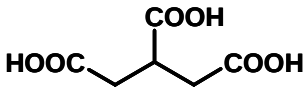
** Selected product ion for the subsequent LC-MS/MS method development

Table 26. Characteristic ions of product ions spectra of fumonisins C series (relative abundances of mass ions in brackets, asterisks indicate the selected product ions).

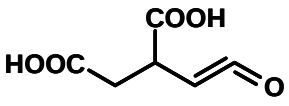
Analyte	**Parent Ion**	**Analyte**	**Parent Ion**
FC_1	707.8	**FC_2, FC_3**	692.2
Fragment ions	**Corresponding losses**	**Fragment ions**	**Corresponding losses**
689.3 (39)	[M+H-H_2O]+	674.8 (44)	[M+H-H_2O]+
495.9 (10)	[M+H-TCA-2H_2O]+	656.3 (18)	[M+H-2H_2O]+
355.8 (55)	[M+H-2TCA]+	515.8 (23)	[M+H-TCA]+
337.8 (71)**	[M+H-2TCA-H_2O]+	498.1 (22)	[M+H-TCA-H_2O]+
319.1(23)**	[M+H-2TCA-2H_2O]+	480.3 (13)	[M+H-TCA-2H_2O]+
Analyte	**Parent Ion**	358.6 (22)	[M+H-TCA-TCAK]+
FC_4	676.1	340.1 (100)**	[M+H-2TCA]+
Fragment ions	**Corresponding losses**	322.1 (94)**	[M+H-2TCA-H_2O]+
657.9 (8)	[M+H-H_2O]+	304.2 (41)	[M+H-2TCA-2H_2O]+
517.9 (9)	[M+H-TCAK]+		
500.2 (12)	[M+H-TCA]+		
342.1 (6)	[M+H-TCA-TCAK]+		
324.1 (100)**	[M+H-2TCA]+		
306.1 (38)**	[M+H-2TCA-H_2O]+		

** Selected product ion for the subsequent LC-MS/MS method development

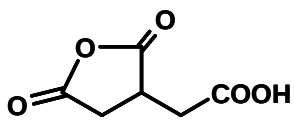
As shown, the neutral losses obtained upon the fragmentation of the molecular ions are very similar for all the toxins studied. Bartók et al. (34) proposed the theoretical CID fragmentation patterns of $[FB_1+ H]^+$, $[FA_1+ H]^+$ and $[FC_1+ H]^+$. The three fragmentation patterns suggested are very similar: in the course of fragmentation TCA units were gradually eliminated along with H_2O, through the concomitant formation of the corresponding anhydride (TCAD, 158 Da) or via their conversion in the form of ketene (TCAK, 158 Da). Molecular structures of tricarballylic acid and its derived anhydride and ketene are reported in Figure 45. In each case, all the fragmentation pathways leads to obtain the central backbone deprived of the terminal amino group.



Tricarballylic acid
(TCA, 176 Da)



Ketene form of tricarballylic acid
(TCAK, 158 Da)



Tricarballylic acid anhydride
(TCAD, 158 Da)

Figure 45. Molecular structures of the neutral losses of 176 and 158 corresponding, respectively, to the deprivation of a tricarballylic acid unit or its anhydride or the ketene form from parent ions (34).

5.4.2 Set up of the MS conditions for MRM monitoring

In addition to molecular characterization, MS/MS experiments allow to detect the most abundant product ions for each analyte (see Table 24, Table 25, Table 26). For most mycotoxins, the strongest transitions were the loss of both the tricarballylic units together with one or two water molecules. Thus, in order to develop a LC-MS/MS method based on MRM acquisition, MS conditions were adjusted to maximize the signals corresponding to the selected product ions.

Due to the lack of analytical standard solutions of the minor fumonisins studied in this work, MS parameters were optimized by performing several MS/MS experiments at different collision energy by directly using the growing broth. The engagement of in-column separation allows for the optimization of MS parameters by working directly with the conditions wherein analytes were eluted from the column, such as solvent composition. Moreover, avoiding the directly MS infusion of a mycotoxin mixture, the effects of ionic suppression and competition for charges exerted by each analyte toward others has been minimized.

Thus, product ion spectra were collected by applying a collision energy ranging from 20 eV to 40 eV to the precursor ion of each analyte and the intensities of the selected ions produced were compared. As example, product ion spectra recorded for FA_1 and FC_1 at different collision energy are reported in Figure 46 and Figure 47.

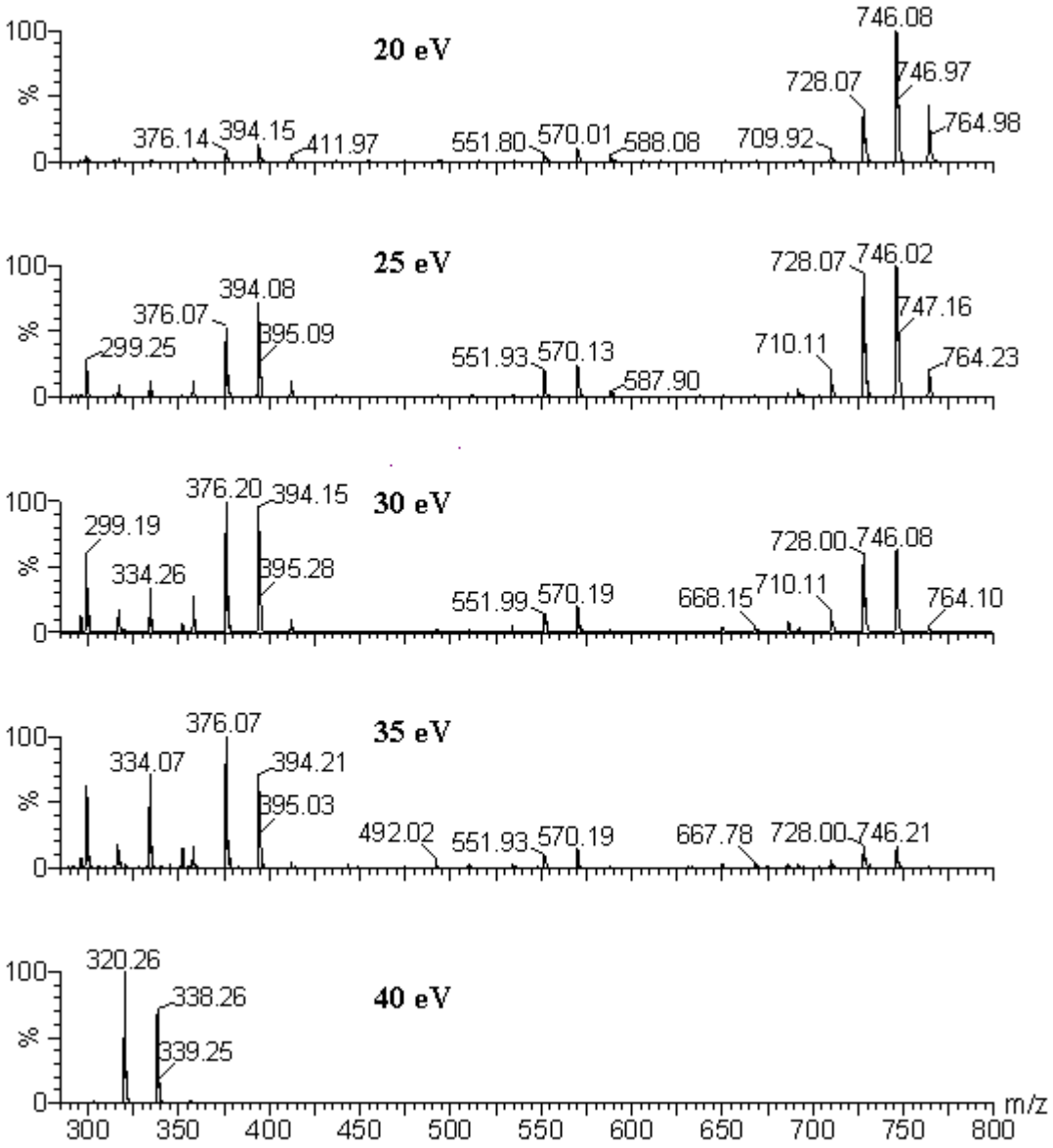


Figure 46. Product ion spectra recorded for FA_1 (Daughter Scan mode). Influence of the collision energy applied to the precursor ion on the profile of the produced fragments.

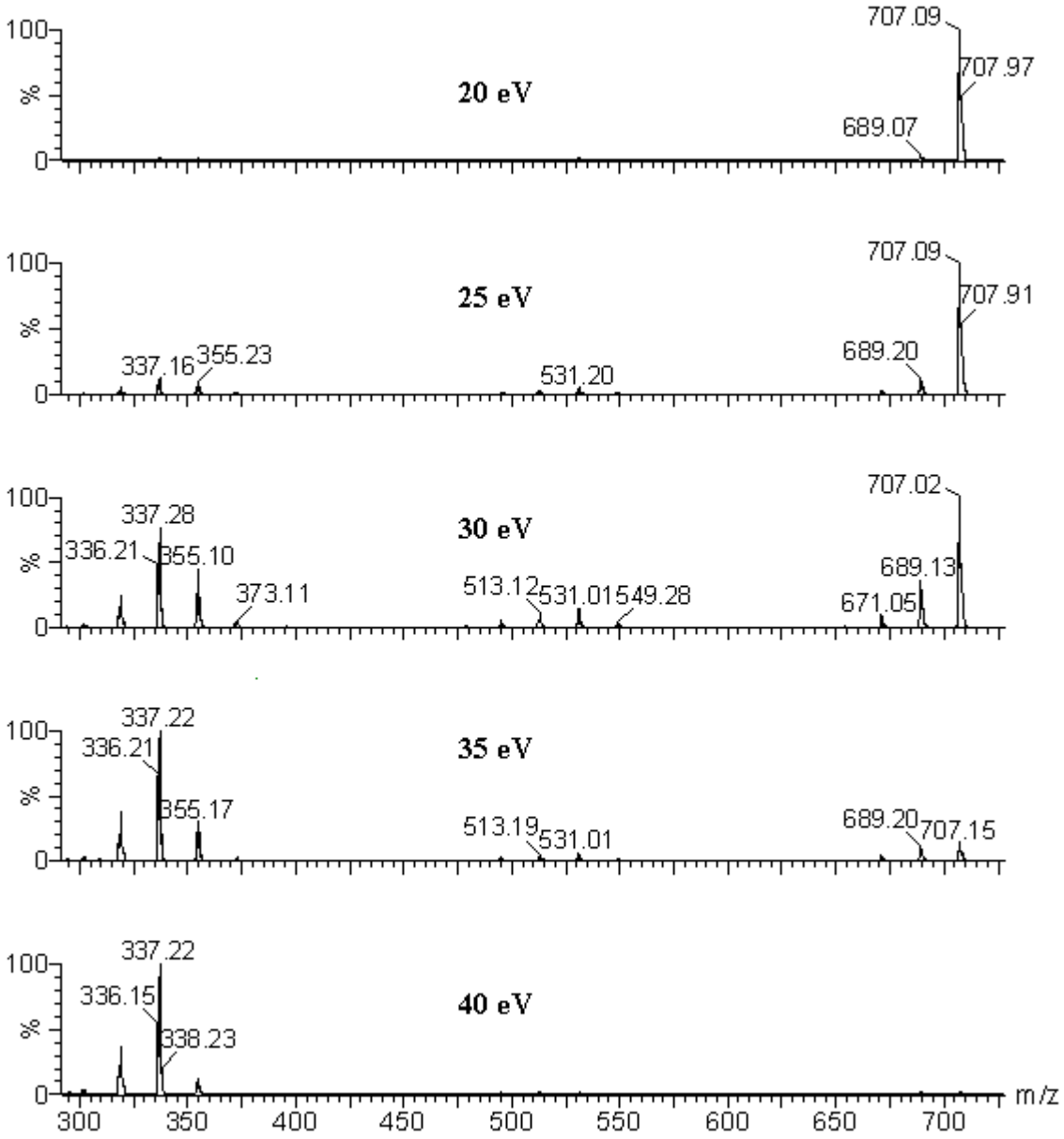


Figure 47. Product ion spectra recorded for FC_1 (Daughter Scan mode). Influence of the collision energy applied to the precursor ion on the profile of the produced fragments.

Finally, appropriate collision energy to optimize the chosen transitions was selected and a MS/MS method based on MRM acquisitions has been developed. The transition monitored for each analyte and the collision energy used are reported in "Material and methods" (see Table 22). A typical LC-MS/MS chromatogram is reported in Figure 48. In addition to fumonisins belonging to the B-series, also FAs and FCs have been detected. Multiple peaks observable for FC_1 and $FC_{2,3}$ are probably due to the presence of iso-forms.

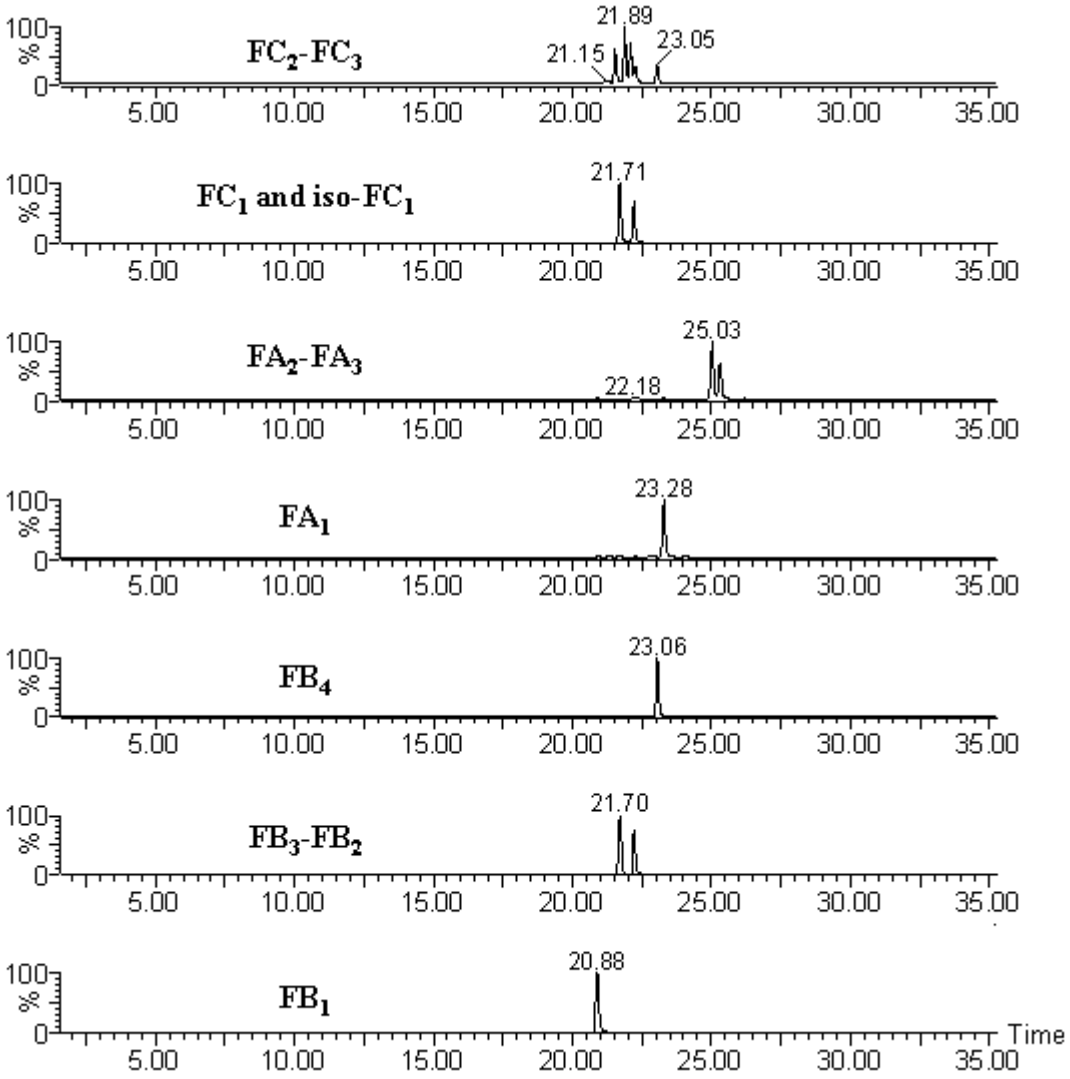


Figure 48. Typical LC-ESI-MS/MS chromatogram obtained from the analysis of a *Fusarium verticillioides* culture broth.

5.4.3 Detection and characterization of fumonisins derivatives in *Fusarium* broth cultures: developed of a LC-ESI-MS/MS method for the analysis of partially-hydrolyzed fumonisins

Fumonisins derivatives were researched in the same *F. verticillioides* broth cultures previously analyzed for parent fumonisins analogue determination. Since hydrolysis or partial hydrolysis are the main modifications that involve fumonisin structure, with the cleavage of one or both tricarballylic moieties, hydrolyzed (HFs) and partially hydrolyzed (PHFs) forms have been determined. As example, the structure of a generic partially hydrolyzed fumonisin B is reported in Figure 49.

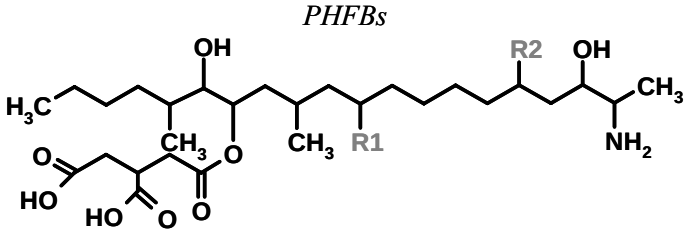


Figure 49. Partially hydrolyzed fumonisin B (PHFBS) structure.

HFs and PHFs were researched by extracting the *m/z* values corresponding to the molecular weights of the protonated molecules from the Total Ion Current chromatogram.

Whereas partially-hydrolyzed derivatives of group B fumonisins have been detected (see Figure 50), PHFAs and PHFCs do not found. Likewise, hydrolyzed fumonisins have not been detected.

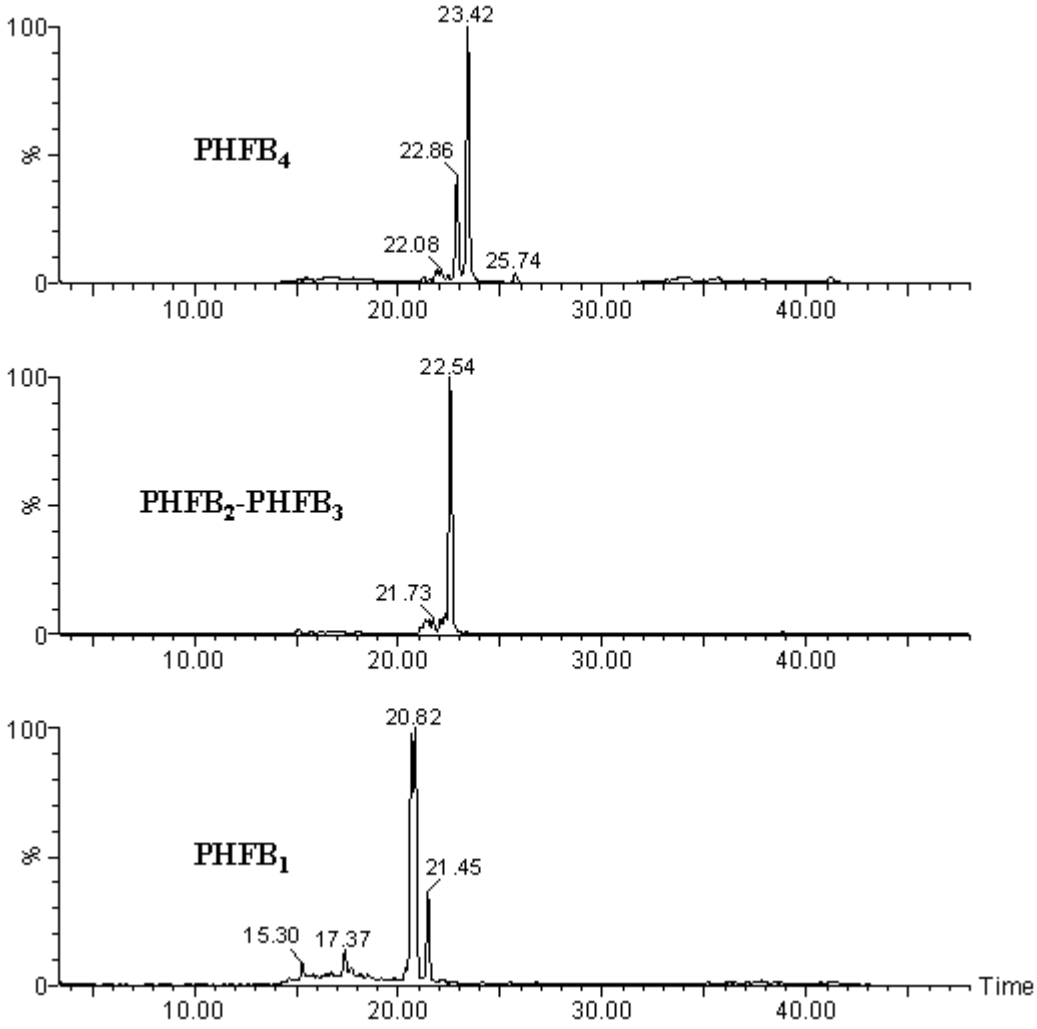


Figure 50. EXtracted Ion Current (XIC) chromatograms of four partially-hydrolyzed fumonisins..

Then, fumonisins derivatives detected were characterized by means of MS/MS experiments, acquiring data in Daughter Scan mode. The fragmentation pattern of each analyte, obtained upon the collection of product ion spectra, has been compared with those reported by Bartók et al. (34), allowing both spectra interpretation and a univocal identification of each form. Product ion spectra obtained upon the fragmentation of each precursor ion are reported in Figure 51, while the assignment of the losses corresponding to each fragment is reported in Table 27.

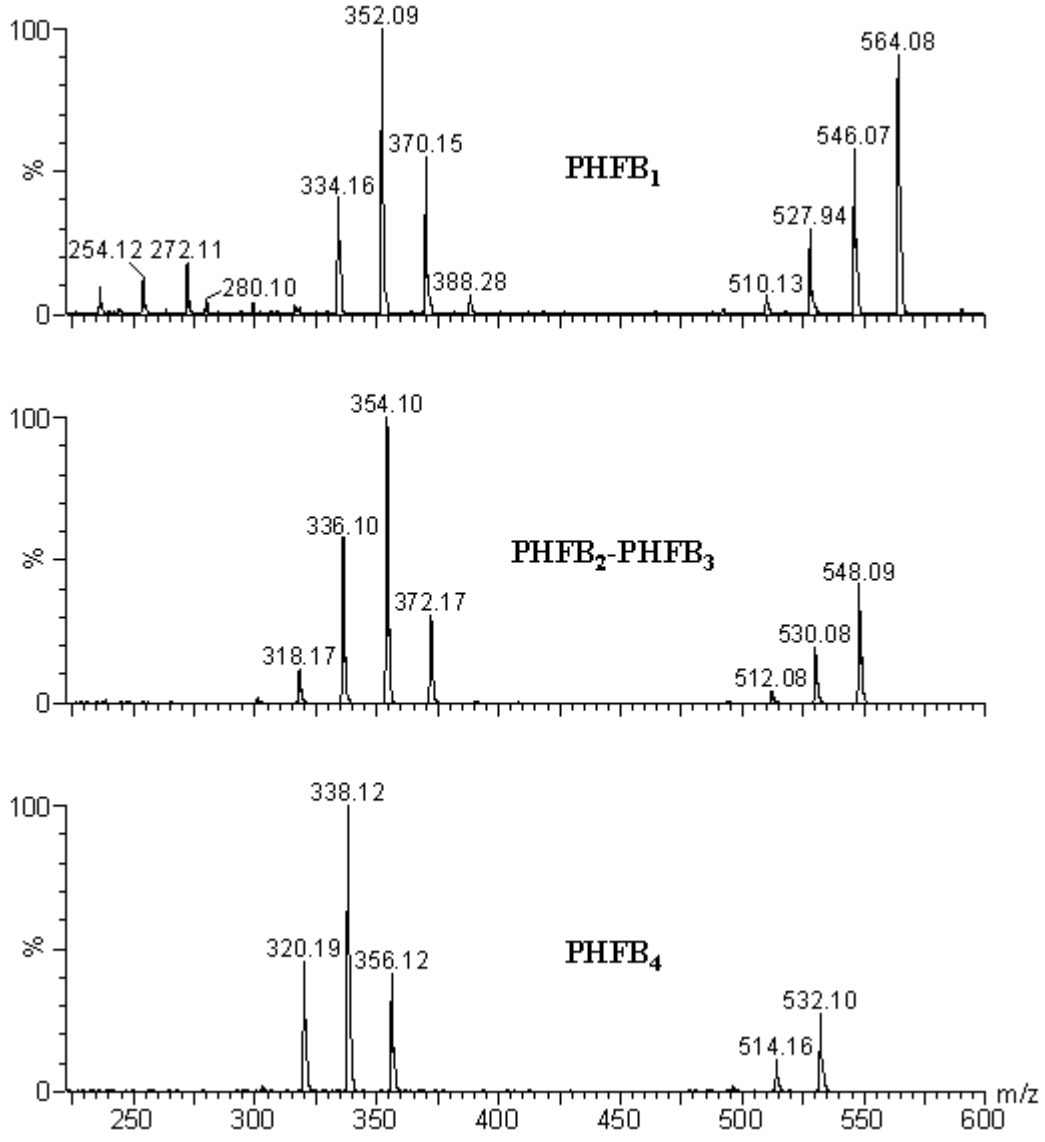


Figure 51. Product ion spectra obtained from $PHFB_1$, $PHFB_{2-3}$ and $PHFB_4$ upon the application of the collision energy to the precursor ion. Acquisition in Daughter scan mode.

Table 27. Characteristic ions of product ions spectra of partially hydrolyzed fumonisins (relative abundances of mass ions in brackets asterisks indicate the most abundant product ions).

Analyte	**Parent Ion**
$PHFB_1$	564.4
Fragment ions	**Corresponding losses**
546.1 (54)	$[M+H-H_2O]+$
527.9 (27)	$[M+H-2H_2O]+$
510.1 (16)	$[M+H-3H_2O]+$
388.2 (9)	[M+H-TCA]+
370.1 (59)	$[M+H-TCA-H_2O]+$
352.1 (62)**	$[M+H-TCA-2H_2O]+$
334.2 (60)**	$[M+H-TCA-3H_2O]+$
Analyte	**Parent Ion**
$PHFB_2$-$PHFB_3$	548.1
Fragment ions	**Corresponding losses**
530.1 (27)	$[M+H-H_2O]+$
512.1 (6)	$[M+H-2H_2O]+$
372.2 (32)	[M+H-TCA]+
354.1 (100)**	$[M+H-TCA-H_2O]+$
336.1 (59)**	$[M+H-TCA-2H_2O]+$
318.2 (19)	$[M+H-TCA-2H_2O]+$
Analyte	**Parent Ion**
$PHFB_4$	532.1
Fragment ions	**Corresponding losses**
514.1 (20)	$[M+H-H_2O]+$
356.1 (50)	[M+H-TCA]+
338.1 (100)**	$[M+H-TCA-H_2O]+$
320.2 (62)**	$[M+H-TCA-2H_2O]+$
** Selected product ion for the subsequent LC-MS/MS method development	

Similarly to what observed for parent FBs, also partially-hydrolyzed forms are characterized by comparable losses during the fragmentation of their molecular ions. Moreover, like to their precursors, most abundant signals were provided by fragments deriving upon the loss of the tricarballylic unit in conjunction with water molecules.

After MS/MS characterizations of derivatives, most abundant product ions were selected and MS conditions were adjusted to maximize the abundance of their signals, as already reported for their parent compounds (see paragraph 5.4.2): different collision energy values were applied to each precursor ion and the intensities of previously selected fragments have been compared.

Figure 52 shows a typical LC-MS/MS chromatogram obtained by the analysis of partially hydrolyzed fumonisins in a *F. verticillioides* culture broth. In this case, positional isomers $PHFB_2$ and $PHFB_3$ were not separated under the selected LC conditions, while two iso-forms of $PHFB_4$ can be easily distinguished

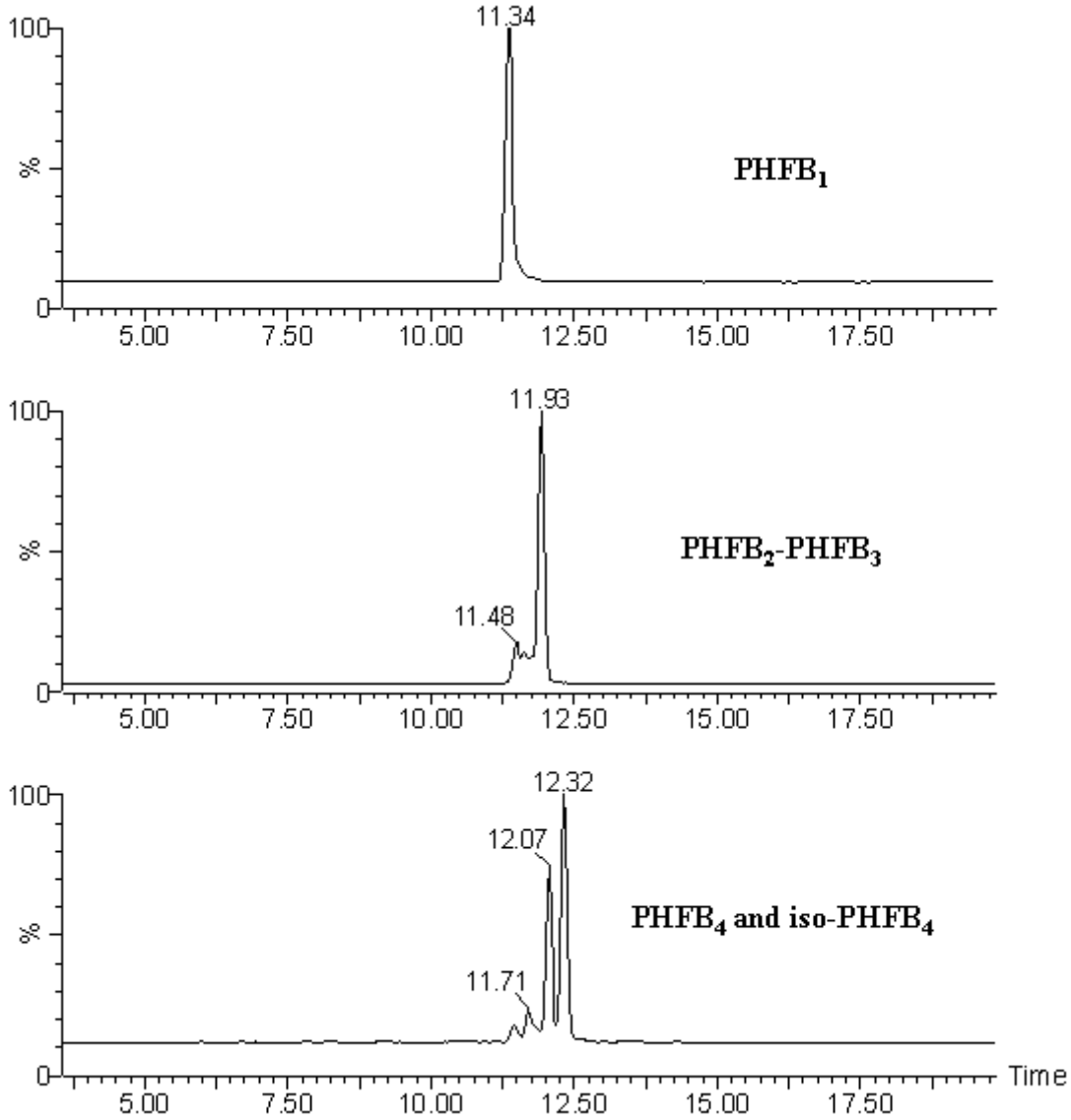


Figure 52. Typical LC-ESI-MS/MS chromatogram obtained from the analysis of partially hydrolyzed derivatives of fumonisins in *F. verticillioides* culture broth.

5.5 Conclusions

The LC-ESI-MS/MS method for the analysis of fumonisin analogues here described allows for the multiresidual determination of fumonisins belonging to the B-series, as well as to minor series, A and C. Indeed, in addition to the main fumonisins produced by *F. verticillioides*, also secondary compounds such as FB_4, FB_5, FAs and FCs can be detect. Although these toxins are less abundant than the three main analogues, their analysis in *Fusarium* cultures may play an important role in fumonisins biosynthetic studies. For example, FB_4 is the main precursor of FB_2 and FB_3, from which FB_1 derives. Concerning FAs and FCs, any information is available for their biosynthetic pathway, but their biosynthesis

may be strictly related to the production of B-series fumonisins. Thus, the availability of a method of analysis that allows the simultaneous detection of several fumonisins analogues and precursors into the synthetic media used to grown the mould of interest, may lead to better define the biosynthetic pathway of these compounds.

During this study, also a LC-ESI-MS/MS method for the determination of partially-hydrolyzed forms has been developed, allowing the analysis of such modified forms. Similarly to the method described earlier, this one may be employed to better define the fate of mycotoxins during fungal growth, their modifications and the reasons for which their conversion takes place.

CHAPTER 6 MONITORING FUMONISIN ANALOGUES PRODUCTION BY *FUSARIUM* SPECIES UNDER DIFFERENT GROWTH PARAMETERS.

6.1 INTRODUCTION

Fusarium verticillioides and *F. proliferatum* are fungal pathogens known to colonize maize, producing fumonisins as toxic secondary metabolites (2). Whereas *F. verticillioides* is able to produce several fumonisin analogues, such as fumonisins B (FBs), fumonisins A (FAs), fumonisins C (FCs) and fumonisins P (FPs), *F. proliferatum* is known to produce only fumonisins A- and B-series and FC_1 (1).

Among all the fumonisins analogs grouped into the four series early mentioned, FB_1, FB_2 and FB_3 are the major mycotoxins produced in corn and are able to cause severe diseases in animals. Moreover, consumption of fumonisins has been associated with elevated human oesophageal cancer incidence in various part of Africa, Central America and Asia (47). For these reasons FBs are the widely studied analogues, while FAs, FCs and FPs have never received a great attention.

Fumonisin B synthesis is regulated by 17 genes grouped in the so-called *FUM* cluster, 10 of which act directly on the biosynthetic pathway (7). The biosynthesis starts with a linear polyketide with 2 methyl groups and a terminal carbonyl one that is condensed with alanine; the following steps comprise the reduction of the carbonyl group to a hydroxyl one, hydroxylation of 2-4 polyketide carbons and esterification of 6 carbon tricarboxylic acids to 2 of the hydroxyls to finally form the active FBs (6).

To date, several authors reported the influence of various factors such as fungal genotype, pH, activity water (a_w), oxygen, temperature, incubation period and presence of fungicides on the FBs production by *F. verticillioides* and *F. proliferatum* (22, 25, 26, 29, 53, 56), however focusing the attention on the naturally occurring B analogues.

Indeed, concerning FAs, FCs and FPs, no information are available on their biosynthetic pathway as well as the influence of the cited factors. Very recently Bartók et al. (34) reported the occurrence of FAs and FCs in solid cultures of *F. verticillioides* strains, but there are no

data concerning the dynamic of their production in time and in comparison with the production of B-series.

In recent years the occurrence of many structurally related compounds generated by plants metabolism or by food processing, called “masked” or “hidden” fumonisins has been demonstrated both in raw maize and in maize-based products, thus posing a serious problem concerning food safety (48). Hidden fumonisins, in fact, are fumonisins derivatives covalently or not covalently linked with various food macro constituents, which can co-exist with parent forms. Since these compounds may a have different chemical behaviour compared with their precursors, they easily escape routine analyses, thus they are detectable only using an hydrolytic approach. This treatment allows both the complete hydrolysis of all matrix constituents and, at the same time, the separation of the tricarballilic moieties from the central backbone of the toxin, thus releasing hydrolyzed forms (49). The most interesting aspect of this phenomenon is that the nature of fumonisins-matrix interactions (covalent or not) is still not clearly explained. Until recently, the formation of covalent bonds among the functional groups of fumonisins and the hydroxyl groups of starch or the amino or sulfidryl groups of the side chains of amino acids in proteins during thermal treatments was considered the main cause of masking phenomenon (44, 50, 51). Nevertheless, the occurrence of hidden fumonisins in raw maize and in mild treated products has been recently reported in literature (49, 52), suggesting that other types of interaction may take place between the target compound and maize macromolecules. Moreover, the hidden fumonisins occurrence in raw maize indicates that masking takes also in field, as a result of plant-pathogen interaction. Therefore, the probable role played both by mould and plant is a topic of great interest in order to obtain more information concerning which are the factors involved in fumonisins masking phenomenon and how it is governed.

6.2 Aim of the work

The present study aimed to evaluate the occurrence of FBs produced by *F. verticillioides* both into a synthetic medium and a maize-based medium, investigating also hidden forms to assess the role of a complex matrix lacking of enzymatic activity in masking phenomenon.

Moreover, the production of FBs as well as of other analogues, A- and C- series, both in *F. verticillioides* and *F. proliferatum* culture broths in different a_w regimes and incubation time

was evaluated, in order to collect more information regarding the production pattern of minor analogs in relation to the biosynthesis of FBs.

6.3 Materials and methods

6.3.1 Chemicals

Fumonisin B_1 in powder, 5 mg, was purchased from Romerlabs (Tulln, Austria). Methanol (LC grade) was obtained from Carlo Erba (Milan, Italy) and acetonitrile (LC grade) was from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA).

6.3.2 Fungal isolates and media

The fungal isolates used in this study are two FB-producers belonging to the species *F. verticillioides* and *F. proliferatum* and collected in the fungal collection of the Institute of Entomology and Plant Pathology-UCSC, Piacenza (294 and 289 MPVP), and of the Institute of Sciences of Food Production-CNR, Bari (10027 and 10026 ITEM; http://server.ispa.cnr.it/ITEM/Collection) and preserved by crio-conservation in water and glycerol (18%) at –80°C and liquid nitrogen. Both strains have been isolated on maize crop from South Tuscany, Italy.

Strains were grown on Potato Dextrose Agar (PDA, Oxoid, Cambridge , UK) at 25°C for 7 days in the dark, then 10 ml of sterile distilled water was added to each plate and the mycelium was gently scraped to collect fungal conidia. The suspension was adjusted to 10^6 conidia ml^{-1} and 100 µl of conidial suspension were used as inoculum for liquid cultures. A tassel, Ø 2 mm, was collected from the whole PDA plate and used as inoculum for solid cultures.

6.3.3 Experimental procedures

Fumonisins production on synthetic media

The conidial suspension of both strains was inoculated on static liquid culture of Malt Extract Agar (MEA), known to be a FB-inducing medium (45, 46), and incubated 21-30-45 days at 25°C in the dark, adjusting the aw in the range 0.955-0.990 with the addition of glycerol. The trial was run in triplicate.

At the end of incubation, the fresh mycelium was separated from the liquid medium, dried under vacuum (Whatman #4 filter, Ø 24 cm, Dassel, Germany) and frozen in liquid nitrogen. The medium was stored at -20°C and then used for FB analysis.

Fumonisins production on maize-based media

The two strains were inoculated on a solid maize-based medium obtained by boiling 108 g maize meal (polenta) in 1 L sterile distilled water until reaching thickness, then cooled in 90 mm Ø plates. Two inoculation methods were used: the tassel was directly inoculated on the medium surface, or on the medium covered by a cellophane sheet (P400; Cannings, Ltd., Bristol, UK) to facilitate removal of the fungal biomass.

Strains were incubated for 21 days at 25°C in the dark, making triplicates for each ecological condition; cultures where stored at -20°C.

Water activity of both liquid and solid media were measured in triplicate using the Aqualab LITE (Decagon, Pullman, WA, USA), according to the manufacturer instructions.

Sample preparation for the analysis of fumonisins in synthetic media

An aliquot (4 mL) of liquid medium (previously separated from fungal conidia) was subjected to a clean-up through Sep-Pak C18 cartridges before LC-ESI-MS/MS analysis, as described in 5.3.3.

Sample preparation for the analysis of total fumonisins after hydrolysis in synthetic media

An aliquot (2 mL) of liquid medium was stirred with 18 mL of 2M KOH for 3 min using a high-speed blender (Ultraturrax T18; IKA, Stauffen, Germany) and then stirred for 60 min at room temperature. Then, 20 mL of acetonitrile was added and after stirring for 3 min two layers were formed which were separated by centrifugation at 3500 rpm for 15 min. A 4mL portion of the acetonitrile-rich upper layer was evaporated to dryness under a stream of compressed air, and the residue was redissolved in 200 µL of water/methanol, 30:70 v/v, prior to LC-MS/MS. Fumonisins obtained after sample hydrolysis were measured as the sum of hydrolyzed FB_1, FB_2, and FB_3. All of the results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of parent and hydrolyzed compounds and referred to as "total fumonisins after hydrolysis".

LC-MS/MS analysis for the determination FB, FA and FC analogs.

LC-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Quattro API triple quadrupole mass spectrometer with

an electrospray source (Micromass; Waters, Manchester, U.K.). Chromatographic conditions were the following: the column was a 250 mm x 2.1 mm i.d., 5 μm, XTerra C18; 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 acetonitrile (eluent B) both acidified with 0.2% formic acid: initial condition at 100% A, 0-5 min isocratic step, 5-30 min linear gradient to 100% B, 30-35 min isocratic step, 35-36 min linear gradient to 100% A and reequilibration step at 100% A for 14 min (total analysis time: 50 min). MS parameters: ESI+ (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 30 V; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow (nitrogen), 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 and 722.4→352.4 for FB_1, 706.4→336.4 and 706→318.4 for FB_2 and FB_3, 690.10→320.10 and 690.10→338.20 for FB_4, 738.4→368.1 and 738.4→350.1 for FB_5, 764.1→334.1 and 764.1→394.1 for FA_1, 748.1→336.1 and 748.1→378.1 for FA_2 and FA_3, 707.8→337.1 and 707.8→319.1 for FC_1, 692.2→322.2 and 692.2→340.2 for FC_2 and FC_3, 676.7→324.1 and 676.7→306.1 for FC_4. A Collision Energy of 35 eV was chosen for each transition. For each analyte, the first transition was used for quantification, while the second transition was chosen as qualifier. All the analytes were quantified using FB_1-calibration curves (calibration range 250-5000 μg/kg).

LC-MS/MS conditions for the analysis of partially-hydrolyzed fumonisins.

. LC-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, U.K.). Chromatographic conditions were the following: the column was a 250 mm x 2.1 mm i.d., 5 μm, XTerra C18; 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 acetonitrile (eluent B) both acidified with 0.2% formic acid: initial condition at 100% A, 0-2 min isocratic step, 2-5 min linear gradient to 50% B, 5-20 min linear gradient to 100% B, 20-25 min isocratic step, 25-27 min linear gradient to 100% A and reequilibration step at 100% A for 13 min (total analysis time: 40 min). MS parameters: ESI+ (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 30 V; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow (nitrogen), 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 and 722.4→352.4 (CE 35 eV) for FB_1, 564.1→334.4 and 564.1→352.2 (CE 30 eV) for $PHFB_1$, 548.2→336.4 (CE 30 eV) and 548.2→354.20 (CE 25 eV) for $PHFB_2$ and $PHFB_3$, 532.2→338.2 (CE 25 eV) and 532.2→320.2 (CE 30 eV) for $PHFB_4$. For each analyte, the first transition was was used for quantification, while the second transition was chosen as qualifier. All the analytes were quantified using FB1-calibration curves (calibration range 250-5000 μg/Kg).

Sample preparation for fumonisins determination in maize-based media

2g of previously homogenised sample were weighted in a centrifuge tube, blended in a high-speed blender (Ultraturrax T18; IKA, Stauffen, Germany) using 8 mL of water/methanol, 30:70 v/v, for 1 min at 14000 rpm and then centrifuged at 3500 rpm for 15 min. Next, an aliquot (50 μL) of supernatant was diluted with 450 μL of water/methanol, 30:70 v/v, before LC-MS/MS analysis.

Sample preparation for the analysis of hydrolyzed fumonisins in maize-based media

Aliquots (2 g) of the maize-based media were blended in high-speed blender (Ultraturrax T18; IKA, Stauffen, Germany) with 20 mL of 2 M KOH for 1 min at 14000 rpm and then stirred for 60 min. Then, 20 mL of acetonitrile were added and after stirring for 10 min, two layers were formed which were separated by centrifugation at 3500 rpm for 15 min. A μL portion of the acetonitrile-rich upper layer was evaporated to dryness under a stream of compressed air, and the residue was redissolved in 800μL water/methanol, 30:70 v/v and analyzed by LC-MS/MS. Fumonisins obtained after sample hydrolysis were measured as the sum of hydrolyzed FB_1, FB_2, and FB_3. All of the results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of parent and hydrolyzed compounds and referred to as "total fumonisins after hydrolysis".

LC-MS/MS Analysis for the determination of parent and total fumonisins after hydrolysis

LC-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, U.K.). Chromatographic conditions were the following: the column was a 250 mm x 2.1 mm i.d., 5 μm, XTerra C18; the flow rate was 0.2mL/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, 0-2 min isocratic step, 2-5

min linear gradient to 45% B, 5-25 min linear gradient to 90% B, 25-35 min isocratic step at 90%B, 35-36min linear gradient to 70%A, and reequilibration step at 70%A for 15min (total analysis time: 50 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 (nitrogen), 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), 722.4→352.3 (CE 35 eV) for FB_1, 706.4→336.4 and 706.4→318.4 (CE 35 eV) for FB_2 and FB_3, 406.5→334.4 and 406.5→353.4 (CE 30 eV) for HFB_1, 390.5→336.4 and 390.5→354.4 (CE 30 eV) for HFB_2 and HFB_3. The first transition reported was used for quantification, while the second transition was chosen as qualifier. Calibration curves (calibration range 10-1000 μg/kg) were used for extractable fumonisins (FB_1, FB_2 and FB_3) and hydrolyzed fumonisin (HFB_1, HFB_2 and HFB_3) quantification.

pH measurements of synthetic media

pH measurements were performed using a pH 212 Microprocessor pH Meter (Hanna Instruments, Modena, Italy). The range of pH measurement was firstly established by submitting each sample to a litmus test and then, before measurements, pH meter has been calibrated using appropriate buffer solution.

Statistical analyses

Statistical analyses 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.

6.4 Results and discussion

6.4.1 Preliminary results: fumonisins B production on synthetic media and investigation of hidden forms

To date, most studies performed to evaluate fumonisins production on synthetic media by *Fusarium* species under different conditions such as incubation time, a_w and media composition, have been carried out by extracting the target analyte from the mycelia previously separated from the culture media (4, 22, 25, 34). In this work, the role of a_w and incubation time on fumonisin production were evaluated by extracting fumonisins from the liquid broth. Moreover, the occurrence of hidden forms has been investigated in order to verify whether fungal enzymes are able to drive a possible binding of fumonisins to the matrix constituents. Thus, several broth cultures of *F. verticillioides* grown on Malt Extract Agar (MEA), adjusting a_w value to 0.99, and incubated at 25°C for 21, 30 and 45 days have been analyzed in order to determine the amount of free fumonisins. At the same time, to evaluate the occurrence of hidden forms, an aliquot of each broth culture underwent to alkaline hydrolysis. Figure 53 shows the amount of free FBs (expressed as sum of FB_1, FB_2 and FB_3) detected from 21 to 45 days.

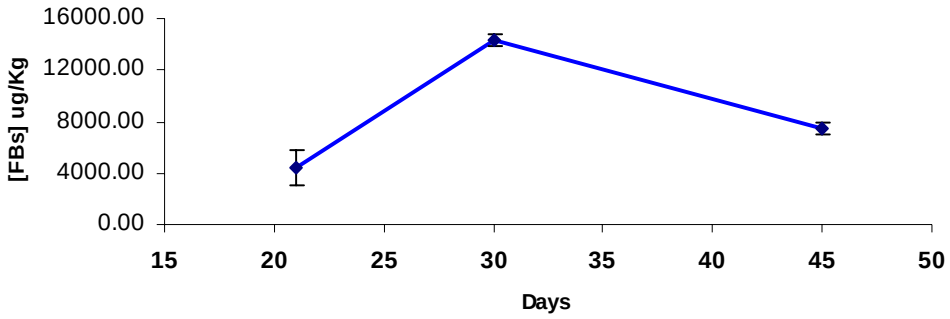


Figure 53. Fumonisins (expressed as sum of FB_1, FB_2 and FB_3) production from 21 to 45 days by *F. verticillioides* cultivated on synthetic media.

The production pattern of FBs here observed is very intriguing: the maximum production of B-fumonisins is reached after 30 days of incubation, while at 45 day an important decrease can be observed. Although the formation of hidden forms might explain such a decrease, data obtained after alkaline hydrolysis do not support this hypothesis, thus proving that the

masking cannot occur in synthetic media under the applied fungal growing conditions. Further studies should be thus performed in order to evaluate the changes occurring in the biosynthetic pathways after a certain time of incubation.

6.4.2 Hidden fumonisins occurrence in maize-based media

Since preliminary results indicated that hidden fumonisins are not contained in synthetic media, masked forms have been searched into a maize-based medium employed for the incubation of two *Fusarium* strains, belonging to the species *F. verticillioides* and *F. proliferatum.* Thus, a maize-based medium has been prepared using a maize meal and then was inoculated with the two *Fusarium* strains,. The inoculation was performed directly either on the medium surface or using a cellophane as cover, to avoid the invasion of the substrate by the growing mycelium. At the end of incubation, mycelia were separated from the growth medium and samples were extracted and hydrolyzed to determine free extractable fumonisins as well as total fumonisins after hydrolysis, and thus the amount of hidden forms (Table 28).

Table 28. Comparison of free extractable FBs, total FBs after hydrolysis and hidden fumonisins (calculate difference among total FBs after digestion and free extractable FBs) found in maize based media inoculated with *Fusarium* strains (n = 3; * p < 0.05;).

Sample	Cellophane (✓=used; × = not used)	[free FBs] (µg/Kg)	[Total FBs] (µg/Kg)	[Hidden FBs] (µg/Kg)	Tukey test (p)
F. proliferatum	✓	138080	166626	28546	*
F. proliferatum	×	123346	161221	37875	*
F. verticillioides	✓	19198	23657	4460	> 0.05
F. verticillioides	×	95349	121738	26389	*

Fumonisin B_1, B_2, B_3 were measured in both strains: *F. proliferatum* was the highest producer (average of FB_{tot}: 2630260 *vs* 200694 µg/Kg of *F. verticillioides*). In almost all cases, hidden fumonisins were detected in the media (Tukey test, $\alpha = 0.05$), being the masking rate the 25% in *F. proliferatum* and the 32% in *F. verticillioides*.

As far as hidden fumonisins is concerned, although they have not been detected in the liquid medium, a considerable amount of these forms was quantified after the incubation of the two selected strains on a maize-based medium, suggesting that the masking phenomenon requires

the presence of macromolecular compounds, such as proteins or starch, to take place. The liquid medium was composed, indeed, mainly by low molecular weight peptides and simple sugars, which seem to be unable to hide or bound fumonisins under the experimental conditions. These results clearly indicate that the masking phenomenon can occur only in the presence of a complex matrix, giving rise thus to a fungus-matrix interaction.

6.4.3 FUMONISINS B, A AND C PRODUCTION IN BROTH CULTURES OF *F. VERTICILLIOIDES* AND *F. PROLIFERATUM*: ROLE OF A_W AND INCUBATION PERIOD

In addition to FB_1, FB_2 and FB_3, *Fusarium* species are able to produce minor analogues, such as FB_4, FB_5 and fumonisins belonging to A- and C-series (1). Thus, with the aim to better investigate the fumonisin biosynthetic pathway and the influence of a_w and incubation period on fumonisin production pattern, the *in vitro* production of FBs, FAs and FCs in *F. proliferatum* and *F. verticillioides* strains at optimal growth temperature and a_w conditions for FBs production (53) has been investigated. From this purpose, two *Fusarium* FB-producers belonging to the species *F. verticillioides* and *F. proliferatum* (identified, respectively, with codes 10027 and 10026) were inoculated on synthetic media and incubated at 25°C for 21, 30 and 45 days in the dark, adjusting a_w to two defined values (0.995 and 0.990). Then, FBs (such as FB_1, FB_2, FB_3, FB_4 and FB_5), FAs (FA_1, FA_2 and FA_3) and FCs (FC_1, FC_2, FC_3 and FC_4) were determined by LC-ESI-MS/MS analysis. The experimental plan is resumed in Table 29.

Table 29. Schematic representation of the experimental design. *Fusarium* strains, condition of a_w and incubation time used and number of repetition of each experiment.

Strain	*Fusarium* species	a_w	Incubation time (days)	Number of trial
10027	*Fusarium verticillioides*	0.955	21	3
		0.955	30	3
		0.955	45	3
		0.99	21	3
		0.99	30	3
		0.99	45	3
10026	*Fusarium proliferatum*	0.955	21	3
		0.955	30	3
		0.955	45	3
		0.99	21	3
		0.99	30	3
		0.99	45	3

The obtained data are resumed in Table 30 and Table 31. While Table 30 resumes the concentrations found for B-series fumonisins after each incubation period for both strains grown in different conditions of a_w, Table 31 shows the amounts of minor analogues (FAs and FCs) detected in the same samples.

Table 30. Middle concentrations of each FB analogs detected in *F. verticillioides* (10027) and *F.proliferatum* (10026) broth cultures incubated from 21 to 45 days, changing a_w.

Incubation period (days)	[FB_1] (µg/Kg)	[FB_2] (µg/Kg)	[FB_3] (µg/Kg)	[FB_4] (µg/Kg)	[FB_5] (µg/Kg)	[FBs] (µg/Kg)
10027, a_w 0.99						
21	2817±1075	337±94	774±193	145±3	23±1	4097±1366
30	10183±654	970±91	2067±65	227±1	89±1	13536±499
45	4993±236	423±54	921±358	91±3	80±41	6508±137
10027, a_w 0.955						
21	1567±13	194±26	360±12	102±18	17±7	2240±50
30	1892±97	223±61	436±7	137±44	23±2	2710±13
45	1480±108	264±57	537±25	202±35	16±11	2499±237
10026, a_w 0.99						
21	196±54	21±29	13±18	32±1	< LOQ	261±102
30	1966±1634	260±210	326±224	31±2	23±33	2607±2099
45	57510±2457	11021±970	7039±302	863±245	250±13	76683±3382
10026, a_w 0.955						
21	104±25	14±0	12±0	< LOQ	< LOQ	131±25
30	21545±17116	1854±1316	932±672	113±48	138±58	24583±19210
45	116±43	14±1	5±8	< LOQ	< LOQ	135±52

Table 31. Middle concentrations of each FA and FC analogs detected in *F. verticillioides* (10027) and *F. proliferatum* (10026) broth cultures incubated from 21 to 45 days, changing a_w.

Incubation period (days)	[FA1] (µg/Kg)	[FA2+FA3] (µg/Kg)	[FAs] (µg/Kg)	[FC1] (µg/Kg)	[FC2+FC3] (µg/Kg)	[FC4] (µg/Kg)	[FCs] (µg/Kg)
10027, aw 0.99							
21	78±9	52±0	130±9	115±12	57±12	35±8	207±32
30	136±1	128±2	264±3	249±6	181±7	116±6	546±5
45	126±10	143±53	269±64	407±384	179±142	116±46	169±180
10027, a_w 0.955							
21	44±6	57±10	101±15	48±8	< LOQ	11±1	59±9
30	66±6	67±2	133±8	49±6	< LOQ	16±3	64±9
45	83±4	111±22	195±26	88±45	< LOQ	25±12	113±58
10026, a_w 0.99							
21	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
30	8±11	< LOQ	8±11	52±74	< LOQ	< LOQ	52±47
45	185±2	388±53	388±53	1498±8	269±86	< LOQ	1762±94
10026, a_w 0.955							
21	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
30	78±5	83±2	161±2	270±23	80±63	17±8	367±194
45	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Fumonisins B together with A- and C- series were produced by both *Fusarium* species included in the study.

F. proliferatum was the major producer of FBs (17400 *vs* 5265 μg/Kg of total FBs) and FCs (364 *vs* 282 μg/Kg of total FCs), while *F. verticillioides* showed the highest production of FAs (182 *vs* 124 μg/Kg of FA_{tot}).

F. proliferatum had a classical pattern of production with FB_1>FB_2>FB_3, as reported in previous studies regarding the chemical characterization of FBs production on *in vitro* maize-based cultures (54). Some strains of *F. proliferatum* have been reported as the highest producers of FBs in culture (more than 6000 μg/g) (55).

On the contrary, *F. verticillioides* production pattern was FB_1>FB_3>FB_2, confirming what observed in a previous study (46).

Information is not available in literature for FA and FC production pattern for both fungal species.

The ANOVA performed considering all factors, species included, underlined the significance of fungal species (data not shown); therefore, the ANOVA was performed separately for the two species.

Water activity was significant for FB_1, FB_1+FB_2 and total FBs production in *F. proliferatum* ($p \leq 0.001$ and 0.005 respectively) while it was significant for FB_1+FB_2 and total FBs ($p \leq 0.001$) and for FCs ($p \leq 0.005$) production in *F. verticillioides*. Low values of a_w (0.955) corresponded to lower values of FBs compared to FBs synthesized at a_w=0.990 for both species (see Table 32). Similarly to what observed for FBs, water activity exerts a comparable effect also on FAs and FCs production. Indeed, their synthesis was always inhibited at a_w=0.955 and conversely enhanced with higher a_w (0.990), as reported in literature by Mogensen et al. (22), Samapundo et al. (23) and Marìn et al. (56, 57). Such authors reported that FB synthesis increased with higher a_w a range of temperature 15-30°C, both for *F. verticillioides* and *F. proliferatum.*

Table 32. ANOVA of the effects of aw, incubation time and their interaction on the production of fumonisin B- A- C- type by *F. proliferatum* (10026) and *F. verticillioides* (ITEM 10027).

Factors	FB_1	FB_2	FB_1+FB_2	FB_{tot}	FA_{tot}	FC_{tot}
F. proliferatum						
a_w	**		*	*		
0.955	7255.2	627.5	7882.7	8283.0	53.6	122.4
0.990	19890.5	3767.0	23657.6	26517.1	193.5	604.7
Time	**	**	**	**	**	**
21	150.1	17.6	167.7	196.1	0.0	0.0
30	11755.7	1057.1	12812.8	13594.8	84.5	209.7
45	28812.8	5517.1	34329.9	38409.2	286.3	880.9
a_w*time	**	**	**	**	**	**
F. verticillioides						
a_w			**	**		*
0.955	1646.3	226.9	1873.2	2483.0	142.9	79.0
0.990	5997.6	576.6	6574.1	8046.7	221.1	484.8
Time			*	**		
21	2191.9	265.3	2457.2	3168.3	115.5	133.1
30	6037.5	596.5	6634.1	8123.1	198.7	305.4
45	3236.4	343.3	3579.7	4503.2	231.8	407.2
a_w*time			*	**		

Incubation time affected significantly all fumonisin series production ($p \leq 0.001$) by *F. proliferatum*, with FBs, FAs and FCs production increased from 21 to 45 days. Concerning *F. vericillioides*, incubation time affected only FB_1+FB_2 and total FBs synthesis ($p \leq 0.005$ and $p \leq 0.001$).

FBs production is markedly different in the two species regarding time-dependence: in *F. proliferatum*, the highest FBs biosynthesis was recorded at 45 days, while the peak of production in *F. verticillioides* was at 30 days (Table 32). As reported in literature, fumonisins B production can vary in time among different *F. verticillioides* strains (58), then differences between species is not surprising.

Regarding FAs and FCs, their synthesis increased with the incubation time in *F. proliferatum* as well as *F. verticillioides*.

The interaction a_w*time had a highly significant effect on all fumonisin series production by *F. proliferatum*, while only FB_1+FB_2 and total FBs synthesis ($p \leq 0.005$ and $p \leq 0.001$) in *F. verticillioides* (see Table 32); however the production pattern of all fumonisin series has been represented for both species for completeness in Figure 54.

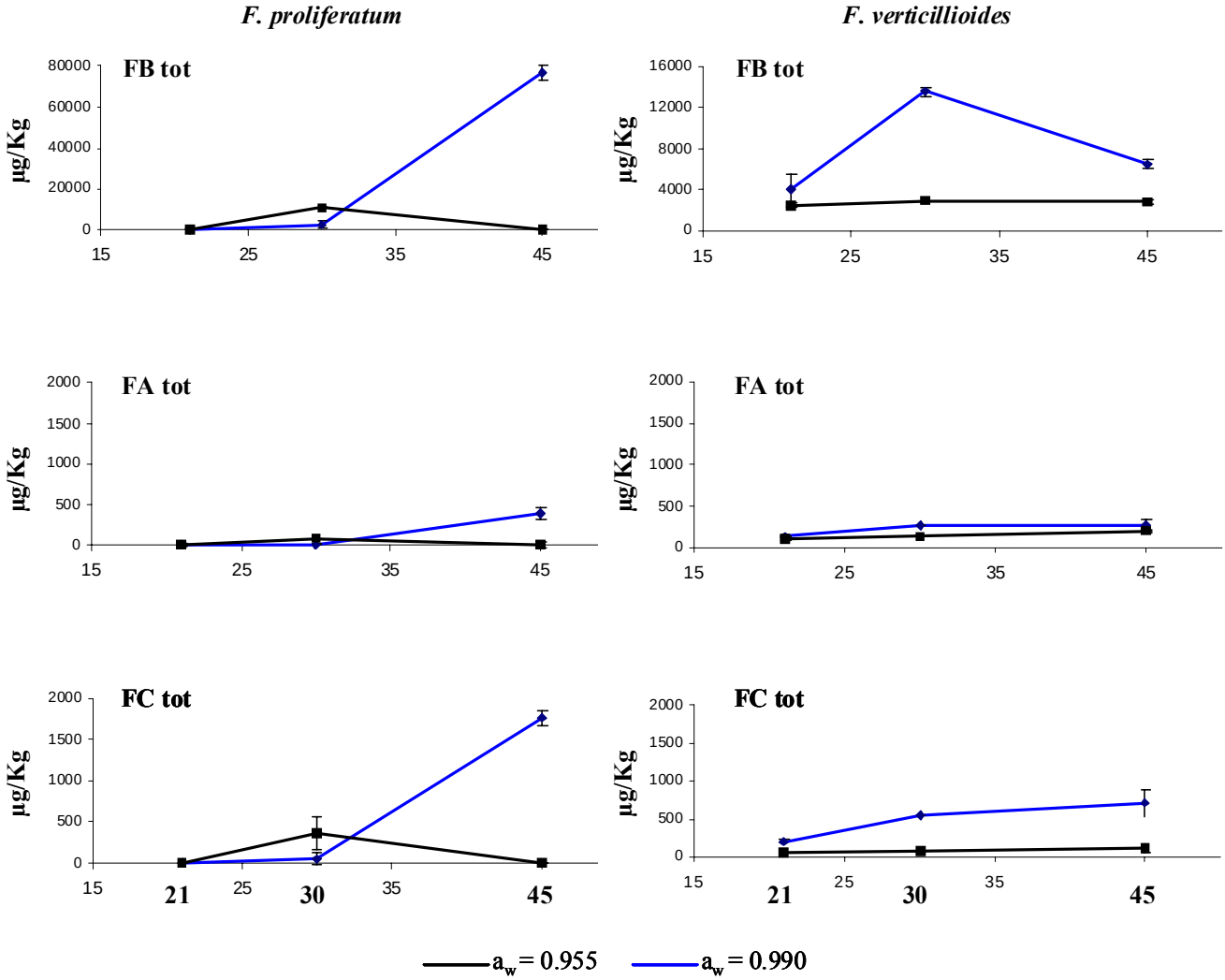


Figure 54. Fumonisins (total FBs, total FAs, total FCs) production under the effect of aw variation (0.955-0.990) from 21 to 45 days in *F. proliferatum* (10026) and *F. verticillioides* (10027).

In *F. proliferatum*, FB_{tot}, FA_{tot} and FC_{tot} production was maximum at 30 days and notably decreased at 45 days with a_w= 0.95; while with a_w=0.99 the production increased from 21 to 45 days, significantly from 30 to 45 days. Even though the trend of production was the same, the quantity was different: the maximum amount detected was 76700 µg/Kg for FB_{tot}, 573 µg/Kg for FA_{tot} and 1760 µg/Kg or FC_{tot}, as reported in Table 30 and Table 31. In *F. verticillioides*, FB_{tot} production was maximum at 30 days and decreased at 45 days at both a_w, with a more marked effect at a_w=0.955. FA_{tot} and FC_{tot} synthesis increased from 21 to 45 days, and again the effect was clearer at a_w=0.99. Both *Fusarium* species produced more FBs than FCs and FAs and fumonisins C were always more abundant than FAs in both species. Since the trend of production of FAs and FCs is very similar to that observed for FBs, it can be suppose that the biosynthesis of minor analogs takes place when the massive production of FBs gets under way. Thus, it can be argue that genes involved in the biosynthetic pathway of

fumonisins belonging to A- and C-series are activated when the biosynthesis of fumonisins B is at the highest levels, and not when it is dimmed.

The ratios between fumonisins in the two fungal species are listed in Table 33 and shown in Figure 55.

Table 33. Ratios between fumonisins calculated for both *Fusarium* species.

Species	FB_2/FB_1	FB_3/FB_1	FB_4/FB_1	FB_5/FB_1	FA_{tot}/FB_{tot}	FC_{tot}/FB_{tot}
F. proliferatum	0.160	0.100	0.010	0.005	0.007	0.002
F. verticillioides	0.100	0.220	0.040	0.010	0.030	0.050

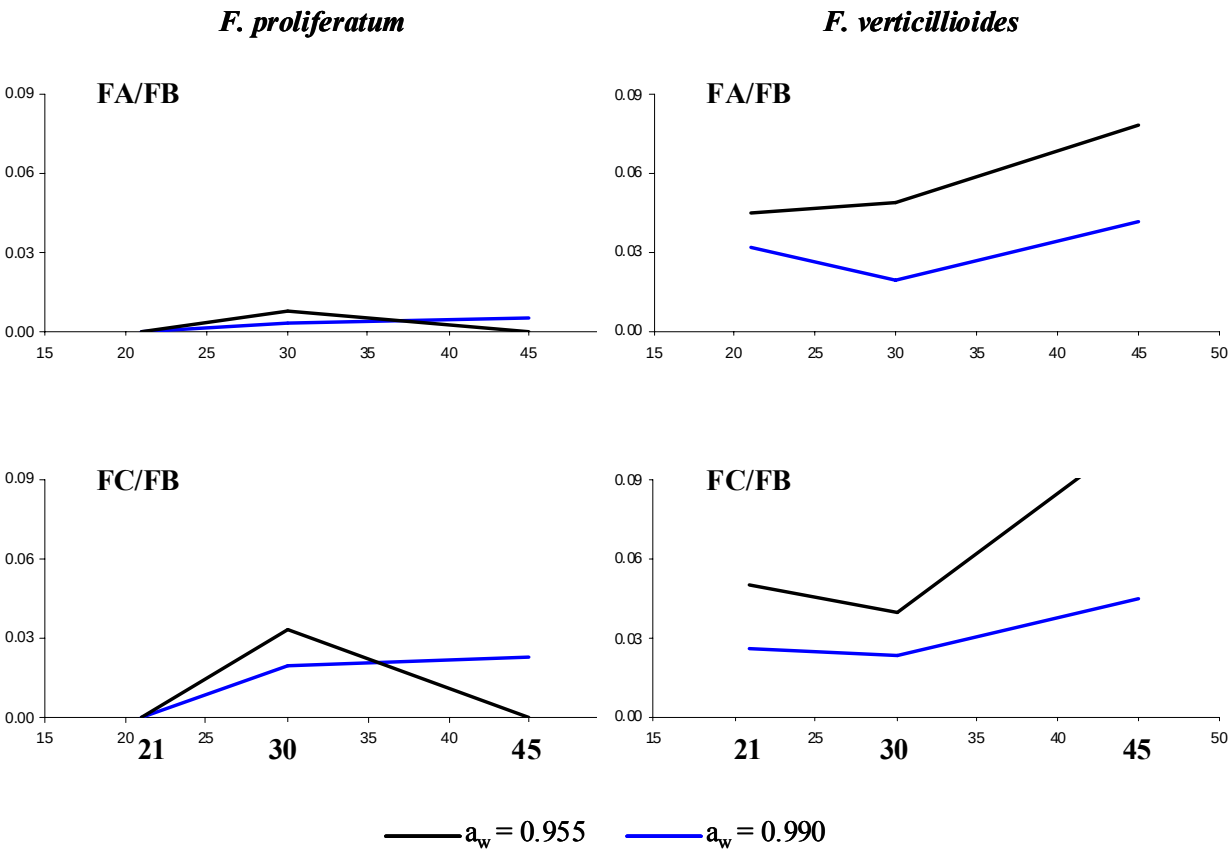


Figure 55. Fumonisins A and C, expressed as ratios to FB, production in *F. proliferatum* (10026) and *F. verticillioides* (10027) from 21 to 45 days.

In *F. proliferatum* FA/FB ratio, as well as FC/FB ratio, were maximum at 30 days and decreased at 45 days at a_w=0.955, while they remain constant from 30 to 45 days at a_w=0.990. Both the considered ratios followed a similar trend also in *F. verticillioides*: after a slight diminution at 30 days, an increment from 30 to 45 days is observed. Even though values were different and related to a_w regimes, the trend measured at a_w=0.955 and a_w=0.990 were similar. The calculation of such ratios provided us a more clear idea about fumonisins production in the two *Fusarium* species used for the present study. Concerning *F.*

proliferatum, we can say that after 45 days of incubation the biosynthesis of minor compounds is surpassed by that of fumonisins B, therefore the production of the main analogues returns predominant. Instead, in *F. verticillioides,* the biosynthesis of FAs and FCs analogs takes from FBs production in both a_w regimes, thus this species seems to shift the biosynthetic pathways to the production of minor mycotoxins.

In order to strictly investigate about the production of FB_1, the FB_4/FB_1 ratio has been calculated. Since FB_4 is the main precursor in FBs biosynthesis, the variation of such ratio from 21 to 45 days of incubation lets us to evaluate if any type of inhibitions may occur at steps involved in the conversion of the dehydroxylated analogue to the final product.

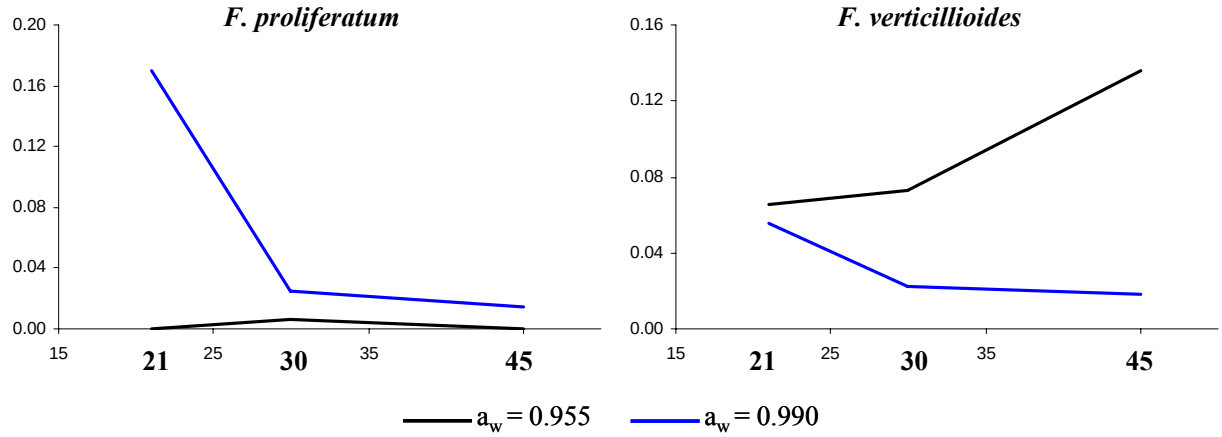


Figure 56. FB_4/FB_1 ratio variation in *F. proliferatum* (10026) and *F.verticillioides* (10027) from 21 to 45 days of incubation.

In *F. proliferatum* FB_4/FB_1 ratio strongly decreases from 21 to 30 days and remains almost constant until the end of incubation with a_w=0.990 while it remains always steady at low levels with a_w=0.995. The FB_4/FB_1 variation in *F. verticillioides* with a_w=0.990 is similar to that observed for *F. proliferatum*, suggesting that longer periods of time lead to a massive conversion of FB_4 to FB_1, thus avoiding the accumulation of the precursor. On the contrary, the great increment of the analyzed ratio observed with a_w=0.955, proposes that in this a_w regimes some inhibition factors can occur at the final steps of FB_1 biosynthesis, leading to an accumulation of FB_4.

Occurrence of partially hydrolyzed fumonisins in broth cultures

In order to explain the decrease of fumonisin levels in media after 45 days of incubation, several degradation forms have been considered, such as partially hydrolysed and totally hydrolysed fumonisins. These derivatives have been directly measured in the growing media, as reported in the Material and Methods section. Totally hydrolyzed FBs were not found in any sample, as well as the corresponding forms belonging to A and C analogs (HFAs and

HFCs). On the other hand, partially hydrolyzed derivatives belonging to the B-series (PHFBs) were detected for both *Fusarium* species at each incubation time (see Table 34). Partially hydrolysed derivatives of minor fumonisins have not been detected under the applied conditions.

Table 34. Middle concentrations of partially hydrolyzed forms of fumonisins belonging to the B-series detected in *F. verticillioides* (10027) and *F.proliferatum* (10026) broth cultures incubated from 21 to 45 days, changing aw.

Incubation period (days)	[$PHFB_1$] (µg/Kg)	[$PHFB_{2-3}$] (µg/Kg)	[$PHFB_4$] (µg/Kg)	[PHFBs] (µg/Kg)
10027, a_w 0.990				
21	138±2	171±6	152±11	462±18
30	229±44	370±80	222±47	822±172
45	448±161	763±804	443±415	1654±1380
10027, a_w 0.955				
21	45±1	78±3	60±4	183±5
30	50±4	86±4	73±15	209±23
45	51±4	85±9	96±5	232±11
10026, a_w 0.990				
21	5±4	12±1	3±5	20±11
30	23±16	126±120	71±63	221±203
45	670±124	2180±403	436±95	3287±432
10026, a_w 0.955				
21	< LOQ	< LOQ	< LOQ	< LOQ
30	307±183	945±869	191±35	1443±1087
45	9±0	10±2	< LOQ	19±2

PHFBs were produced mainly in *F. proliferatum* cultures, reaching the highest level after 45 days (3287 µg/Kg) with a_w=0.990 and at 30 days (1443 µg/Kg) with a_w=0.955. In *F. verticillioides* broth cultures their PHFBs production among 21 and 45 days remains quietly constant at a_w=0.955, while with a_w=0.990 the maximum amount has been detected at 45 days (1654 µg/Kg). Figure 57 shows the production patterns of partially hydrolyzed fumonisins for both *Fusarium* species from 21 to 45 days in different conditions of a_w.

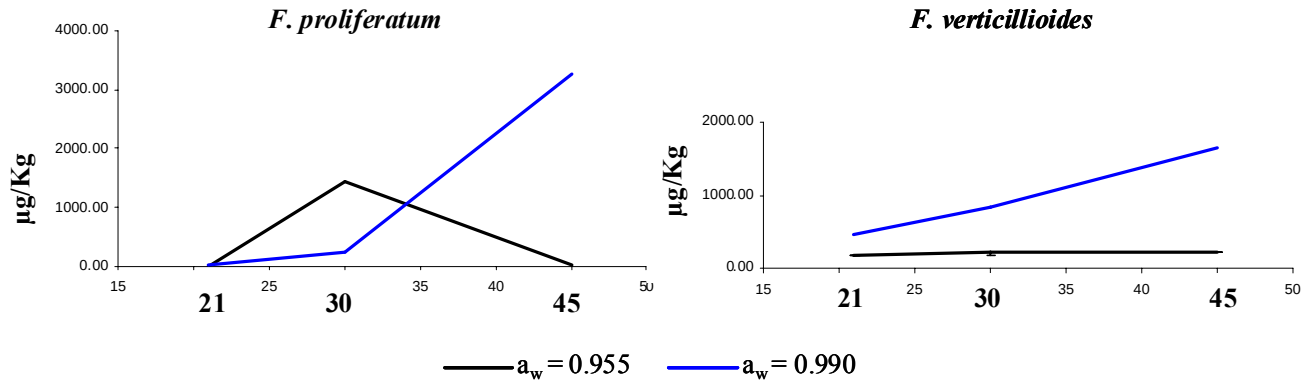


Figure 57. Partially hydrolyzed fumonisins B (PHFBs) production under the effect of a_w variation (0.955-0.990) from 21 to 45 days in *F. proliferatum* (10026) and *F. verticillioides* (10027).

Concerning *F. proliferatum*, the trend of production of partially hydrolyzed forms at two selected a_w regimes is the same than can be observed for their corresponding precursors, thus the occurrence of such derivatives do not explain the lower amounts of parent fumonisins detected after 45 days of incubation. The same situation occurs for partially hydrolyzed fumonisins produced by *F. verticillioides* with a_w=0.955. The only trend that opposes such observed for FBs production, is that concerns PHFBs produced by *F. verticillioides* with a_w=0.955, since their production increment from 21 to 45 days. Nevertheless, the slight increment measured can not explain the abatement of fumonisins concentration at 45 days of incubation.

Since the cleavage of the FB esteric bond giving rise to the formation of partially hydrolysed forms might be due to a significant change in the pH of the medium occurring during the growing period, pH measurement of each broth culture have been performed. The variation of pH in the growth medium from 21 to 45 days in *F.proliferatum* and *F.verticillioides* is shown in Figure 58.

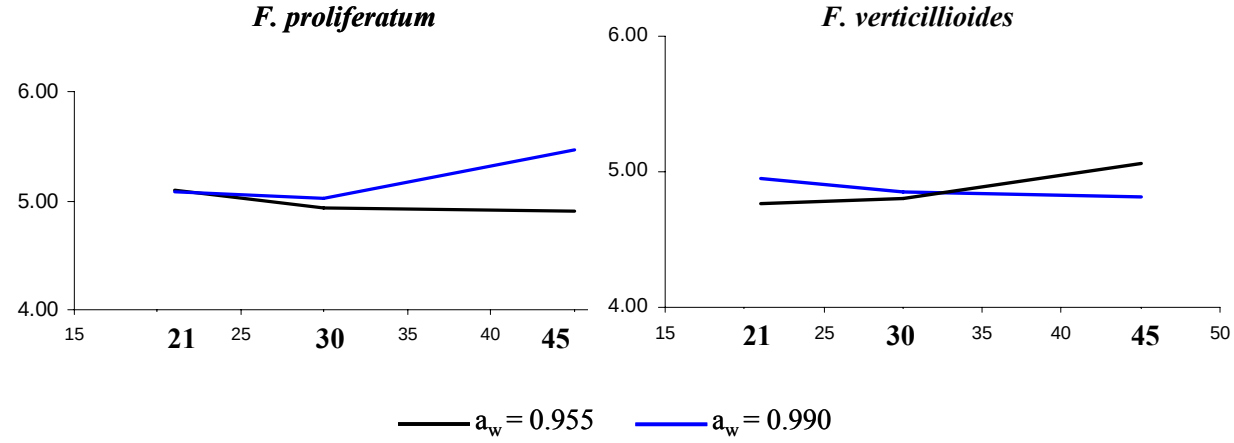


Figure 58. Variation of pH in the growth medium MEA under the effect of a_w variation (0.955-0.990) from 21 to 45 days in *F. proliferatum* (10026) and *F. verticillioides* (10027).

The pH of culture media changed during the incubation time following a different trend in the two species. In *F. proliferatum*, with a_w=0.955, pH decreased (5.10 to 4.91) from 21 to 45 days; while at a_w=0.990 it increased (5.08 to 5.47). In *F. verticillioides* the trend of pH was opposite: with a_w=0.955 pH increased (4.77 to 5.06) from 21 to 45 days, while at a_w=0.990 it decreased (4.96 to 4.82).

The pH of the medium measured before fungal inoculation was 4.61 at a_w=0.990, while the addition of glycerol to modify the a_w value to 0.955 induced a pH increase to 4.90. The variations occurring during the growing period were probably due to a different fungal

metabolism leading to a different catabolic production and/or to a different nutrient consumption under growing conditions.

Since parent fumonisins can be hydrolyzed only in strongly acid or alkaline conditions, their occurrence in broth cultures is not ascribable to the pH variation of the synthetic media during the incubation period, suggesting that the fungal metabolism may be responsible for their occurrence. Nevertheless, PHFBs levels are too low to justify the great decrease of parent fumonisins detected at the end of the incubation time. Thus, further transformations of the target toxins in other derivatives or fungal metabolites may be supposed. This hypothesis may be supported by the recent research presented by Bartók et al. (43), in which the occurrence of FB_1 fatty acid esters in solid cultures of *F. verticillioides* incubated for 30 days has been demonstrated. In these forms the carboxyl groups on the TCA units are esterified with long-chain fatty acids, such as palmitic acid, oleic acid and linoleic acid, making the molecule more apolar than the parent precursor.

6.5 Conclusions

This is the first work in which the production patterns of FB, FA and FC series by *F. proliferatum* and *F. verticillioides* are compared and studied as regard the influence of a_w and incubation time. The trend of production of the fumonisins belonging to the three series has been observed and several information concerning the biosynthetic pathways of such compounds have been obtained, also demonstrating that the biosynthesis of minor analogues takes place only when the production of FBs reaches its maximum level. Moreover, fumonisins B derivatives such as partially hydrolyzed fumonisins were detected and the trend of their production was studied, showing that the great decrease of parent forms observable after 45 days of incubation may be due to the fungal activity.

For the first time it has been observed that masked fumonisins were not recovered in synthetic media-based cultures, but only on maize-based media. Thus, the FB masking phenomenon takes place only in the presence of macromolecules such as starch and proteins. It will be interesting to further investigate on FB masking-effect in order to understand whether a certain maize component can induce it. The lack of maize enzymatic activity occurring in the media considered for this study suggests, finally, that the masking requires a complex substrate, although the host-plant interaction seems to be not strictly essential.

REFERENCES

(1) Rheeder, J.P.; Marasas, W.F.O.; Vismer, H.F. Production of fumonisins analogs by *Fusarium* species. *Appl. Environ. Microbiol.* **2002**, 68, 2101-2105.

(2) Logrieco, A.; Bailey, J.A.; Corazza, L.; Cooke, B.M. Mycotoxins in plant disease (Special Issue: Mycotoxins in Plant Disease). *Eur. J. Plant Path*. **2002**, 108, 597-734.

(3) Bezuidenhout, S.C.; Gelderblom, W.C.A.; Gorst-Allman, C.P.; Horak, R.M.; Marasas, W.F.O.; Spiteller, G.; Vleggaar, R. Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *J. Chem. Soc., Chem. Commun.* **1988**, 743-745

(4) Sewram, V.; Mshicileli, N.; Shephard, G.S.; Vismer, H.F.; Rheeder, J.P.; Lee, Y.W.; Leslie, J.F.; Marasas, W.F.O. Production of fumonisin B and C analogues by several *Fusarium* species. *J. Agric. Food Chem.* **2005**, 53, 4861-4866.

(5) Marín, S.; Homedes, V.; Sanchis, V.; Ramos, A.J.; Magan N. Impact of *Fusarium moniliforme* and *F. proliferatum* colonization of maize on calorific losses and fumonisin production under different environmental conditions. *J. Stored Prod. Res*. **1999**, 35,15-26.

(6) Butchko, R.A.E.; Plattner, R.D.; Proctor, R.H. Deletion analysis of *FUM* genes involved in tricarballylic ester formation during fumonisin biosynthesis. *J. Agr. Food Chem*. **2006**, 54, 9398-9404.

(7) Desjardins, A.E.; Proctor, R.H. Molecular biology of *Fusarium* mycotoxins. *Int. J. Food Microb.* **2007**,119, 47-50.

(8) López-Errasquín, E.; Vázquez, C.; Jiménez, M.; González-Jaén, M.T. Real-time RT-PCR assay to quantify the expression of *fum*1 and *fum*19 genes from the fumonisin-producing *Fusarium verticillioides*. *J. Microbiol. Meth.* **2007**,68, 312-317.

(9) Jurado, M.; Marìn, P.; Magan, N.; Gonzalez-Jaen, M.T. Relationship between solute and matric potential stress, temperature, growth and *FUM1* gene expression in two *Fusarium verticillioides* strains from Spain. *Appl. Environ. Microbiol.* **2008** ,74, 2032-2036.

(10) Caldas, E.D.; Sadilkova, K.; Ward, B.L.; Jones, A.D.; Winter, C.K.; Gilchrist, D.G. Biosynthetic studies of fumonisin B1 and AAL toxins. *J. Agric. Food Chem.* **1998**, 46, 4734-4743.

(11) Blackwell, B.A.; Edwards, O.E.; Fruchier, A.; ApSimon, J.W.; Miller, J.D. NMR structural studies of fumonisin B1 and related compounds from *Fusarium moniliforme*. *Adv. Exp. Med. Biol.* **1996** 392, 75–91.

(12) Du, L.; Zhu, X.; Gerber, R.; Huffman, J.; Lou, L.; Jorgenson, J.; Yu, F.; Zaleta-Rivera, K.; Wang, Q. Biosynthesis of sphinganine-analog mycotoxins. *J. Ind. Microbiol Biotechnol.* **2008**, 35, 455-464.

(13) Branham, B.E.; Plattner, R.D. Alanine is a precursor in the biosynthesis of fumonisin B1 by *Fusarium moniliforme*. *Mycopathologia* **1993**, 124, 99-104.

(14) Plattner R.D.; Shackelford D.D. Biosynthesis of labeled fumonisins in liquid cultures of *Fusarium moniliforme*. *Mycopathologia* **1992** 117,17–22

(15) Zaleta-Rivera, K.; Xu, C.; Yu, F.; Butchko, R.A.; Proctor, R.H.; Hidalgo-Lara, M.E.; Raza, A.; Dussault, P.H.; Du, L. A bidomain nonribosomal peptide synthetase encoded by *FUM14* catalyzes the formation of tricarballylic esters in the biosynthesis of fumonisins. *Biochem.* **2006** 45, 2561–2569.

(16) Gerber, R.; Lou, L.; Huffman, J.; Zhu, X.; Lin, T.; Li, L.Q.; Arreguin, I.; Butchko, R.A.E.; Proctor, R.H.; Du, L. Advances in understanding the biosynthesis of fumonisins. In: *Mycotoxin prevention and control in agriculture*, Ed. 1, Appell, M.; Kendra, D.F.; Trucksess, M.W.; ACS Symposium Series; American Chemical Society; Washington, DC, **2010**, 1031, 167-182.

(17) Seo, J.A.; Proctor, R.H.; Plattner, R.D. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal. Genet. Biol.* **2001**, 34,155-165.

(18) Proctor, R.H.; Plattner, R.D.; Desjardins, A.E.; Busman, M.; Butchko, R.A. Fumonisin production in the maize pathogen *Fusarium verticillioides*: genetic basis of naturally occurring chemical variation. *J. Agric. Food Chem.* **2006** 54, 2424–2430

(19) Ding, Y.; Bojja, R.S.; Du, L. Fum3p, a 2-ketoglutarate-dependent dioxygenase required for C-5 hydroxylation of fumonisins in *Fusarium verticillioides*. *Appl. Environ. Microbiol.* **2004** 70:1931–1934

(20) Yu, J-H.; Keller, N.; Regulation of secondary metabolism in filamentous fungi. *Annu. Rev. Phytopathol.* **2005**, 43, 437–58.

(21) Reverberi, M.; Ricelli, A.; Zjialic, S.; Fabbri, A.A.; Fanelli, C. Natural function of mycotoxins and control of their biosynthesis in fungi. *Appl. Microbiol. Biotechnol.* **2010**, 87, 899-911.

(22) Mogensen, J.M.; Nielsen, K.F.; Samson, R.A.; Frisvad, J.C.; Thrane, U. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. *BMC Microbiology*. **2009,** 9, 281.

(23) Samapundo, S.; Devliehgere, F.; De Meulenaer, B.; Debevere, J. Effect of water activity and temperature on growth and the relationship between fumonisin production and the radial growth of *Fusarium verticillioides* and *Fusarium proliferatum* on corn**.** *J. Food Prot.* **2005,** 68, 5, 1054-9.

(24) Picot, A.; Barreau, C.; Pinson-Gadais, L.; Caron, D. ; Lannou, C. ; Richard-Forget, F. Factors of the *Fusarium verticillioides*-maize environment modulating fumonisin production. *Crit. Rev. Microbiol.* **2010**, 36, 221-231.

(25) Alberts, J.F.; Gelderblom, W.C.A.; Thiel, P.G.; Marasas, W.O.; Van Schalkwyk, D.J.; Behrend, Y. Effects of temperature and incubation period on production of fumonisin B1 by *Fusarium moniliforme. Appl. Environ. Microbiol.* **1990**, 56, 1729-173.

(26) Keller, S.E.; Sullivan, T.M.; Chirtel, S. Factors affecting the growth of *Fusarium proliferatum* and the production of fumonisin B1: oxygen and pH. *J. Ind. Microbiol. Biotechnol.* **1997,** 19, 305-309.

(27) Tilburn, J.; Sarkar, S.; Widdick, D.A.; Espeso, E.A.; Orejas, M.M.; Mungroo; J.; Peñalva, M.A.; Arst, H.N. Jr. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acidic- and alkaline-expressed genes by ambient pH. *Eur. Mol. Biol. Organ.* **1995**, 14, 779–90.

(28) Jiménez, M.; Mateo. J.J.; Hinojo, M.J.; Mateo, R. Sugars and amino acids as factors affecting the synthesis of fumonisins in liquid cultures by isolates of the *Gibberella fujikuroi* complex. *Int. J. Food Microbiol.* **2003**, 89, 185–93.

(29) Falcão, V.C.A.; Ono, M.A.; de Ávila Miguel, T.; Vizoni, E.; Hirooka, E.Y.; Ono, E.Y.S. *Fusarium verticillioides*: evaluation of fumonisin production and effect of fungicides on *in vitro* inhibition of mycelial growth. *Mycopathologia* **2011**, 171, 77-84.

(30) Marín, S.; Ramos, J.D.; Cuevas, D.; Sanchis, V.; *Fusarium verticillioides* and *Fusarium graminearum* infection and fumonisin B1 and zearalenone accumulation in resveratrol-treated corn. *Food Sci. Technol. Int. (London, UK)*. **2006**, 12, 353-359.

(31) Gelderblom, W.C.A.; Jaskiewicz, K.; Marasas, W.F.O.; Thiel, P.G.; Horak, R.M.; Vleggaar, R.; Kriek, N.P.J. Fumonisins-Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl. Environ. Microbiol.* **1988**, 54, 1806-1811.

(32) Gelderblom, W.C.A.; Marasas, W.F.O.; Vleggaar, R.; Thiel, P.G. & Cawood, M.E. Fumonisins: isolation, chemical characterization and biological effects. *Mycopathologia* **1992**, 112, 11-16.

(33) ApSimon, J.W.; Blackwell, B.A.; Edwards, O.E.; Fruchier, A.; Miller, J.D.; Savard, M.; Young, J.C. The chemistry of fumonisins and related compounds. Fumonisins from *Fusarium moniliforme*: chemistry, structure and biosynthesis. *Pure & Appl. Chem.* **1994**, 66, 2315-2418.

(34) Bartók, T.; Szecsi, Á.; Szekeres, A.; Mesterhazy, Á.; Bartók, M. Detection of new fumonisin mycotoxins and fumonisin-like compounds by reversed-phase high-performance liquid chromatography/electrospray ionization ion trap mass spectrometry. *Rapid Comm. Mass Spectrom.* **2006**, 20, 2447-2462.

(35) Musser, S.M.; Gay, M.L.; Mazzola, E.P.; Plattner, R.D. Identification of a new series of fumonisin containing 3-hydroxypiridine. *J. Nat. Prod.* **1996**, 59, 970-972.

(36) Seo, J-A.; Lee, Y-W. Natural occurrence of the C series of fumonisins in moldy corn. *Appl. Environ. Microbiol.* **1999**, 65, 1331-1334.

(37) Shephard, G.S.; van der Westhuizen, L.; Sewram, V.; van Zyl, J.; Rheeder, J.P. Occurrence of the C-series fumonisins in maize from the former Transkei region of South Africa. *Food Addit. Contam., Part A.* **2011**, 28, 1712-1716.

(38) Zachariasova, M.; Lacina, O.; Malachova, A.; Kostelanska, M.; Poustka, J.; Godula, M.; Hajslova, J. Novel approaches in analysis of Fusarium mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. *Anal. Chim. Acta.* **2010**, 662, 51-61.

(39) Maragos, C.M.; Busman, M. Rapid and advanced tools for mycotoxin analysis: a review. *Food Addit. Contam., Part A.* **2010**, 27, 688-700.

(40) Sforza, S.; Dall'Asta, C.; Marchelli, R. Recent advances in mycotoxin determination in food and feed by hyphenated chromatographic techniques/mass spectrometry. *Mass Spectrom. Rev.* **2006**, 25, 54-76.

(41) Zöllner, P.; Mayer-Helm, B. Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. *J. Chromatogr., A.* **2006**, 1136, 123-169.

(42) Sulyok, M.; Berthiller, F.; Krska, R.; Schuhmacher, R. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Commun. Mass Spectrom.* **2006**, 20, 2649-2659.

(43) Bartók, T.; Tölgyesi, L.; Mesterházy, A.; Bartók, M.; Szécsi, A. Identification of the first fumonisin mycotoxins with the three acyl groups by ESI-ITMS and ESI-TOFMS following RP-HPLC separation: palmitoyl, linoleoyl and oleoyl EFB1 fumonisin isomers from a solidi culture of *Fusarium verticillioides*. *Food Addit. Contam.* **2010**, 27, 1714-1723.

(44) Seefelder, W.; Knecht, A.; Humpf, H.U. Bound fumonisins B1: analysis of fumonisin-B1 glyco and amino acid conjugates by liquid chromatography-electrospray ionization-tandem mass spectrometry. *J. Agric. Food Chem.* **2003**, 51, 5567-5573.

(45) Frisvad, J.C. & Samson, R.A. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and airborne *terverticillate Penicillia* and their mycotoxins. *Stud. Mycology*. **2004**, 49, 1-173.

(46) Lazzaro, I.; Susca, A.; Mulè, G.; Ritieni, A.; Ferracane, R.; Marocco, A.; Battilani, P. Effects of temperature and water activity on FUM2 and FUM21 genes expression and fumonisins B production in *Fusarium verticillioides*. Submitted.

(47) Bennet, J.W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, 16, 497-516.

(48) Galaverna, G.; Dall'Asta, C.; Mangia, M.; Dossena, A.; Marchelli, R. Masked mycotoxins: an emerging issue for food safety. *Czech. J. Food Sc*i**. 2009**, 27, 89-92.

(49) Dall'Asta, C.; Mangia, M.; Berthiller, F.; Molinelli, A.; Sulyok, M.; Schuhmacher, R.; Krska, R.; Galaverna, G.; Dossena, A.; Marchelli, R. Difficulties in fumonisin determination: the issue of hidden fumonisins. *Anal. Bioanal. Chem.* **2009**, 395, 1335-1345.

(50) Howard, P.C.; Churchwell, M.I.; Couch, L.H.; Marques, M.M.; Doerge, D.R. Formation of N-(Carboxymethyl)fumonisin B1, following the reaction of fumonisin B1 with reducing sugars. *J. Agric. Food Chem.* **1998**, 46, 3456-3557.

(51) Poling, S.M.; Plattner, R.D.; Weisleder, D. N-(1-Deoxy-D-fructos-1-yl) fumonisin B1, the initial reaction product of fumonisin B1 and D-glucose. *J. Agric. Food Chem.* **2002**, 50, 1318-1324.

(52) Dall'Asta, C.; Galaverna, G.; Aureli, G.; Dossena, A.; Marchelli, R. A LC/MS/MS method for the simultaneous quantification of free and masked fumonisins in maize and maize-based products. *World Mycotoxin J.* **2008**, 1, 237-246.

(53) Marìn, P.; Magan, N.; Vazquez, C.; González-Jaén, M.T. Differential effect of environmental conditions on the growth and regulation of the fumonisin biosynthetic gene *FUM1* in the maize pathogens and fumonisin producers *Fusarium verticillioides* and *Fusarium proliferatum*. *FEMS Microbiol. Ecol.* **2010**, 73, 303-311.

(54) Fadl-Allah, E.M. Occurrence and toxigenicity of *Fusarium moniliforme* from freshly harvested maize with special reference to fumonisins production in Egypt. *Mycophathologia.* **1997**, 140, 99-103.

(55) Leslie, J. F.; Zeller, K. A.; Logrieco, A.; Mulè, G.; Moretti, A.; Ritieni, A. Species diversity of and toxin production by *Gibberella fujikuroi* species complex strains isolated from native prairie grasses in Kansas. *Appl. Environ. Microbiol.* **2004**, 70, 2254-2262.

(56) Marín, S.; Sanchis, V.; Vinas, I.; Canela, R.; Magan, N. Effect of water activity and temperature on growth and fumonisin B1 and B2 production by *Fusarium proliferatum* and *F. moniliforme* on maize grain. *Lett. Appl. Microbiol.* **1995**, 21, 298-301.

(57) Marín, S.; Homedes, V.; Sanchis, V.; Ramos, A.J.; Magan N. Impact of *Fusarium moniliforme* and *F. proliferatum* colonization of maize on calorific losses and fumonisin production under different environmental conditions. *J. Stored Prod. Res*. **1999**, 35,15-26.

(58) Bailly, J.D.; Querin, A.; Tardieu, D.; Guerre P. Production and purification of fumonisins from a highly toxigenic *Fusarium verticillioides* strain. *Rev. Med. Vet.* **2005,** 156, 547-554.

SUMMARY

In the present Ph.D. thesis the occurrence of free and hidden fumonisins in raw maize as well as the nature of masking phenomenon have been investigated.

In the first section the masking mechanism has been largely studied through several *in vitro* experiments.

The digestion assay allowed both to evaluate the potential risk associated to the presence of hidden forms in a matrix and to obtain information concerning masking mechanism. Indeed, the release of hidden fumonisins from a matrix after a process simulating the human gastrointestinal digestion and their availability for intestinal absorption has been demonstrated. Thus, since the amount of mycotoxin released in the small intestine may be higher than that estimated through common analytical procedures, a new issue concerning the risk assessment has been introduced, also revealing the needs of methods able to detect the real amount of fumonisin contained into a food sample.

Moreover, the release of hidden fumonisins as parent forms as well as the complete stability of covalent adducts were the most important experimental evidence in a perspective of understanding masking mechanism, supporting the hypothesis based on the existence of non-covalent interaction between the target toxin and maize constituents.

The subsequent experiments performed allowed to better study fumonisins masking mechanism and the conditions in which it takes place.

The association of fumonisin with zein (the main maize prolamin) and starch has been demonstrated in mono component model systems, perceiving a great affinity of fumonisins for starch rather than zein. The contribution of each starch fraction in masking phenomenon has been investigated demonstrating that both amylose and amylopectin are able to hide fumonisins. Nevertheless, the partial loss of masking observed in binary *in vitro* systems suggests that in these conditions there is any synergic effects among macromolecules in fumonisins masking, thus is intuitive that in more complex system the presence of the mould is required to guarantee the entrapment or the complexation of such mycotoxin into maize macro constituents.

Finally, the reliability of all the data concerning masking phenomenon has been demonstrated by means of the last experimental set performed. Indeed, the lack of analytical artefacts generated by supramolecular structure formed during solvent extraction and able to host

fumonisins has been demonstrated for the extraction mixture employ in this work, showing also that the increase of fumonisins levels found after alkaline hydrolysis of a maize sample is mainly due to forms that really interact with matrix macro constituents.

Subsequently, the role of maize hybrids and kernel composition as factors affecting fumonisins production in raw maize, widely studied as concern the incidence of free fumonisins, has been considered for the first time in a new context, that is masking phenomenon and hidden fumonisins occurrence. Through an extensive study, starting from information collected under field conditions and over two years of observation, solid data that confirm the presence of hidden forms in raw maize have been obtained, also pointing out that different maize genotypes support masking phenomenon at a different extent. Moreover, maize hybrid seems to have a role also in FBs biosynthesis modulation, particularly with poorly conducive conditions for fungal infection, as confirmed by data obtained by evaluating the contamination of same hybrids over two years.

This is the first work in which the composition of the substrate in terms of macro constituents, comprising the lipid fraction, has been considered to clarify the role of hybrid-related characteristics in *Fusarium* infection and FBs production in corn, and a close relationship between lipids and both free and hidden fumonisins was found. Indeed, significant positive correlations were obtained between free and total FBs levels and the main lipid related factors (total fat percentage and C18:2 amounts): the highest the fat and the linoleic acid amount in the considered maize genotypes the highest the FBs contamination result. Since unsaturated fatty acids play a key role in plant defence strategies during infection, it may be suppose that the plant-pathogen system cross-talk is involved in fumonisins accumulation and masking phenomenon.

The fate of both fungal infection and fumonisin contamination was followed during the entire maize chain, collecting several new information about the dynamic of contamination occurring under storage conditions. Also in this case, this is the first paper in which hidden fumonisins were considered in a study aimed to evaluate the effect of storage conditions and technological treatments on mycotoxin accumulation.

Whereas microbial contamination remains slightly uniform in a whole mass stored within a silo over time and after flour production, a gradient of mycotoxin concentration seems to take place moving from lower and inner parts toward outer layers. Moreover, although the milling seems to have only little effects on overall levels of mycotoxins, a release of hidden forms after the mechanical rupture of the matrix was observed, suggesting that the consequences of

such operation are reflected on the form in which analytes occur into the matrix rather than their amount.

The last section was dedicated to the investigation of the biosynthetic pattern of fumonisins, considering not only those belonging to the main B-series but also minor analogues appertaining to the A- and C- series. Thus, several fumonisin analogues and their corresponding partially hydrolyzed forms were initially detected and identified in *Fusarium* broth cultures by means of LC-ESI-MS/MS experiments, developing in a second time two LC-MS/MS methods able to allow the simultaneous detection of all the analytes previously detected. Then, the method here developed was employed to monitoring the production of fumonisins in broth cultures by two *Fusarium* species and the influence of growing parameters on FA and FC synthesis and accumulation over the incubation period has been evaluated for the first time. Through the observation of the trend of production of the fumonisins belonging to the three series several information concerning the biosynthetic pathways of such compounds have been obtained, also demonstrating that the biosynthesis of minor analogues takes place only when the production of FBs reaches its maximum level. Besides several fumonisins analogues, partially hydrolyzed metabolites were found and the trend of their production was studied, demonstrating that their presence in liquid cultures was due mainly to fungal activity.

Finally, the production of fumonisin in *Fusarium* liquid cultures was compared with that obtained by cultivating the same strains on a maize-base medium specifically developed for this study. The data obtained from this experiment were very interesting, since the occurrence of hidden forms was demonstrated only in the more complex substrate such as maize-based medium. This confirms that during fungal development and mycotoxin production fumonisins are not covalently linked with matrix constituents such as oligosaccharides and peptides (the main components of a liquid substrate) by the enzymatic activity of the mould. Moreover, it was established that the concomitant presence of the microorganism and macromolecules such as proteins or starch is a prerogative so that masking phenomenon takes place.

In conclusion, this thesis tackled in different ways the issue of fumonisins and their respective hidden forms, contributing to extend the state-of-the-art concerning such phenomenon and giving a particular attention towards the conditions that affect the occurrence of both forms in raw maize, also trying to understand in which conditions masking takes place in a new risk assessment point of view.

At the same time, whereas the studies performed have provided exhaustive answers to several questions concerning masking phenomenon and fumonisin production and accumulation, new

research perspectives have been introduced by the experimental evidences obtained. Indeed, many of the results achieved may be taken as starting point for the development of new research studies.

In particular, concerning masking mechanism new *in vitro* experiments could be developed in order to demonstrate in a direct way that fumonisin is complexated by starch or zein, also by using other analytical techniques rather than LC-MS/MS, such as x-ray, UV and fluorescence spectroscopy that allow to study the phenomenon from a more specific points of view.

As far as the study of the conditions that affect the occurrence of hidden fumonisins in raw maize is concerned, further studies are necessary in order to confirm the results obtained and to clarify the role of plant-pathogen interaction in fumonisin production and masking, looking also confirms of the hypotheses retained about the FB-fatty acids relationship.

Moreover, it would be very interesting to evaluate the influence of the considered factors over the entire ripening period, by monitoring also minor analogues in order to obtain additional information regarding the biosynthetic pattern of such compounds and to investigate about their natural occurrence in maize, since these are topics that have never been addressed.

ATTACHMENT

Attachment Table 1. Chemical data obtained from the analysis of maize samples collected in 2009. All the data have been reported on the dry matter. Free and total fumonisins are expressed as µg/Kg. Each analysis was performed in duplicate.

Sample	Hybrid	Starch (%dm)	Lipids (%dm)	Proteins (%dm)	Free FUM (%dm)	Total FUM (%dm)	C18:0	C18:1	C18:2	C18:1/C18:2
01	HYB 7	71.76	2.98	7.39	465	1001	11.05	31.12	57.83	0.54
02	HYB 1	72.76	2.46	7.37	799	1459	16.51	26.06	57.43	0.45
03	HYB 8	67.63	4.72	8.92	2179	3364	10.69	35.76	53.55	0.67
04	HYB 8	70.39	3.18	7.33	176	171	11.52	33.22	55.25	0.60
05	HYB 8	68.85	4.07	8.63	3948	9138	11.32	35.44	53.24	0.67
06	HYB 7	69.78	3.69	7.95	608	1665	12.03	32.25	55.72	0.58
07	HYB 1	69.65	3.13	8.52	377	970	14.74	29.46	55.80	0.53
08	HYB 6	69.10	3.94	8.32	1920	2113	12.25	23.54	64.21	0.37
09	HYB 4	71.16	3.33	7.56	845	811	13.69	32.09	54.21	0.59
10	HYB 4	69.88	3.83	7.83	934	1923	13.73	31.57	54.70	0.58
11	HYB 9	68.78	4.16	8.07	276	387	12.18	32.59	55.23	0.59
12	HYB 9	69.69	3.70	8.02	LOQ	LOQ	11.93	25.13	62.94	0.40
13	HYB 4	70.17	3.72	7.95	600	2742	12.23	26.16	61.61	0.42
14	HYB 5	71.73	3.37	7.06	81	2207	10.92	28.66	60.42	0.47
15	HYB 2	69.63	3.83	8.15	8020	9678	12.16	27.56	60.29	0.46
16	HYB 2	68.87	4.22	8.21	2432	4741	12.40	27.32	60.29	0.45
17	HYB 2	69.51	4.14	7.94	4218	4720	12.37	27.23	60.40	0.45
18	HYB 2	69.65	4.1	8	2187	6548	12.16	27.14	60.70	0.45
19	HYB 2	70.01	3.93	8.04	4351	7268	12.22	27.65	60.13	0.46
20	HYB 5	69.30	3.89	8.36	2348	2323	12.60	31.58	55.82	0.57
21	HYB 5	69.57	3.95	8	542	2826	12.80	33.14	54.06	0.61
22	HYB 7	71.16	3.32	7.24	809	783	11.35	30.81	57.84	0.53
23	HYB 3	68.45	3.67	9.08	584	1074	12.05	26.70	61.26	0.44
24	HYB 2	68.75	3.72	8.52	7392	9186	12.29	27.09	60.62	0.45
25	HYB 2	69.76	3.38	8.42	956	1932	12.26	27.18	60.56	0.45
26	HYB 2	69.74	3.87	8.22	1774	1650	12.23	25.39	62.37	0.41
27	HYB 2	69.67	3.77	8.16	1076	1575	12.02	26.07	61.91	0.42
28	HYB 2	69.49	3.94	7.97	3049	5089	11.99	25.87	62.14	0.42

Attachment Table 2. Chemical data obtained from the analysis of maize samples collected in 2010 in the district of BOLOGNA. All the data have been reported on the dry matter. Free and total fumonisins are expressed as µg/Kg. The dash designates that the fatty acids profile has not been evaluated. Each analysis was performed in duplicate.

Sample	Hybrid	Starch (%dm)	Lipids (%dm)	Proteins (%dm)	Free FUM (%dm)	Total FUM (%dm)	C18:0	C18:1	C18:2	C18:1/C18:2
01	HYB 2	70.49	3.35	8.23	12486	28035	1.51	30.64	53.98	0.57
02	HYB 6	74.22	2.39	6.12	7580	11121	1.63	29.88	53.71	0.56
03	HYB 6	73.00	2.80	6.59	4113	7437	1.50	29.92	54.99	0.54
04	HYB 2	70.95	2.86	7.97	18303	19588	1.52	32.17	53.66	0.60
05	HYB 6	71.55	2.80	6.87	4198	7182	1.49	30.82	54.97	0.56
06	HYB 3	71.17	3.21	8.82	3882	7052	1.44	29.42	56.09	0.52
07	HYB 3	70.75	3.31	7.93	1982	3489	-	-	-	-
08	HYB 5	72.95	3.28	6.88	2253	4663	1.41	27.65	57.06	0.48
09	HYB 5	73.27	2.54	7.19	14256	20684	1.45	25.49	57.37	0.44
10	HYB 5	69.70	4.02	7.88	3015	5154	1.46	30.63	54.71	0.56
11	HYB 5	69.42	3.39	7.60	6621	6864	1.41	30.52	55.09	0.55
12	HYB 3	67.79	4.11	8.36	7868	12311	-	-	-	-
13	HYB 3	66.43	4.18	8.78	4798	7263	-	-	-	-
14	HYB 3	70.95	2.91	8.14	18755	25502	-	-	-	-
15	HYB 3	70.05	2.90	8.00	25067	37675	-	-	-	-
16	HYB 6	69.72	3.22	8.02	< LOQ	< LOQ	1.66	31.37	53.35	0.59
17	HYB 6	71.29	2.51	7.35	< LOQ	< LOQ	1.88	32.09	51.64	0.62
18	HYB 6	73.11	2.65	7.47	7775	19951	1.84	32.84	50.59	0.65
19	HYB 6	70.24	3.04	7.92	3968	4512	1.85	33.71	49.53	0.68
20	HYB 4	72.77	2.48	7.72	373	436	1.32	25.48	57.19	0.45
21	HYB 5	70.96	3.36	7.47	969	1443	1.37	26.34	56.40	0.47
22	HYB 5	72.38	3.03	7.39	2132	2105	1.47	27.67	56.32	0.49

Attachment Table 3. Chemical data obtained from the analysis of maize samples collected in 2010 in the district of MODENA. All the data have been reported on the dry matter. Free and total fumonisins are expressed as µg/Kg. The dash designates that the fatty acids profile has not been evaluated. Each analysis was performed in duplicate.

Sample	Hybrid	Starch (%dm)	Lipids (%dm)	Proteins (%dm)	Free FUM (%dm)	Total FUM (%dm)	C18:0	C18:1	C18:2	C18:1/C18:2
01	HYB 3	74.05	2.62	7.41	1830	4684	-	-	-	-
02	HYB 3	73.85	2.36	7.77	3687	6834	-	-	-	-
03	HYB 3	75.20	2.24	7.52	926	1471	1.49	31.22	54.09	0.58
04	HYB 2	73.86	2.70	7.12	1488	2543	1.43	31.68	52.94	0.60
05	HYB 2	72.44	3.15	8.41	8586	10428	-	-	-	-
06	HYB 2	72.64	3.73	8.72	7649	13255	1.46	31.66	52.66	0.60
07	HYB 6	74.09	2.50	8.35	494	1173	1.46	31.54	52.50	0.60
08	HYB 6	72.41	2.63	7.94	< LOQ	< LOQ	1.34	28.52	55.65	0.51
09	HYB 6	71.87	2.71	7.90	575	731	1.28	25.82	58.98	0.44

Attachment Table 4. Chemical data obtained from the analysis of maize samples collected in 2010 in the district of FERRARA. All the data have been reported on the dry matter. Free and total fumonisins are expressed as µg/Kg. The dash designates that the fatty acids profile has not been evaluated. Each analysis was performed in duplicate.

Sample	Hybrid	Starch (%dm)	Lipids (%dm)	Proteins (%dm)	Free FUM (%dm)	Total FUM (%dm)	C18:0	C18:1	C18:2	C18:1/C18:2
01	HYB 1	72.09	2.74	7.49	5455	8847	1.50	25.75	57.13	0.45
02	HYB 4	74.57	2.38	7.18	4059	4643	-	-	-	-
03	HYB 10	73.25	2.73	6.39	14976	14039	1.50	30.08	55.07	0.55
04	HYB 6	72.36	2.65	7.03	32770	33269	1.62	30.28	54.97	0.55
05	HYB 6	73.72	2.31	7.43	4613	11819	1.58	28.63	56.37	0.51
06	HYB 3	72.29	2.60	7.20	9453	16109	1.54	26.98	57.77	0.47
07	HYB 5	68.72	3.45	7.96	15572	28014	1.47	28.62	56.51	0.51
08	HYB 6	74.00	2.42	7.49	5449	6234	1.39	30.26	55.25	0.55
09	HYB 6	72.89	2.05	7.31	14782	20723	1.48	30.56	55.18	0.55
10	HYB 5	72.24	2.78	6.80	8083	13342	1.48	26.25	57.37	0.46
11	HYB 5	69.70	3.30	7.51	39692	68693	1.39	25.33	57.67	0.44
12	HYB 5	70.41	3.75	7.29	3394	6249	1.29	24.60	58.35	0.42
13	HYB 5	69.34	3.79	7.53	2368	5765	1.38	27.50	56.76	0.48
14	HYB 4	68.59	3.19	8.49	455	123	1.48	30.40	55.17	0.55
15	HYB 3	70.92	2.48	7.50	16753	23725	1.42	30.15	55.17	0.55
16	HYB 10	72.54	2.70	6.24	10143	17529	1.37	29.89	55.18	0.54

Attachment Table 5. Chemical data obtained from the analysis of maize samples collected in 2010 in the districts of PARMA and PIACENZA. All the data have been reported on the dry matter. Free and total fumonisins are expressed as µg/Kg. The dash designates that the fatty acids profile has not been evaluated. Each analysis was performed in duplicate.

Sample	Hybrid	Starch (%dm)	Lipids (%dm)	Proteins (%dm)	Free FUM (%dm)	Total FUM (%dm)	C18:0	C18:1	C18:2	C18:1/C18:2
01	HYB 1	72.59	3.26	6.96	3015	4294	1.29	27.89	56.31	0.50
03	HYB 4	71.94	3.70	7.18	1743	1785	1.45	27.66	57.53	0.48
07	HYB 4	70.54	3.38	7.54	9554	13908	-	-	-	-
08	HYB 4	70.84	3.46	7.46	13267	33599	-	-	-	-
11	HYB 4	72.49	2.72	7.70	1601	2331	1.32	28.99	55.36	0.52
15	HYB 2	72.78	2.43	7.89	1233	5202	-	-	-	-
17	HYB 2	71.59	3.10	8.83	9964	11532	-	-	-	-
20	HYB 2	72.24	3.31	7.31	4852	6722	-	-	-	-
23	HYB 5	72.88	2.89	7.40	5580	8061	1.39	28.33	56.45	0.50
27	HYB 2	68.40	3.37	9.60	15607	28369	-	-	-	-
31	HYB 6	74.98	2.18	6.80	2856	3939	1.49	27.56	57.33	0.48
34	HYB 4	72.30	2.53	7.18	3744	5302	-	-	-	-
38	HYB 4	68.51	2.95	7.96	988	2509	-	-	-	-
41	HYB 10	68.96	3.46	7.13	21697	28682	1.53	27.46	57.13	0.48
45	HYB 2	67.32	3.70	8.10	15107	25263	-	-	-	-
46	HYB 10	73.13	2.43	6.60	1998	1778	-	-	-	-
47	HYB 10	72.48	2.86	7.17	3102	4962	-	-	-	-
48	HYB 5	71.53	2.99	7.70	4567	5558	1.61	29.02	55.68	0.52
49	HYB 10	70.98	2.85	7.35	12286.19	17036	1.30	26.20	58.15	0.45
50	HYB 6	71.96	2.42	7.16	1029	1335	1.26	26.53	57.20	0.46

Attachment Table 6. Microbiological and chemical data concerning samples collected in February 2010 during the emptying of the silo core cylinder. The sampling time indicates in which moment sampling takes place respect to the beginning of the discharge. All the data were reported on the dry matter. Each analysis was performed in duplicate.

Sample	Sampling Time (min)	CFU *Fusarium*	CFU *A. flavus*	CFU *A. niger*	CFU *Penicillum*	Free FUM (µg/Kg)	Total FUM (µg/Kg)
1	0	2.E+06	3.E+02	7.E+02	1.E+06	1219	1479
2	0	5.E+06	3.E+02	4.E+05	0.E+00	1811	2335
3	0	7.E+06	0.E+00	8.E+03	0.E+00	1904	3987
4	15	6.E+05	3.E+05	0.E+00	9.E+03	352	860
5	15	2.E+05	0.E+00	0.E+00	7.E+03	111	255
6	15	9.E+06	0.E+00	0.E+00	0.E+00	1381	1645
7	30	5.E+05	3.E+04	0.E+00	0.E+00	1977	2526
8	30	1.E+07	0.E+00	0.E+00	0.E+00	744	1255
9	30	2.E+07	0.E+00	0.E+00	1.E+06	260	851

Attachment Table 7. Microbiological and chemical data concerning samples collected in 2010 during the emptying of the silo. All the data were reported on the dry matter. Each analysis was performed in duplicate.

Sample	Day of sampling	CFU *Fusarium*	CFU *A. flavus*	CFU *A. niger*	CFU *Penicillum*	Free FUM (µg/Kg)	Total FUM (µg/Kg)
FLOUR 1	08/03/2010	3.45E+05	1.00E+07	0.00E+00	5.05E+04	2234	2386
FLOUR 2	08/03/2010	4.09E+06	5.05E+05	0.00E+00	0.00E+00	3121	3151
FLOUR 3	08/03/2010	4.83E+06	0.00E+00	0.00E+00	4.80E+06	2240	2260
FLOUR 4	08/03/2010	6.81E+05	0.00E+00	0.00E+00	1.26E+07	1420	1468
FLOUR 5	14/04/2010	2.47E+06	0.00E+00	0.00E+00	2.00E+04	894	1686
FLOUR 6	14/04/2010	2.94E+05	0.00E+00	0.00E+00	0.00E+00	1563	3116
FLOUR 7	14/04/2010	3.22E+05	1.00E+03	0.00E+00	0.00E+00	242	959
FLOUR 8	14/04/2010	2.18E+05	0.00E+00	0.00E+00	0.00E+00	799	1858
FLOUR 9	14/06/2010	2.98E+05	9.25E+03	1.00E+03	3.34E+06	819	1356
FLOUR 10	14/06/2010	1.90E+05	1.00E+03	2.00E+03	2.00E+03	459	578
FLOUR 11	14/06/2010	5.83E+05	1.13E+04	1.12E+04	6.75E+03	831	1130
FLOUR 12	14/06/2010	2.83E+05	1.00E+03	3.00E+03	1.10E+04	801	799
TRUCK 1	08/03/2010	4.01E+06	6.50E+03	1.00E+03	0.00E+00	3982	4309
TRUCK 2	08/03/2010	5.13E+05	0.00E+00	0.00E+00	1.60E+07	3159	3223
TRUCK 3	08/03/2010	3.08E+07	0.00E+00	0.00E+00	0.00E+00	2883	3523
TRUCK 4	08/03/2010	1.92E+05	0.00E+00	0.00E+00	1.00E+06	3072	3147
TRUCK 5	14/04/2010	2.79E+05	0.00E+00	0.00E+00	0.00E+00	1322	2937
TRUCK 6	14/04/2010	2.84E+04	0.00E+00	0.00E+00	0.00E+00	703	1199
TRUCK 7	14/04/2010	1.17E+05	0.00E+00	0.00E+00	0.00E+00	203	278
TRUCK 8	14/04/2010	4.11E+05	0.00E+00	2.00E+03	0.00E+00	89	162
TRUCK 9	14/06/2010	6.56E+04	1.00E+03	1.00E+03	1.07E+04	734	2529
TRUCK 10	14/06/2010	1.29E+06	3.00E+03	1.00E+03	5.74E+05	78	178
TRUCK 11	14/06/2010	1.99E+06	0.00E+00	8.67E+03	6.33E+03	85	125
TRUCK 12	14/06/2010	5.09E+05	0.00E+00	1.00E+03	2.33E+03	794	879
TRAIL 1	08/03/2010	4.61E+06	0.00E+00	0.00E+00	7.33E+05	1140	4034
TRAIL 2	08/03/2010	2.71E+07	0.00E+00	0.00E+00	1.01E+06	1187	4164
TRAIL 3	08/03/2010	4.38E+06	0.00E+00	0.00E+00	6.96E+06	LOQ	1485
TRAIL 4	08/03/2010	3.02E+05	0.00E+00	0.00E+00	1.82E+07	552	3630
TRAIL 5	14/04/2010	1.18E+05	0.00E+00	0.00E+00	1.00E+04	2990	3685
TRAIL 6	14/04/2010	5.26E+05	0.00E+00	0.00E+00	0.00E+00	1486	2281
TRAIL 7	14/04/2010	7.72E+05	0.00E+00	0.00E+00	0.00E+00	741	944
TRAIL 8	14/04/2010	1.98E+05	0.00E+00	0.00E+00	0.00E+00	881	4520
TRAIL 9	14/06/2010	9.37E+05	1.00E+04	0.00E+00	1.00E+04	68	188
TRAIL 10	14/06/2010	1.04E+06	6.83E+03	1.00E+03	1.00E+03	1008	1102
TRAIL 11	14/06/2010	4.27E+06	0.00E+00	1.00E+04	1.00E+04	333	351
TRAIL 12	14/06/2010	1.10E+06	0.00E+00	0.00E+00	1.42E+04	309	317

Attachment Table 8. Amounts of fumonisins B, A and C detected in *Fusarium* broth cultures. Data were reported on the weight unit, by correcting the concentration of each analyte for the dry weight of the mycelium.

Incubation period (days)	[FBs] (µg/Kg)	Std. Dev.	[FAs] (µg/Kg)	Std. Dev.	[FCs] (µg/Kg)	Std. Dev.	[FUM] (µg/Kg)	Std. Dev.
Strain 10027, a_w 0.990								
21	40967	1366	130	9	207	32	4434	1407
30	13536	499	264	3	546	5	14346	508
45	6508	137	269	64	701	180	7478	499
Strain 10027, a_w 0.955								
Incubation period (days)	[FBs] (µg/Kg)	Std. Dev.	[FAs] (µg/Kg)	Std. Dev.	[FCs] (µg/Kg)	Std. Dev.	[FUM] (µg/Kg)	Std. Dev.
21	2240	50	101	15	59	9	2400	74
30	2710	13	133	8	65	9	2908	14
45	2499	237	195	26	113	58	2807	269
Strain 10026, a_w 0.990								
Incubation period (days)	[FBs] (µg/Kg)	Std. Dev.	[FAs] (µg/Kg)	Std. Dev.	[FCs] (µg/Kg)	Std. Dev.	[FUM] (µg/Kg)	Std. Dev.
21	261	102	<LOD	-	<LOD	-	261	102
30	2607	2099	8	11	52	74	2667	2184
45	76683	3382	388	53	1762	94	79018	3531
Strain 10026, a_w 0.955								
Incubation period (days)	[FBs] (µg/Kg)	Std. Dev.	[FAs] (µg/Kg)	Std. Dev.	[FCs] (µg/Kg)	Std. Dev.	[FUM] (µg/Kg)	Std. Dev.
21	131	25	<LOD	-	<LOD	-	131	25
30	11000	-	83	2	367	194	11388	-
45	135	52	<LOD	-	<LOD	-	135	52

Attachment Table 9. Amounts of partially hydrolyzed fumonisins detected in *Fusarium* broth cultures. Data were reported on the weight unit, by correcting the concentration of each analyte for the dry weight of the mycelium.

Incubation period (days)	[$PHFB_1$] (µg/Kg)	Std. Dev.	[$PHFB_2$] (µg/Kg)	Std. Dev.	[$PHFB_4$] (µg/Kg)	Std. Dev.	[PHFBs] (µg/Kg)	Std. Dev.
Strain 10027, a_w 0.990								
21	139	2	171	6	152	11	462	18
30	229	44	370	80	222	47	822	172
45	448	161	763	804	443	415	1654	1380
Strain 10027, a_w 0.955								
Incubation period (days)	[$PHFB_1$] (µg/Kg)	Std. Dev.	[$PHFB_2$] (µg/Kg)	Std. Dev.	[$PHFB_4$] (µg/Kg)	Std. Dev.	[PHFBs] (µg/Kg)	Std. Dev.
21	45	1	78	3	60	4	183	5
30	50	4	86	4	73	15	209	23
45	51	4	85	9	96	5	232	11
Strain 10026, a_w 0.990								
Incubation period (days)	[$PHFB_1$] (µg/Kg)	Std. Dev.	[$PHFB_2$] (µg/Kg)	Std. Dev.	[$PHFB_4$] (µg/Kg)	Std. Dev.	[PHFBs] (µg/Kg)	Std. Dev.
21	5	7	12	1	3	5	20	11
30	23	16	126	125	71	63	221	203
45	671	124	2180	403	436	95	3287	432
Strain 10026, a_w 0.955								
Incubation period (days)	[$PHFB_1$] (µg/Kg)	Std. Dev.	[$PHFB_2$] (µg/Kg)	Std. Dev.	[$PHFB_4$] (µg/Kg)	Std. Dev.	[PHFBs] (µg/Kg)	Std. Dev.
21	<LOD	-	<LOD	-	<LOD	-	<LOD	-
30	307	183	945	869	191	35	1443	1087
45	<LOD	-	<LOD	-	<LOD	-	19	2

AUTHOR

Claudia Falavigna

Born in Suzzara (Mantova, Italy), on November 5th, 1983.

e-mail: claudia.falavigna@nemo.unipr.it

STUDIES

Claudia Falavigna got the Bachelor Degree in Food Science and Technology (University of Parma, 110/110) in July 2006, with a thesis titled "Fumonisin analysis in maize-based food products". In October 2008 she got the Master Degree in Food Science and Technology (University of Parma, 110/110, Honours) with a graduation thesis titled "Multiresidual analysis of trichothecenes and zearalenone in wheat".

In January 2009 Claudia Falavigna starts the PhD in Food Science and Technology (University of Parma), under the supervision of Prof.ssa Chiara Dall'Asta. The PhD work has been focused on the study of the occurrence of free and hidden fumonisins in raw maize (*Zea Mays L.*), focusing the attention on the conditions able to affect masking phenomenon as well as the mechanism of interaction between fumonisins and maize macroconstituents.

SCIENTIFIC ACTIVITY

Manuscripts in preparation

Fumonisins B, A and C profile and masking in Fusarium verticillioides and F. proliferatum in different water activity regimes
C. Falavigna, I. Lazzaro, C. Dall'Asta, G. Galaverna, P. Battilani

Submitted publications

Role of maize hybrids and their chemical composition in Fusarium infection, fumonisin production and masking.
C. Dall'Asta, C. Falavigna, G. Galaverna, P. Battilani.
Submitted to Journal of Agricultural and Food Chemistry.

Publications in peer-reviewed ISI Scientific Journals

In vitro digestion assay for determination of hidden fumonisins in maize.
C. Dall'Asta, C. Falavigna, G. Galaverna, A. Dossena, R. Marchelli.

Journal of Agricultural and Food Chemistry, 2010, *58*, 12042-12047, ISSN 0021-8561.

A multiresidual method for the simultaneous determination of the main glycoalkaloids and flavonoids in fresh and processed tomato (Solanum lycopersicum L.) by LC-DAD-MS/MS.
C. Dall'Asta, C. Falavigna, G. Galaverna, S. Sforza, A. Dossena, R. Marchelli.
Journal of Separation Science, 2009, *32*, 3664-3671, ISSN 1615-9306.

Publications in Proceedings

Oral Communications

Hidden fumonisins in maize hybrids and their correlation with chemical composition.
C. Falavigna, C. Dall'Asta, G. Galaverna.
Conference Abstracts of 33rd Mycotoxin Workshop 2011, Freising, Germany, 30th May-1st June 2011, p. 7, L05.

Hidden fumonisins: a step beyond the analytical issue
C. Dall'Asta, C. Falavigna, A. Tonelli, G. Galaverna, A. Dossena
Book of Abstracts of 5th International Symposium on recent Advances in Food Analysis, Prague, Czech Republic, 1-4 November 2011, p. 119, L-81. ISBN 978-80-7080-795-8.

Poster communications

Monitoring fumonisin analogues production in liquid cultures by LC-MS/MS
C. Falavigna ,C. Dall'Asta, P. Battilani, A. Dossena.
Proceedings of 2nd MS Food Day 2011, University of Trieste, Italy, 19-21 October 2011, Vol. 1, pp. 152-153, P33, ISBN: 978-88-8420-708-1.

Production of fumonisin analogues in Fusarium verticillioides broth cultures under different growth parameters.
C. Falavigna, C. Dall'Asta, G. Galaverna, P. Battilani, A. Dossena.
Atti del XXIV Congresso Nazionale della Società Chimica Italiana, Lecce, Italy, 11-16 September 2011, Vol.1, p. 942, ORG-PO-53, eISBN: 978-88-8305-085-5.

In vitro models for studying the fumonisin masking mechanism in maize.
C. Falavigna, C. Dall'Asta, G. Galaverna.
Conference Abstracts of 33rd Mycotoxin Workshop 2011, Freising, Germany, 30th May-1st June 2011, p. 84, P40.

Free and masked fumonisins in maize hybrids and their correlation with chemical composition
C. Dall'Asta, C. Falavigna, G. Galaverna, A. Dossena, M. Zatti, A. Rossi, P. Battilani.
Book of Abstracts of ISM Conference 2011, Cape Town, South Africa , 4-6 April 2011.

Free and hidden fumonisins in corn: occurrence and masking mechanism.
C. Falavigna
Proceedings of 15th Workshop on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, University of Napoli, 15-17 September 2010, pp. 239-240, ISBN: 978-88-95028-62-0.

Hidden fumonisins: a study of the masking mechanism in raw maize.
C. Falavigna, A. Dall'Erta, C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli.
Conference Abstracts of 32nd Mycotoxin Workshop 2010, Lyngby, Denmark, 14-16 June 2010, p. 82, P38.

Simulated digestion assay for hidden fumonisin evaluation.
A. Dall'Erta, C. Falavigna, C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli.
Conference Abstracts of 32nd Mycotoxin Workshop 2010, Lyngby, Denmark, 14-16 June 2010, p. 46, P02.

A LC-ESI-MS/MS method for the simultaneous determination of native and masked Fusarium mycotoxins in grains.
C. Falavigna, C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli.
Book of Abstract of 1st MS Food Day 2009, Parma, Italy, 2-3 December 2009, pp.226-229.

Valutazione della presenza di micotossine nascoste nella filiera dei cereali mediante digestione gastrointestinale simulata.
Falavigna C., Dall'Asta C., Galaverna G., Dossena A., Marchelli R.
Rapporti ISTISAN 10/32, 3° Congresso Nazionale Istituto Superiore di Sanità, Le micotossine nella filiera agro-alimentare e zootecnica, Rome, Italy, 28-30 September 2009, Vol. 1, pp. 16-25, ISSN: 1123-3117.

Deossinivalenolo, zearalenone e loro metaboliti nella filiera dei cereali.
Falavigna C., Dall'Asta C., Galaverna G., Dossena A., Marchelli R.
Rapporti ISTISAN 10/32, 3° Congresso Nazionale Istituto Superiore di Sanità, Le micotossine nella filiera agro-alimentare e zootecnica, Rome, Italy, 28-30 September 2009, Vol. 1, pp. 26-32, ISSN: 1123-3117.

Hidden fumonisins: an emerging issue
C. Dall'Asta, M. Mangia, C. Falavigna, G. Galaverna, A. Dossena, R. Marchelli.
Book of Abstracts of ISM Conference 2009, Tulln, Austria, 9-11 September 2009, p. 168, Poster 143.

A digestion assay for masked mycotoxin evaluation in cereal.
C. Falavigna, A. Dall'Erta, C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli.
Book of Abstracts of ISM Conference 2009, Tulln, Austria, 9-11 September 2009, p. 109, Poster 084.

Participation to international conferences

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

33rd Mycotoxin Workshop. Freising, Germany, 30th May-1st June 2011

Sostanze tossiche negli alimenti: un approccio multidisciplinare. Parma, Italy, 9th July 2010.

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

1st MS Food Day. Parma, Italy, 2-3 December 2009.

ISM Conference 2009. Global Discussion Forum on: *Worldwide Mycotoxin Reduction in Food and Feed Chains.* Tulln, Austria, 9-11 September 2009.

Applicazioni in Spettrometria di Massa per la valutazione della qualità degli alimenti e della sicurezza alimentare. Parma, Italy, 30th April 2009.

Participation to PhD schools and workshops

15th Workshop on the Developments in the Italian phD Research on Food Science Technology and Biotechnology. Portici, Italy, 15-17 September 2010

14° Corso di Spettrometria di Massa per Dottorandi di Ricerca 2010. Certosa di Pontignano, Siena, Italy, 21-26 March 2010.