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Fumonisins and their modified forms, a matter of concern in future scenario?

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REVIEW ARTICLE

Abstract

Masked mycotoxins are found in grains and derived foods as a result of plant phase II metabolism. Recently, masked mycotoxins *senso strictu,* together with other covalently or non-covalently conjugated forms, even formed upon processing, have been classified as modified mycotoxins. In this context, the issue of modified fumonisins is of great interest, on account of the wide range of factors affecting their formation and accumulation in maize pre- and postharvest. Fumonisins, indeed, may undergo modification in plants, along the growing season, but also during storage and drying of maize kernels, and upon processing. All these modifications strongly affect the analytical outcome, thus making more difficult the assessment of maize compliance. Since the ratio between free and modified fumonisins is affected by maize composition and environmental factors, a deeper knowledge on the phenomena driving the production and accumulation of free and modified forms in plants may support the selection of resistant hybrids. This review provides a critical picture of the state of the art on this topic, mainly focusing on those events occurring in field, identified as crucial in determining amount and partitioning of contamination. Nevertheless, knowledge on modified fumonisins is still in its dawn, on account of the wide range of factors involved. Anyway, reported results, taking altogether, clearly indicate that modified fumonisins should be included in the monitoring plans to have an overview of the possible contribution to human exposure. Furthermore, next efforts should focus on the events occurring in field and on the cross-talk between the plant and the fungus, to support the identification of resistant hybrids and to provide data for predictive models, the most suitable tool to forecast what is going to happens in the future changing climate.

Keywords: hidden fumonisins, *Fusarium verticillioides*, maize, climate change

1. From masked mycotoxins to modified fumonisins

Mycotoxins are secondary metabolites produced by fungi that may contaminate crops worldwide, mainly cereals. Advance in mycotoxin determination have shown that in addition to parent mycotoxins, cereals also contain mycotoxin conjugates. These compounds are commonly known as 'masked mycotoxins', since they originally escaped routine methods on account of their different chemical properties compared to parent forms (Berthiller *et al.*, 2005).

Masked mycotoxins

Masked mycotoxins are found in grains and derived foods as a result of plant phase II metabolism. More specifically, the plant may respond to the pathogen infection through a detoxification pathway, leading to the conjugation of the mycotoxin with a polar compound such as a glucose, glutathione or sulphate. Conjugates of trichothecenes and zearalenone (ZEA) have been identified so far (Berthiller *et al.*, 2013). While occurrence data on masked deoxynivalenol (DON) and ZEA have been reported in the literature, less is known to date about other trichothecenes such as nivalenol and T-2 and HT-2 toxins (Meng-Reiterer *et al.*, 2015; Nathanail *et al.*, 2015a,b; Yoshinari *et al.*, 2014).

Besides conjugation, there is evidence that some mycotoxins can be covalently or non-covalently bound to the matrix macromolecules, or physically entrapped within the matrix structure (Berthiller *et al.*, 2013). According to a more recent classification, masked mycotoxins *senso strictu* are part of the wider group 'modified mycotoxins', also including those compounds derived from processing and metabolic transformations (Rychlik *et al.*, 2014). Starting from the literature, masked/modified mycotoxins in cereals and product thereof account for an additional amount up to 40-60% of the parent compounds (EFSA, 2014). This suggests that the mycotoxin burden in cereals could be significantly higher if the 'masked' forms are taken into account. In this context, a peculiar role is played by fumonisins, as their modified forms are still to be univocally characterised (Dall'Asta *et al.*, 2010).

Parent and masked fumonisins

Fumonisins are a group of mycotoxins produced by various *Fusarium* sp., and are frequently found in maize worldwide, mainly associated to *Fusarium verticillioides*, a member of *Fusarium fujikuroi* species complex. Structurally, they are long-chained aliphatic amines carrying methyl and hydroxyl groups at various positions of the aliphatic chain. Two of the hydroxyl groups are esterified with tricarballylic acids. Although a large number of fumonisins have been identified so far, the B group (i.e. fumonisin B_1 to B_4 (FB_{1-4}) is the most prevalent in food and feed commodities (Figure 1).

Unlikely other mycotoxins, FBs are reactive compounds that can undergo modification *via* the primary amino group, and the hydroxyl and the carboxylic groups. In addition, tricarballylic side chains are commonly cleaved upon treatment with alkali releasing the aminopentol backbone, commonly known as hydrolysed FBs. It has been observed that alkaline hydrolysis of contaminated maize products leads to an often higher amount of released hydrolysed FBs than that stoichiometrically derived by the conversion of the FBs detectable by the routine analysis (Dall'Asta *et al.*, 2009, 2010; Kim *et al.*, 2003; Park *et al.*, 2004). Starting from this observation, studies on the possible occurrence of modified forms, both covalently or not covalently bound, have been performed over the last decade.

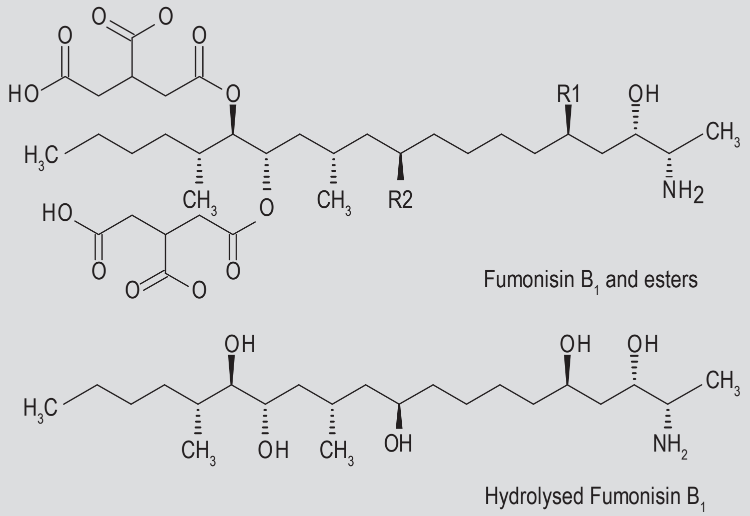
Over the years, a number of unidentified bound forms were detected in thermally treated food products such as cornflakes (Kim *et al.*, 2003; Park *et al.*, 2004). The nature of this masking mechanism was initially attributed to the formation of covalent bonds between the tricarballylic groups of FBs and the hydroxyl groups of starch or the amino or sulfidryl groups of the side chains of amino acids in proteins (Seefelder *et al.*, 2003). However, the so called 'hidden fumonisins' are not restricted to thermally treated products, but have been proven to occur with mild-temperature exposure as well as in maize kernels harvested in commercial fields (Dall'Asta *et al.*, 2010).

This evidence is actually related to the empirical observation, often reported by farmers, that different FB contamination levels may be found before and after maize drying, confirmed by some research data (Giorni *et al.*, 2015a).

The masking mechanism

The mechanism of FB modification is still an issue, as the chemical structure of these compounds let them interact and/or react in a number of modes with the matrix biopolymers. Possible modifications are reported in Figure 2.

The parent compounds can be found physically entrapped into the structure of macromolecular components, such as



	R1	R2
FB1	OH	OH
EFB1OA	Oleoyl	H
isoEFB1OA	H	Oleoyl
EFB1LA	Linoleoyl	H
isoEFB1LA	H	Linoleoyl

Figure 1. Chemical structures of fumonisin B_1, hydrolysed fumonisin B_1, and fatty acid esters of fumonisin B_1.

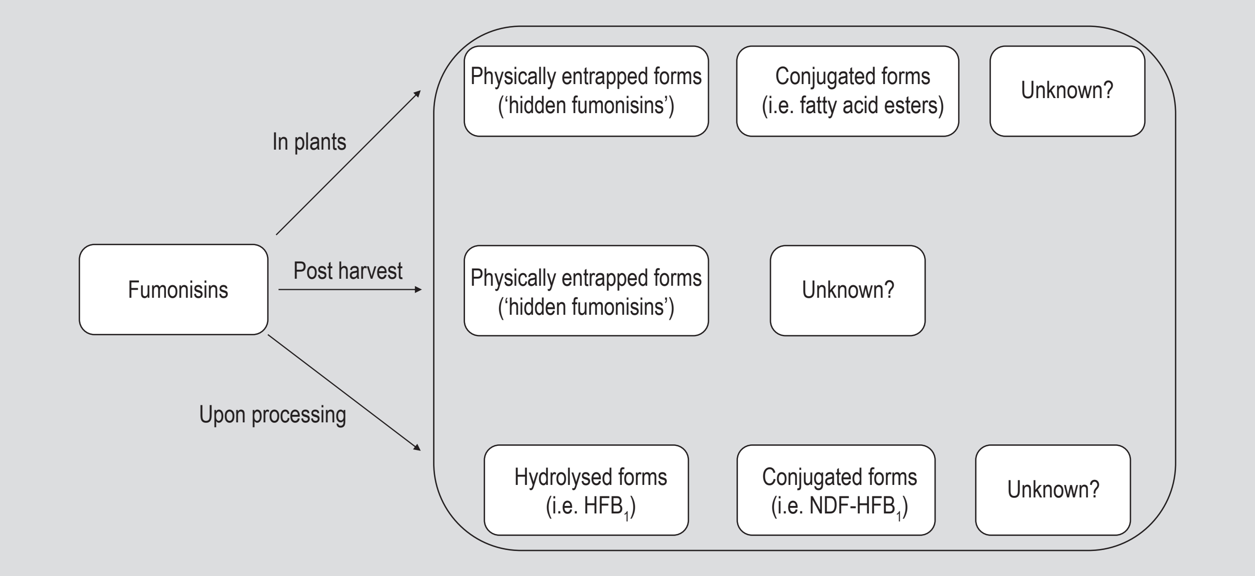


Figure 2. Modified forms of fumonisins formed in maize at various stages. The occurrence of possible unknown compounds is expected in consideration of the mass unbalance.

starch or proteins. Thus, these forms are only extractable after alkaline hydrolysis of the matrix (Dall'Asta *et al.*, 2009). The entrapping mechanism is still to be clarified from a physicochemical side, but data collected so far indicate that biopolymers – preferentially amylose and amylopectine – may form inclusion complexes with FBs. These complexes are stable under the routine extraction conditions, but can be easily destroyed under *in vitro* digestion conditions, when biopolymers are enzymatically degraded (Dall'Asta *et al.*, 2010). Although the nature of the complexation is not described, it is known that amylose is able to bind lipid compounds. These supramolecular structures have been elucidated by physical methods (Genkina *et al.*, 2015; Manca *et al.*, 2015; Marinopoulou *et al.*, 2016).

In addition, due to the presence of a primary amino group and of four carboxyl groups, FBs can undergo various chemical reactions, e.g. Maillard-type reactions with carbohydrates or ester/amide with proteins, lipids or carbohydrates. Such reactions have been reported in thermally treated maize products (Humpf and Voss, 2004), giving rise to a multitude of compounds, which causes major analytical problems. Further investigation performed on model system showed that such conjugation may occur at 140-150 °C (Seefelder *et al.*, 2003). In consideration of the low yield reported in the model system, the occurrence of these compounds at significant amount in a more complex food product is likely to be very low.

Finally, the hydroxyl groups and the amino group are involved in the formation of fatty acid conjugates in plants. The structures of 3-*O*- and 5-*O*-linoleoyl-FB$_1$, 3-*O*- and 5-*O*-oleoyl-FB$_1$, and 3-*O*- and 5-*O*-palmitoyl-FB$_1$ were proposed for six esterified FB$_1$ isomers detected in small amounts in a *F. verticillioides*-inoculated rice culture (Bartók *et al.*, 2010, 2013). Similarly, the occurrence of *N*-palmitoyl, *N*-oleoyl- and *N*-linoleoyl-FB$_1$ derivatives in rice cultures of *F. verticillioides* has been reported so far (Bartók *et al.*, 2013).

2. Fumonisins: formation and modification in plants

Formation of fumonisins

The formation and accumulation of FBs during maize growth is a very complex process, affected by both the interaction of the fungal pathogen with the host plant and the environment. Many ecological studies confirmed the key role of temperature and a_w in modulating *F. verticillioides* growth and FB production. From these observations, descriptive and predictive models have been derived (i.e. Battilani *et al.*, 2003; Maiorano *et al.*, 2009; Samapundo *et al.*, 2005).

More recently, it has been stated that FB biosynthesis is regulated by the *FUM* gene cluster whose mechanism has not yet been totally clarified (Butchko *et al.*, 2006; Desjardins and Proctor, 2007; Proctor *et al.*, 2003). However, regulating action of ecological factors in eliciting the expression of genes belonging to the cluster was newly considered.

In *in vitro* studies, differences in the expression of *FUM* genes, depending on the ecological condition of exposure, were described. The effect of temperature, i.e. was significantly higher on *FUM21* than on *FUM2* expression (Lazzaro *et al.*, 2012b). *FUM3* and *FUM14* were the most highly expressed among the 5 *FUM* genes studied in different water activity (a_w) conditions and a positive correlation with FB production has been shown. However, the partitioning FB_3 (precursor) to FB_1 (resulting compound) is in favour of the former when *FUM14* is more highly expressed than FUM3, possibly due to the role of *FUM3* in the hydroxylation of FB_3 to FB_1 (Lazzaro *et al.*, 2012a).

The relative expression of nine biosynthetic *FUM* cluster genes and FB production, in relation to the environmental factors, was also considered by Medina *et al.* (2013). They developed a predictive model which provided a good linear regression fit between the predicted and observed FB production based on the expression rate of the studied genes in different temperature and a_w regimes. This approach was considered in view of examining the impact of climate change factors on toxin production (Magan *et al.*, 2011).

The expression of *FUM1* gene (fumonisin polyketide synthase) is induced in *F. verticillioides* in response to water stress. Therefore, drought conditions are supposed to result in increased risk of FB contamination (Marìn *et al.*, 1999). In particular, significant and prolonged increase in water stress (e.g. 0.93 a_w) enhanced *FUM1* expression, while a mild water stress (0.98-0.95 a_w) did not significantly affect gene transcripts (Jurado *et al.*, 2008).

After many studies *in vitro* that quantitatively described the role of ecological parameters in FB synthesis, the formation of FBs in plant is still to be completely clarified. However, it is confirmed that the rate of water loss during maize ripening seriously affects FB accumulation (Battilani *et al.*, 2011). The decrease to 30-35% moisture content in grains at the mid-dough stage (approximately 0.95 a_w) open the window for *F. verticillioides* to maximise FB synthesis (Medina *et al.*, 2013). Time and length of these optimal conditions in field is strictly weather dependent.

In addition, drought, insect damages and, in general, the wide concept of stress induce a great increase in fungal growth and in FB accumulation in maize kernels (Wu *et al.*, 2011). Rain and irrigation are important limiting factors in FB accumulation (Pitt *et al.*, 2013), while pest attacks are enhancing factors. Moreover, the use of cultivars developed for particular climates is important; the choice of hybrid outside the recommended growing areas increases stress and FB production (Doko *et al.* 1995; Visconti, 1996).

Fumonisin accumulation is influenced by nutritional factors such C:N ratio, amylopectin content in kernels (Bluhm and Woloshuk, 2006; Giorni *et al.*, 2015b; Picot *et al.*, 2012) and pH (Flaherty *et al.*, 2003). However, the chemical composition of maize kernels, mainly the lipid fraction, has been shown to influence FB production by *F. verticillioides* (Dall'Asta *et al.*, 2012). In particular, fatty acids, such as oleic and linoleic acids, seem to have a key role in driving the FB accumulation in plants. Notably, maize hybrids with high linoleic content displayed higher FB contamination (Dall'Asta *et al.*, 2012). This has been related to the involvement of these compounds in the plant pathogen cross-talk (Scala *et al.*, 2013). Unsaturated and polyunsaturated fatty acids, such as oleic and linoleic acids, are precursors of oxylipins, a class of oxidised metabolites responsible for the signalling pathways of both plants and fungi (Reverberi *et al.,* 2012). In agreement, it was recently found that the content of the phytoceramides lignoceric phytosphingosine, and hydroxyl lignoceric phytosphingosine, as well as the oxylipins 9- and 13-hydroxyoctadecenoic acid, are significantly modified upon FB accumulation in maize hybrids under natural field conditions (Giorni *et al.*, 2015a).

The cropping system as a whole is the tool available for farmers to try mitigate FBs, with hybrid selection, irrigation, pest control and harvest time choice being the main driving factors; however, even when optimised they are not able to prevent, but only to mitigate, FB contamination.

Modification of fumonisins

All mechanisms and factors involved in FB production in maize ears are not yet completely clarified, but many steps forward have been done; instead, regarding modified FB, knowledge is still poor. Nonetheless, most of the studies have been performed under field conditions, and univocally pointed out the crucial role of maize hybrids. In consideration of the open field design of these studies, bigger efforts and longer time are requested to obtain reliable results compared to *in vitro* studies.

Maize hybrids, water activity and amylose/amylopectin ratio in grain are affecting the extent of masking (Giorni *et al.*, 2015b), which is however mostly related to lipid compounds. Fatty acids play a key role not only in FB production, but also in its modification in plants; the ratio oleic/linoleic acid is in fact negatively related to FB modification (Dall'Asta *et al.*, 2012). As both oleic and linoleic acids are precursors of oxylipins – a class of chemical mediators between the plant and the fungus – the correlation of these compounds with the accumulation of mycotoxins underlines that the plant-fungus cross talk strongly affects FB modification.

Interestingly, the only conjugated forms of FB that have been isolated in naturally infected kernels so far are due to the esterification of FBs with oleic and linoleic acids (Falavigna *et al.*, 2013). Studies performed *in vitro* on different growing substrates (maize vs rice) showed that the distribution in terms of oleic and linoleic moieties in fatty acid esters produced *by F. verticillioides* strongly resemble the fatty acid composition of the growing medium. In addition, comparing the trend of production, it is clearly demonstrated that the conjugated forms increase in both media when the free forms reach a threshold (Falavigna *et al.*, 2016). This statement suggests that fatty acid esters of FB_1 are produced by the fungus itself at a late stage of its growth, or at a certain level of FB accumulation, using fatty acids from the plant.

Similarly, the formation in maize of *N*-acyl derivatives of FB_1 with oleic and linoleic acids has been proved *in vitro* (Bartók *et al.*, 2013). However, no information about the possible occurrence of similar compounds in plants are reported so far.

The involvement of lipid composition of the maize kernels in toxin accumulation was further confirmed by different studies (Christensen and Kolomiets, 2011; Scala *et al.*, 2014). Maize lipids seemed to play a pivotal role in FB accumulation; the oxylipin 9-HODE ((±)-9-hydroxy-10E,12Z-octadecadienoic acid) and three sphingolipids were identified as able to differentiate high-contaminated from low contaminated hybrids (cut-off = 2,000 μg/kg of FBs: Dall'Asta *et al.,* 2015). The correlation between FB accumulation and plant lipid profile was further supported by Giorni *et al.* (2015a), although the cause-effect relation between FB and plant lipidome is still to be clarified. It was recently suggested that trapping endo- and exotoxins within lipid droplets (LD) is a resistance mechanism in fungi (Chang *et al.*, 2015), leading to a quenching of the intracellular ROS production. Although the trapping of mycotoxins in such structures has never been investigated, a similar mechanism might occur. LD are intracellular organelles containing neutral lipids, mainly triacylglicerols and sterol esters. Although they are mainly lipid storage structures, they take part to a number of cellular functions linked to lipid metabolism, among them membrane trafficking and cell signalling (Walther and Farese, 2012). These are important input for further studies aimed to clarify the mechanism and to answer open questions.

The possible role of oxylipins was also studied *in vitro* using a mutant strain of *F. verticillioides*. Oxilipins are supposed to regulate gene expression in *F. verticillioides* and to be able to re-shape the transcriptional profile by inducing chromatin modifications. They appear to exert thus a direct control on the transcription of secondary metabolism (Scala *et al.*, 2014).

In a field study managed in 2010, hidden FBs were detected in 9 out of 10 hybrids, accounting for ~57% of the free form at harvest. Interestingly, the only hybrid negative to hidden FBs was found highly infected by *Aspergillus flavus* (Dall'Asta *et al.*, 2015). Therefore, interaction between fungi, and the mycoflora associated to the grains, should be considered as relevant in the complex pathosystem, although the mode of action is actually totally unknown.

3. Modified fumonisins: formation and fate postharvest

Effect of drying and storage

Although modified FBs already occur in field, the ratio between free and hidden forms is likely affected by technological treatments postharvest. Information about the distribution of FBs into the maize fractions clearly indicate that mycotoxins are concentrated in the surface layers of the maize kernel. Accordingly, germ, hull, pericarp and tip cap fractions are expected to have high levels of FBs (Brera *et al.*, 2004; Burger *et al.*, 2013; Pietri *et al.*, 2009). The fungal colonisation progresses from the outer layer of the kernel to the inner layer. This could be due to the bran that acts as a physical barrier against the fungal penetration into the endosperm (Burger *et al.*, 2013). A lower surface wax content on the pericarp has been also associated with higher FB amounts in the kernel (Sampietro *et al.*, 2009).

Very recently, several studies addressed not only the occurrence of free and hidden FBs in maize preharvest, but also their possible redistribution into the milling fractions post-harvest. Under a true-scale perspective, a significant increase in the free-to-total FB ratio was observed moving from stored kernels to commercial flour, suggesting that milling leads to a reduction of the masked fraction with respect to the free forms (Falavigna *et al.*, 2014). A consistent trend was reported by Giorni *et al.* (2015a), by measuring the free and total forms into maize samples before and after drying treatments (two conditions applied: 70 °C, 24 h or 95 °C, 9 h). In particular, entrapped forms were higher in samples analysed at harvest, while an opposite trend was observed after drying (Giorni *et al.*, 2015a), indicating that the free forms are dominant only after heat treatment. The chemical and/or physical changes affecting the distribution are actually still to be understood.

According to some authors, the fumonisin contamination seems to increase in lower particle size final products (Brera *et al.*, 2004; Pietri *et al.*, 2009). This could be induced by a major extractability of the free FBs fraction when the particle size decreases, and thus the sample surface available to the solvent increases.

A role of starch was hypothesised, related to its gelatinisation during maize drying, that presumably make FBs more easily extractable, thus increasing their detectable content. The possible involvement of starch in FB accumulation and modification postharvest has been pointed out also by Bryla *et al.* (2015). By comparing the concentration of free and total FBs in flour and bran upon maize milling, the authors showed that the components interacting with FBs are non-uniformly distributed throughout the kernel. They also stated that there is an overall reduction in FB contamination moving from raw kernels to flour, while comparable levels of contamination were reported by Dall'Asta *et al.* (2012). The different outcomes could be explained by the different technological parameters applied during the trials. In addition, the starch composition in terms of amylose/amylopectine ratio is affected by both the hybrid and the weather conditions. It was reported, indeed, that high temperature during the growing season may lead to a reduction in the amylose content (Hizukuri, 1969).

Bryla *et al.* (2015) pointed out that the free-to-total FBs ratio is flour (0.61) < bran (0.64). The values are quite similar, even in consideration of possible experimental bias. Nonetheless, once the trend will be confirmed by further experiments, it suggests that an enrichment in starch, as obtained in flour, leads to a decrease of the free forms. From these results, it could be argued that physically entrapped FBs are more present in the endosperm than in the outer layers. In agreement, another study from the same group showed that maize-based starch concentrates apparently found as blank upon routinely analysis, contained on the contrary low amount of entrapped forms when alkali-treated (Bryła *et al.*, 2016).

Although apparently contradictory, results from studies reported above (Bryla *et al.,* 2015; Giorni *et al.,* 2015a) indicated that both the particle size and the starch amount may affect the free-to-total FBs ratio. Comparing corn flour to the grinded kernels, the stronger association of FBs to the matrix components (greater starch amount) is partially balanced by the higher extractability due to lower particle size. Therefore, since the experiments were performed independently and under different conditions, the relative contribution of each parameter to the overall effect cannot be extrapolated.

With regard to changes occurring upon ensiling, a very recent study showed a similar trend (Latorre *et al.*, 2015). The authors analysed a number of maize silage samples (n=124) finding that 65 and 39% of the total FB_1 and FB_2 amount were due to hidden FBs, respectively. Moreover, the same study followed the changes in free-to-total ratio in laboratory maize silos upon aerobic fermentation, confirming that the aerobic conditions have a significant influence on the amount of total FB_1 and FB_2 in silage, while free forms seemed to be unaffected.

Effect of thermal treatments

A number of studies reported the degradation of FBs upon processing, when temperature of 160 °C or more are applied (Bullerman *et al.,* 2008; Castells *et al.,* 2009; Jackson *et al.,* 2011). Similarly, FB degradation is often associated to alkali-treatments commonly applied for the production of tortillas or masa flour (Humpf and Voss, 2004; Jackson *et al.,* 2012). The main reaction described upon thermal treatments and in the presence of reducing sugars involves Maillard-type reactions and further rearrangement. The main products are *N*-carboxymethyl-fumonisin (NCM-FB_1) and *N*-deoxyfructosyl-fumonisin (NDF-FB_1). In addition, the hydrolysed form due to the cleavage of both carballylic moieties is commonly obtained upon alkali treatment (Humpf and Voss, 2004). Possible chemical structures are reported in Figure 3.

Jackson *et al.* (2011) proposed a twin-screw extrusion study to investigate the fate of fumonisins in flaking maize grits. FB_1 and its analogues were measured with a mass balance approach. Results showed that FB content was reduced up to 72% without sugar addition, and up to 94% with added glucose. After extrusion the authors detected little amount of NCM-FB_1 and hydrolised fumonisin (HFB_1), while the formation of NDF-FB_1 via Maillard-type reaction was a major modification pathway upon sugar addition. Nonetheless, unrecovered unknown forms were predominant (>50%) when comparing the FB_1 originally present in maize and the amount of FB-related compounds measured after extrusion. Concerning the non-covalently bound forms, very recent studies demonstrated that thermally processed food products like flakes and snacks exhibit higher ratio of hidden-to-free FBs than low processed products, like flour, groats or popcorn grains (Bryła *et al.*, 2016). This result indicates that a possible modification of the masking rate may occur upon thermal process, according to the technological treatment and the food properties.

Finally, a lower degradation of hidden forms compared to free FBs upon baking (260 °C, 38 min) was reported Bryla *et al.,* (2014). Accordingly, the associative interaction with macroconstituents may lead to a stabilisation of the hidden forms compared to free FBs. Again, the formation of supramolecular structures may prevent FBs from further degradative reactions: this effect is commonly exploited for the chemical stabilisation of reactive species (Galan and Ballester, 2016). It should be mentioned, however, that sourdough fermentation was performed before the baking treatment, and this could lead to additional side reactions involving matrix constituents.

4. Modified fumonisins: toxicological relevance

The toxicological role of masked mycotoxins is still to be fully clarified, but preliminary results strongly suggest their inclusion in risk assessment studies (EFSA, 2014). Although most of the modified forms are stable under gastric conditions, they are prone to cleavage in the gut upon large intestinal microbial activity (Dall'Erta *et al.*, 2013; Gratz *et al.*, 2013; McCormick *et al.*, 2015). This will lead to the release of free, unbound mycotoxins within the human gut and might add to the toxicological burden. Therefore, the presence of masked metabolites of mycotoxins in foods and their release inside the human gut

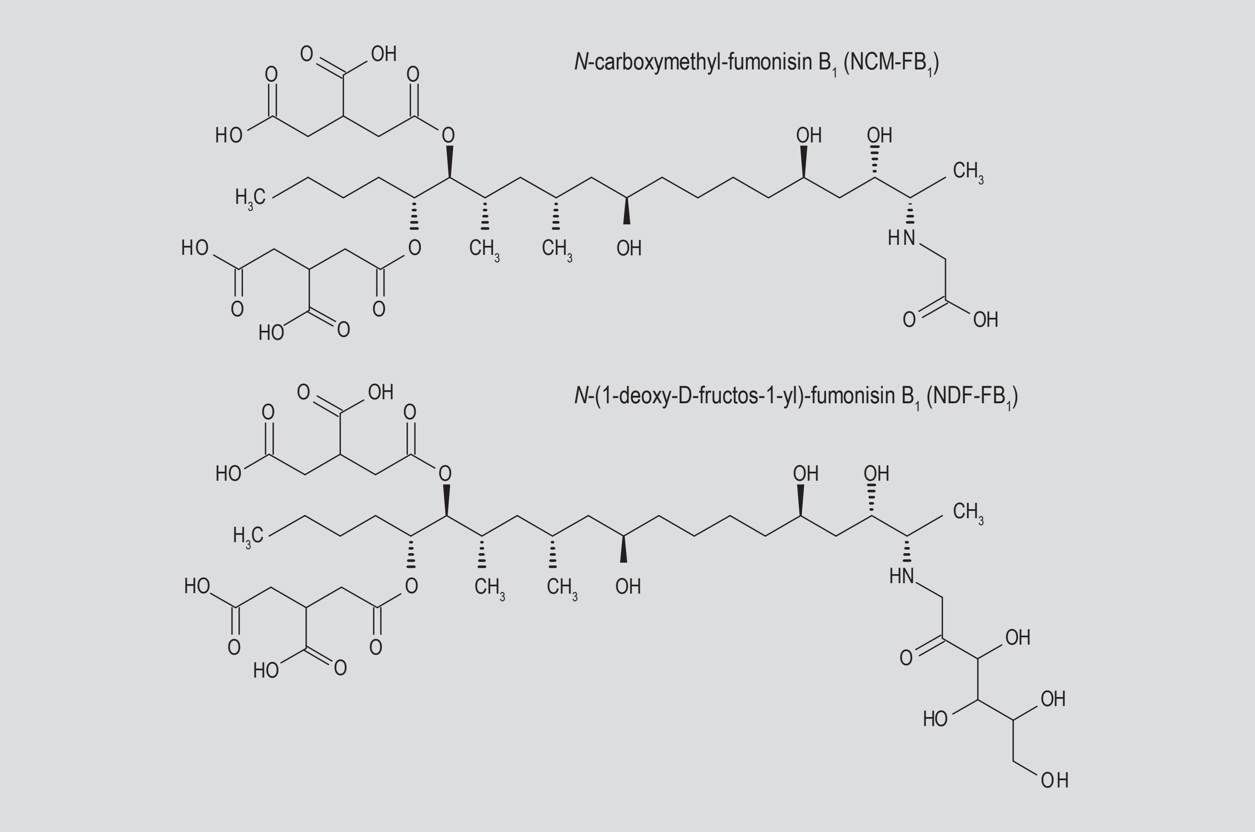


Figure 3. Chemical structure of the main *N*-conjugates of fumonisins formed upon thermal process in the presence of reducing sugars.

need to be assessed to get a full picture of the risk posed by these compounds.

Studies performed *in vitro* on the bioaccessibility and bioavailability of modified FBs in maize showed that their release is strongly affected by the nature of the modification. Physical entrapment leading to hidden FBs can be easily disrupted *in vitro* under digestive conditions, using a digestion assay that simulates human gastrointestinal conditions (Dall'Asta *et al.*, 2010; Falavigna *et al.*, 2012). Fumonisin amount detected in the sample before the digestive assay was lower than that found in the chyme after the treatment. This result suggested the complete release of hidden FBs from the matrix, likely due to the enzymatic degradation of its macroconstituents (Dall'Asta *et al.*, 2010). However, it must be underlined that this outcome was obtained under *in vitro* conditions, while *in vivo* other factors (i.e. gut microbiome, gastrointestinal pathologies, etc.) may play a relevant role.

A different behaviour has been shown, on the contrary, by covalently bound FBs, such as NDF- and NCM-FB_1 conjugates. These compounds are stable upon *in vitro* digestion, not representing thus an additional contribution to the overall FB load (Falavigna *et al.*, 2012). In particular, the catabolic fate of NDF-FB_1 was studied *in vitro* by Cirlini *et al.* (2015), showing that this compound is rather stable upon digestion and is not biotransformed by human gut microbioma. Hahn *et al.* (2015) studied the metabolic fate in rats of HFB_1 and NDF-FB_1 compared to a FB_1-treated control group (Hahn *et al.*, 2015). In agreement with previous literature, positive control group individuals showed an increased sphinganine-to-sphingosine ratio after 1 week of exposure (10 mg/kg FB_1 by diet), indicating a disruption of the sphingoid bases metabolism. On the contrary, neither HFB_1 nor NDF-FB_1 showed significant effects. In addition, NDF-FB_1 was mainly recovered in the faeces of rats, unchanged or as FB_1 (up to 30%), suggesting a partial release of the precursor in the gut of rats due to resident microbiota.

The formation of histopathological damages in kidneys was studied by Voss *et al.* (2013) in rats fed with nixtamalised maize containing FB_1. Nixtamalisation is an alkali-treatment leading to the hydrolyses of FB_1 to form mainly HFB_1 together with other unknown modified forms. According to the authors, nixtamalisation effectively reduces histological lesions in kidney, thus indicating a protective effect of some chemical modification reactions towards FB_1 related nephropathy. In addition, nothing is known so far about the possible toxic effects due to *N*-acyl conjugates formed in plants (Bartók *et al.,* 2013). Since these compounds are structurally related to ceramides, and in consideration of the ability of FBs to alter ceramide metabolism in humans (Riley *et al.*, 2015), the toxicological relevance of these modified forms should be further considered.

5. Climate change, more than a warmer world

Talking about climate change the pillars agreed by scientists are the increase in mean temperature and a different distribution of rainfall, abundant during winter and scarce/absent during summer time; to summarise, climate in temperate zones will become more similar to tropical conditions, and extreme events, like drought periods or episodes of very intense precipitation are expected. This will be combined with an increasing carbon dioxide concentration in the air (IPCC, 2007).

Climate change is expected to result in strong effects on crop geographic distribution and crop production. From one side, several crops, maize included, are expected to enlarge their growing area, thanks to temperature increase that make their life cycle possible at higher latitudes (Battilani *et al.,* 2016). On the other side, extreme events will cause relevant stress to the crops and their production is expected to decrease, both in term of quantity and quality. This is confirmed with field data on wheat (Asseng *et al.*, 2014) and maize (Tao and Zang, 2011). Further, the predicted increase in CO_2 (up to 600/700 ppm over 1000 ppm) and mean air temperature (+2 to +5 °C) are expected to modify the phyllosphere mycoflora of cereals during ripening (Magan and Baxter, 1996). Therefore, climate change has been related to emerging food and feed safety and security issues worldwide (Miraglia *et al.*, 2009), with mycotoxin occurrence playing a crucial role both in food and feed.

Impact of climate change in field

The impact of climate change on mycotoxin producing fungi in field counts really few studies. It is worth mentioning Magan *et al.* (2011). They reported the important example of *Fusarium culmorum* that has been gradually outcompeted by *Fusarium graminearum* in *Fusarium* Head Blight of wheat. A further fundamental example regards *A. flavus* that caused severe aflatoxin contamination in maize in Italy in 2003 (Piva *et al.*, 2006) and in several European maize growing areas in 2012 and 2015 (Pleadin *et al.*, 2015), attributable to the ongoing climate change (Battilani *et al.*, 2016). This will be a relevant topic to be studied in the next future, being reported that FB modification is apparently absent with relevant incidence of *A. flavus*.

The interest towards *F. verticillioides* behaviour in climate change scenario seems scarce, probably due to the more immediate link between warmer conditions and aflatoxin contamination. It is anyway stressed by Wu *et al.* (2011) that climate extremes should result in altered composition of *Fusarium* species associated to maize kernels. Based on surveys managed i.e. in Iowa, *F. verticillioides* is predominant in warm areas, but *Fusarium subglutinans* incidence increases in cooler northern areas. Differences in maize associated fungal population was also noticed in Italy in a two year study where *Fusarium proliferatum* was largely dominant compared to *F. verticillioides* only in the dryer year, mainly in organic, but also in conventionally managed crops (Lazzaro *et al.*, 2015). Therefore, the impact of a changing climate on *F. verticillioides* will be complicated by the concomitant effects on the partitioning between *Fusarum* species. Recent studies evidenced that preceding infection of *F. graminearum* favours *F. verticillioides*, in addition to its known competitive advantage over the former (Picot *et al.*, 2012). Moreover, the prevalence of *F. graminearum*, in a mild and rainy season, was recently highlighted in north Italy, a maize growing area where *F. verticillioides* is traditionally the key fungus (Andreotti, 2014).

Focusing on *F. verticillioides*, because it maximises FB production at marginal a_w around 0.95, this will be surely affected by the supposed warmer season. Further, it has been shown that in warm conditions, the systemic transmission of *F. verticillioides* from plant to kernels, commonly considered almost irrelevant, increased and could contribute to FB contamination in grain (Murillo-Williams and Munkvold, 2008).

The neglected aspect of CO_2 increase in future scenario was suggested as relevant for maize susceptibility to *F. verticillioides*, even with a reduction in FB produced per unit pathogen. Interestingly, in maize grown at elevated CO_2, the accumulation of some compounds is modified, i.e. sugars, free fatty acids, lipoxygenase (LOX) transcripts. In particular, the attenuation of maize 13-LOXs and jasmonic acid production correlates with increased susceptibility to the fungus. Furthermore, the diminished induction of 9-LOXs, involved in mycotoxin biosynthesis, is consistent with reduced FB per unit fungal biomass (Vaughan *et al.*, 2014). As a general comment, elevated CO_2 seems to compromise maize LOX dependent signalling and, based on available knowledge, this could be speculated to affect also FB modification.

Apart the direct effect of climate change on the pathosystem maize-*F. verticillioides* and the related mycoflora, pests will be surely involved (Porter *et al.*, 1991). Insect life cycle is strictly dependent on temperature, and its increase result in a faster completion and possibly in the increase of cycle number per year, with more damages to the crops.

Finally, as a further comment regarding the effect of climate change in field, it is mandatory to remember that all stresses, both involving the host plant and the fungus, enhance FB accumulation and, even not yet detailed, also their modified forms.

Impact of climate change post-harvest

The post-harvest stage in the maize chain is less connected to climate change than the pre-harvest period and only general comments are reported in literature regarding the effect on mycotoxins. The supposed impact mainly regards the residual effect from field, the ecosystem inside silos where maize grains are stored and the complex interactions between abiotic and biotic factors. The effect of increasing temperature and CO_2 on grain quality, with protein and micronutrient content reduction, could influence the interaction with fungi and their secondary metabolism, protracted during post-harvest (Chakraborty and Newton, 2011).

The more rapid pest development under elevated temperature can modify the water balance inside the grain mass and create spots of wet grains supporting fungal activities and therefore spot increase in mycotoxins, that are very difficult to be correctly detected (Magan *et al.*, 2011). This is especially true in poorly-stored grain, as supported by *in vitro* studies where suitable conditions for mycotoxin production were considered. On the contrary, it is well known that grain stored with proper a_w is almost a guarantee of no increase in FB content, also in very simple storage tools, like silobags, that strongly reflect in their inside the fluctuation of external temperatures (Gregori *et al.*, 2013).

6. Modified fumonisins: the known and the unknown

Although the issue of masked mycotoxins is still scientifically young, an increasing number of studies have been published over the last decade. Questions related to the toxicological relevance and the role in plants of these compounds are still open, but great efforts have been spent for the characterisation of DON-3-glucoside and other masked forms of major trichothecenes, as well as for zearalenone-14-glucoside. DON-3-glucoside is commercially available, other compounds can be easily synthesised, and a number of in-house validated methods for their quantification have been proposed so far.

On the other side, modified FBs have been overlooked over recent years. Fumonisin contamination is often regarded as a less relevant problem compared to other mycotoxins, since these compounds are mainly found in maize, and maize is less frequently consumed by humans than other cereals, at least in developed countries. Nowadays, the scenario is changing. *F. verticillioides,* or in a wider view, *F. fujikuroi* species complex, infection is affecting a large range of crops over a wide geographical area (Leslie *et al.,* 1996). Among the relevant crops apart maize, rice (Desjardins *et al.,* 1997), asparagus (Elmer, 1990), date palm (Abdalla *et al.,* 2000) and *Allium* crops, including onion and garlic (Dugan *et al.,* 2003; Du Toit *et al.,* 2003), can be mentioned. Even if data regarding their contribute to human exposure to FBs are not available, it is worth to cite them. Further, other fungi, i.e. *A. niger,* have been recognised as FB producers. The industrial use of maize is no longer limited to feed industry, but there is an increasing number of biotechnological applications based on maize-derived raw materials, and the interest of food industries in maize fractions for the production of innovative foods is exponentially growing. As an example, maize flour itself is often used as main ingredient for gluten free products.

In this frame, the issue of modified FBs and the possible effect of technological process have to be deeper investigated. Among the open questions, urgent is the analytical one. Besides conjugated forms such as NDF-FB_1 or fatty acid esters, that can be included in routinely LC-MS/MS methods, physically entrapped FBs are difficult to be quantified, as the associative interaction between FBs and the matrix must be destroyed before extraction. The ratio between free and 'hidden' FBs is thus strongly affected by analytical parameters (i.e. sample particle size, extraction solvent, pH), and by the matrix itself (i.e. chemical composition, pH, physical structure). As it has been demonstrated, the same sample may return different results if analysed by different laboratories (Dall'Asta *et al.*, 2009). Even reference materials – when naturally incurred – may return different results according to the different protocols of analysis, as often shown by higher variability encountered within FBs proficiency tests.

The methods applied today for the determination of total FBs rely on an alkaline hydrolysis of the sample, which is often cumbersome and may be easily affected by bias. Calculation is then applied for obtaining free and hidden FB amounts (Bryla *et al.*, 2014, 2015; Dall'Asta *et al.*, 2009). As an alternative approach, the sample could undergo digestion to completely degrade the matrix (Dall'Asta *et al.*, 2010). According to previous studies, *N*-acyl and *N*-sugar derivatives seem to be stable under digestive conditions, and thus could be totally recovered in the chime (Falavigna *et al.*, 2012). Nothing is known, on the other side, on the stability of *O*-esters in such analytical conditions. Although more accurate and elegant than alkaline hydrolysis, this procedure is time-consuming and actually not feasible for routine analysis. In order to include modified FBs in monitoring plans – and thus to obtain a larger set of occurrence data for exposure studies – a simpler and quicker procedure has to be implemented.

Besides the analytical determination, some intriguing issues are still on the table. As first, a fascinating issue is the role played by the plant in the formation of modified FBs. Fatty acid esters of FBs are indeed the only conjugated forms isolated in the field so far. However, unlikely other masked mycotoxins, these compounds are formed by the fungus itself and not by the plant, but the substrate is used as source of fatty acids (Falavigna *et al.*, 2016). Furthermore, the production of esters seems to start when FB_1 accumulation has reached a certain threshold (Falavigna *et al.*, 2013, 2016). Which is the biological role of these compounds? Why is the fungus conjugating FBs only at a later stage of infection? These questions will require efforts in the next years, but the answer will possibly offer an opportunity to increase knowledge about the *F. verticillioides – Zea mays* pathosystem, also in terms of enhancing the plant resistance to pathogens. Further, the role played by the environment will became crucial in the ongoing climate change.

Another aspect to be clarified is the nature of the associative interaction occurring between FBs and maize macroconstituents. According to the more recent studies (Bryła *et al.*, 2015, 2016; Giorni *et al.*, 2015a), starch seems to be the key compound, and its physical modification occurring pre- and postharvest are driving the ratio between free and hidden FBs. It is thus crucial to understand the mutual interaction between these compounds, and how external parameters can affect this interaction. A deeper understanding of this phenomenon will explain the changes in contamination often reported by maize chain professionals, and may eventually support the design of fit-for-purpose technological processing, mainly in terms of mitigation.

Finally, gathering all the data related to modified FBs will support a deeper comprehension of the toxicological role played in mammals. Besides physically entrapped FBs, whose occurrence may lead to a significant underestimation of the exposure in animals and humans, other conjugated forms of FBs could contribute to the overall toxic load. Most of them are still to be structurally elucidated, while little is known about the metabolism and bioactivity of the already described *N*-acyl compounds that strongly resemble ceramides (Bartók *et al.*, 2013).

7. Conclusions

In the recent years an increasing number of studies on masked/modified mycotoxins have been published, providing information and data for stakeholders and regulatory agencies. Advances in the analytical field have made available multi-toxin methods not only for research, but also for routinely monitoring plans. Although still preliminary, toxicological studies are moving forwards as well.

In this context, however, knowledge on modified FBs is still in its dawn, on account of the wide range of interactions the toxin may have with the matrix, and of the complexity of the *F. verticillioides – Zea mays* pathosystem. The production and accumulation of FBs and their modified forms are affected by climate, agronomic factors, and maize hybrid composition pre-harvest, while storage conditions and further technological processing postharvest may cause changes in their ratio. Extreme events, mentioned in climate change scenario, make more reliable the alternation, in the same growing season, of weather conditions favourable to different mycotoxin producing fungi, strongly suggesting to focus in future studies on the interaction between these microorganisms and the effect on mycotoxin production and modification.

These results, taking altogether, clearly indicate that modified FBs should be included in the monitoring plans to have an overview of the possible contribution to human exposure. Furthermore, next efforts should focus on the events occurring in field and on the cross-talk between the plant and the fungus, to support the identification of resistant hybrids and to provide data for predictive models, the most suitable tool to forecast what is going to happens in the future changing climate.

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