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Free and Hidden Fumonisins in Maize and gluten-free products

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“Solo la morte m'ha portato in collina
un corpo fra i tanti a dar fosforo all'aria
per bivacchi di fuochi che dicono fatui
che non lasciano cenere, non sciolgon la brina.
Solo la morte m'ha portato in collina.

Da chimico un giorno avevo il potere
di sposare gli elementi e di farli reagire,
ma gli uomini mai mi riuscì di capire
perché si combinassero attraverso l'amore.
Affidando ad un gioco la gioia e il dolore.
…
Ma guardate l'idrogeno tacere nel mare
guardate l'ossigeno al suo fianco dormire:
soltanto una legge che io riesco a capire
ha potuto sposarli senza farli scoppiare.
Soltanto la legge che io riesco a capire…”

F. De Andrè

per aspera ad astra

Abstract

The work has been focused on the development of a LC-MS/MS method for the evaluation of hidden Fumonisins. The application of the method to several batches of raw maize and gluten free products let us to discover that hidden FBs are widespread along the maize chain. Moreover, further experimental data collected in the work strongly suggest a non-covalent interaction between FB_1 and the α-Zein (the principal endosperm protein of maize), with an inclusion-like mechanism, allowing us to partially explain the masking mechanism of Fumonisins.

Keywords: hidden Fumonisins, masked mycotoxins, LC-MS/MS, non-covalent masking, α-Zein.

Abstract

Nel presente lavoro di tesi è stato sviluppato un metodo per la valutazione delle Fumonisine nascoste. L'applicazione del metodo a diversi lotti di campioni di mais grezzo e prodotti gluten-free, ha permesso di scoprire una diffusa presenza di Fumonisine nascoste lungo la filiera del mais. Tutti i dati sperimentali successivamente ottenuti nel presente lavoro suggeriscono fortemente una interazione di tipo non covalente tra FB_1 e l'α-Zeina (la principale proteina dell'endosperma di mais), per spiegare il fenomeno di mascheramento delle Fumonisine.

Parole chiave: Fumonisine nascoste, micotossine mascherate, LC-MS/MS, mascheramento non covalente, α-Zeina.

1. Introduction

1.1 Mycotoxins

The term mycotoxin was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died (1). It is difficult to define mycotoxins in a few words, all mycotoxins are non-antigenic, low molecular weight natural products produced as secondary metabolites by filamentous fungi. Many mycotoxins display overlapping toxicity to invertebrates, plants and microorganisms (2). When the mysterious turkey X disease was linked to a peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus flavus* (aflatoxins), it sensitized scientists to the possibility that other occult mould metabolites might be deadly. Soon, the mycotoxin rubric was extended to include a number of previously known fungal toxins (e.g., the ergot alkaloids), some compounds that had originally been isolated as antibiotics (e.g., patulin), and a number of new secondary metabolites revealed in screens targeted at mycotoxin discovery (e.g., Ochratoxin A).

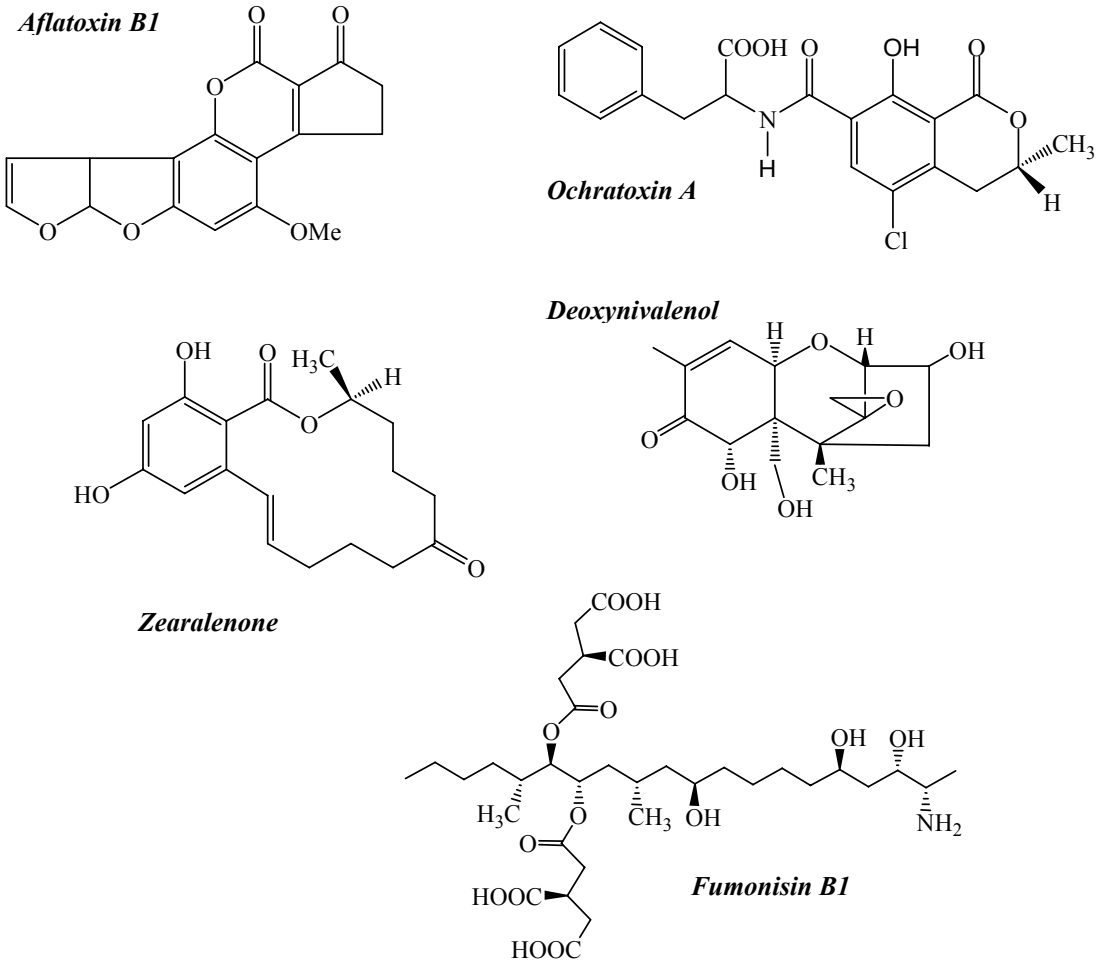


Fig. 1: Structures of the most important mycotoxins related to food.

While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins. The target and the concentration of the metabolite are both important. Fungal products that are mainly toxic to bacteria (such as penicillin) are usually called antibiotics. Other low-molecular weight fungal metabolites, such as ethanol, that is toxic only in high concentrations are not considered mycotoxins (3). Finally, although mushroom poisons are definitely fungal metabolites that can cause disease and death in humans and other animals, they are rather arbitrarily excluded from discussions of mycotoxicology. The distinction between a mycotoxin and a mushroom poison is based not only on the size of the producing fungus, but also on human intention. Mycotoxin exposure is almost always accidental. In contrast, mushroom poisons are usually ingested by amateur mushroom hunters who have collected, cooked, and eaten what was misidentified as a delectable species (4). Mycotoxins are not only hard to define, they are also challenging to classify, due to their different chemical structures and biosynthetic origins, biological effects and their production by a wide number of different fungal species. Mycotoxin-producing mould species are extremely common, and they can grow on a wide range of substrates under a wide range of environmental conditions (Tab.1 and 2).

For agricultural commodities, the severity of crop contamination tends to vary from year to year based on weather and other environmental factors. Mycotoxins occur, with varying severity, in agricultural products all around the world. The estimate usually given is that one quarter of the world's crops are contaminated to some extent with mycotoxins (5,6).

Mycotoxins can enter in human and animal dietary systems by indirect or direct contamination, (Tab. 3); direct contamination occurs when the food or feed becomes infected with a toxigenic fungus with subsequent toxin formation. In contrast, indirect contamination can take place when a raw material of a formulation has previously been contaminated with toxin producing fungi, while the fungus itself may be killed or removed during the processing, the mycotoxin will mostly remain in the final products.

Tab. 1: Fungal genera and species of major significance and their associated mycotoxins.

Fungi	Mycotoxins
Aspergillus* :** ***A.flavus ***A.parasiticus*** ***A. nidulans*** ***A. carbonarius*** ***A. ochraceus*** ***A. niger***	• Aflatoxin B_1, B2 • Aflatoxin G1, G2 • Aflatoxin M (metabolite of B in mammals) • Sterigmatocistin • Ochratoxin A
Fusarium* :** ***F. graminearum ***F. culmorum*** ***F. sporotrichioides*** ***F. proliferatum*** ***F. verticillioides***	• Trichothecenes type A (e.g. T-2, HT-2, DAS) • Trichothecenes type B (e.g. Nivalenol, Deoxynivalenol and acetylated derivatives) • Zearalenon • Fumonisins
Penicillium*:** ***P. verrucosum ***P. expansus*** ***P. roqueforti***	• Ochratoxin A • Patulin • Citrinin • Roquefortines • Cyclopiazonic acid

Methods for controlling mycotoxins are largely preventive. They include good agricultural practice and sufficient drying of crops after harvest (7). There is considerable on-going research on methods to prevent preharvest contamination of crops. These approaches include developing host resistance through plant breeding and through enhancement of antifungal genes by genetic engineering, use of biocontrol agents, and targeting regulatory genes in mycotoxin development (8).

Tab. 2: Principal features of the fungal genera of major mycotoxicological significance concerning food.

Genus	Growth Range	Areas of Major Incidence	Substrates
Aspergillus spp.	**10 - 46 °C** **aw 0.77 - 0.99** **pH 2 - 11**	**tropical and sub-tropical climates**	oilseed cereals species dried fruit grapes
Penicillium spp.	**0 - 40 °C** **aw 0.80 - 0.99** **pH 2 - 10**	**temperate and continental climates**	cereals fruit/vegetables chilled food
Fusarium spp.	**0 - 40 °C** **aw 0.80 - 0.99** **pH 2 - 10**	**temperate and continental climates**	cereals other crops (phytopathogen)

However, none of these methods has solved the problem. Because mycotoxins are "natural" contaminants of foods, their formation is often unavoidable. The end result is that mycotoxins are commonly found in foods. Mycotoxins are ranked as the most important chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives, or pesticide residues (9). The economic consequences of mycotoxin contamination are profound.

Many efforts to address the mycotoxin problem simply involve the diversion of mycotoxin contaminated commodities from the food supply through government screening and regulation programs. Crops with large amounts of mycotoxins often have to be destroyed. Alternatively, contaminated crops are sometimes diverted into animal feed. Giving contaminated feeds to susceptible animals can lead to reduced growth rates, illness, and death. Moreover, animals consuming mycotoxin contaminated feeds can produce meat and milk that contain toxic residues and biotransformation products. Thus, aflatoxins in cattle feed can be metabolized by cows into aflatoxin M1, which is then secreted in milk (10). Ochratoxin in pig feed can accumulate in porcine tissues (11).

Tab. 3: Routes for mycotoxins contamination of food and feed.

Mould-damaged foodstuffs
• Agricoltural products : cereal oilseed fruit vegetables • Consumer's products (secondary infections) • Animal feeds (secondary infections)
Residues in animal tissues and animal products
• Milk (human and animal) • Dairy products • Meat (liver, kidney)
Mould-ripened foods
• Cheeses • Fermented meat products • Oriental fermentations
Fermented-derived products
• Microbial proteins • Food additives

Court actions between grain farmers livestock owners, and feed companies can involve considerable amounts of money. The ability to diagnose and verify mycotoxicoses is an important forensic aspect of the mycotoxin problem (12). People that have enough to eat normally avoid foods that are heavily contaminated by mould, so it is believed that dietary exposure to acute levels of mycotoxins is rare in developed countries. Nevertheless, many mycotoxins survive processing into flours and meals. When mould-damaged materials are processed into foods and feeds, they may not be detectable without special assay equipment. It is important to have policies in place that ensure that such "hidden" mycotoxins do not pose a significant hazard to human health.

1.2 Fumonisins

Fumonisins were first described and characterized in 1988 (13,14). The most abundantly produced members of the family are Fumonisin B_1, B_2 and B_3. Since then, more than 28 homologues have been discovered and more are likely to be found (15,16). FB_1 is the most common and, from a toxicological standpoint, the most thoroughly studied. FB_2 and FB_3 in order less prevalent and differ structurally from FB_1 in the number and position of hydroxyl groups on the hydrocarbon backbone of the molecule.

Fumonisins are produced by a number of *Fusarium* species, notably *Fusarium verticillioides, Fusarium proliferatum,* and *Fusarium nygamai,* as well as *Alternaria alternata* f.sp. *lycopersici* (17,18). The major species of economic importance is *Fusarium verticillioides*, which grows as a maize endophyte in both vegetative and reproductive tissues, often without causing disease symptoms in the plant. However, when weather conditions, insect damage, and the appropriate fungal and plant genotype are present, it can cause seedling blight, stalk rot, and ear rot (19). *Fusarium verticillioides* is present in virtually all maize samples (20,21).

Most strains do not produce the toxin, so the presence of the fungus does not necessarily mean that Fumonisin is also present (22). Although it is phytotoxic, Fumonisin B_1 is not required for plant pathogenesis (23, 24).

Higher incidences of severe pathologies in humans, such as primary hepatocellular carcinoma (25), esophageal cancer (26,27), and neural tube defects (28), were found in populations chronically exposed to diets contaminated with FB_1. However, neither an illness–exposure relationship nor the mechanisms involved in the toxicity of this mycotoxin were clearly established yet.

Some pathological changes appear to be a consequence of the immunotoxic action of FB_1, which was observed in several experimental models in animals (29,30).

Beyond the above-mentioned evidences, the knowledge of the early events that trigger FB_1 action mechanism is scarce.

1.2.1 Chemistry and biosynthesis

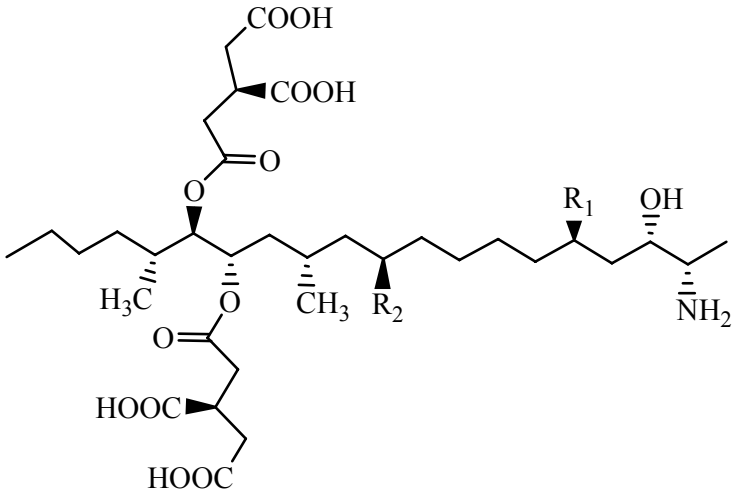
At least 15 different Fumonisins have so far been reported and other minor metabolites have been identified, although most of them have not been shown to occur naturally. They have been grouped into four main categories (31):

Fumonisins A (and AK), Fumonisins B, Fumonisins C and Fumonisins P.

FB_2, FB_3 and FB_4 differ from FB_1 in that they lack hydroxyl groups present in FB_1; FA_1, FA_2 and FA_3 are like FB_1, FB_2 and FB_3, but are *N*-acetylated; FAK_1 is like FA_1 but is 15-keto functionalized; FCs are like FBs but lack the methyl group adjacent to the amino group;

FPs have a 3-hydroxypyridium group instead of the amine group in the FBs (31).

FB_1 is chemically described as polyhydroxy alkyl amine, esterified on C14 and C15 with 2 molecules of tricarballylic acid (CAS No 116355-83-0) (32). The structure of Fumonisin B_1, the most significant of the Fumonisins in terms of toxicity and occurrence, is reported in the following figure :



	FB_1	**FB_2**	**FB_3**
R1	**OH**	**OH**	**H**
R2	**OH**	**H**	**OH**
MW [g/mol]	**721.39**	**705.39**	**705.39**

Fig. 2: Structures and molecular weights of Fumonisins B_1, B_2 and B_3.

Fumonisins B_1 has 10 stereogenic centers and their absolute configurations were clarified in the last years (33,34). Fumonisin B_2 and B_3 are deoxy analogues of FB_1 in which the corresponding epimeric units on the backbone have the same configuration (35,36).

No report concerning the pKs of Fumonisin B_1 has been found in the available literature. The pKa values for tricarballylic acid are 3.49, 4.56, and 5.83. The aliphatic amino group would be expected to have a pK greater than 9; therefore, Fumonisin B_1 will be a zwitterion at physiological pHs between 6 and 9 (37,38).

Wild-type strains of *F. verticillioides* produce predominantly four B-series Fumonisins (B_1, B_2, B_3, and B_4), with Fumonisin B_1 making up to approximately 70% of the total content (39).

These compounds have a linear 20-carbon backbone with hydroxyl, methyl, and tricarballylic acid moieties at various positions along the backbone (40). The backbone originates from a C18 polyketide chain and one amino acid. Isotope feeding experiments have shown that the carbons 3-20 of the backbone are derived from acetate and that the amino group and C-1 and C-2 are derived from alanine (41,43). The origin of several moieties attached to the backbone has also been studied. The two methyl groups on C-12 and C-16 are derived from methionine (44).

The hydroxyl group on C-3 is from an acetate-derived carbonyl group, whereas the hydroxyl groups on C-5, C-10, C-14, and C-15 are likely derived from molecular oxygen (45). The origin of the tricarballylic acid is generally believed to be from the citric acid cycle (46). Despite these previous biochemical studies, however, little is known about the biosynthetic sequence for these individual steps.

Recently, a 15-gene cluster (*FUM1* and *FUM6-FUM19*) required for the biosynthesis of Fumonisins in *F. verticillioides* was cloned and characterized (47,48).

The identification of this cluster has facilitated direct studies of the biosynthetic genes by genetic and biochemical approaches. The proposed pathway of Fumonisin B_1 biosynthesis in reported in Fig. 3. Among the *FUM* genes, *FUM13* has been characterized by a genetic approach (49).

Butchko et al. (49) found that a *FUM13*-deleted mutant accumulated the C-3 keto form of Fumonisins B_3 and B_4, indicating that this gene encodes a C-3 keto reductase of Fumonisins (49).

More recently, Butchko et al. (50) generated a *FUM3* (also *FUM9*) deletion mutant and showed that this gene is required for the C-5 hydroxylation of Fumonisins.

It was also demonstrated that, via heterologous expression in yeast, *FUM3* encodes a

2-ketoglutarate-dependent dioxygenase that catalyzes the conversion of Fumonisin B_3 to B_1 (51), directly confirming that *FUM3P* catalyzes the C-5 hydroxylation (52). Several other *FUM* disruption mutants, including ¢*FUM1*, ¢*FUM6*, ¢*FUM8*, ¢*FUM17*, ¢*FUM18*, and ¢*FUM19*, have also been generated by Proctor and co-workers (47,48).

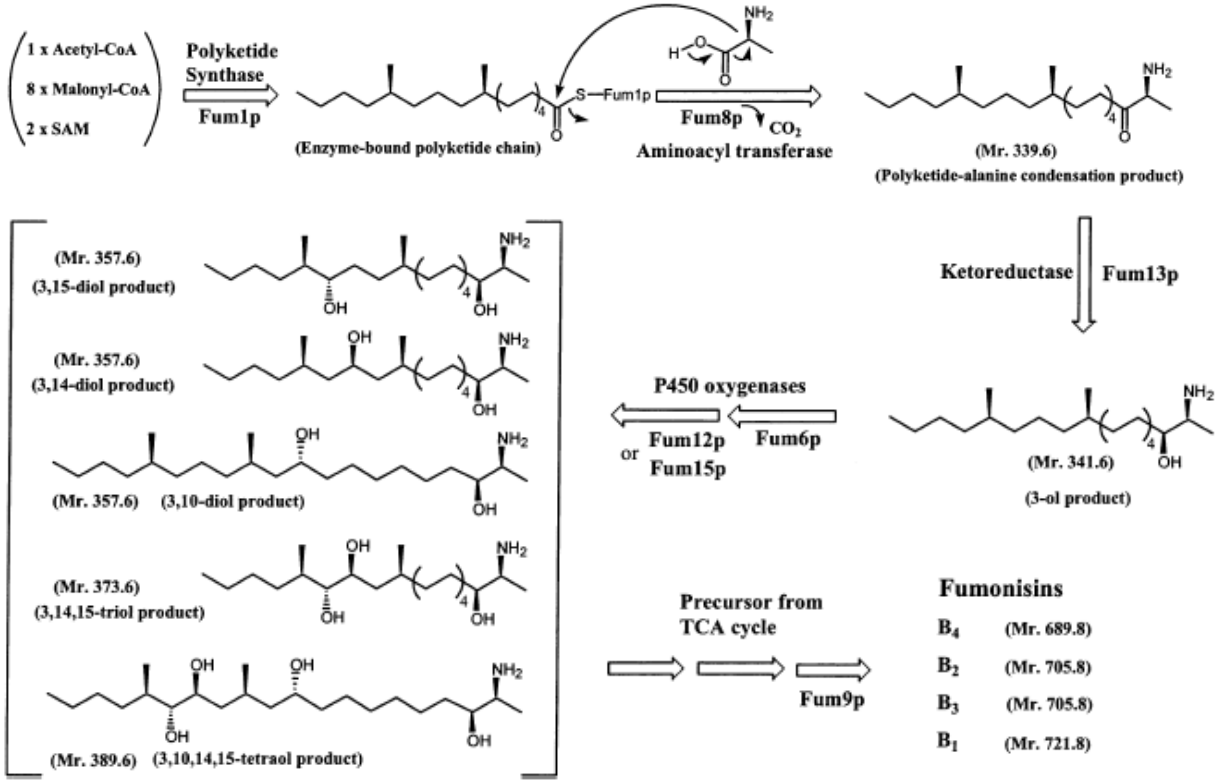


Fig. 3: Proposed biosynthetic pathway of Fumonisins B. (Bojja et al., 2001)

The first three mutants did not accumulate any identifiable intermediates that could have provided additional information about the function of these genes, while the latter mutants were not or only subtly affected in their ability to produce Fumonisins.

Very recently, Frisvad et al. (2007) have reported the production of FB_2 from strains of *Aspergillus niger*, showing how the biosynthetic pathways for secondary metabolites production can be conserved among different fungal genus, as in the case of Ochratoxin A.

1.2.2 Toxicology

When purified FB_1 became available, it was possible study its role played by FB_1 in mediating some agriculturally-important diseases, which had previously been shown to be associated with consumption of *F. verticillioides*-contaminated foods and feeds, including were demonstrations that FB_1 mediates equine leukoencephalomalacia (ELEM) and porcine pulmonary edema (PPE) (53,54). However, most of the research on the toxic effects of FB_1 was devoted to studying its cancer-causing potential (55,56). Initially both field samples of maize and culture materials infested with *F. verticillioides* isolated from field samples, were shown to induce benign and malignant tumors in the liver after feeding to rats (57). These observations have prompted the International Agency for Research on Cancer (IARC) working group to label toxins from *F. Verticillioides* as possible carcinogenic to humans (Group 2B) (57).

Progress has been made in understanding the mechanism of action of FB_1, but unanswered questions remain, particularly with respect to the mechanism of tumor promotion. It was initially recognized by Riley (81) that FB_1 is a structural analog of sphingosine (Fig. 4), and this observation led to the discovery that FB_1 is an effective inhibitor of ceramide synthetase (82,83) a key enzyme in the biosynthesis of sphingolipids (84).

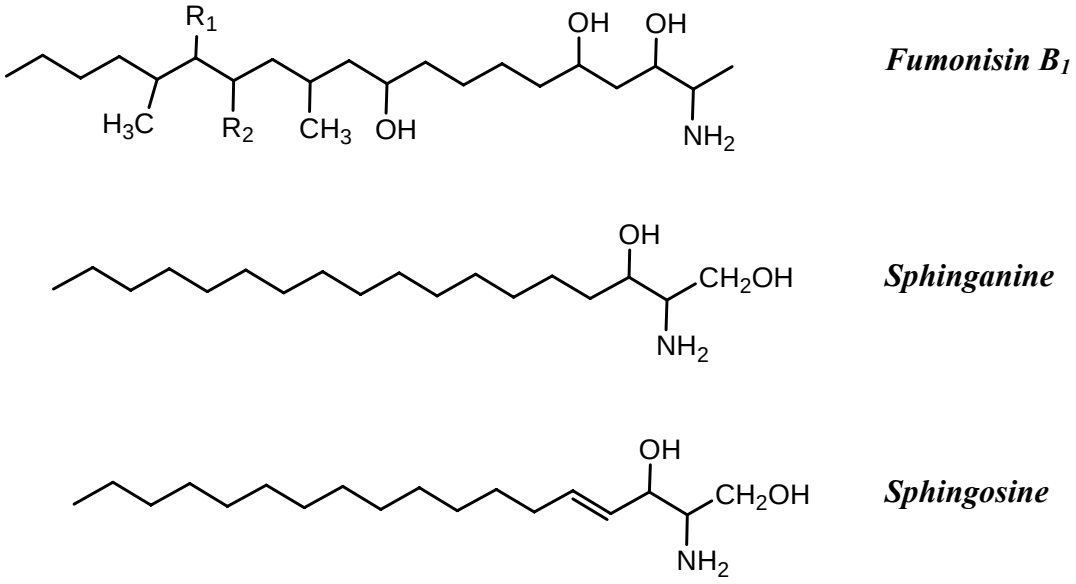


Fig. 4 : Comparison of the chemical structure of Fumonisin B_1 and the sphingoid bases sphinganine and sphingosine.

Ceramide synthase appears to interact with FB_1 at both the free amino group (as a sphingosine analog), and at the side chains (consistent with the negative charges of one or both side chains interacting with the CoA-fatty acid binding site) (83). Consistent with this, intact FB_1 is a better inhibitor than hydrolysed FB_1 (83). Disruption of sphingolipid metabolism by FB_1 has been confirmed *in vivo* by measuring elevated levels of sphinganine, the normal ceramide synthase substrate, and sphingosine in intact animals (85), tissue slices (86), cultured mammalian cells (87–90) and plants (91) in response to exposure to FB_1. Altered tissue and serum ratios of sphinganane to sphingosine have been proposed as an early biomarker for exposure of domesticated animals to Fumonisins in feeds (92).

Inhibition of ceramide synthase can affect cells in a variety of ways. Ceramide synthase is a key enzyme for the *de novo* synthesis of sphingomyelin and other sphingolipids (84). Sphingomyelin is known to be required for membrane stability, because destruction of sphingomyelin in membranes by treatment with sphingomyelinase results in cell lysis. (93). Blocking sphingomyelin biosynthesis provides a mechanism for delayed necrosis, such as occurs in ELEM and PPE. The recent observation that hydrolysed FB_1 can be converted by the action of ceramide synthase to an FB_1 analog of ceramide with markedly enhanced cytotoxic activity (66,67), suggests another plausible mechanism of action.

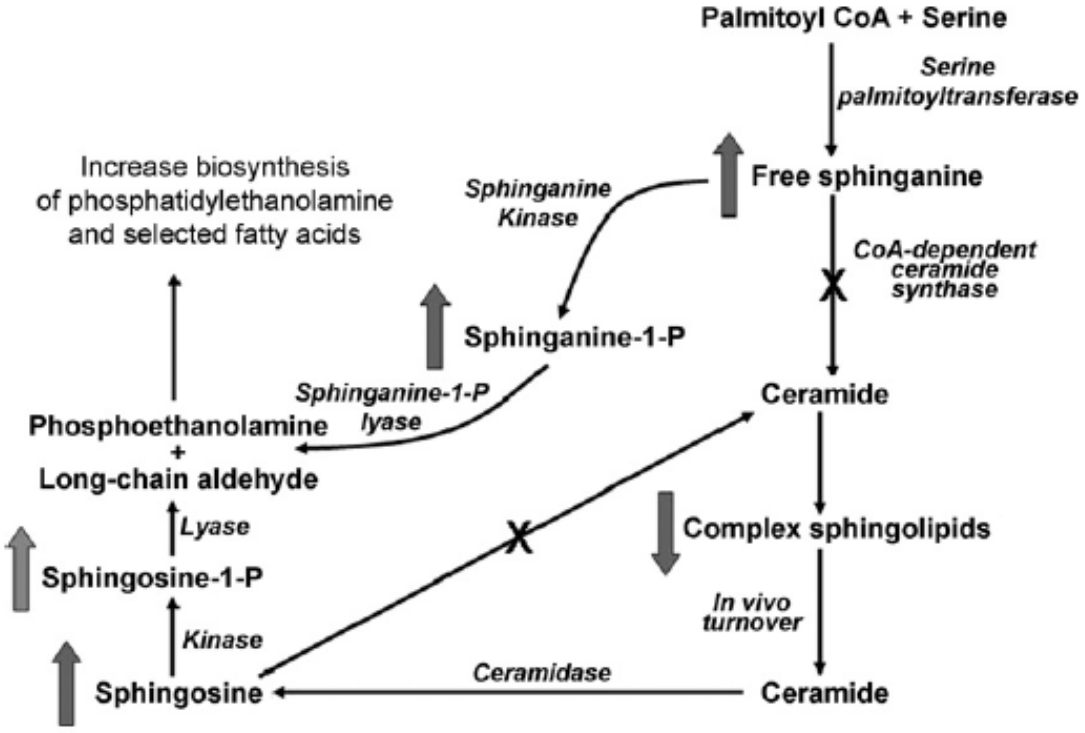


Fig. 5: Schematic representation of sphingolipid metabolism showing the inhibition of ceramide synthase (×) by Fumonisins. (Merrill et al., 2001; Riley and Voss, 2006).

While accumulating ceramide analogs could provide a plausible mechanism of action for HFB_1, it is not likely to be important in the mechanism of action of FB_1, given the lack of demonstrable esterase-mediated hydrolysis of FB_1 in target tissues. The results of studies on the toxicokinetics of FB_1, performed during several years, are in general agreement with the following conclusions:

→ FB_1 has very low oral bioavailability in rats, pigs, chickens, cows, and monkeys;

→ almost all FB_1 administered by any route (oral/intragastric, intraperitoneal or intravenous) is rapidly cleared by the biliary route and excreted largely unchanged in the feces;

→ even after feeding periods as long as 12 days, only traces of FB1 are found in liver and kidney, and less is found in other organs.

Thus, a very low oral bioavailability of FB_1 seems to be the result of low uptake coupled with efficient biliary excretion (94).

It has been well established that eating *F. verticillioides* -contaminated foods and feeds produces diseases such as ELEM and PPE in domesticated animals, and that administration of purified FB_1 replicates the diseases in domesticated and experimental animals. However, extensive studies on the toxicokinetics of radiolabelled FB_1 in rats, pigs, chickens, cows and monkeys indicates that orally administered FB_1 is absorbed very poorly, if at all (94).

For this reason Shier, in the 2000, proposed the term ‘‘fumonisin paradox’’ to describe the apparent paradox of FB_1 causing disease despite being very poorly absorbed.

No evidence for functional metabolism of Fumonisins has ever been reported. The most probable predicted metabolic processes are first, removal of the free amino group by monoamino oxidase action, and then removal of one or both propane-1,2,3-tricarboxylic acid side chains by esterase action. Structure-activity relationship studies on FB_1 and a series of natural and synthetic structural analogs indicated that the free amino group of Fumonisins is required for biological activity (58,59–65). Therefore, monoamine oxidase action, if occurred, would be a metabolic inactivation process, not activation. Esterase action on FB_1 would produce HFB_1, which retains its biological activity (58), although it is not as effective as an inhibitor of ceramide synthase. HFB_1 does act as a substrate for ceramide synthase, which converts it to a ceramide analog, which is approximately 10 times as more cytotoxic as intact FB_1 (66,67). Thus, if FB_1 were efficiently metabolized to HFB_1, a mechanism does exist for metabolic activation. However, in extensive studies on the fate of radiolabeled FB_1 administered orally or by injection, metabolism is generally not detected (68–79). In one case in which a modest amount of metabolism was observed in vervet monkeys, it was partial

hydrolysis to remove the propane-1,2,3-tricarboxylic acid group from the C-14 position, and the source of the enzyme was traced to anaerobic bacteria in the gut flora (80).

Plausibile explanations for the Fumonisin paradox are reported in the original paper (94), and only summarised here :

. 1: An Unknown Bioavailable Toxin

ELEM, PPE and other disease states may not be caused by FB_1, but rather by another as yet unknown *F. verticillioides* toxin, which has good oral bioavailability.

. 2: Dose-dependent Bioavailability

FB_1 bioavailability may actually be substantially higher than the reported values when the toxin is administered at dose levels typically encountered in nature.

. 3: Dose-dependent Metabolism

The percent of FB_1 metabolized may actually be substantially higher than the reported values when the toxin is administered at dose levels typically encountered in nature. Diseases such as ELEM and PPE could be mediated by a highly active metabolite of FB_1 which is being formed in amounts too small to be detectable above background when it is formed from FB_1 radiolabeled at low specific activity.

. 4: Bioaccumulation of Toxin

Very little net absorption and metabolism of FB_1 occurs, but the little that does occur bioaccumulates as the free toxin or an active metabolite(s) until toxic levels are achieved. Published studies on bioavailability have been short-term studies, and they were not designed to detect low levels of bioaccumulated toxin or metabolite.

. 5: Uptake of FB_1 Derivatives with High Bioavailability

FB_1 in contaminated corn may be converted during cooking and other normal food processing treatments into a derivative or derivatives that have high oral bioavailability, and are effectively converted back to FB_1 or to another active metabolite after the bioavailable derivatives have been transported into the body.

In the last years the last hypothesis have gain supports from several authors, especially after that the *in vitro* reaction between lysine, as a protein model compound, and FB_1 was demonstrated (120).

1.2.3 Methods of analysis and legislation

Fumonisins mostly occur in maize and maize based products and the development of analytical methods has concentrated on this commodity. FB_1 and FB_2 are the main target molecules although the methods must be valid for FB_3 as well. Little is known about FB_4 and its natural occurrence. Fumonisins are produced by *Fusarium spp.* from the *Liseola* section including *F. verticillioides* (syn. *F. moniliforme)* and *F. proliferatum*. Analytical procedures differ in extraction, clean up and determination steps, and the method of choice depends on available equipment and analytical requirements such as sensitivity and time required for analysis. Most analytical methods include: thin layer chromatography (TLC), liquid chromatography (LC), gas chromatography (GC) and immunochemical methods. Derivatisation is necessary before fluorescent detection can be performed, as Fumonisins do not contain a fluorophore (95). TLC and immunochemical methods such as ELISA tests are among the most frequent screening methods. A number of ELISA kits, which are commercially available. Additionally dipstick tests have been developed, which claim a detection limit of FB_1 as low as 0.04-0.06 µg/g in corn based foods (95). Reversed phase TLC (on C_{18} modified silica plates) has also been employed with acidic vanillin or fluorescamine/sodium borate buffer as spray reagents (96).

Methods suitable for quantification usually include extraction and clean-up steps prior to determination of the analyte. Various methods can be employed, but all require varying degrees of experience in the field of analytical chemistry and use considerable amounts of reagents. While a clean up is usually not necessary for immunoassays, extensive clean-up procedures are required before physicochemical methods can be employed. Among the commonly used solvent mixtures for extraction are methanol/water (3:1) and acetonitirile/water (1:1). Mixtures can be used either with reversed phase cartridges (C_{18}) or strong anion exchange columns (SAX). More recently immunoaffinity columns (IAC) have become available featuring high selectivity. The analyte molecules are bound to antibodies and the toxin can be eluted subsequent by a washing step the toxin can be eluted. Reversed phase cartridges (C_{18}) can be also used (97). Reversed Phase (RP-) HPLC separation and detection is the most widely used technique for the analysis of Fumonisins, because of their polar character. The majority of researchers reported using pre-column derivatisiation with o-phthaldialdehyde / mercaptoethanol (OPA), despite its limited stability. Naphthalene-2,3-dicarboxaldehyde/potassium cyanide (NDA) has been proposed as a viable alternative, although handling is more tedious requiring additional safety precautions. GC methods have

also been developed by determining aminopolyol after clean-up on a XAD-2 column. The amino and hydroxy groups are protected with trimethylsilyl (TMS) or trifluoroacetate (TFA) and FBs are detected by flame ionisation or mass spectroscopy. The advances being made in LC methods caused a shift away from GC methods, as the latter require multiple sample handling steps. With the availability of liquid chromatography systems with mass spectrometric detection (LC-MS), a powerful method has been established for identification and quantification (98). High sensitivity can be achieved, although disadvantages include high running and maintenance costs (98). Two LC-methods and an ELISA have received AOAC Official Method status (AOAC International, 2000). They were validated for maize and certain maize products (99). Detailed reviews of available analytical methods have been given by Shephard (100), Dutton (101), Scott (102), Norred (103) and Krska (104). While in 1995 Fumonisins were only subject of regulations in one country (FAO, 1997), actually in Europe specific EU-harmonised limits for Fumonisins in food or feed have has been established with the last decision approved from the European Commision (105) concerning the *Fusarium* toxins (Tab.4).

Tab.4: Limits of FB_1+FB_2 in maize and maize based food, EC No. 1126/2007.

Material	Sum of FB_1 and FB_2 (µg/Kg)
Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	4000
Maize intended for direct human consumption, maize-based foods for direct human consumption	1000
Maize-based breakfast cereals and maize-based snacks	800
Processed maize-based foods and baby foods for infants and young children	200
Milling fractions of maize with particle size > 500 micron and other maize milling products with particle size > 500 micron not used for direct human consumption	1400
Milling fractions of maize with particle size ≤ 500 micron and other maize milling products with particle size ≤ 500 micron not used for direct human consumption	2000

1.3 Masked mycotoxins

An emerging issue on mycotoxin research is represented by masked mycotoxins. The terms 'masked', 'bound', 'hidden' or 'conjugated' are generally used in literature to indicate that forms of mycotoxins undetectable by the commonly employed methods of analysis, but chemically related to the native toxic compounds. The lack of detection, due to slight modifications of the chemical structure or to the interactions with the food matrix, occurs because the analyte is not extracted, separated or detected. Mycotoxins can undergo modifications in each step of the their presence in the food chain, Berthiller et al. reported all the forms of conjugated mycotoxins know so far in a very recent review (105).

The steps whereas mycotoxins can be 'masked' are :

1) During the fungal production.

Mould do not produce only one form of mycotoxins, but a range of chemically related compounds (106) (for Fumonisins it was told that more than 20 analogues can be isolated from the fungal culture). Usually the law regulate the presence of few forms of each mycotoxins, generally the most abundant and more toxic.

2) During the plant-pathogen interactions.

Plants protective system against xenobiotic compounds (e.g. pesticides, mycotoxins) consist in converting them to more polar metabolites, which can be stored in vacuoles or conjugated to biopolymers such as cell wall components (107,110).

3) During the food processing.

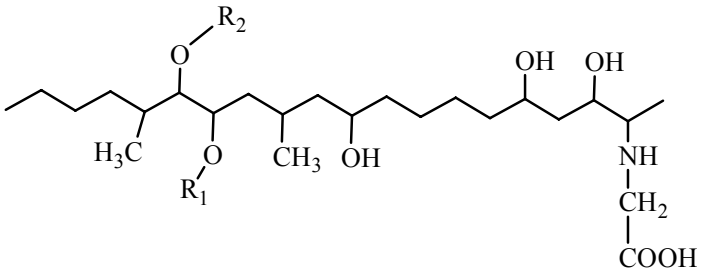
Modifications of the chemical structure and behavior of mycotoxins, can occurs during the food processing (upon heating or fermentation) (111,112). Heat treatment of food can promote reactions between the mycotoxin and the component of the food matrix.

The undetected forms are ingested together with the food, with the possibility that the native toxic compound be released during digestion. For this reason masked mycotoxins must be studied in order to undestand the potential hazard that they represent.

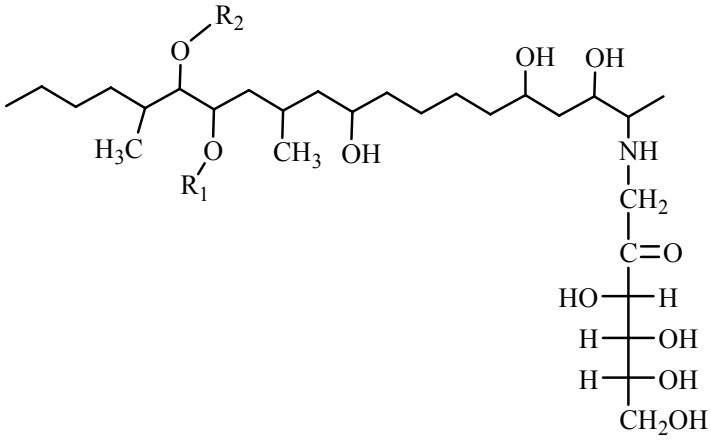
1.3.1 Hidden Fumonisins

Hidden Fumonisins were early identified as conjugates with sugars, after heating treatments. Heating of Fumonisin B1 with reducing sugars can yield N-(carboxymethyl) Fumonisin B_1 (113). This substance, together or N-(1-deoxy-D-fructos-1-yl)Fumonisin B_1 (114), was

later found also in corn products (115) (Fig.6). This compounds are well known and, as already told, less toxic due to the modification of the amino group, which is essential for the biological activity.



N-(carboxymethyl) Fumonisin B_1



N-(1-deoxy-D-fructos-1-yl)Fumonisin B_1

Fig. 6: Chemical structures of the reaction products of FB_1 with reducing sugar upon heating.

More recently, other unidentified bound forms were detected in thermally treated food products such as corn-flakes (116,118). These forms are not directly detectable, but their presence has been inferred from the observation that, upon SDS-extraction and alkaline hydrolysis of the food matrix, the amount of released hydrolysed Fumonisins is often higher than that expected by the hydrolysis of the Fumonisins detected in the sample using the routine procedure (119). The nature of this masking mechanism has been attributed to the formation of covalent bonds between the tricarballylic groups of Fumonisins and the hydroxyl groups of starch or the amino or sulfidryl groups of the side chains of amino acids in proteins. The possibility of such an interaction has been supported by the evidence that the two tricarballylic acid (TCA) groups can react with methyl glucose (as a starch model compound)

upon heating, leading to the formation of the diester of FB_1 (120). The TCA groups were also found to react with protected lysine and cysteine methyl esters (as protein model compounds), demonstrating that the free thiol or amino groups of proteins may covalently bound to FB_1 (Fig.7). Thermal processing of food contaminated with Fumonisin mycotoxins studied and the effect on the chemical structures and toxicity, was reviewed extensively, for what concerns the N-derivatives and the hydrolysed forms of FBs (121).

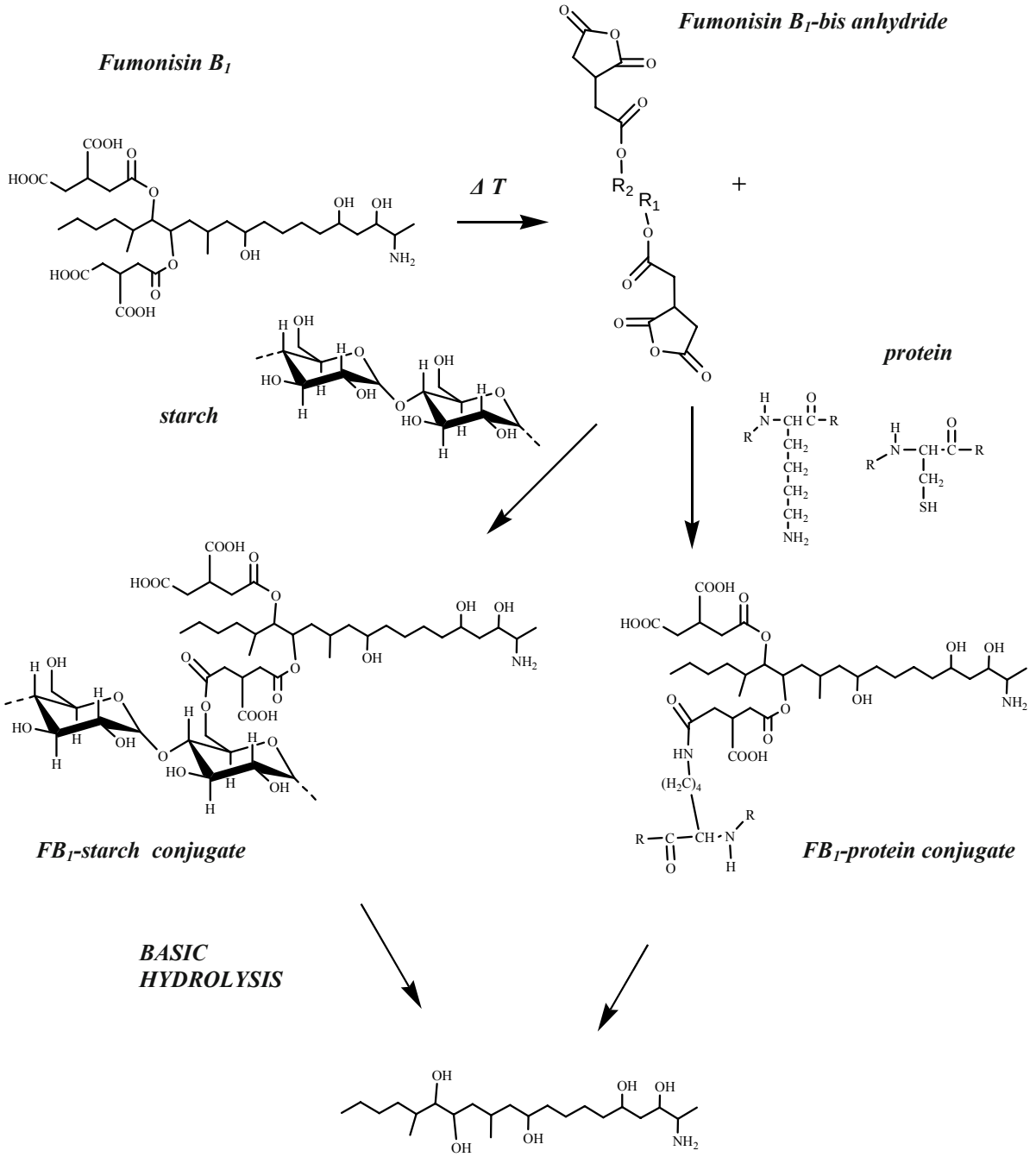


Fig. 7: Proposed binding of Fumonisin B_1 to proteins and starch, by TCA groups, and its release, by basic hydrolysis to the hydrolysed derivative HFB_1.

However the pararadox of FBs, concerning their toxicology, is still unsolved, altought TCA groups of FBs seems to play a critical role in both toxicology and masking mechanism of Fumonisins.

The overall knowledge of the masking mechanism that involve the TCA groups of Fumonisins is still not totally understood.

Thus more studies are required to improve the knowledge of FBs behavior and their interactions with the maize matrix .

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1.4 The maize chain

Maize (syn. corn) is widely cultivated throughout the world, and a higher amount of maize is produced each year than any other grain. While the United States produce almost half of the world's harvest(~42.5%), other top producing countries include China, Brazil, Mexico, Argentina, India and France. Italy is the tenth world producer with about 10 millions tonnes per year. Worldwide production was around 800 million tonnes in 2007—just slightly more than rice (~650 million tonnes) or wheat (~600 million tonnes). In 2007, over 150 million hectares of maize were planted worldwide. Because it is cold-intolerant, in the temperate zones maize must be planted in the spring. Its root system is generally shallow, so the plant is dependent on soil moisture. As a C4 plant (a plant that uses C4 carbon fixation), maize is a considerably more water-efficient crop than C3 plants (plants that use C3 carbon fixation) (122-125). Maize can undergo basically 3 different types of processes: dry-milling, wet-milling, and masa-type process. The principal products of dry milling are cornmeal, flour, and grits (Fig.8) (126).

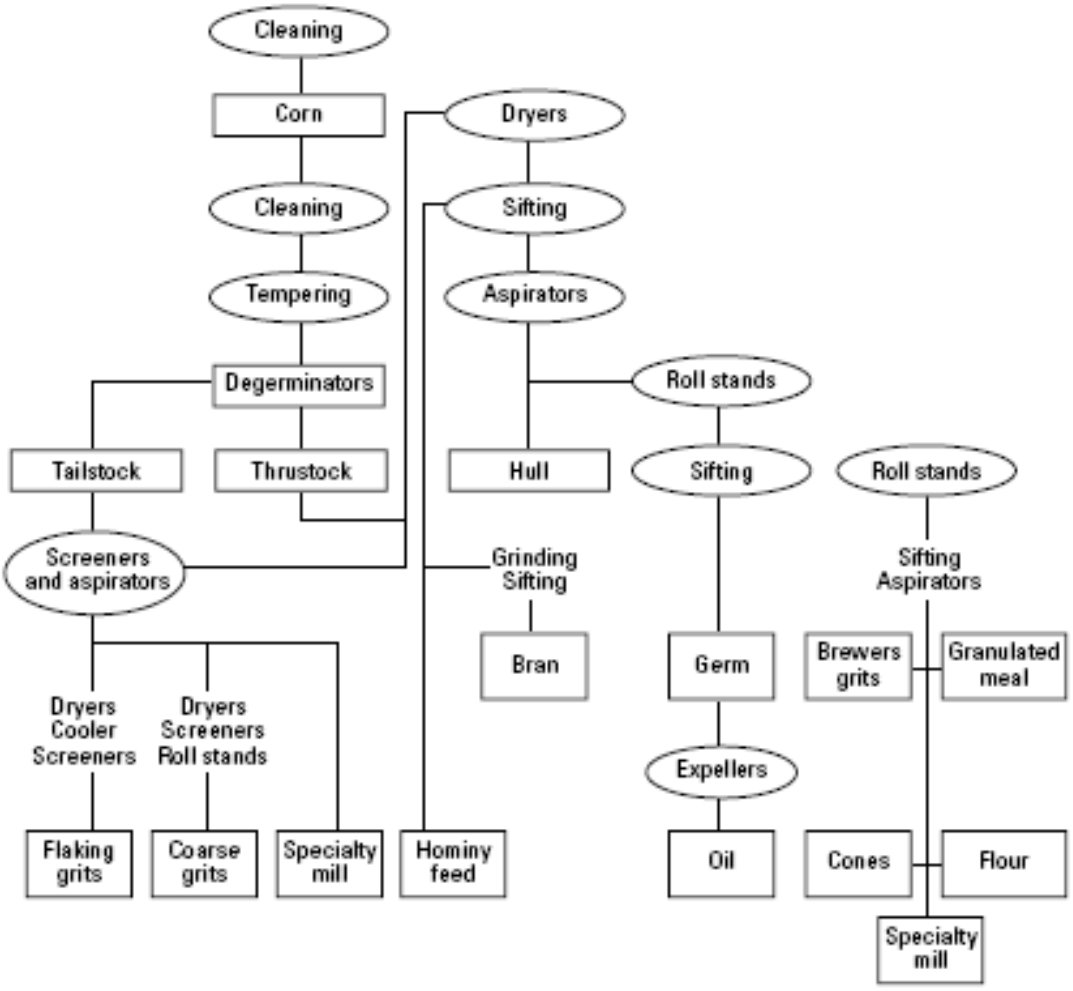


Fig. 8: Flow diagram of a dry-milling process. (Illinois Cereal Mills, Inc.)

These products are ingredients for foods such as pasta, breakfast cereals and snack foods, and baked goods such as corn bread and muffins. The corn is first cleaned to remove dirt and debris and to separate fine and broken kernels from whole corn. The corn is tempered by increasing moisture, and the hull (pericarp), germ, and tip cap are removed, leaving the endosperm. The endosperm is then converted into flours, meals, and grits through a series of mills, sifters, and gravity tables. Corn germ is the raw material for corn oil; bran is generally an animal feed item but with further processing is used in some specialty foods as a source of fiber. The bran fraction is an animal feed item, and FB residues from the whole corn are largely associated with this fraction (127). The principal products resulting from the wet-mill process are cornstarch, high-fructose corn syrup (a sweetener), chemicals (zein, xanthophyl) and ethanol from fermentation (Fig. 9) (126).

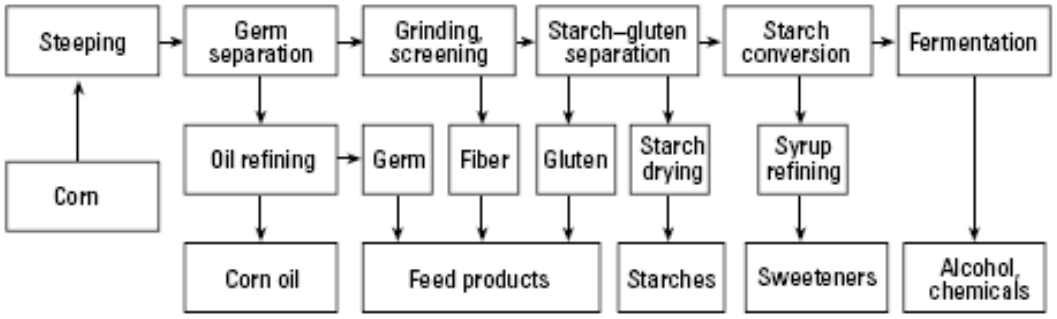


Fig. 9: Flow diagram of a wet-milling process. (Corn Refiners Association).

The masa-type process is used to produce tortillas and tortilla chips, especially common in Latin America and in the Hispanic communities of the United States. In the traditional method the corn is

cooked in alkaline water for a short period of time and then steeped overnight. The steeped liquor is discarded, and the cooked-steeped corn (nixtamal) is washed to remove alkali and loose pericarp, then ground to form masa. The masa can then be formed into flat discs and cooked on a griddle. Although technology and automation have brought greater efficiency to the process, most modern food-processing techniques involving masa can be traced in some form to ancient Native

American inhabitants (*126*). During nixtamalization, FB_1 may be converted to its hydrolysed form, HFB_1, and this compound has been reported in some processed foods (*119*).

2. *Aim of the thesis*

In this thesis we intend to approach the problem of masked Fumonisins in maize and maize-products. The problem is very relevant on account of several implications: scientific, toxicological and regulative. The mechanism of masking is still unknown and it may occur at two levels: on the plant and following technological treatments.

More data are required to improve the knowledge of the behaviour of FBs also considering their interactions with the maize matrix .

This PhD work is divided in three principal parts:

1) development of a LC-MS/MS method for the evaluation of hidden Fumonisins, and application of the method to several batches of maize derived materials : raw cereal, gluten-free foods, reference materials;

2) screening of the occurence of masked Fumonisins in commercially avalilable products, in particular products dedicated to celiacs.

3) study of the masking mechanism, with particular regard to the non-covalent hypothesis, using an endosperm protein as model for the maize matrix.

3. Results and Discussion

3.1 Hidden Fumonisins : the general approach

The presence of hidden forms of mycotoxins in a sample, such as Fumonisins, can be assessed in two ways: by using a direct method by extraction and analysis, whenever the structures and the chemical properties of the molecules of interest are already known; if this information is not available, by using an indirect method based on a hydrolysis step and evaluating the concentration of analytes before and after hydrolysis.

For Fumonisins (FBs), since information regarding hidden forms is still limited, it was decided to proceed with an alkaline hydrolysis, in order to achieve several goals :

→ Cleavage of the FB side chains, which are potential sites of covalent modification;

→ Disruption of the secondary/primary structures of the food macromolecules eventually complexing the toxin;

The hydrolysis, performed with 2 M KOH at room temperature for 1 hour, gives the hydrolysed derivatives of Fumonisins (HFBs), which are stable in alkaline media.

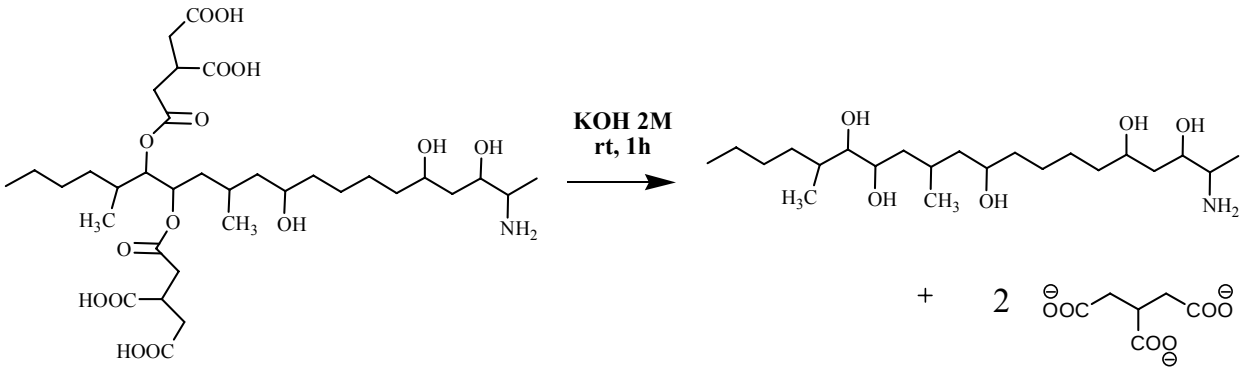


Fig. 10 : Hydrolysis of Fumonisins.

Moreover, the use of a strong base can lead to the disruption of the macromolecular network formed among the main matrix macroconstituents, thus removing thus all the non-covalent interactions which could give rise to the formation of inclusion complexes which could prevent the extraction.

In particular, by this reaction we can obtain :

→ protein denaturation

→ starch swelling

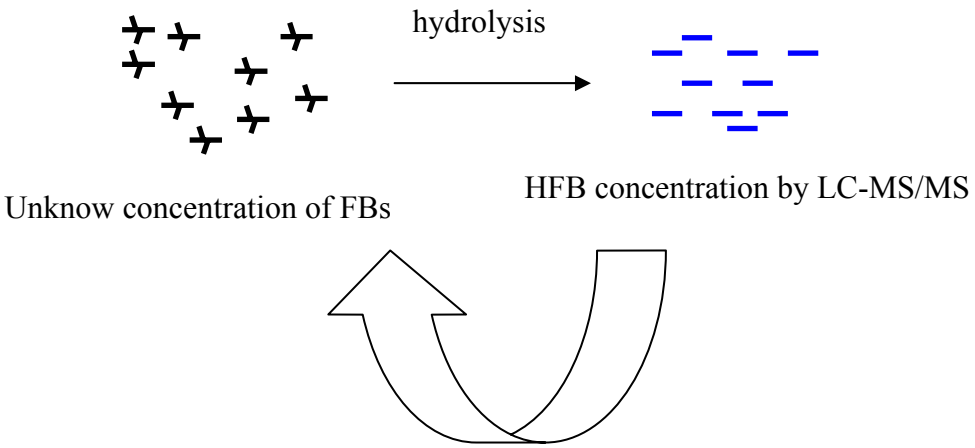
→ saponification of the saponificable lipidic fractions

We assume that, also in the food matrix, the reaction has a yield of 100%.

After hydrolysis it is possibile to extract the hydrolysed forms, HFBs, which are then quantified by LC-MS/MS; starting from these data, we can calculate the concentration of the native Fumonisins present in the sample.

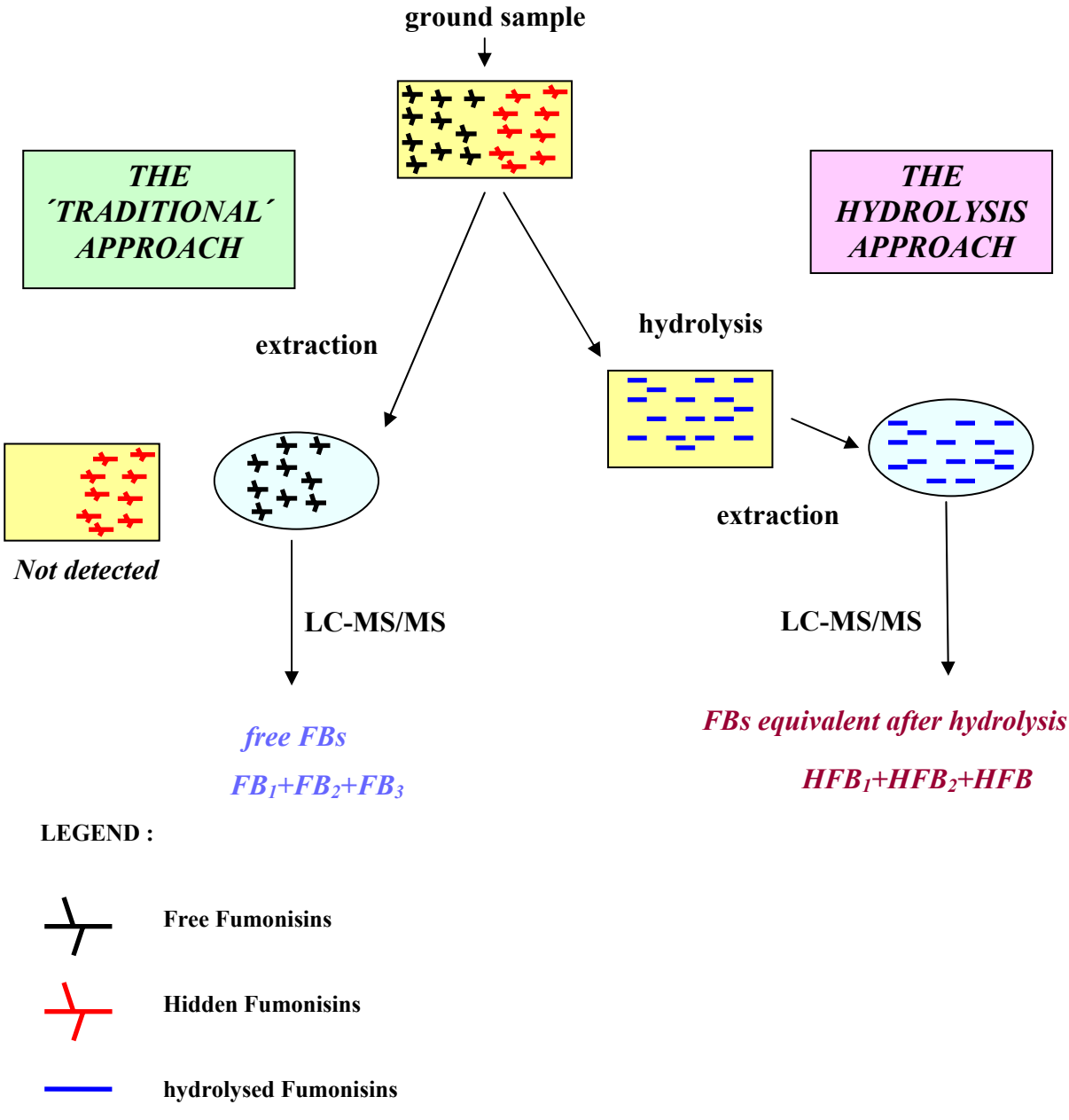
Thus, in order to develop this idea, we divided a sample in two aliquots: the first underwent to the "traditional" extraction and analysis for free Fumonisin quantification; the second aliquot underwent to hydrolysis with 2 M KOH, followed by an extraction step with acetonitrile and finally analysed by LC-MS/MS, thus obtaining the concentration of the hydrolysed FBs.

From this value we can calculate the total amount of FBs before hydrolysis, expressed as Fumonisin equivalents. The amount of hidden Fumonisins occurring in the sample was then obtained by the difference between the total Fumonisins amount and the free Fumonisin concentration (Fig. 11)



FBs equivalent = native FBs in the sample before hydrolysis (total FBs)

Fig. 11: Scheme of the approach.



Equivalent FBs after hydrolysis - free FBs = hidden FBs

Fig. 12: Proposed approach for the quantification of hidden Fumonisins.

3.2 Fumonisin analysis

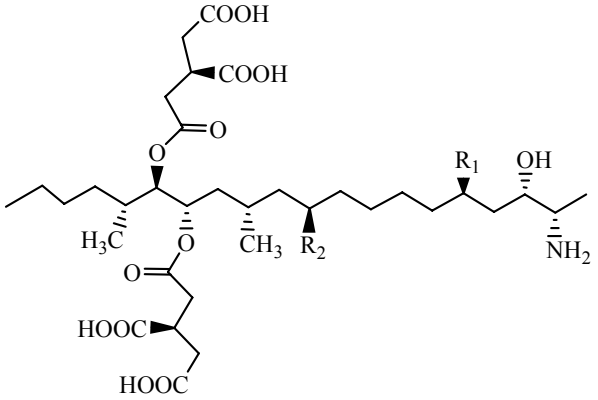
Due to the absence of cromophores or fluorescent group in the molecules, the most appropriate technique for analysis of FBs and of their relative hydrolysed form, is represented by high pressure liquid cromathography iphenated with mass spectrometry detection, with electospray interface (LC-ESI-MS). A particular application of this technique, is the Multiple Reaction Monitoring (MRM) with triple quadrupole or ion trap detector, which allowed a very high specificity and sensitivity. The MRM mode is based on the selection of fragments, produced from the analyte molecular ions, by collision versus an inert gas, as Argon. Mass spectrometry detection require the selection of the ionization mode (positive o negative) for the analytes of interest. In this work, for Fumonisins, the positive mode $(M+H)^+$ was chosen on the basis of the experience of our research group and in agreement with the literature, since it is facilitated by the protonation of the primary amino group of the analytes.

3.2.1 MS/MS detection

In order to develop the MS/MS detection of Fumonisins, it was necessary to have reference standard solutions. A working standard solution at a proper concentration was infused in the mass spectrometer, in order to optimise the ionisation parameters such as voltages and gas flows applied in the source.

The optimization of the source parameters is necessary to maximise response of the analytes, so that as many molecules can be protonated and taken to the gas phase.

The structures of FB_1, FB_2, FB_3 and their molecular weights are reported in the next figure.



	FB1	FB2	FB3
R1	OH	OH	H
R2	OH	H	OH
MW [g/mol]	721.39	705.39	705.39

Fig. 13: Structures and molecular weights of Fumonisins B_1, B_2 and B_3.

Capillary and cone potentials must be chosen in an ESI interface.

The former is required to produce a good ionization of the analyte, while the latter regulates the strenght whereby the ions, once in the gas phase, are attracted from the source at atmospherical pressure, to the zone kept at middle vacuum.

In this zone the ion packet is collimated by lens, in order to send it compact to the first quadruple, for the selection of the ion of interest. As it is possible to see in the next figures, the higher is the applied potential to the capillary and cone, the higher is the signal relative to the molecular ion of Fumonisin B_1 (m/z 722.03), until a maximum value is reached after which the signal decreases, due probably to the increase of the so called 'in source fragmentation'.

The optimization procedure was then made for Fumonisin B_2 and B_3.

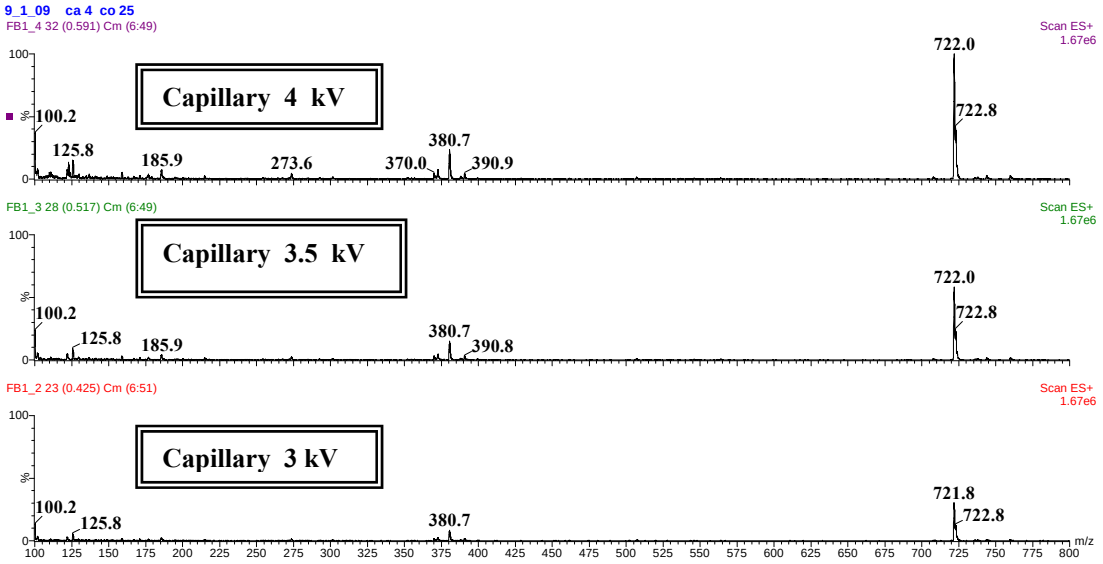


Fig. 14: Influence of the capillary potential (kV) on the intensity of the m/z 722.03 $(M+H)^+$ signal of the Fumonisin B_1 molecular ion. Infusion of the standard (5 ppm) in MeOH, Full scan mode.

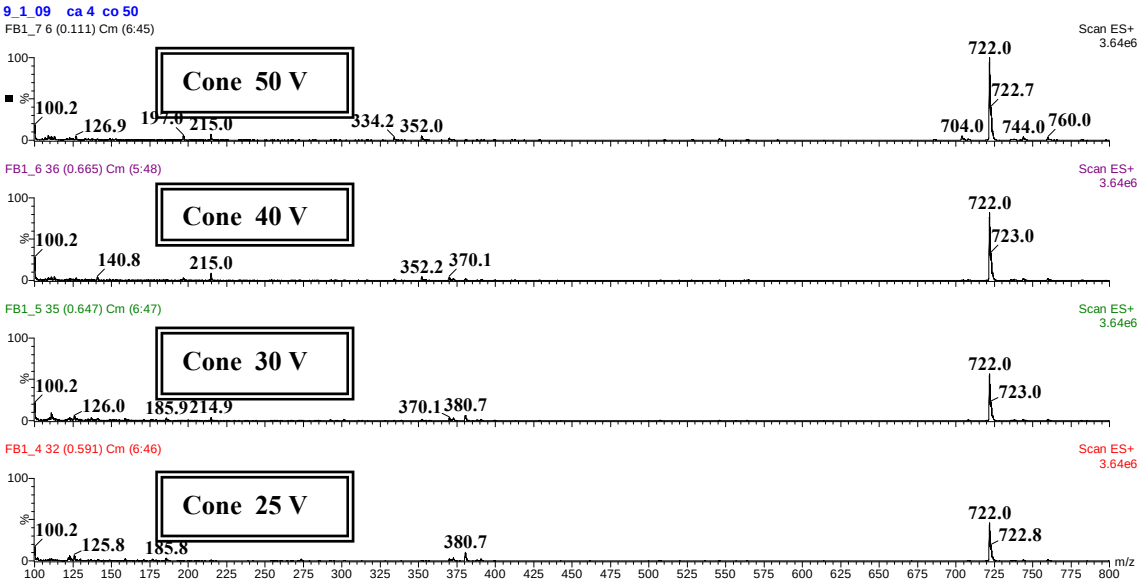


Fig. 15: Influence of the cone potential (V) on the intensity of the signal m/z 722.03 $(M+H)^+$ of the Fumonisin B_1 molecular ion. Infusion of the standard (5 ppm) in MeOH, Full scan mode.

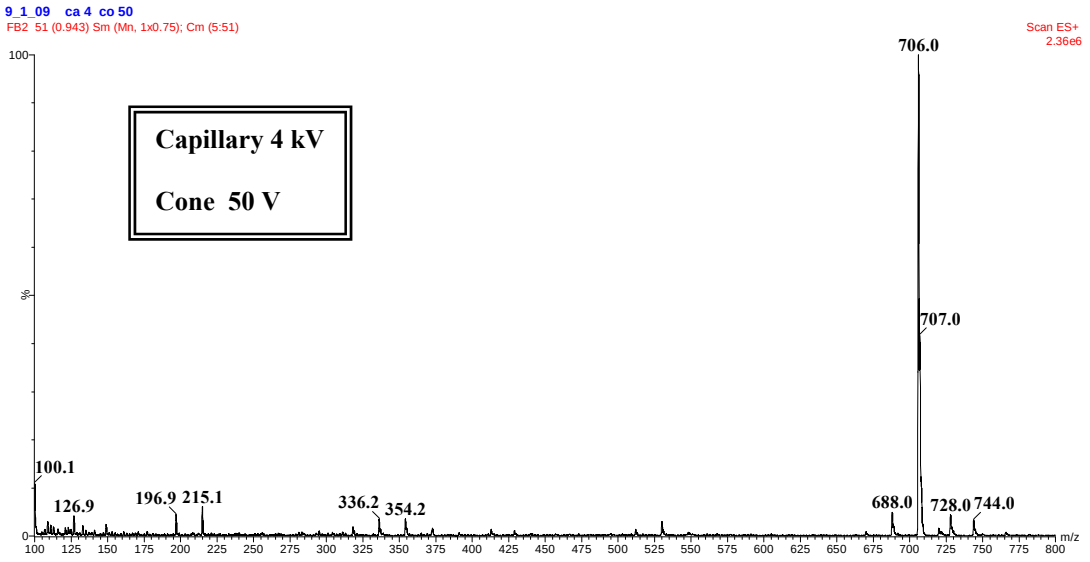


Fig. 16: Signal 706.03 $(M+H)^+$ relative to the molecular ion of Fumonisin B_2. Infusion of the standard (5 ppm) in MeOH, Full scan mode.

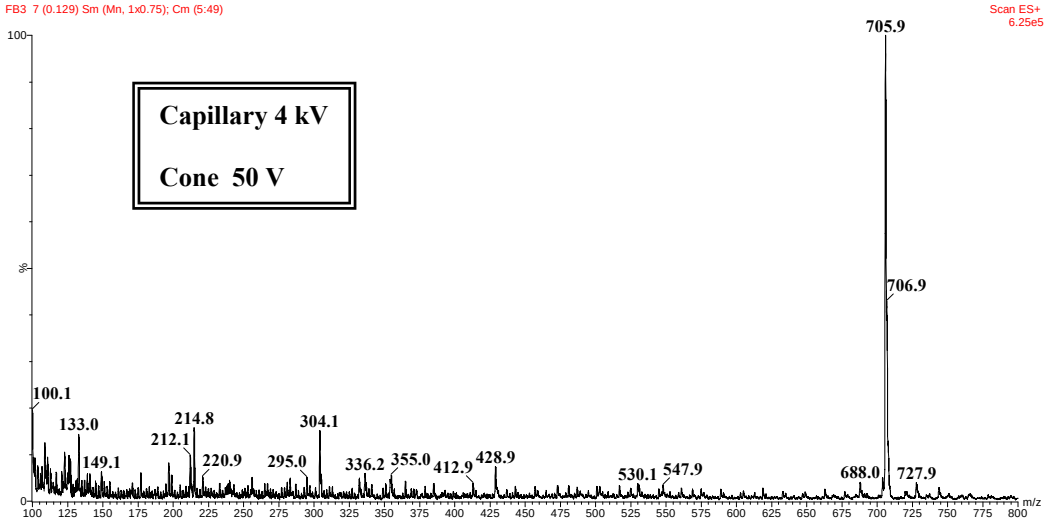


Fig. 17: Signal 705.9 $(M+H)^+$ relative to the molecular ion of Fumonisin B_3. Infusion of the standard (5 ppm) in MeOH, Full scan mode.

Once the source parameters are optimized, so as the molecular ion formation of the analytes is maximized, the fragmentation can be developed.

In this phase, the molecular ion coming from the first quadrupole is fragmented, after acceleration, versus molecules of an inert gas (Argon).

The fragmentation magnitude depends from the energy given to the precursor ion (collision energy), and from the pressure of the inert gas in the collision cell, (higher pressure corresponds, with equal volume and temperature, to a higher number of gas molecules and then higher frequencies of collision).

The parameters are optimized in order to obtain intense and stable signals of the daughter ions, which are next analyzed from the third quadrupole. The selected ions arrive to the photomultiplier which, representing the detector in sensu strictu, transforms and amplifies the electric current signal, into a digital electric signal.

The step sequence just described represents what happens in the MRM technique, where production and selection of ions are repeated twice (source and collision cell); this double selection brings about a significant reduction of the background noise and an increase of the technique sensitivity. Moreover, the choice of the fragment produced specifically from the precursor ion of interest (previously produced in the source) minimizes the possibility that other molecules can be detected togheter with the analytes, making the MRM technique extremely specific.

In theory, it is sufficient to choose one daughter ion from those produced from the precursor fragmentation, in order to get this benefit. In practice at least two ions are choosen for different purposes. The former, usually called 'QUANTIFIER', is the most intense; it is generally used for quantification purposes. Under controlled conditions, the most intense ion is assumed to be the most stable, thus contributing in a predominant way to the total ion current; for this reason, it results very useful for quantification purposes. The latter fragment chosen is called 'QUALIFIER': it is generally less intense then the quantifier, and its presence is used as further proof of the identity of the precursor ion.

In the following figures the results of the fragmentation test for FB_1, FB_2 and FB_3 are reported In order to develop the MRM detection, standard solutions of known concentration were infused in the mass spectrometer, and the parameters were changed in order to find the conditions to produce intense, stable and specific fragment of the analytes.

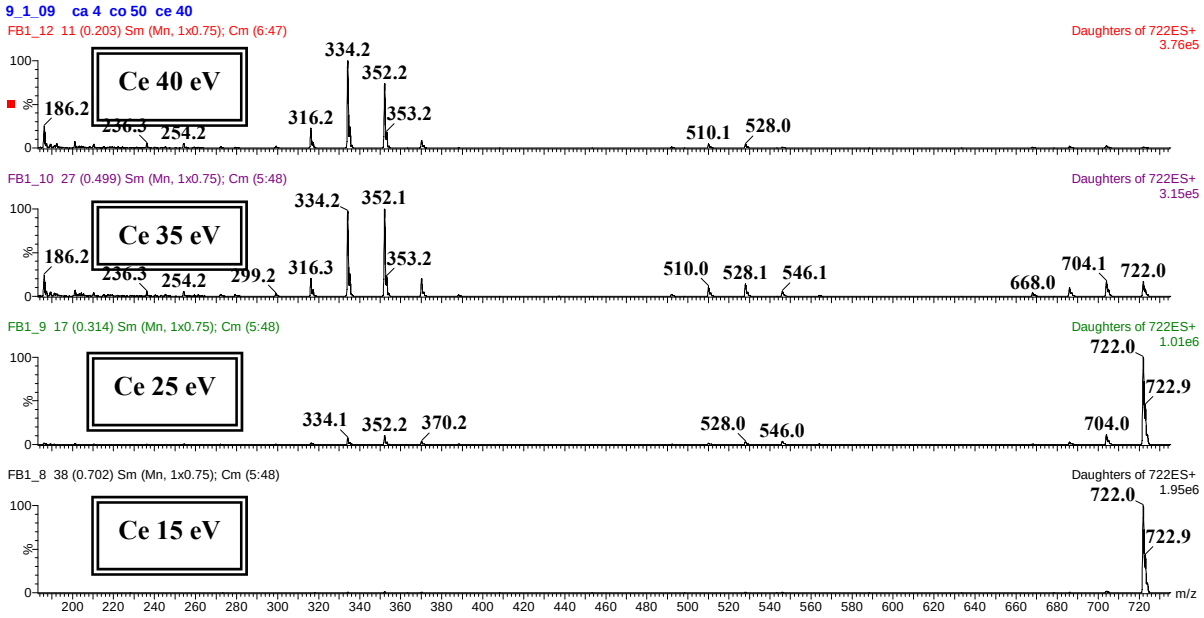


Fig. 18: Infusion of the Fumonisin B_1 standard, (5 ppm) in MeOH; daughter ion scan mode. Influence of the collision energy (eV) applied to the precursor ion m/z 722.03 $(M+H)^+$ of Fumonisin B_1 on the profile of the produced fragments.

Tab. 5: Proposed assignment for FB_1 ion fragments.

m/z	ion
722.03	$(M + H)^+$: molecular ion
704.02	$(M - H_2O + H)^+$
668.01	$(M - 2H_2O + H)^+$
546.10	$(M - TCA + H)^+$
528.11	$(M - TCA - H_2O + H)^+$
510.11	$(M - TCA - 2H_2O + H)^+$
370.19	$(M - 2TCA + H)^+$
352.19	$(M - 2TCA - H_2O + H)^+$
334.19	$(M - 2TCA - 2H_2O + H)^+$
316.19	$(M - 2TCA - 3H_2O + H)^+$

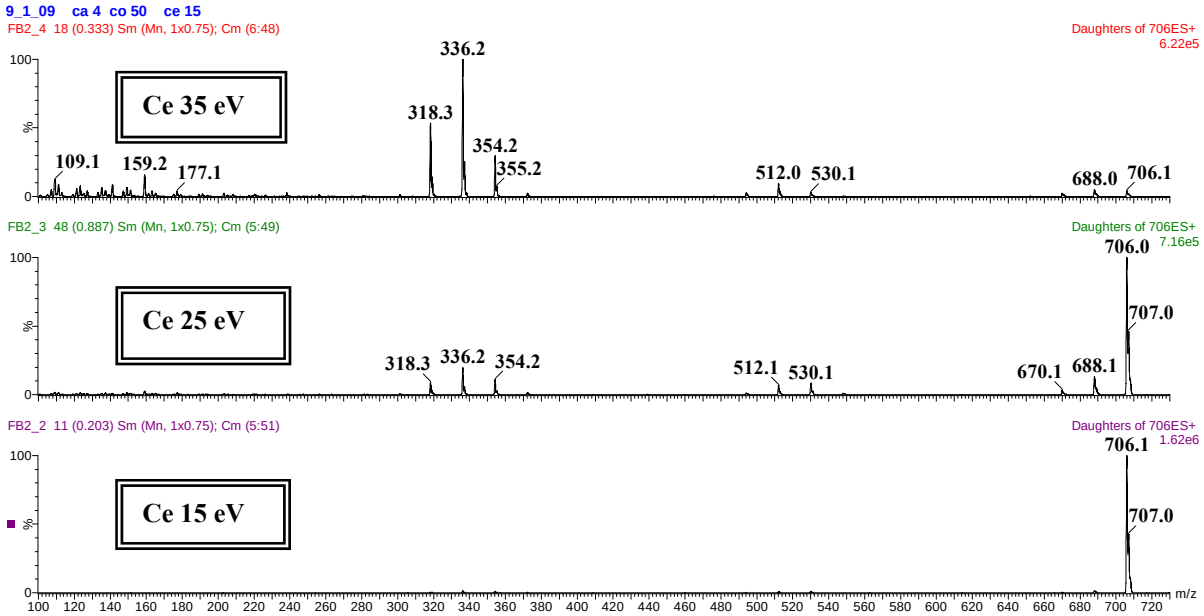


Fig. 19: Infusion of the Fumonisin B_2 standard, (5 ppm) in MeOH; daughter ion scan mode. Influence of the collision energy applied (eV) to the precursor ion m/z 706.03 $(M+H)^+$ of Fumonisin B_2 on the profile of the produced fragments.

Tab. 6: Proposed assignment for FB_2 ion fragments.

m/z	ion
706.03	$(M + H)^+$: molecular ion
688.12	$(M - H_2O + H)^+$
670.13	$(M - 2H_2O + H)^+$
530.12	$(M - TCA + H)^+$
512.09	$(M - TCA - H_2O + H)^+$
354.19	$(M - 2TCA + H)^+$
336.19	$(M - 2TCA - H_2O + H)^+$
318.26	$(M - 2TCA - 2H_2O + H)^+$

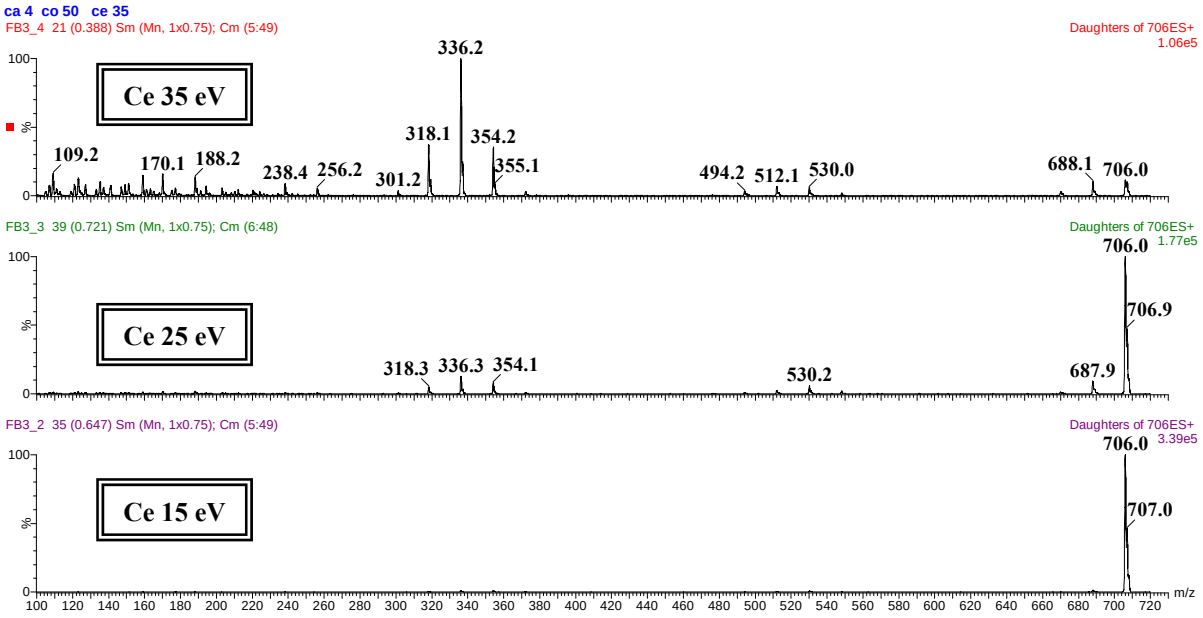


Fig. 20: Infusion of the Fumonisin B_3 standard, (5 ppm) in MeOH; Daughter scan mode. Influence of the collision energy applied to the precursor ion m/z 706.03 $(M+H)^+$ of Fumonisin B_3 on the profile of the produced fragments.

Tab. 7: Proposed assignment for FB_3 ion fragments.

m/z	ion
706.03	$(M + H)^+$: molecular ion
688.12	$(M - H_2O + H)^+$
670.13	$(M - 2H_2O + H)^+$
529.97	$(M - TCA + H)^+$
512.07	$(M - TCA - H_2O + H)^+$
494.18	$(M - TCA - 2H_2O + H)^+$
354.19	$(M - 2TCA + H)^+$
336.19	$(M - 2TCA - H_2O + H)^+$
318.26	$(M - 2TCA - 2H_2O + H)^+$

For all Fumonisins it is possible to see a common fragmentation pathway, the molecular ion can lose several molecules of water and the two tricarballylic chains.

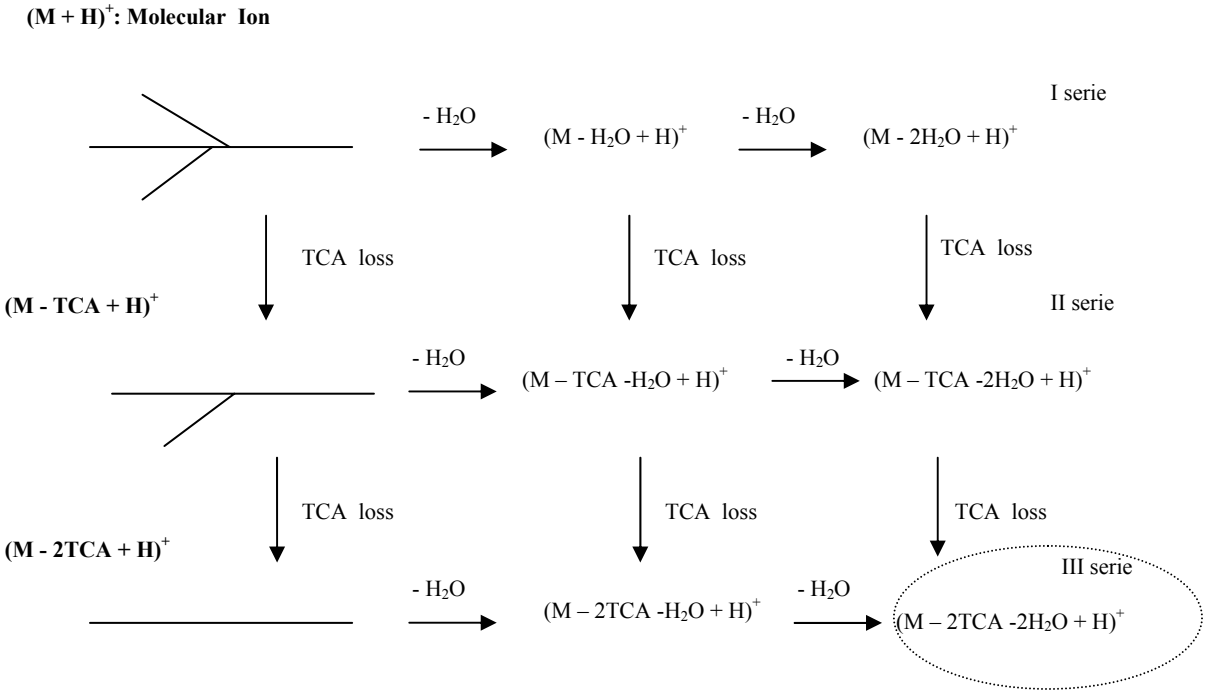


Fig. 21: Proposed pathways for Fumonisin B_1, B_2 and B_3 fragmentation.

By increasing the collision energy provided to the precursor (molecular) ion, the fragments of the third serie increase significantly, while those of the first and second series remain approximatively low for all the tested energies. This may suggest that all the routes of possible fragmentation of Fumonisins lead to the formation of the third series fragments, which are thermodynamically favored.

Once the parameters of collision are optimized, the most suitable transition can be chosen in order to perform the method MRM detection. In this work two transitions per analyte were chosen; the former for quantification purposes, while the latter for the identification of the molecular ion.

In the following table the transitions used for Fumonisins MRM detection are reported. Is possible to see that while FB_1 has a specific channel, the detection of FB_2 and FB_3 require one common channel. This fact is due to the isomeric nature of Fumonisins B_2 and B_3, which differ only for the position of an hydroxy group on the backbone. This little difference implies that this mycotoxins respond to the same transition, at least in a MS/MS experiment; maybe with more fragmentation should be possible to distinguish them in two single channels. Thus, the common response of two analytes require their prevuoius separation, in order to prevent co-elution and then the co-detection.

Tab. 8: MRM detection method: Transitions, potentials and collision energies used for Fumonisin B_1, B_2 and B_3.

Transition	Cone potential (V)	Collision energy (eV)	function
FB_1			
722.40 → 334.40	50	40	QUANTIFIER
722.40 → 352.40	50	35	QUALIFIER
FB_2 - FB_3			
706.40 → 318.40	50	35	QUANTIFIER
706.40 → 336.40	50	35	QUALIFIER

Once the detection method has been developed, it is necessary to set the parameters for the chromatographic separation of the analytes. A standard solution of the three Fumonisins was prepared and several test were performed in order to achieve the baseline separation of the analytes in HPLC using a 2.5 x 25 mm, 5µ, C_{18} X-Terra column and H_2O-MeOH eluents as reported in the experimental part. In the following figure is reported a chromatogram obtained by injection of 1 ppm standard of Fumonisin B_1 B_2 and B_3. It is possible to see the peaks of Fumonisins B_2 and B_3 well separetd despite the isomeric nature of the two analytes. The elution order is $FB_1 < FB_3 < FB_2$.

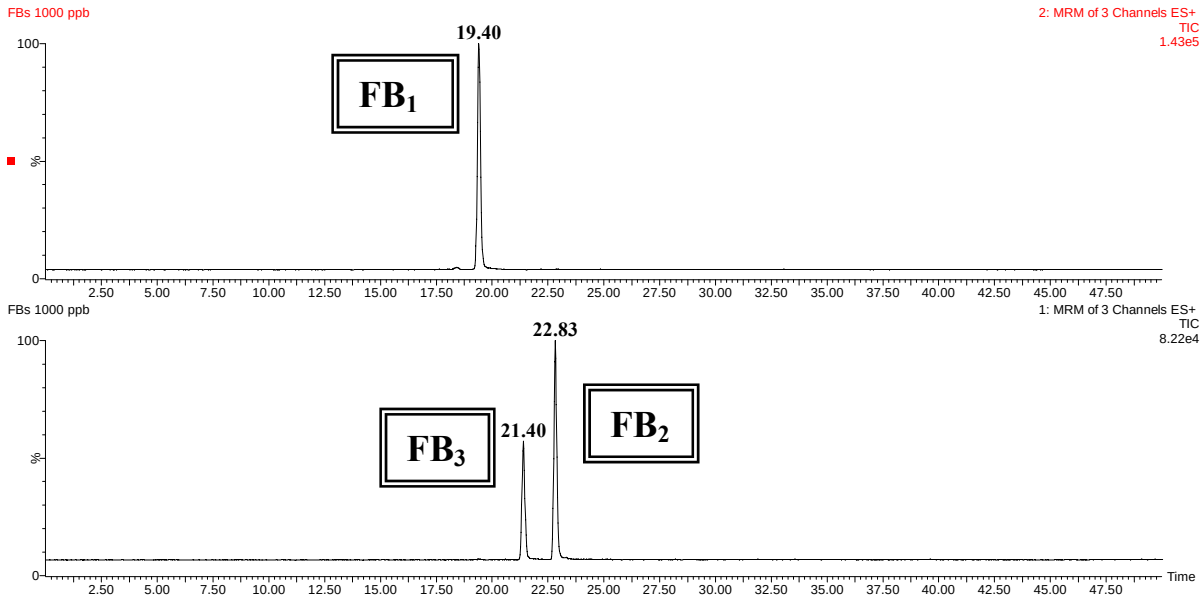


Fig. 22: MRM, TIC, cromathograms of Fumonisin B_1, B_2 and B_3, (1 ppm) standard in MeOH.

Tab. 9 : Retention times of Fumonisin B_1, B_2 and B_3.

analyte	retention time (min.)
Fumonisin B_1	19.4
Fumonisin B_2	22.8
Fumonisin B_3	21.4

By comparing the retention times it is possible to observe that FB_1 is the most polar compound, due to the presence of two hydroxyl groups in the backone and is eluted first. Fumonisin B_2 and B_3, which only differ for the position of one hydroxy group, elute slighlty later with the higher retention time for FB_2, suggesting that the latter is better retained on the column, probably due to a different conformation which interact better with the stationary phase.

3.2.2 Calibration and matrix effect

Calibration and matrix effects must be evaluated in order to quantify the analytes. First, the linearity response of the detector is checked by the external standard method. Then the matrix effect can be evaluated by means of matrix matched calibrations, produced with a blank maize extract. Based on the past experience, we know that Fumonisins are affected from matrix effect, at least on the instrument used for this work. Matrix effects concern the variation of the analyte response in the presence of co-eluting compounds. For this reason we decide to use directly matrix matched calibration in order to correct the matrix effect and to get a more accurate Fumonisin quantification. All the three analytes show a good linearity in the 50-2000 ppb range. Matrix matched calibration curves were determined by diluting an opportune stock of the three Fumonisins, with an extract of blank sample. This procedure was repeated with each batch of samples (three replicates for each point), according to the procedure reported in the Experimental Part.

Tab. 10: Matrix matched calibration curves for Fumonisin B_1, B_2 and B_3.

analyte	curve equation	R^2
Fumonisin B_1	y = 14.31x + 239.45	0.9988
Fumonisin B_2	y = 6.66x + 267.46	0.9931
Fumonisin B_3	y = 5.58x + 264.32	0.9932

3.2.3 Recovery and Limits of Detection

The efficiency of the extraction step can be evaluated by means of spiking experiments or using a certified reference material. In the former case, a blank sample is spiked at proper concentration levels with an analyte standard solution and then analysed according to the described procedure. After quantification, the concentration found for each analyte is compared with that initially added to the sample. This method is commonly used for method validation, but it shows some limitations: the spiked analyte is subject to a different environment in the matrix when compared to a natural contaminant. The use of a certified reference material may thus overcome this limitation, since it is a naturally contaminated sample, already measured by several certified laboratories, and provided of a certified contamination level which should be considered as a "true value". The method recovery is thus obtained by comparison of the concentration measured with the given or nominal concentration.

Assuming that the recovery evaluation by means of reference material could be affected from error due to the unknown nature of the analyte masking phenomenon (hidden Fumonisins), we decided to evaluate the extraction recovery by means of the spiking procedure, reported in the Experimental Part (Fig. 23).

Recovery% = (experimental concentration / reference concentration) x 100

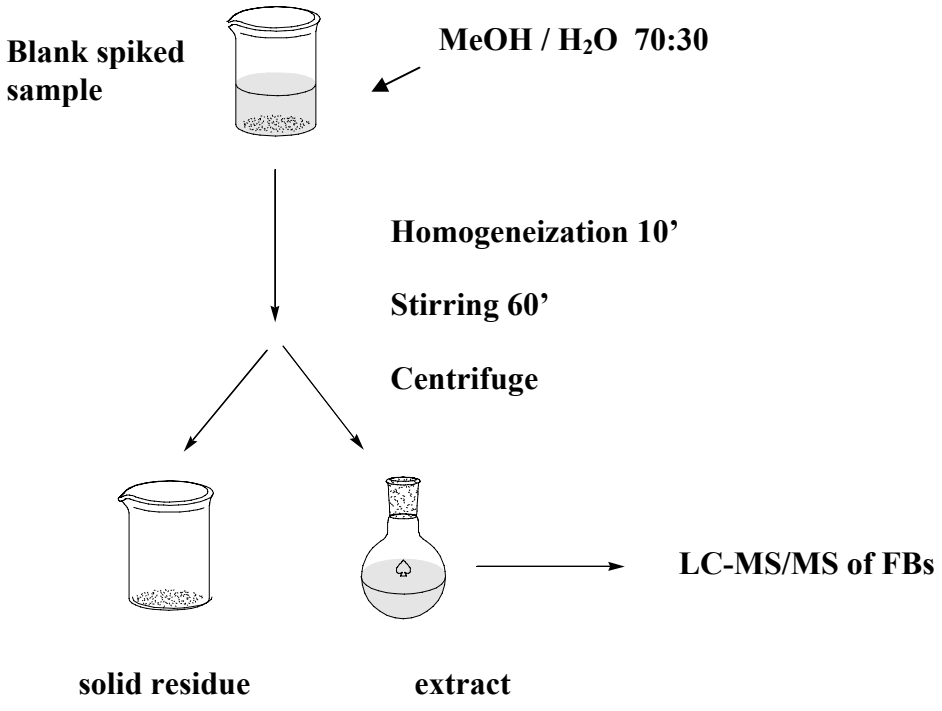


Fig. 23: Schematic procedure of Fumonisin extraction for the recovery evaluation.

Blank maize was spiked with an opportune volume of Fumonisins stock solution, on two levels, 250 and 1000 ppb, and then analyzed with two injections per level, with a matrix matched calibration. In the following tables the results for the extraction recovery of the three Fumonisins are reported.

Tab. 11: Recovery evaluation for Fumonisin B_1.

analyte	expected µg/Kg	found µg/Kg	recovery	RSD %
FB_1	248.98	262.25	105.3 %	4.5
FB_1	996.91	865.4	86.8 %	4.3

Tab. 12: Recovery evaluation for Fumonisin B_2.

analyte	expected µg/Kg	found µg/Kg	recovery	RSD %
FB_2	250.45	277.25	110.9 %	4.7
FB_2	1002.84	895.6	89.3 %	1.7

Tab. 13: Recovery evaluation for Fumonisin B_3.

analyte	expected µg/Kg	found µg/Kg	recovery	RSD %
FB_3	250	228.4	91.4 %	7.9
FB_3	1000	829.1	82.9 %	1.4

Tab. 14: Mean recovery for Fumonisin B_1 B_2 and B_3.

analyte	mean recovery
FB_1	96.1 %
FB_2	100.1 %
FB_3	87.3 %

It is possible to see that the recovery values are higher for the lower levels, and lower by increasing the tested concentrations. Although we used a matrix matched calibration, several recovery values were higher than 100%: they can be explained by a slight imprecision in the spiking volume of the common standard solution for FB_1 and FB_2. However the mean values are satisfactoy and have been used to correct the data.

A reference material was also tested in order to check the results of the spiking procedure.

In the following table the results of the recovery for FB_1 and FB_2, obtained by analysis of a reference material are reported.

Tab. 15: Reference material mean recovery for Fumonisin B_1 and B_2.

analyte	certified µg/Kg	found µg/Kg	recovery	RSD %
FB_1	2406 ± 630	1937.8	80.5 %	8.9
FB_2	603 ppb ± 114	747.5	118.7 %	5.3

The results concerning FB_1 are consistent with those obtained from spiking experiments, according to which a high analyte concentration usually corresponds to a lower extraction efficiency. On the other hand, the results obtained for FB_2 do not agree with those previously obtained: this is probably due to the high standard deviation in the reference material. The reliability of certified material, for extraction efficiency evaluation, in Fumonisins analysis, will be further discussed in the chapter of maize analysis.

The limit of detection was evaluated by the signal to noise ratio (S/N), calculated by MASSLYNX software (Waters Inc.), at 10 and 5 ppb levels. Matrix matched standard were used, in order to correct matrix effects.

Tab. 16: Evaluation of the limit of detection (LOD) and quantification (LOQ) for Fumonisin B_1.

analyte	level	LOD µg/Kg	LOQ µg/Kg
FB_1	10 ppb	2	6
FB_1	5 ppb	1	7

Tab. 17: Evaluation of the limit of detection (LOD) and quantification (LOQ) for Fumonisin B_2.

analyte	level	LOD µg/Kg	LOQ µg/Kg
FB_2	10 ppb	1	4
FB_2	5 ppb	1	4

Tab. 18: Evaluation the limit of detection (LOD) and quantification (LOQ) for Fumonisin B_3.

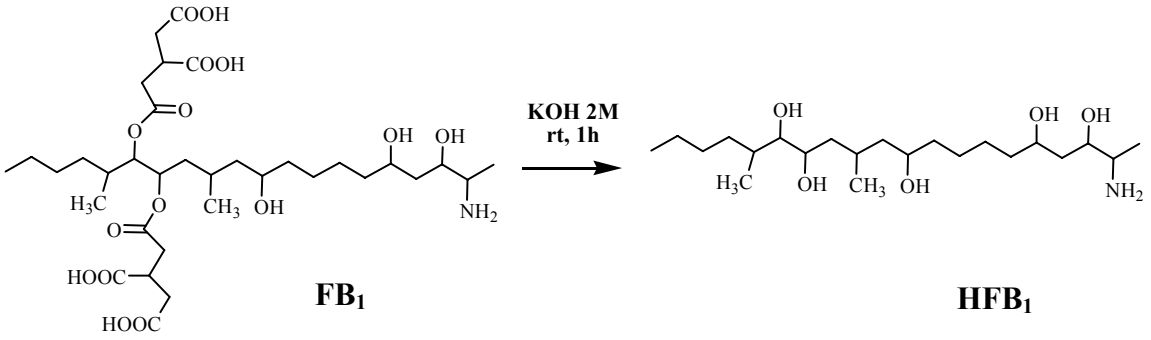
analyte	level	LOD µg/Kg	LOQ µg/Kg
FB_3	10 ppb	2	6
FB_3	5 ppb	2	5

It's generally accepted to consider a S/N = 3 for calculating the LOD and S/N = 10 for calculating the LOQ. The LODs and LOQs were evaluated as reported in Tab. 16,17 and 18. However In the present work, 10 ppb was considered as limit of quantification for Fumonisin B_1 B_2 and B_3.

3.3 Analysis of Hydrolysed Fumonisins: optimization of the method

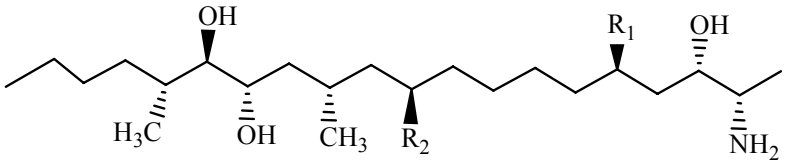
3.3.1 Standard preparation

The evaluation of hidden Fumonisins requires the comparison of the toxin concentration before and after alkaline hydrolysis of the sample. Standards of hydrolysed Fumonisins (HFBs) are not commercially available, thus we prepared the standards as described in the Experimental Part, according to the following reaction :



FB_1 **HFB_1**

Fig. 24: Hydrolysis of Fumonisin.



	HFB1	HFB2	HFB3
R1	OH	OH	H
R2	OH	H	OH
MW [g/mol]	405.35	389.35	389.35

Fig. 25: Structures and molecular weights of hydrolysed Fumonisins HB_1, HB_2 and HB_3.

The standard preparation was performed by alkaline hydrolysis of a proper amount of a FBsstandard solution, as reported in the experimental part. The hydrolysed derivatives are stable also in alkali solution, while an acidic pH can promote loss of water, by protonation of the hydroxyl groups.

For the reaction, we used potassium hydroxyde (KOH) at a concentration of 2 M. The choice of concentration and type of alkali was based on several observations:

- When NaOH is used as alkali, HFBs tend to form sodium adducts, $(M+Na)^+$ during the ionization step in the MS detector, decreasing then the analyte response as molecular ion. This phenomenon is remarkly lower with the potassium cation. The use of potassium hydroxyde instead sodium hydroxyde can thus minimize adducts formation, increasing the sensitivity of the technique.
- High concentration of the base is needed firstly to disrupt the matrix and then to reach a complete analyte hydrolyzation in a reasonable time.
- The high concentration is also useful during the liquid phase extraction of the hydrolysed derivatives of Fumonisins, since acetonitrile doesn't mix with concentrated aqueous solutions at high ionic strenght and the organic phase is easily recoverable.

Production of hydrolysed Fumonisins (HFBs) for standard preparation or for total Fumonisins evaluation, requires some preliminary considerations.

Although the use of mass concentration (ppm, ppb), which is generally accepted for risk assessment and contamination limit definition, is also useful for calibration purposes, the hydrolysis of Fumonisins is performed on the basis of reaction molar yields, and thus molar concentrations of analytes are required.

The "Hidden Fumonisins" quantification sequence is as follows:

1) HFBs mass concentration in the sample, after hydrolysis, is found by LC-ESI-MS/MS analysis;
2) The corresponding moles of HFBs are calculated starting from the found mass concentration;
3) the moles of HFBs after hydrolysis are considered equal to the total FBs moles before hydrolysis;
4) FBs moles can be used to calculate the mass concentration of total FBs in the sample.

The procedure is reported in the following scheme.

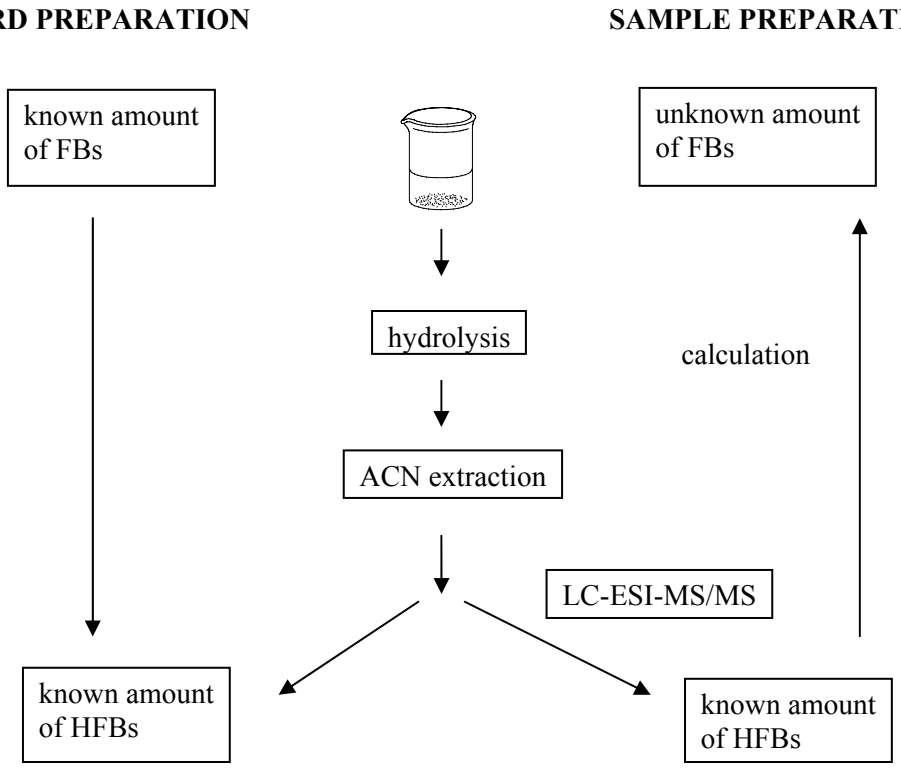


Fig. 26: Analogies of HFB production in standard and sample preparation.

Tab. 19: Molecular weight ratios for Fumonisin B_1, B_2 and B_3 and their hydrolysed analogues .

	FB (g/mol)	HFB (g/mol)	Mass ratio MW_{HFB}/MW_{FB}
1	721.39	405.35	0.56
2	705.39	389.35	0.55
3	705.39	389.35	0.55

3.3.2 MS/MS detection

Once the standards were obtained, the LC-ESI-MS/MS analysis of hydrolysed Fumonisins was developed.

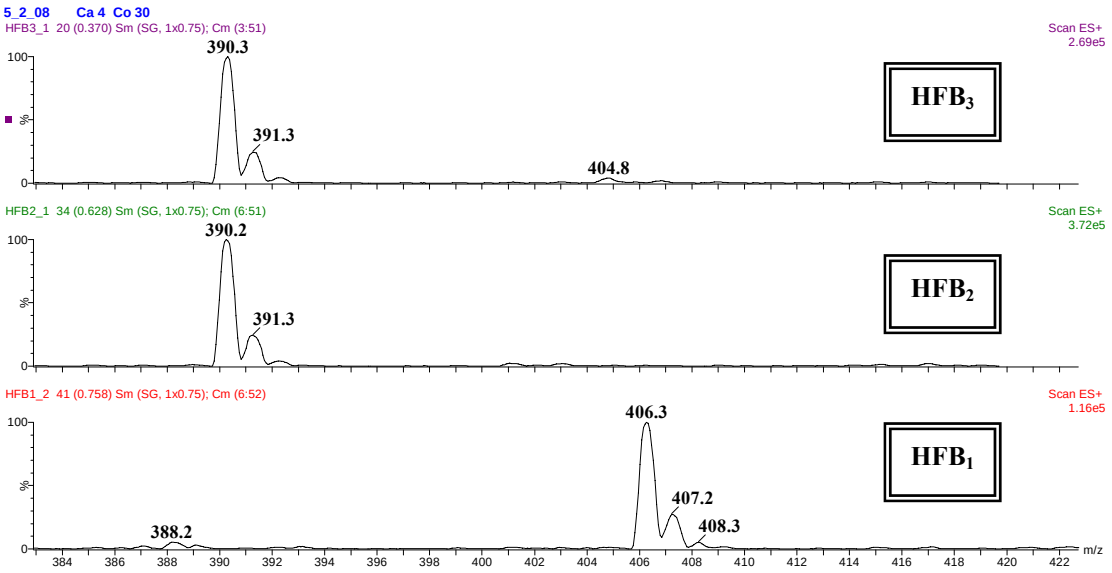


Fig. 27: Zoom of the molecular ion $(M+H)^+$ of hydrolysed Fumonisin B_1, B_2 and B_3. Capillary: 4 kV, Cone: 30 V. Infusion of the standard (5 ppm) in MeOH, Full scan mode.

As far as the ionization process of hydrolysed derivatives of Fumonisins is concerned, it's interesting to note that the optimal capillary voltage is approximately the same as for native Fumonisins; this can be explained by assuming that the positive charge carried by the amino group (for $(M+H)^+$), in both forms of Fumonisins, native and hydrolysed, provide a common ionization pathway, in positive mode. The comparison of cone voltages show a significant lower value for HFBs in respect to those of FBs. In the following figures the results of the fragmentation test for HFB_1, HFB_2 and HFB_3 are reported. In order to develop the MRM detection, standard solutions of know concentration were infused in the mass spectrometer, and the analyser parameters were changed in order to obtain intense, stable and specific fragments of the analytes. Fragmentation comparison between Fumonisins and their hydrolysed derivatives reflects the lack of the TCA chains of HFBs. It's possible to see that the HFBs fragmentation derives only from the loss of the hydroxyl groups, as a water molecules.

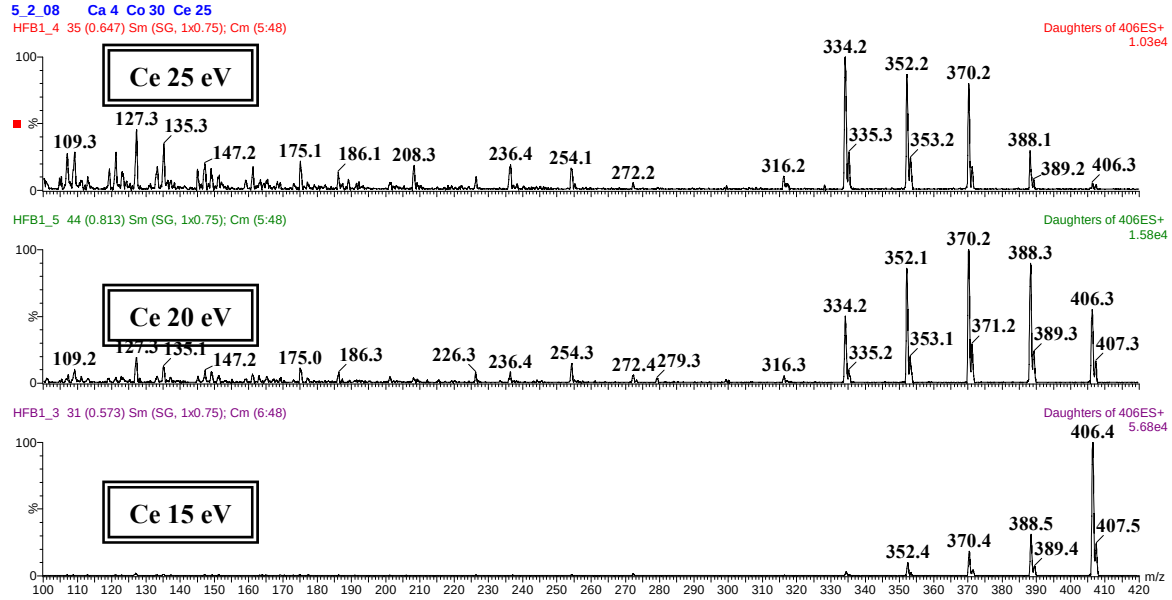


Fig. 28: Infusion of the hydrolysed Fumonisin B_1 standard, (5 ppm) in MeOH, daughter ion scan mode. Influence of the collision energy (eV) applied to the precursor ion m/z 406.3 $(M+H)^+$ of HFB_1 on the profile of produced fragments.

Tab. 20: Proposed assignment for HFB_1 ion fragments.

m/z	ion
406.3	$(M + H)^+$: molecular ion
388.3	$(M - H_2O + H)^+$
370.2	$(M - 2H_2O + H)^+$
352.1	$(M - 3H_2O + H)^+$
334.1	$(M - 4H_2O + H)^+$
316.3	$(M - 5H_2O + H)^+$

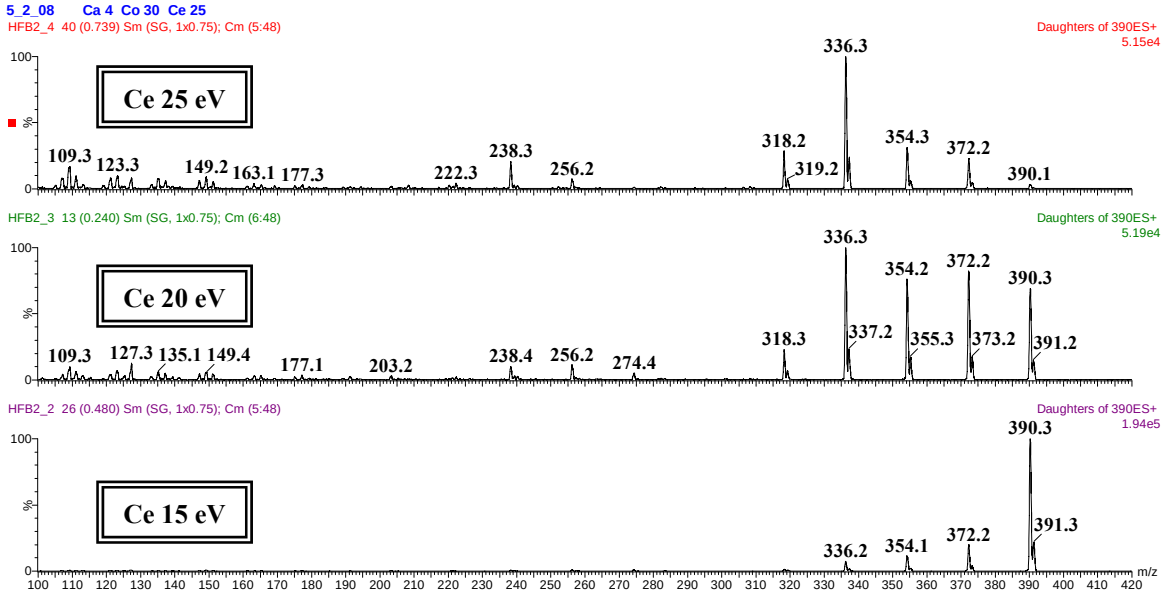


Fig. 29: Infusion of the hydrolysed Fumonisin B_2 standard, (5 ppm) in MeOH, daughter ion scan mode. Influence of the collision energy (eV) applied to the precursor ion m/z 390.3 $(M+H)^+$ of HFB_2 on the profile of produced fragments.

Tab. 21: Proposed assignment for HFB_2 ion fragments.

m/z	ion
390.3	$(M + H)^+$: molecular ion
372.2	$(M - H_2O + H)^+$
354.2	$(M - 2H_2O + H)^+$
336.3	$(M - 3H_2O + H)^+$
318.2	$(M - 4H_2O + H)^+$

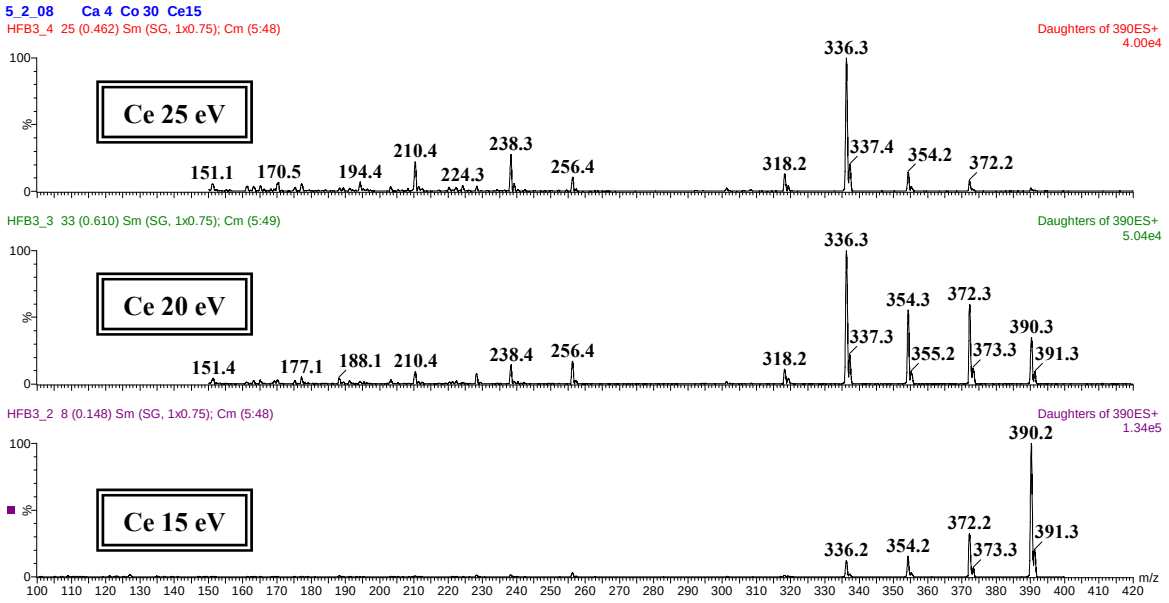


Fig. 30: Infusion of the hydrolysed Fumonisin B_3 standard, (5 ppm) in MeOH, daughter ion scan mode. Influence of the collision energy (eV) applied to the precursor ion m/z 390.3 $(M+H)^+$ of HFB_3 on the profile of produced fragments.

Tab. 22: Proposed assignment for HFB_3 ion fragments.

m/z	ion
390.3	$(M + H)^+$: molecular ion
372.2	$(M - H_2O + H)^+$
354.2	$(M - 2H_2O + H)^+$
336.3	$(M - 3H_2O + H)^+$
318.2	$(M - 4H_2O + H)^+$

Since the HFB fragmentation is due to the loss of water, which is a thermodynamically preferred process in comparison to the loss of TCA side chains recorded for FBs, a lower collisional energy is required for the MRM analysis.

Once the collision parameters are optimized, the most suitable transition can be chosen in order to perform the MRM detection. In the following tables the transitions used for hydrolysed Fumonisins MRM detection are reported. It is possible to see that, as for native

Fumonisins, HFB_1 has a specific channel, while HFB_2 and HFB_3 require one common channel. Being isomers HFB_2 and HFB_3 give rise to the same transitions.

Tab. 23: Transitions, potentials and collision energies used for hydrolysed Fumonisin B_1, B_2 and B_3 MRM detection method.

Transition	Cone potential (V)	Collision energy (eV)	function
HFB_1			
406.4 → 334.1	30	25	QUANTIFIER
406.4 → 352.1	30	20	QUALIFIER
HFB_2 - HFB_3			
390.4 → 354.2	30	25	QUANTIFIER
390.4 → 336.3	30	20	QUALIFIER

Once the detection method is developed, it is necessary to optimize the chromatographic separation of the analytes. A standard solution of the three hydrolysed Fumonisins was prepared and analyzed with the optimized conditions.

The column used for the analytical separation, as for the native Fumonisins, is a 2.5 x 25 mm, 5µ, C_{18} X-Terra, with a flow rate of 0.2 ml/min.

The chromatograms obtained by injection of 1 ppm standard of hydrolysed Fumonisin B_1, B_2 and B_3 are reported in the following figure. It is possible to see the peaks corresponding to HFB_2 and HFB_3 on the same channel.

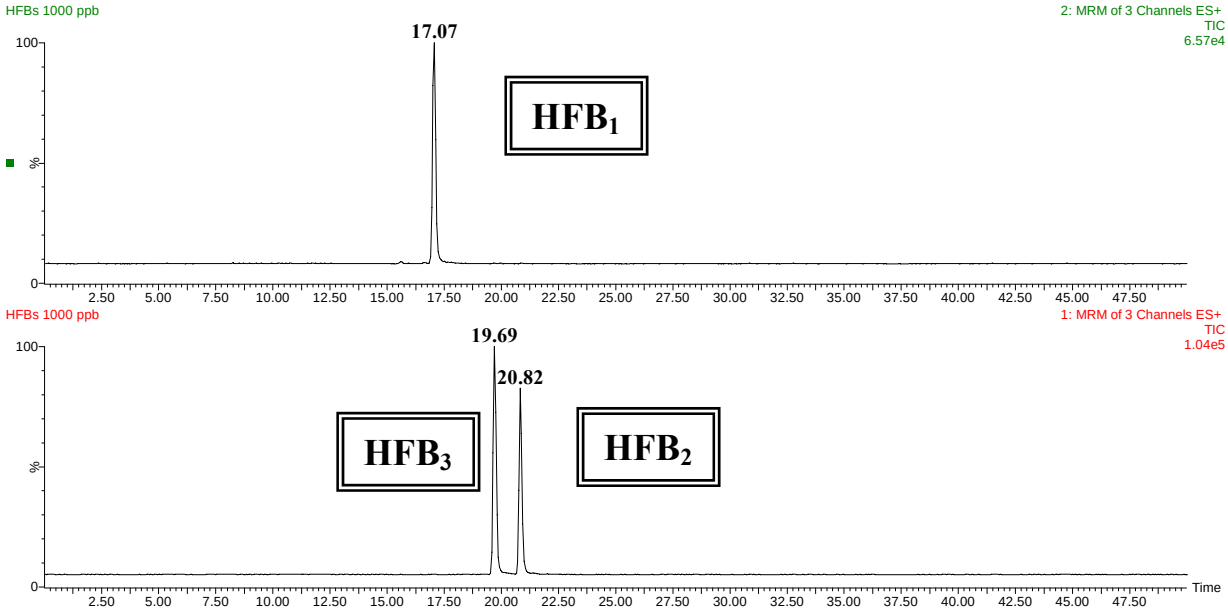


Fig. 31: MRM, TIC, chromathogram of hydrolysed Fumonisin B_1, B_2 and B_3, 1 ppm standard in MeOH.

Tab. 24: Retention time of hydrolysed Fumonisin B_1, B_2 and B_3.

analyte	retention time (min.)
hydrolysed Fumonisin B_1	17.6
hydrolysed Fumonisin B_2	20.3
hydrolysed Fumonisin B_3	21.4

The elution order is $HFB_1 < HFB_3 < HFB_2$. Comparison of the retention times shows that, as in the case of the native Fumonisins, HFB_1 is the most polar compound, due to the presence of two hydroxyl group in the backbone. Hydrolysed Fumonisin B_2 and B_3, which only differs for the position of one hydroxyl group, elute slightly later with a little difference between

them. It's interesting to note that the retention times for the hydrolysed derivatives of Fumonisins, are always slightly lower, if compared with the respective native analogues. Removal of TCA chains of Fumonisins diminish the interactions with the column.

3.3.3 Calibration and matrix effect

The same approach adopted for the calibration experiment performed for the native forms was used for hydrolysed Fumonisins. Matrix matched calibration was used in order to correct the matrix effect and get a more accurate data. Also hydrolysed Fumonisins show a good linearity in the 50-2500 ppb range. Matrix matched calibration curves were prepared by adding a proper amounts of HFBs stock solution in a hydrolysed blank sample extract. The spiked extracts were then analyzed (three replicates for each point), as reported in the Experimental Part.

Tab. 25: Matrix matched calibration curves for hydrolysed Fumonisin B_1 B_2 and B_3.

analyte	curve equation	R^2
hydrolysed Fumonisin B_1	y = 15.46x - 555.39	0.9958
hydrolysed Fumonisin B_2	y = 9.18x - 273.6	0.9962
hydrolysed Fumonisin B_3	y = 3.01x - 109.1	0.9968

3.3.4 Recovery and Limits of Detection

The efficiency of extraction step regarding HFBs cannot be evaluated by analysis of the reference material, because we assume the presence of masked mycotoxins. Then the extraction recovery can be evaluated only by spiking of free FBs and assuming 100% of hydolysis. However, also this procedure, could not be effective representative of the real samples; until the mechanism of masking remain unknown, or cannot be assumed with a reasonable certainty, recovery can only be indirectly evaluated.

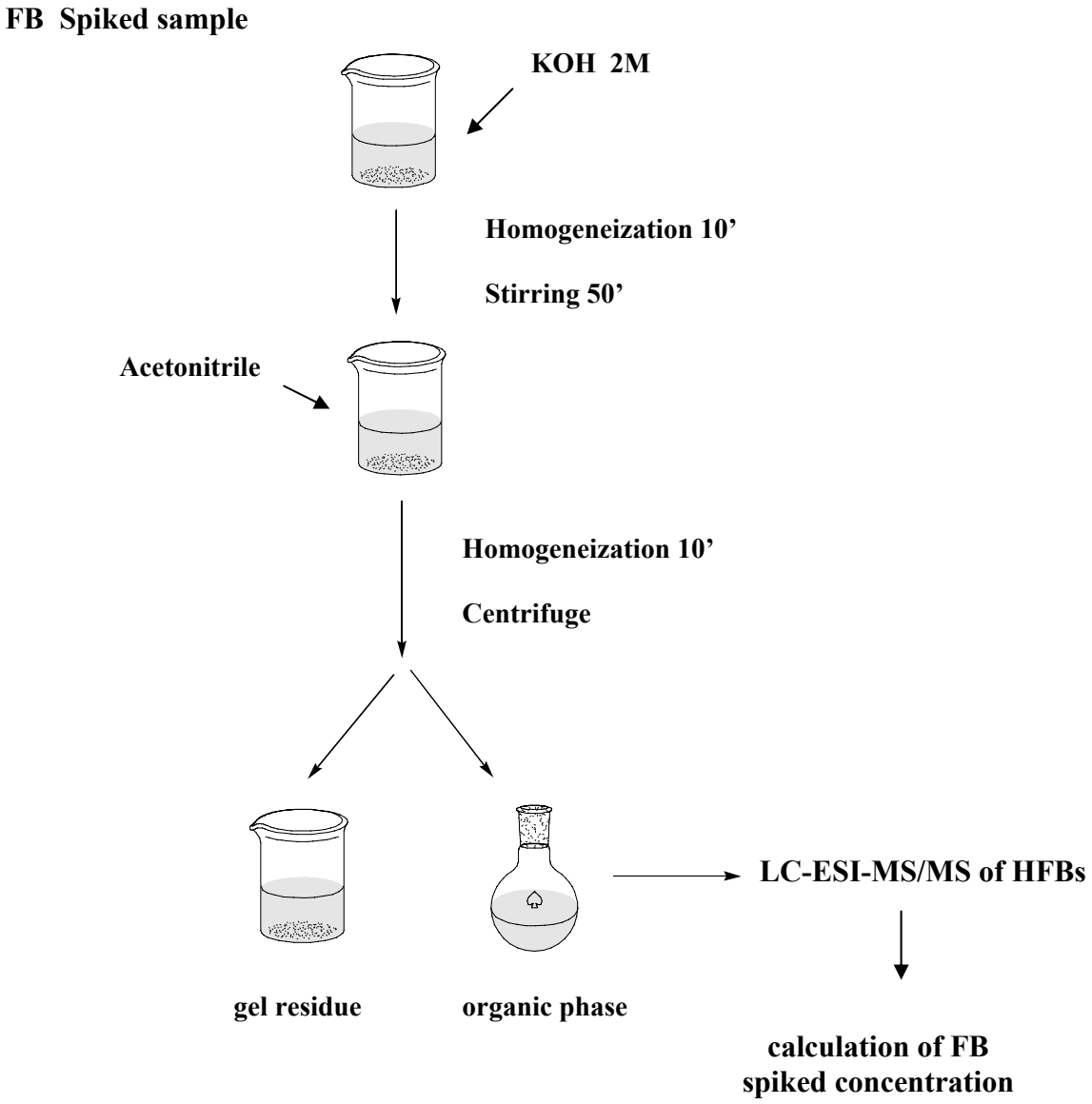


Fig. 32: Schematic procedure of sample hydrolysis for HFB recovery evaluation.

The spiking experiment was performed at two concentration levels (250 - 1000 ppb for HFB_1 and HFB_2 and 500 - 2000 ppb for HFB_3) and each level was analysed in duplicate using a matrix matched calibration.

In the following tables the results obtained for the extraction recovery of the three hydrolysed Fumonisins are reported.

Tab. 26: Recovery evaluation for hydrolysed Fumonisin B_1.

analyte	expected	found	recovery	RSD %
HFB_1	250.00 ppb	204.45 ppb	81.8 %	3.0
HFB_1	1000.00 ppb	731.25 ppb	73.1 %	9.7

Tab. 27: Recovery evaluation for hydrolysed Fumonisin B_2.

analyte	expected	found	recovery	RSD %
HFB_2	250.45 ppb	244.15 ppb	97.7 %	2.8
HFB_2	1002.84 ppb	987.05 ppb	98.7 %	6.3

Tab. 28: Recovery evaluation for hydrolysed Fumonisin B_3.

analyte	expected	found	recovery	RSD %
HFB_3	500.00 ppb	475.05 ppb	95.0 %	14.4
HFB_3	2000.00 ppb	2192.3 ppb	109.6 %	5.9

Tab. 29: Mean recovery for hydrolysed Fumonisin B_1 B_2 and B_3.

analyte	mean recovery
HFB_1	77.4 %
HFB_2	98.2 %
HFB_3	102.3 %

It's important to note that recovery is influenced by two factors, the hydrolysis of the sample, and the extraction in *sensu stricto*.

Differences in recovery could be also explained by the slight different polarity of the analytes, and then by their solubility in acetonitrile.

Limits were evaluated by the signal to noise ratio (S/N), calculated by MASSLYNX software (Waters Inc.), on 10 ppb level for HFB_1 and HFB_2, and 50 ppb for HFB_3. Matrix matched standard were used in order to correct matrix effect.

Tab. 30: Evaluation of the limits of detection (LOD) and quantification (LOQ) for hydrolysed Fumonisin B_1 B_2 and B_3.

analyte	level	LOD µg/Kg	LOQ µg/Kg
HFB_1	10 ppb	2	6
HFB_2	10 ppb	3	10
HFB_3	50 ppb	10	35

It's generally accepted to consider a S/N = 3 for calculating the LOD and S/N = 10 for calculating the LOQ. The LODs and LOQs were evaluated as reported in Tab. 26, 27 and 28.

However, in the present work, 10 ppb were considered as limit of quantification (LOD) for hydrolysed HFB_1 and HFB_2, while 50 ppb was considered for HFB_3.

3.4 The indirect approach

As already discussed in the introduction chapter, hidden Fumonisins were first identified in corn flakes as N-derivatives of the amino group of FBs (115), following the reaction with reducing sugars, giving rise to the formation of Shiff base like compounds.

This covalent modification of Fumonisins is well know, and is promoted by high temperature like that used in some maize based foodstuff preparation. The investigation proposed in this work is not focused on this reaction, but rather on masking mechanisms involving the TCA chains, which contain 4 carboxylic acid groups.

These groups are essential for some observed properties of FBs (i.e.: ceramide synthase irreversible inhibition). Moreover the FBs derivatives produced by covalent modification of the amminic group, are usually less toxic compound (58,59–65).

When performing the basic hydrolysis, several effects are obtained:

- ⇨ the elimination of direct covalent modifications at the carboxylic groups
- ⇨ the loss of TCA chains by cleaving the ester of FBs, producing the hydrolysed form (HFBs);
- ⇨ the disruption of non covalent interactions among matrix macroconstituents with the consequent loss of the matrix tridimensional structure.

On the other side, chemical hydrolysis has a low specificity and doesn't offer much information about the type of modification undergone by the toxin.

Thus, we decided to use an indirect approach to investigate the masking phenomenon: firstly the extraction of free FBs was performed on a maize sample aliquot, then a second aliquot underwent to the hydrolysis procedure to obtain the total FBs.

The presence of hidden Fumonisins in a sample can be calculated by the following formula:

Total FBs (calculated from HFBs) - free FBs = hidden FBs

If the sample doesn't contain hidden FBs (hidden FBs = 0):

Total FBs (calculated from HFBs) = free FBs

Then the total FBs concentration, found by analysis of HFBs, should be a value similar to free FBs.

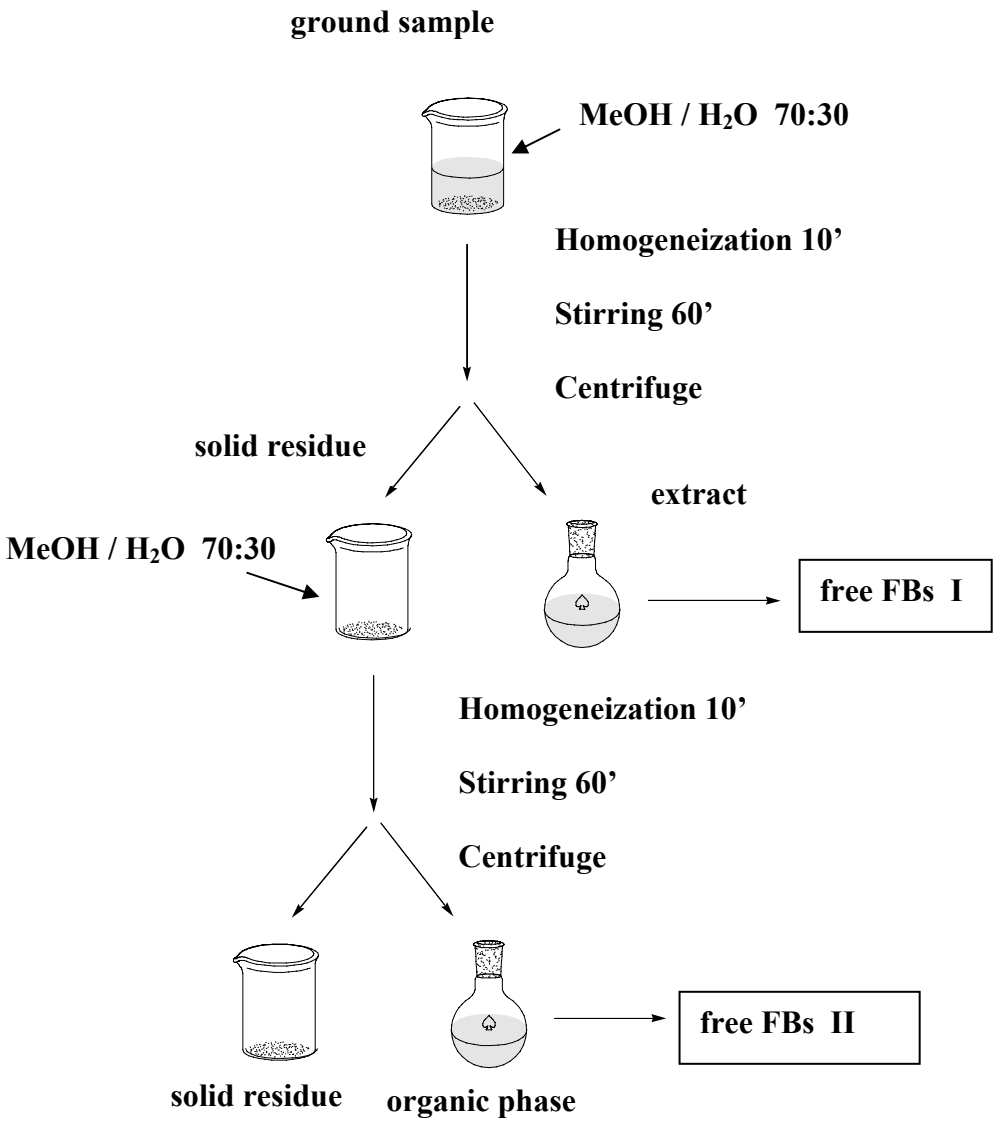


Fig. 33: Schematic procedure of sample preparation for free Fumonisin evaluation.

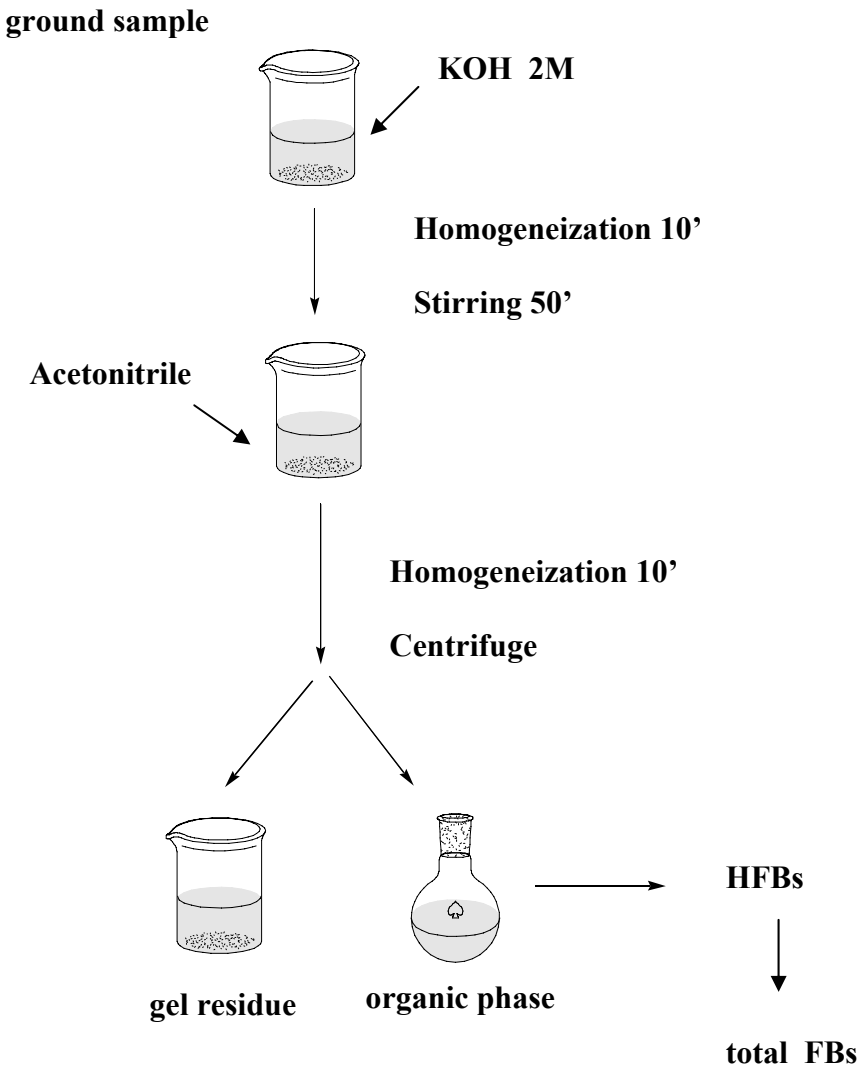


Fig. 34: Schematic procedure of sample hydrolysis for total Fumonisins evaluation, by HFB analysis.

3.5 Analysis of maize based food

3.5.1 The first screening

The presence of hidden Fumonisins was checked as first in corn-based food. The products were purchased from traditional and organic retails, and included pasta, snack and breakfast cereals, in order to analyse both low and high temperature-treated foods.

The protocol of the method is schematized in the previous figures (33,34) and described in the Experimental Part. The extraction step was repeated twice for each sample, in order to allow for the highest extraction yield of the target analytes (free FBs). Each extract was preliminary

analyzed in order to check the presence of HFBs before the hydrolysis, although the occurrence of HFBs is generally negligible in corn-based food. Starting from these preliminary data, a proper sample concentration/dilution step was set to fit in the range of matrix matched calibration.

Five samples of corn pasta (low temperature treated products), and five corn-based snacks and corn flakes (high temperature treated products) were analysed for free and total FBs.

A chromatogram is reported in fig. 35 as example. The results obtained for corn-based products are reported in the following figures.

The collected data underlined a widespread FBs contamination in corn-based food, which had been randomly selected from different shops and brands. When free Fumonisins are considered, all the analysed products coped with the EU legal limits. After hydrolysis, the total Fumonisin concentration was increased and, for several samples, turned out to be higher than the legal limit.

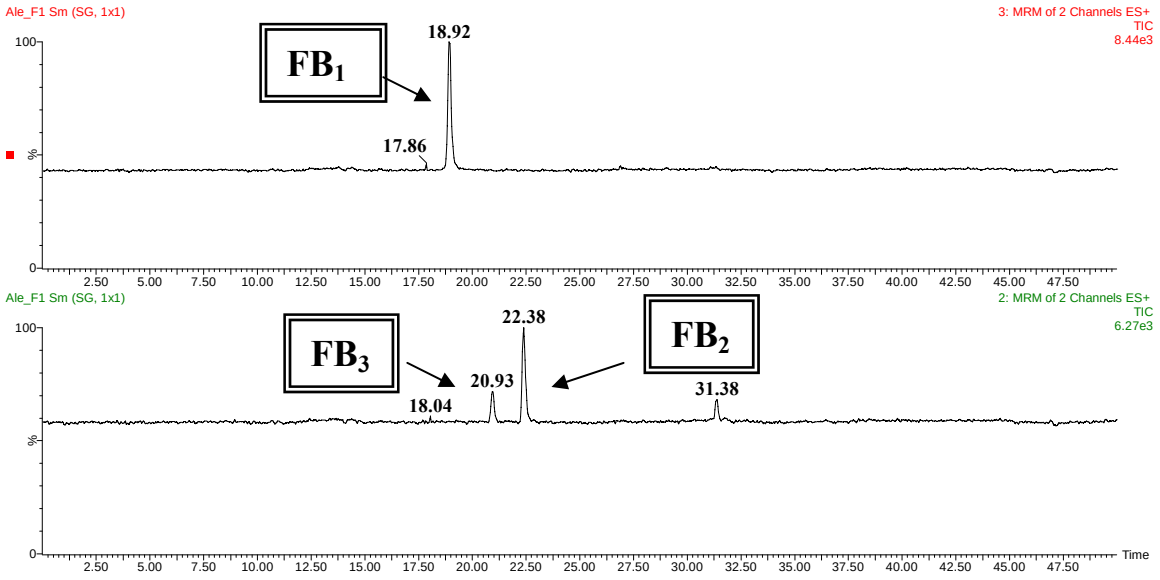


Fig. 35 : MRM (TIC) cromathogram of a FBs naturally contaminated sample of maize based food.

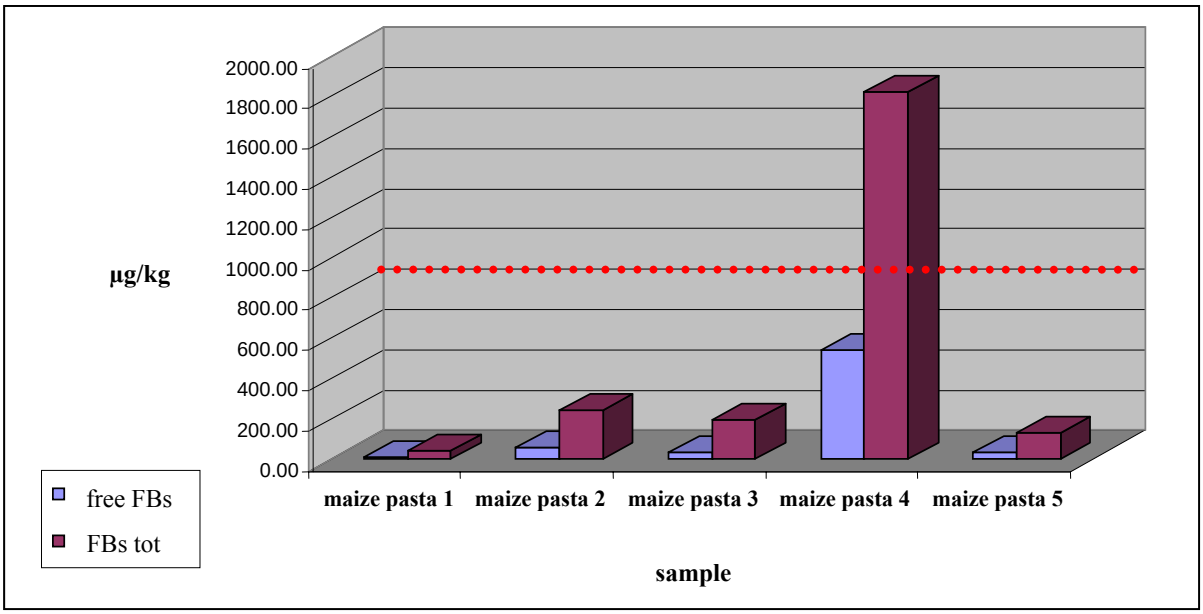


Fig. 36: Results of a preliminary screening of maize pasta samples. Graphic comparison of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis). The dotted red line represents the legal limit of FBs contamination in maize based food (1000 µg/kg), except corn-flakes (800 µg/kg).

Tab. 31: Result of a preliminary screening on maize pasta samples. Contamination of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3) I extraction* (µg/kg)	*Free FBs (FB_1+FB_2+FB_3) II extraction* (µg/kg)	*FBs tot (HFB_1+HFB_2+HFB_3)* (µg/kg)	*Hidden FBs (FBs tot - free FBs)* (µg/kg)
Maize pasta 1	10	< LOD	44	33
Maize pasta 2	55	< LOD	240	185
Maize pasta 3	35	< LOQ	193	159
Maize pasta 4	445	96	1822	1281
Maize pasta 5	29	< LOQ	127	98

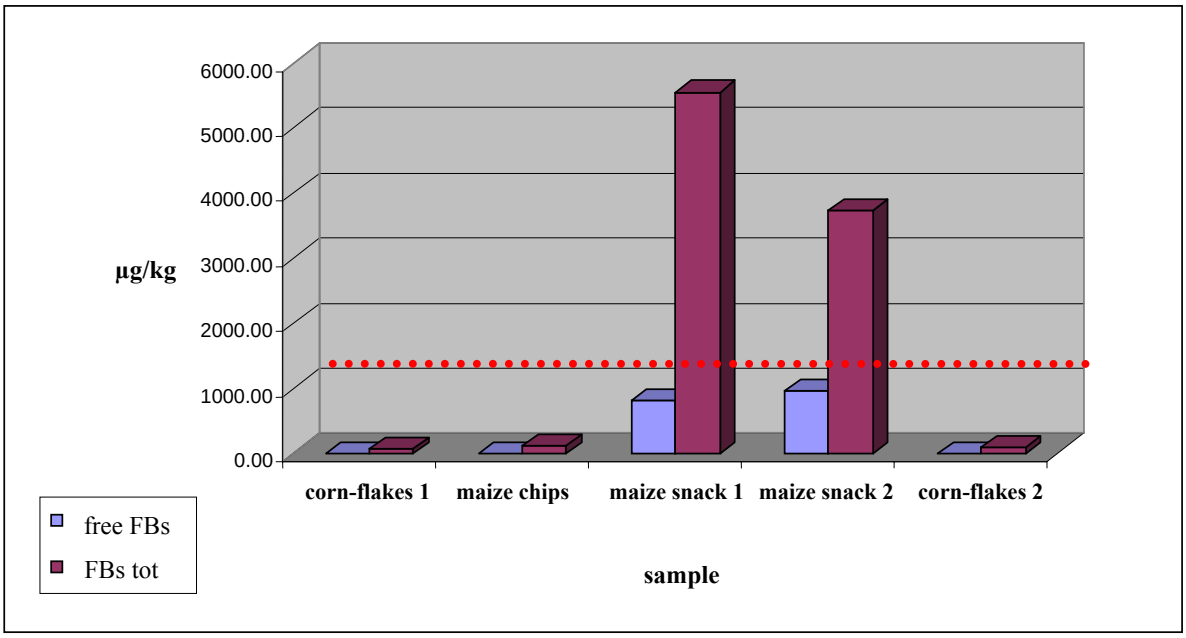


Fig. 37: Results of a preliminary screening on maize based food. Graphic comparison of free FBs (FB_1+FB_2+FB_3, blue bars) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis, purple bars). The dotted red line represents the legal limits of FB contamination in maize based food (1000 µg/kg), excluded corn-flakes (800 µg/kg).

Tab. 32: Results of a preliminary screening on maize based food. Contamination of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3) I extraction* (µg/kg)	*Free FBs (FB_1+FB_2+FB_3) II extraction* (µg/kg)	*FBs tot (HFB_1+HFB_2+HFB_3)* (µg/kg)	*Hidden FBs (FBs tot - free FBs)* (µg/kg)
corn flakes 1	< LOD	< LOD	70	70
maize chips	< LOD	< LOD	108	108
maize snack 1	660	152.53	5549	4736
maize snack 2	712	242.14	3733	2769
corn flakes 2	< LOQ	< LOD	94	94

The following figure summaryzed the preliminary results obtained.

Tab. 33: Summary of preliminary screening on maize based food samples.

Samples analysed	**Positive samples for free FBscontamination**	**Mean free FB contamination** ***(FB_1+FB_2+FB_3)*** **(µg/kg)**	**Mean tot FB contamination** ***(HFB_1+HFB_2+HFB_3)*** **(µg/kg)**
10	7	305.87	1198.1

From this preliminary screening we can draw the following considerations.

I) The method proposed for the indirect evaluation of hidden Fumonisins seems to work well; in all cases the Fumonisin concentrations calculated after sample hydrolysis increase when compared with the concentrations obtained from the analysis of the free FBs; thus the presence of some forms of FBs that are not extracted by conventional method is proven.

II) In agreement with Kim et al. (2003), hidden Fumonisins seem to be present in corn-based food. Probably the reaction between FBs and the matrix components, so far unknown, is promoted by heating steps applied during processing.

III) Since FB N-derivatives are stable under alkaline hydrolysis, the hidden forms detected by our procedure indirectly detected by this method should be different; in particular, the FB tricarballylic chains could be involved in the masking mechanism.

IV) The presence of hidden FBs also in low temperature treated food raises new questions.

It is known that high temperatures are not required for pasta production in contrast with snack and corn-flakes. Thus, other mechanisms could be involved in the masking of Fumonisins.

Tab. 34: Ratio of hidden and free FBs in low temperature processed products.

Low T treated products	Positive sample for free FBs contamination	hidden FBs / free FBs
5	5	3.4

Tab. 35: Ratio of hidden and free FBs in high temperature processed products.

High T treated products	Positive sample for free FBs contamination	hidden FBs / free FBs
5	2	4.4

The values of the hidden / free FBs ratios indicate the amount of hidden form compared with the free ones. While the ratio observed for the products heated at high temprature is as expected, the one obtained at low temperature is still high. Probably a different mechanism is occuringin the two cases.

3.5.2 Survey on gluten free products

After the first screening, other products were collected from retail among organic food, especially those sold with the claim 'gluten free', intended for people with celiac disease.

In order to widen the screening and get more data, several samples of corn pasta, crackers, corn-flakes and polenta were analysed. In particular, the following analyses have been performed:

I) Two extractions of free FBs from each sample;

II) Two sample preparations for both free FB and total FB evaluation;

III) Two injections for each preparation.

The survey results are reported in the following figures.

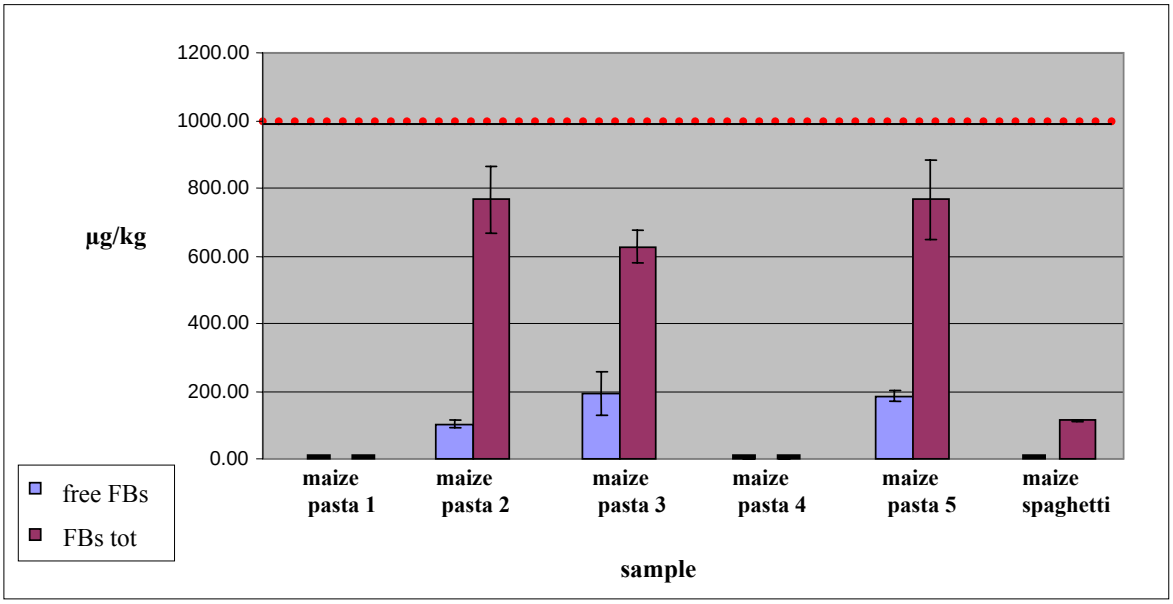


Fig. 38: Results of the survey on gluten free maize pasta samples. Graphical comparison of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis, purple bar). The dotted red line represents the legal limit of FBs contamination in maize based food (1000 µg/kg), excluded corn-flakes (800 µg/kg). (Error bars = RSD%).

Tab. 36: Results of the survey on gluten free maize pasta samples. Contamination of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (4 replicates)*	*tot FBs (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD %) (4 replicates)*
Maize pasta 1	< LOD	0	< LOD	0
Maize pasta 2	103	12	767	13
Maize pasta 3	194	32	625	8
Maize pasta 4	< LOD	0	< LOD	0
Maize pasta 5	186	9	767	15
Maize spaghetti	< LOD	0	113	3

Tab. 37: Hidden Fumonisin estimation in gluten free maize pasta samples.

sample	*Hidden FBs (FBs tot - free FBs)* (µg/kg)
Maize pasta 1	N.D.
Maize pasta 2	664
Maize pasta 3	431
Maize pasta 4	N.D.
Maize pasta 5	581
Maize spaghetti	113

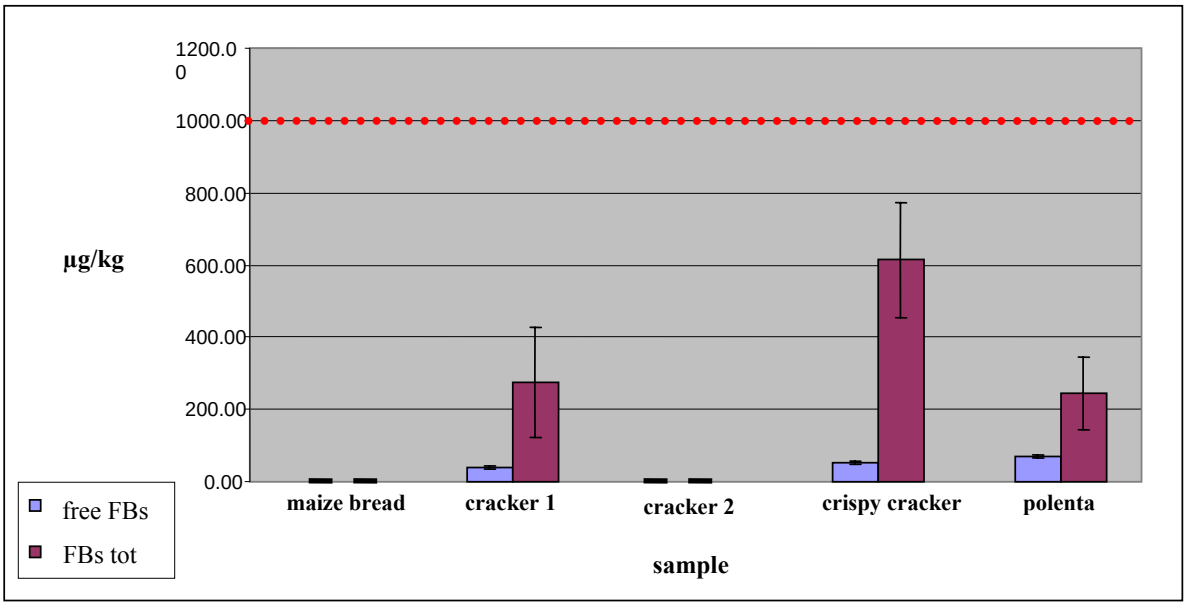


Fig. 39: Results of the survey on gluten free maize based food samples. Graphic comparison of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3). The dotted red line represents the legal limits of FBs contamination in maize based food (1000 µg/kg), excluded corn-flakes (800 µg/kg). (Error bars = RSD%).

Tab. 38: Results of the survey on gluten free maize based food samples. Contamination of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (4 replicates)*	*FBs tot (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD % (4 replicates)*
maize bread	< LOD	0.00	< LOD	0.00
cracker 1	38	13	276	55
cracker 2	< LOD	0.00	< LOD	0.00
crispy cracker	54	7	613	26
polenta	70	5	245	42

Tab. 39: Hidden Fumonisin estimation in gluten free maize based food samples.

sample	*Hidden FBs (FBs tot - free FBs)* (µg/kg)
bread	N.D.
cracker 1	238
cracker 2	N.D.
crispy cracker	560
polenta	175

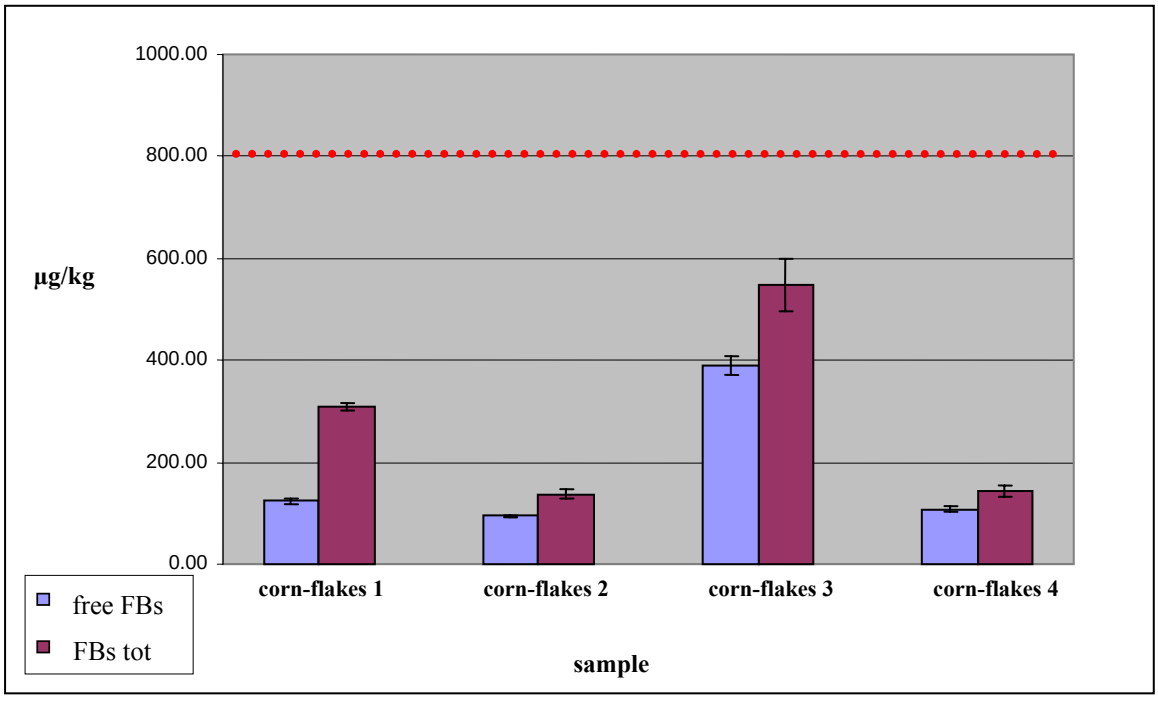


Fig. 40: Results of the survey on gluten free corn flakes samples. Graphic comparison of free FBs (FB_1+FB_2+FB_3) and total FB (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis). The dotted red line represents the legal limits of FBs contamination in corn-flakes (800 µg/kg). (Error bars = RSD%).

Tab. 40: Results of the survey on gluten free corn flakes samples. Contamination of free FBs ($FB_1+FB_2+FB_3$) and total FBs (calculated from $HFB_1+HFB_2+HFB_3$ after sample hydrolysis).

sample	*Free FBs ($FB_1+FB_2+FB_3$)* (µg/kg)	*RSD % (2 replicates)*	*FBs tot ($HFB_1+HFB_2+HFB_3$)* (µg/kg)	*RSD % (2 replicates)*
corn flakes 1	124	1	308.55	3
corn flakes 2	95	2	137.56	6
corn flakes 3	390	4	548.24	9
corn flakes 4	108	5	143.33	8

Tab. 41: Hidden Fumonisin estimation in gluten free corn flakes.

sample	*Hidden FBs (FBs tot - free FBs)* (µg/kg)
corn flakes 1	184
corn flakes 2	43
corn flakes 3	158
corn flakes 4	35

Also in the second batch of products it is possible to see a widespread FB contamination among maize based food, randomly selected from different shops and brands. The mean concentration found of free Fumonisins, 90.84 ppb, is under the fixed limit; unfortunately hidden FBs are still present at higher concentration in almost all samples, reaching in some case values close to the limit. The results are reported in Fig. 52.

Tab. 42: Summary of the survey on maize based food samples.

Samples analysed	*Positive samples for free FB contamination*	*Mean free FB contamination (FB_1+FB_2+FB_3) (µg/kg)*	*Mean tot FB contamination (HFB_1+HFB_2+HFB_3) (µg/kg)*	*Mean hidden FBs / free FBs*
15	10	91	303	3.52

The new analysis has confirmed the results obtained with the first screening: hidden Fumonisins are present in maize based food; where the term 'hidden' means not extractable, or not directly analysable.

The proposed method for indirect evaluation of hidden Fumonisin is a first approach to estimate the amount of hidden FBs in a sample. Concerning the reproducibility of the method, it is possible to see that the relative standard deviation can reach high values in some cases, especially in the analysis of total Fumonisins, on account of the close dependence of the hydrolythic step from the matrix.

The presence of some additives, necessary for these class of products, as stabilyzers, emulsifers, swelling agents and lipids can affect the hydrolysis and the acetonitrile extraction. However, the difference between total and free FBs is significative and the hidden FBs appear to be constantly in a ratio 3:1 relative to free FBs. The widespread presence of hidden Fumonisin in gluten free products can represent a problem for celiac consumers, that must consume this type of food. Moreover the survey has shown that also low temperature treated food can contain considerable amounts of hidden FBs.

As already discussed in the Introduction chapter, some authors have proved the *in vitro* reaction of Fumonisin B_1 with lysine and glucose upon heating above 100°C, as models of the reaction with proteins and starch. This reaction can lead to covalent TCA-derivatives of FBs, amidation and transesterification respectively. However, the temperatures reached in the maize pasta production technology are not so high to justify such a high formation of conjugates FBs. During processing, indeed, the technological transformation is aimed at

starch gelification. Moreover maize proteins, like cereal proteins in general, lack or have a poor content of lysine, which is the limiting amino acid for this product.

Although the heat-induced degradation may contribute to the loss or masking of free Fumonisins, the limited contribution of the reaction at low temperature suggests the occurring of other types of masking mechanisms in corn, as already said before. On the other side metabolites of Fumonisins are not known in maize, making poorly reliable then the hypothesis of covalent modification of FBs by endogenous maize enzymes. In order to check if hidden Fumonisin are already present in the raw material, we applied the indirect approach also to raw maize samples.

3.6 Analysis of raw maize

3.6.1 The first screening

The presence of hidden Fumonisins was checked also in raw maize (harvest 2007). For a first screening two samples of dry cereals were kindly provided by the farmers of Pianura Padana. The sample preparation procedure is the same previously used for food products, and reported in the experimental part.

The extraction step was repeated twice consecutively to extract as much as possible of the native (free) forms of FBs and each extract was preliminarly analysed injected in order to check the presence of hydrolysed Fumonisins before the hydrolysis. The results obtained for maize are reported in the following figures. It is possible to see that free forms of Fumonisins have been detected in both samples in high concentration, moreover seem that their high contamination so that make them unsuitable for direct consuption. Surprisingly, hidden Fumonisins seem to be present already in the raw cereal, as shown by the analysis of total FBs after hydrolysis.

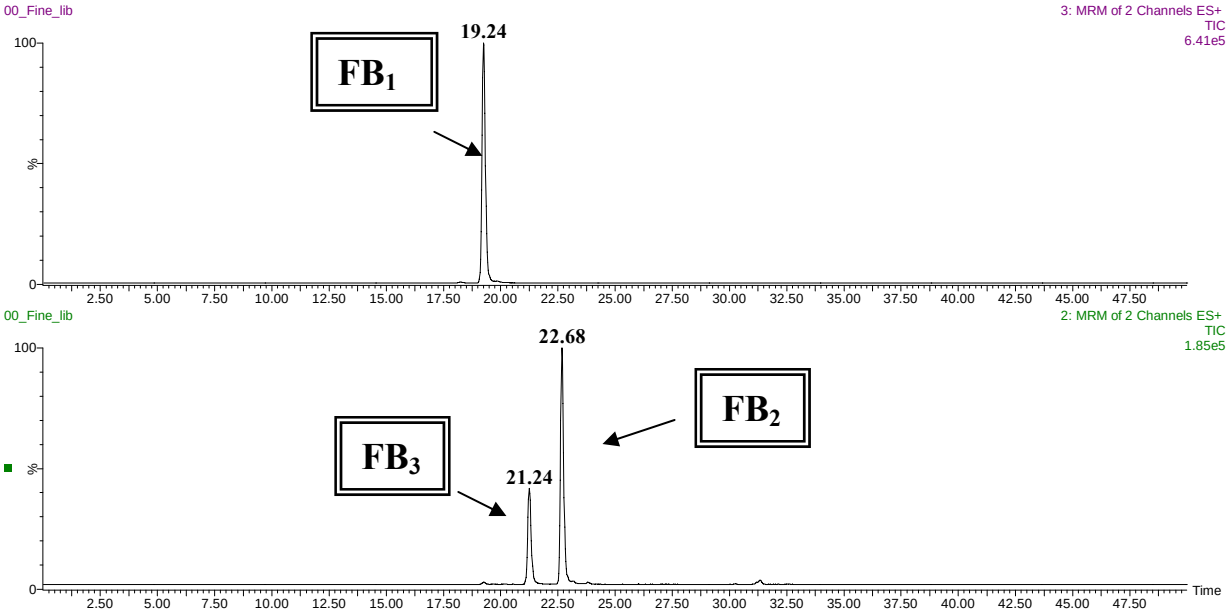


Fig. 41: MRM (TIC) chromathogram of a Fumonisin naturally contaminated sample of raw maize.

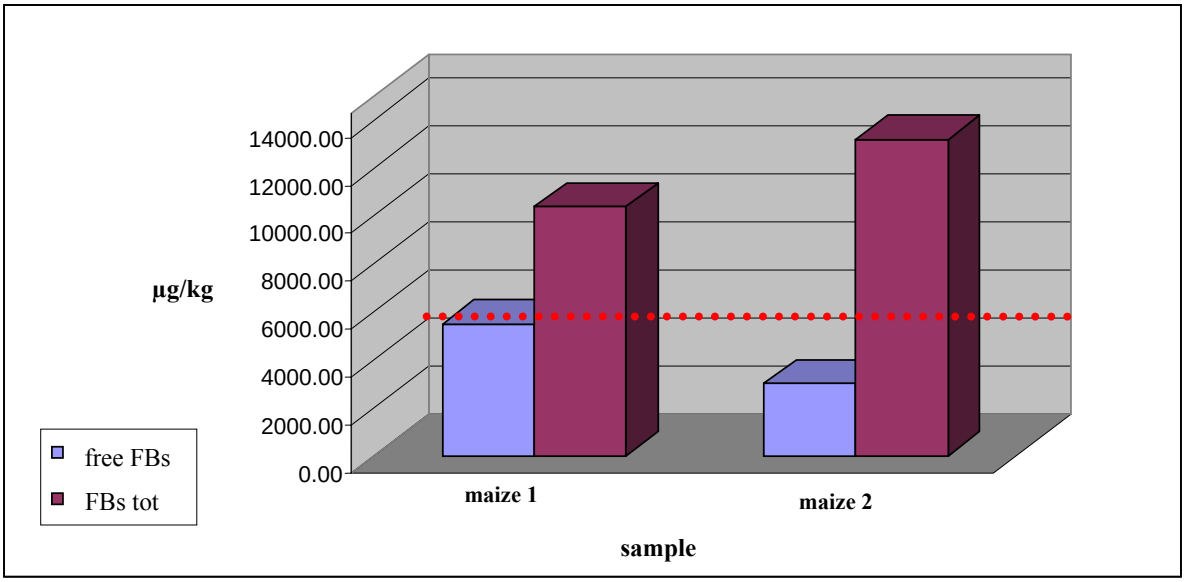


Fig. 42: Results of the analyses of raw maize samples. Graphic comparison of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3). The dotted red line represents the legal limits of FB contamination in raw maize for food industries (4000 µg/kg), except maize for direct human consumption (1000 µg/kg).

Tab. 43: Results of the preliminary screening of raw maize samples. Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3).

sample	*Free FBs (FB_1+FB_2+FB_3) I extraction (µg/kg)*	*Free FBs (FB_1+FB_2+FB_3) II extraction (µg/kg)*	*FBs tot (HFB_1+HFB_2+HFB_3) (µg/kg)*	*Hidden FBs (FBs tot - free FBs) (µg/kg)*
maize 1	5283	209	10379	4887
maize 2	2746	255	13183	10182

The results of the preliminary screening confirm the hypothesis previously advanced: hidden Fumonisins are already present in raw maize. For this reason it was decided to proceed with a more extended survey.

3.5.2 Survey on Italian raw maize

In order to get more accurate data, six other samples of raw maize, harvested in 2007 in the Pianura Padana, were collected and analysed by :

I) Double extraction of free FBs from the same sample;

II) Two sample preparation for both free FB and total FB evaluation;

III) Two injections for each preparation.

The results are reported in the following figures.

Once again it is possible to see a widespread presence of Fumonisin in all the samples collected.

All the collected samples are not suitable for human consumption, due to the high contamination, and must be assigned to feed industries.

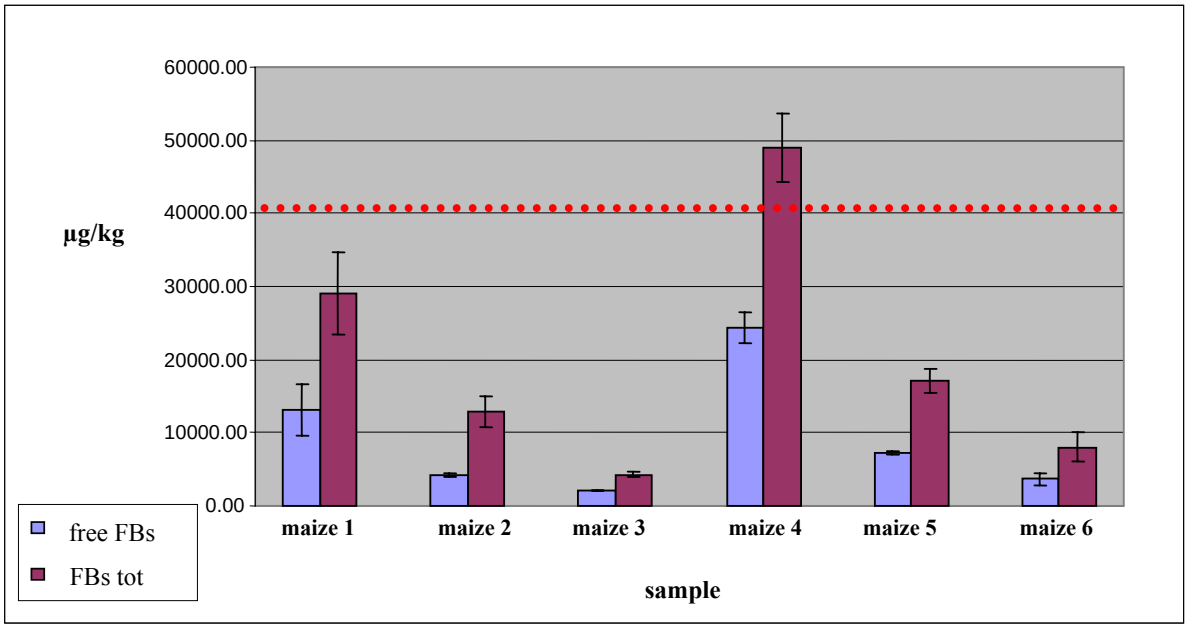


Fig. 43: Results of the survey of italian maize samples. Graphic comparison of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3). The dotted red line represents the legal limits of FB contamination in raw maize for food industries (4000 µg/kg), except maize for direct human consumption (1000 µg/kg).

Tab. 44: Results of the survey of raw maize samples. Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (4 replicates)*	*FBs tot (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD % (4 replicates)*
maize 1	13133	26	29006	20
maize 2	4227	7	12958	16
maize 3	2081	3	4246	9
maize 4	24372	8	49059	10
maize 5	7225	3	17121	9
maize 6	3653	22	8076	24

Tab. 45: Hidden Fumonisin estimation in Italian maize samples.

sample	*hidden FBs (FBs tot - free FBs)* (µg/kg)
maize 1	15874
maize 2	8731
maize 3	2165
maize 4	24688
maize 5	9897
maize 6	4423

As far as the reproducibility of the analysis of raw maize samples the standard deviation can reach high values in some cases. Problems concerning sampling and the homogeneity of the sample may be responsible.

However, the method for the indirect evaluation of hidden Fumonisins seems to shows that also in raw cereals there are some forms of FBs which are not detected by conventional methods. This fact provides new clues to understand the mechanism of FBs masking. If the previous hypothesis was based on the heat dependent reaction between carboxyl group of FBs and the hydroxyl or amino groups of the matrix components, as proteins and starch, the presence of hidden FBs also in raw maize (hidden FBs/free FBs = 1,20), is difficult to explain, altought the ratio between hidden and free forms is significantly lower than that found in maize based products.

Thus, the problem is very complex:

I) hidden Fumonisins are present in high temperature treated products;

II) hidden Fumonisins are present in low temeperure treated products;

III) hidden Fumonisins are present in raw maize (no heat treatment);

The higher ratio between hidden and free forms found in food products is consistent with the idea that there can be at least 2 mechanisms of masking, the former occuring in the field, the latter promoted by the processing temperature.

3.5.3 Survey on non european raw maize

The survey continued with the analysis of several samples of Canadian maize, kindly provided by professor Krska of IFA-Tulln (BOKU. Wien, Austria). The analysis, performed at the IFA-Tulln Department, required the preliminary development and in house validation of the LC-MS/MS method for both free and hydrolysed Fumonisins. For this second batch of sample it was decide to check also the presence of hidden Fumonisin already in the extract of free FBs. Briefly (the procedure is reported in the experimental part), the methanol/water extract obtained for the analysis of free FBs was divided in two aliquots :

I) the former was used for the normal MRM analysis of free Fumonisins (free FBs);

II) the latter was hydrolysed for the indirect evaluation of hidden FBs, by MRM analysis of HFBs (total FBs in the extract). The procedure is reported in the following figure.

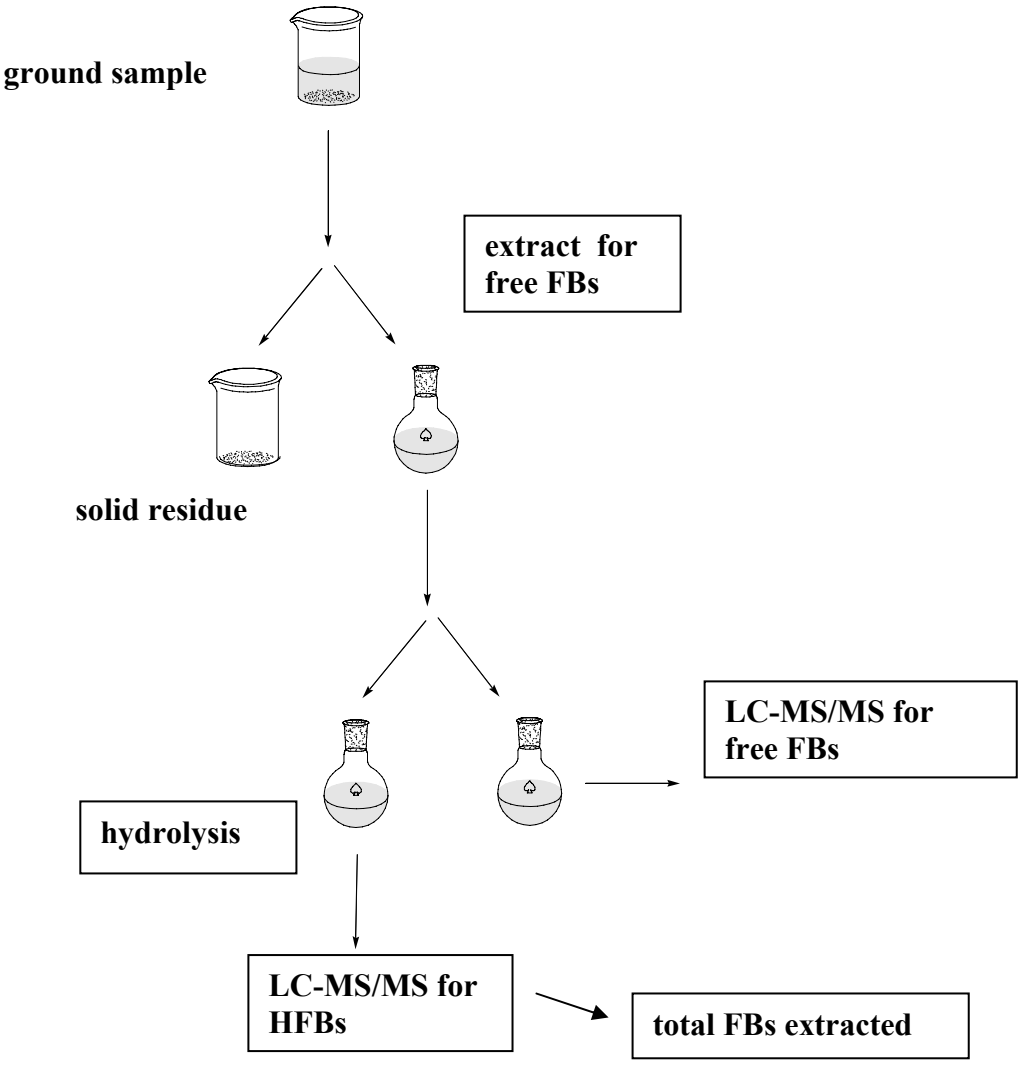


Fig. 44: Schematic procedure of the analysis of total Fumonisins in the extract for free FBs .

The presence of hidden FBs in the extract can be calculated in the same way as before, by the difference of the concentrations found by the two analyses.

The samples of maize has been prepared with the same procedure already used (see Experimental Part).

Results on Canadian maize samples are reported in the following figures;

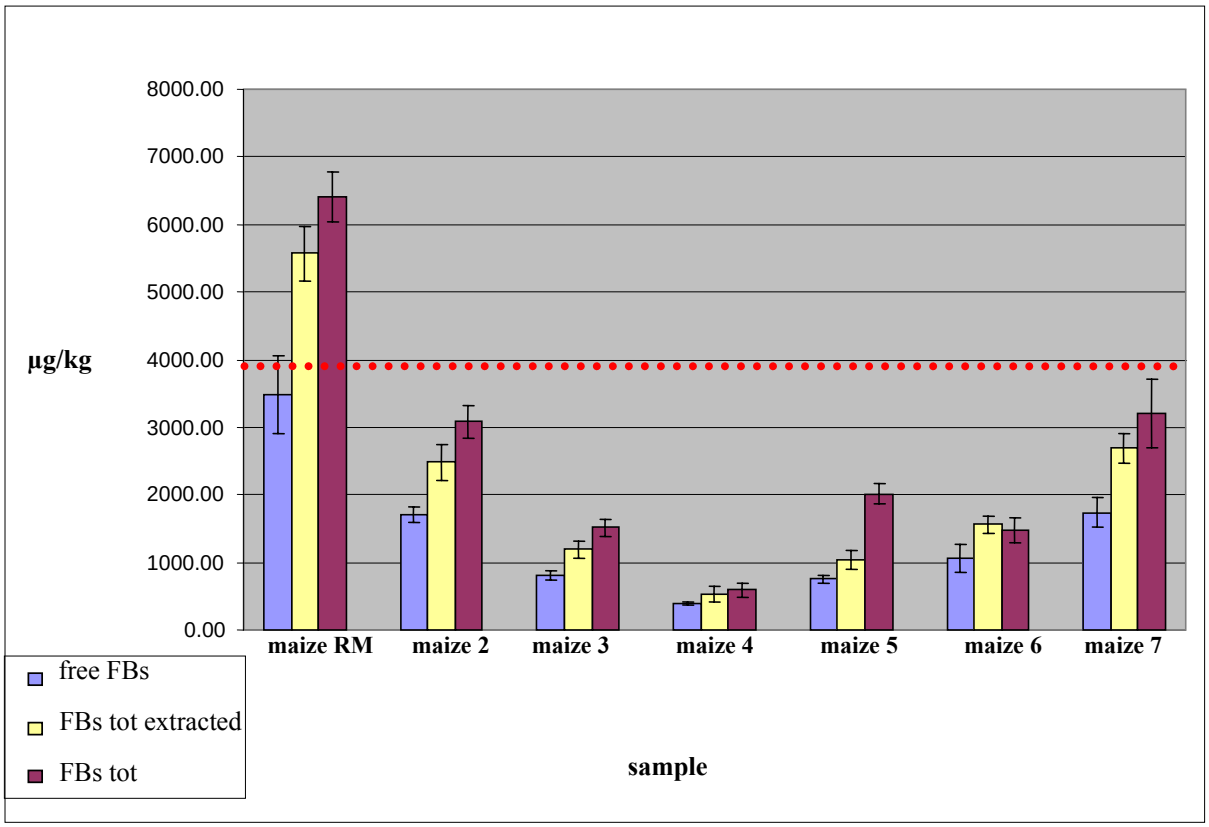


Fig. 45: Results of the survey of Canadian maize samples (maize 2-7), and a reference material (maize RM). Graphic comparison of free FBs (FB_1+FB_2+FB_3), total FBs extracted (calculated from HFB_1+HFB_2+HFB_3, after hydrolysis of the extract) and total FBs (calculated from HFB_1+HFB_2+HFB_3, after sample hydrolysis). The dotted red line represents the legal limits of FB contamination in raw maize for food industries (4000 µg/kg), except maize for direct human consumption (1000 µg/kg).

Tab. 46: Results of the survey on Canadian maize samples (maize 2-7), and a reference material (maize RM). Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (2 replicates)*	*FBs tot (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD %*) *(2 replicates)*
maize RM	3486	16	6406	6
maize 2	1701	7	3079	8
maize 3	810	8	1512	9
maize 4	388	8	590	16
maize 5	752	8	2014	8
maize 6	1059	20	1472	12
maize 7	1739	12	3196	16

Tab. 47: Results of the survey on Canadian maize samples (maize 2-7), and a reference material (maize RM). Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs in the extract (calculated from HFB_1+HFB_2+HFB_3 after extract hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (2 replicates)*	*FBs tot in the extract (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD %*) *(2 replicates)*
maize RM	3486	16	5578	7
maize 2	1701	7	2481	10
maize 3	810	8	1188	12
maize 4	388	8	536	22
maize 5	752	8	1032	14
maize 6	1059	20	1561	9
maize 7	1739	12	2686	8

Tab. 48: Hidden Fumonisin estimation (total and extracted) in Canadian maize samples (maize 2-7), and a reference material (maize RM).

sample	*total hidden FBs (FBs tot - free FBs)* (µg/kg)	*hidden FBs in the extract (FBs tot extract - free FBs)* (µg/kg)
maize RM	2920	2092
maize 2	1378	780
maize 3	702	378
maize 4	202	148
maize 5	1262	280
maize 6	413	502
maize 7	1457	947

The mean concentration found appears significantly lower than that found in Italian maize samples. The new data confirm the results obtained with the first batch of maize samples. Moreover, the hydrolysis of the extract of 'free FBs', revealed that hidden forms are co-extracted with the free forms, being already present in the first extraction altough in a hidden FB /free FB ratio lower than that found on the products. Considerations on this experimental evidence will be made to the next chapter.

The same analysis was then made on grain samples used as a reference material (calibrator) for an ELISA kit for Fumonisins B_1 analysis in raw cereals.

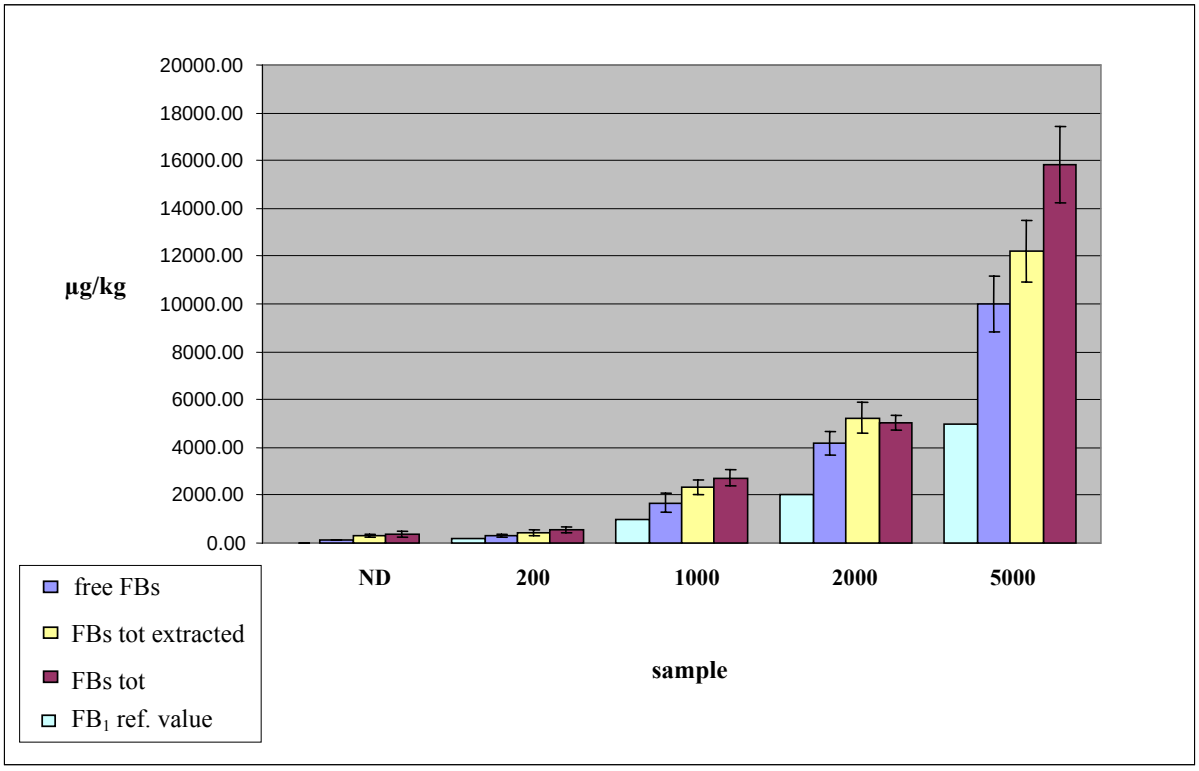


Fig. 46: Results of the analysis of an ELISA kit reference materials (calibrator). Graphic comparison of free FBs (FB_1+FB_2+FB_3), total FBs extracted (calculated from HFB_1+HFB_2+HFB_3, after hydrolysis of the extract) and total FBs (calculated from HFB_1+HFB_2+HFB_3, after sample hydrolysis). The light blue bars represent the reference value of FB_1 concentration, as µg/kg, in each point (200-5000, ND: blank for the ELISA kit).

Tab. 49: Results of the analysis of an ELISA kit reference material (calibrator). Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after sample hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (2 replicates)*	*tot FBs (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD %*) *(2 replicates)*
ND	142	7	363	30
200	310	21	544	26
1000	1680	24	2717	12
2000	4180	11	5036	6
5000	10010	12	15840	10

Tab. 50: Result of the analysis of an ELISA kit reference material (calibrator). Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs extracted (calculated from HFB_1+HFB_2+HFB_3 after extract hydrolysis).

sample	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (2 replicates)*	*FBs tot in the extract (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD %*) *(2 replicates)*
ND	142	7	324	15
200	310	21	439	30
1000	1680	24	2319	13
2000	4180	11	5236	12
5000	10010	12	12220	10

Tab. 51: Hidden Fumonisin estimation (total and extracted) of an ELISA kit reference material (calibrator).

sample	*hidden FBs* *(FBs tot - free FBs)* (µg/kg)	*hidden FBs extracted* *(FBs tot extract - free FBs)* (µg/kg)
ND	221	182
200	234	128
1000	1037	639
2000	855	1056
5000	5830	2210

Is possible to see that the LC-MS/MS is more sensitive than the ELISA kit, the ND (not detectable) sample for the ELISA test being contaminated. Moreover, our method allows to analyse also Fumonisin B_2 and B_3, while the reference value of the ELISA kit only regard FB_1. Results emerging from this survey show the presence of hidden FBs also in the reference materials for the ELISA kit.

The lack of detection of hidden Fumonisin by antibodies can be explained in several ways, further discussed in the next chapter.

3.7 Hidden Fumonisin: the non covalent hypothesis

It is useful at this point to make some considerations, on the basis of the results obtained and of what is actually know from the literature.

I) Hidden Fumonisins were first isolated as N-derivatives at the ammino group of FBs, in heat treated foods. The derivatives are known, isolated, and generally less toxic (114,115).

II) Hidden Fumonisins were also found in corn-flakes, after SDS extraction and basic hydrolysis, as HFBs derivatives. The use of SDS support the idea of protein bound derivatives of FBs.

III) The reaction of Fumonisin B_1 with protected lysine and glucose, after heating, was proved *in vitro*.

From these experimental data the presence of TCA-derivatives is indirectly proved, as a consequence of the reaction of TCA chains of FBs with food matrix components, especially with the protein fraction, in treated maize based foods.

In this work, by application of the newly developed method, it was observed that:

1) Hidden Fumonisins are present in maize based food as corn-flakes, bread substitutives and snacks, in agreement with the previous hypothesis.

2) Hidden Fumonisin are present in maize pasta. This does not agree with the previous hypothesis because the significantly lower temperature involved in pasta production, compared with other processes, was expected to induce a lower incidence of masking. Indeed our results show a hidden / free FBs ratio of the same order of magnitude as observed for high temperature treated foods.

3) Hidden Fumonisins are present in raw maize. This fact seem to account for a reaction occuring at room temperature. However the hidden / free FBS ratio is significantly lower.

4) Hidden Fumonisins seem to be present also in reference materials for ELISA kits. This means that antibodies can't detect them. So, either the structures of FBs have been modified by endogenous enzymes in the plant or are masked by non-covalent inteactions with the matrix components.

The hypothesis of heat-dependent Fumonisin masking seem not to be able to explain all the new experimental data. The presence of hidden FBs also in raw maize could require a mechanism active already at room temperaure. For this reason a non covalent binding mechanism is a better candidate than the covalent bond formation, to explain the presence of hidden Fumonisins.

Moreover, the significantly different ratios of hidden / free FBs in maize based products and in the raw cereals seem to be consistent with the new hypothesis. The non covalent mechanism of FB masking should occur already present in the plants and in the cereal, while the covalent modifications, occuring during the heating process, can explain the higher ratios hidden / free FBs found in products.

In order to explain this possibility we investigated the non covalent hypothesis in the raw cereal. Cereals are complex matrix where several compound, or classes of compounds, such as polysaccharides, proteins and lipids, are potentially candidates for a non covalent interaction with Fumonisin.

In particular, the most used maize fraction for food production is the maize endosperm.

Maize endosperm is composed almost totally of starch and proteins: on account of their chemical properties, we decided to preliminary test proteins in order to understand the masking mechanism of Fumonisins

3.8 Hidden Fumonisins: understanding the masking mechanism

3.8.1 Association with maize protein

Proteins were the best candidates to be studied to prove the "non-covalent binding" hypothesis, being easily separated on the basis of their solubility.

The separation of cereal proteins on the base of their relative solubility in different solvents, the so called 'Osborne fraction', is made by consecutively extracting the sample with different solvents in order to separate selectively one protein class from others.

Accordingly four fractions were obtained as reported in the following table.

Tab. 52 :Osborne fractions, and solvent mixture used in this work.

Fraction	*Solvent*
globulins	**Bidistilled Water**
albumins	**NaCl 0.2 M**
prolammines	**Ethanol/Water 60:40**
glutelins	**Isopropanol/Water 50:50 + 2% DTT**

On each fraction the presence of hidden Fumonisins was checked, by indirect evaluation; then also the solid residue of the extraction was analysed after hydrolysis. The residue of a maize sample, after the Osborne fraction extraction, still contains polysaccharides and lipids, which can also interact with FBs.

The schematic procedure of sample preparation is reported in the next figure, while details are reported the Experimental Part.

The presence of hidden Fumonisins in maize Osborne fraction are reported in following figures.

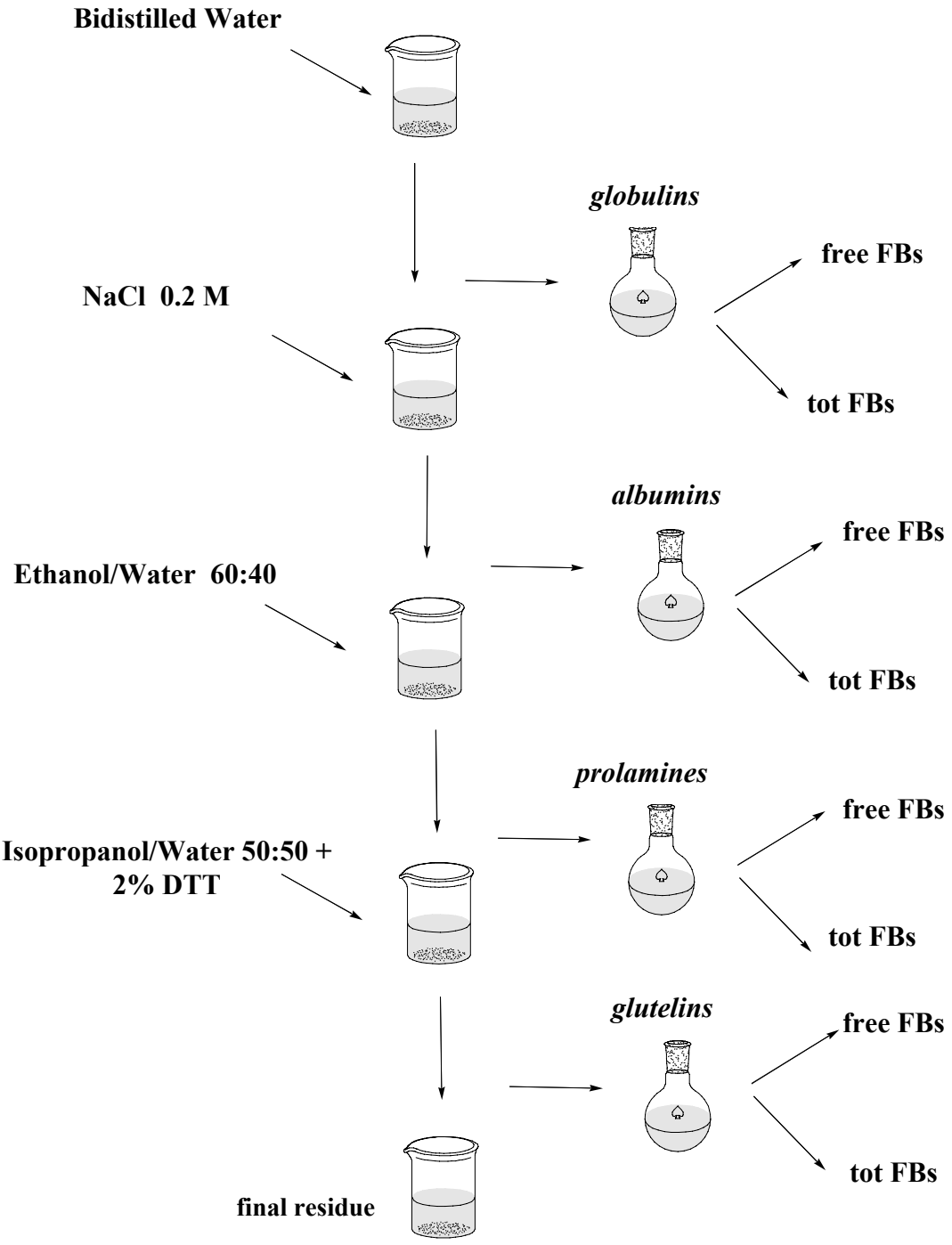


Fig. 47: Schematic procedure for Osborne fractions extraction used in this work.

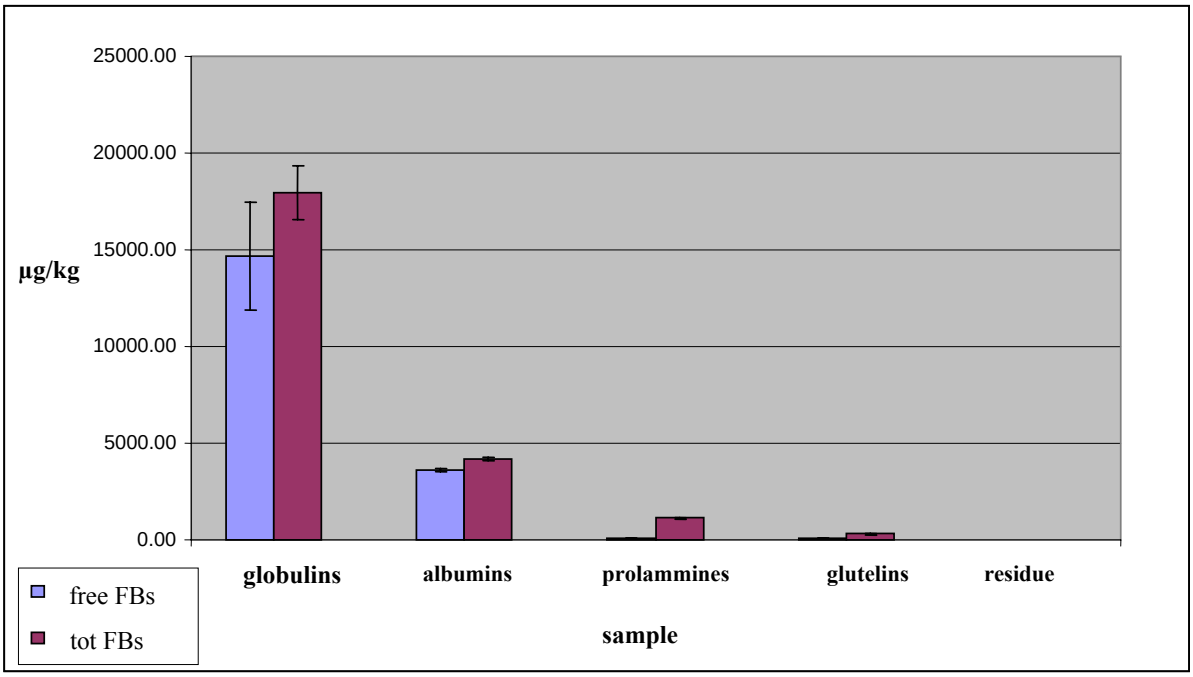


Fig. 48: Results of the survey on the Osborne fractions of maize sample A and the solid residues of the extractions. Graphical comparison of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after fraction hydrolysis).

Tab. 53: Results of the survey on Osborne fractions of maize sample A and the solid residues of the extraction. Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after hydrolysis).

fraction	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (2 replicates)*	*FBs tot (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD %) (2 replicates)*
globulins	14695	19	17923	8
albumins	3604	3	4208	2
prolammines	91	5	1119	3
glutelins	45	9	288	6
solid residue	< LOD	0.00	< LOD	N.D.

Tab. 54: Hidden Fumonisin evaluation on Osborne fractions of maize sample A and the solid residues of the extractions.

fraction	*hidden FBs (FBs tot - free FBs)* (µg/kg)	*hidden FBs / free FBs*
globulins	3228	0.22
albumins	604	0.17
prolamines	1029	11.35
glutelins	243	5.34
solid residue	N.D.	0

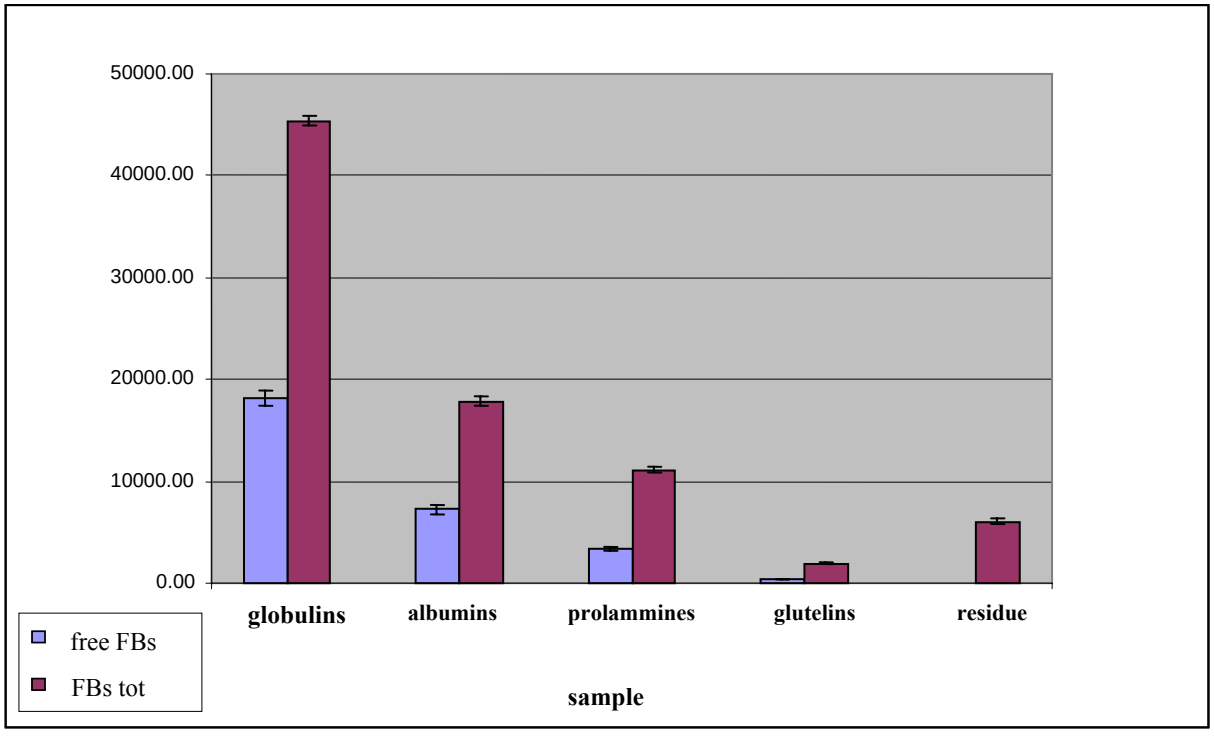


Fig. 49: Results of the survey on Osborne fractions of maize sample B and the solid residues of the extraction . Graphic comparison of free FBs (FB_1+FB_2+FB_3, blue bar) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after hydrolysis, purple bar).

Tab. 49: Result of the survey on Osborne fractions of maize sample B and the solid residues of the extractions. Concentration of free FBs (FB_1+FB_2+FB_3) and total FBs (calculated from HFB_1+HFB_2+HFB_3 after hydrolysis).

fraction	*Free FBs (FB_1+FB_2+FB_3)* (µg/kg)	*RSD % (2 replicates)*	*FBs tot (HFB_1+HFB_2+HFB_3)* (µg/kg)	*RSD %) (2 replicates)*
globulins	18243	4	45338	1
albumins	7281	6	17824	2
prolammines	3422	4	11122	2
glutelins	361	10	1959	3
solid residue	< LOD	0	6060	4

Tab. 56: Hidden Fumonisin evaluaion on Osborne fractions of maize sample B and the solid residues of the extractions.

fraction	*hidden FBs (FBs tot - free FBs)* (µg/kg)	*hidden FBs / free FBs*
globulins	27095	1.49
albumins	10542	1.45
prolammines	7699	2.25
glutelins	1598	4.43
solid residue	6060	0

Tab. 57: Mean hidden FBs / free FBs ratios found in Osborne fractions of maize samples.

sample	*mean found hidden/free FB between samples*
globulins	0.85
albumins	0.81
prolamines	6.80
glutelins	4.88

By considering the amounts found in the diffrent extractions, it appears that hidden Fumonisin seem to be distributed in all the Osborne fraction analysed; moreover hidden FBs seem still present in the final solid residue in one sample.

Althought the amounts of hidden FBs are higher in globulins and albumins, the relative amounts of hidden to total FBs is higher in prolamines and glutelins.

These classes are present almost exclusively in the endosperm being the maize storage proteins. The endosperm fraction of maize is used in the food industries and so prolamines and glutelins seem to be the protein classes more involved in FB masking.

The presence of hidden FBs in the raw cereal and in low temperature treated products can be explained as endogenous to the plant without involving the mechanism supported by heating dependent covalent reaction of Fumonisin TCA chains. The presence of hidden FBs in the final solid residue can imply interactions with other matrix contituents.

Zeins and in particular α-Zein, are group well known maize proteins, belonging to the prolamines class. The α-Zein protein is the most abundant protein, representing almost the 60 % of the maize protein content. Several reasons suggested to use α-Zein as a model protein to investigate the hypothesis of Fumonisin non covalent masking mechanism.

3.8.2 The α-Zein model

The α-Zein protein was chosen for several reasons. It's the most abundant maize protein and seems to be involved, together with the glutelins, in the Fumonisin masking mechanism. Moreover, it is commercially available as standard and this is a not negligible fact, since the availability of a standard for the protein chosen as model can facilitate the study.

The standard is sold with the claim 'alcohol soluble mixture of two polypeptides'. The prolamines of cereal are soluble in aqueous alcohol, and the Osborne fraction is obtained by Ethanol/Water 60:40 mixture. This solvent mixture is similar to that used for free FB extraction, except for the type of alcohol, ethanol instead of methanol. Moreover from the previous data hidden form seem to be already present in the extract of free FBs.

Free Fumonisins, hidden Fumonisins and maize prolamines seems to have the same solubility in quite similar conditions. The α-Zein protein should be the best candidate also in the case of covalent reactions, being the most abundant protein in maize.

The UPLC-MS method used for experiments with α-Zein was adapted from past works of the group on peptides (128). Briefly, the separation of α-Zein is performed on Acquity system (Waters Inc.) with a C18 column, using a gradient elution with water and acetonitrile both acidified with 0.2% of formic acid.

Detection was performed with a single quadrupole mass spectrometer operating in scan mode from 200 to 2000 m/z ratio. The α-Zein prolamine is a large and low polar storage protein of maize, thus it is expected to have high retention time. Moreover, the low polarity can create problems to the electrospray ionisation of analytes, decreasing the sensitivity of the method. The mass spectrometry detection of α-Zein was then optimised by trial and error method. Accurate protein mass measurement, sufficient for protein sequence identification, can't be achieved on a quadrupole instrument, due to the low resolution power of the instrument. Moreover, proteins usually produce multicharged profiles on ESI interfaces, so that the molecular weight must be deconvoluted by means of a software.

However, a sufficient level of accuracy in protein identification can be achieved by measuring at least two attribute of the protein. The former, the average molecular mass of native α-Zein, was evaluated on the standard directly dissolved in methanol and injected in the UPLC.

The chromatograms of the α-Zein standard are reported in the following figures. The α-Zein peak seems to be composed by different peaks, not totally resolved. However more then two polypeptides, as reported from the seller, seem to be present. It must be said that probably the

seller refers to the presence of 2 polypeptides in SDS-PAGE, where conditions are totally different. Moreover the purity of the standard is not declared.

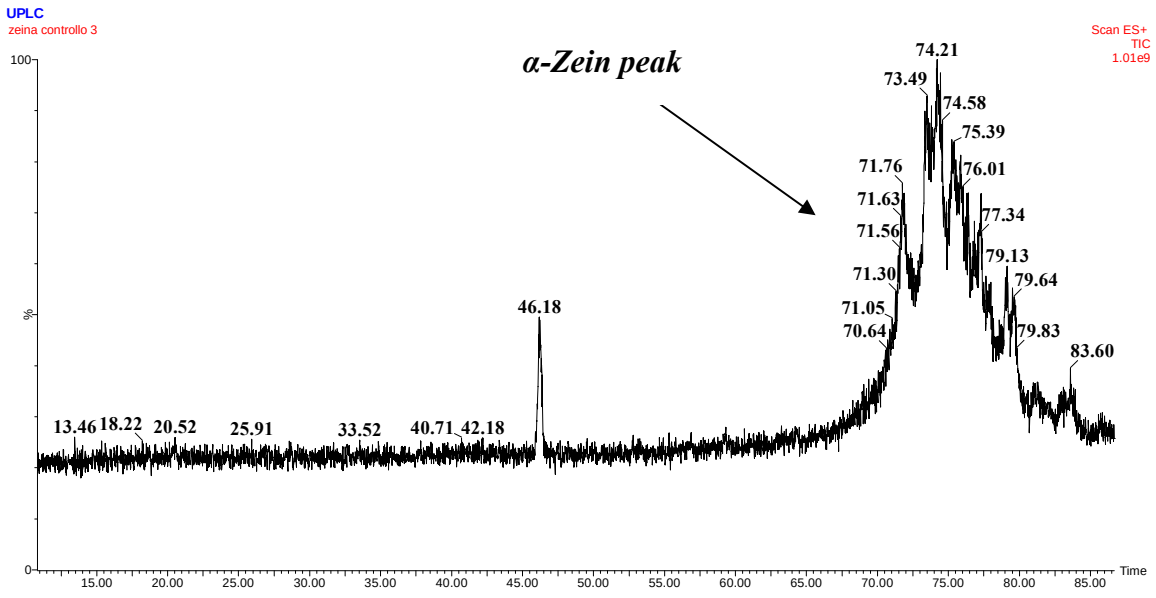


Fig. 50: Zoom of the α-Zein peak in the UPLC-MS chromatogram of a 500 ppm Zein standard in MeOH, 5 µl injection. MS scan (TIC) of the 200-2000 m/z range .

The mass spectra corresponding to the different parts of the α-Zein chromatographic peak were separately analyzed. In this way it was possible to evaluate the different isoforms present in the standard, and their masses can be compared with those reported in the literature.

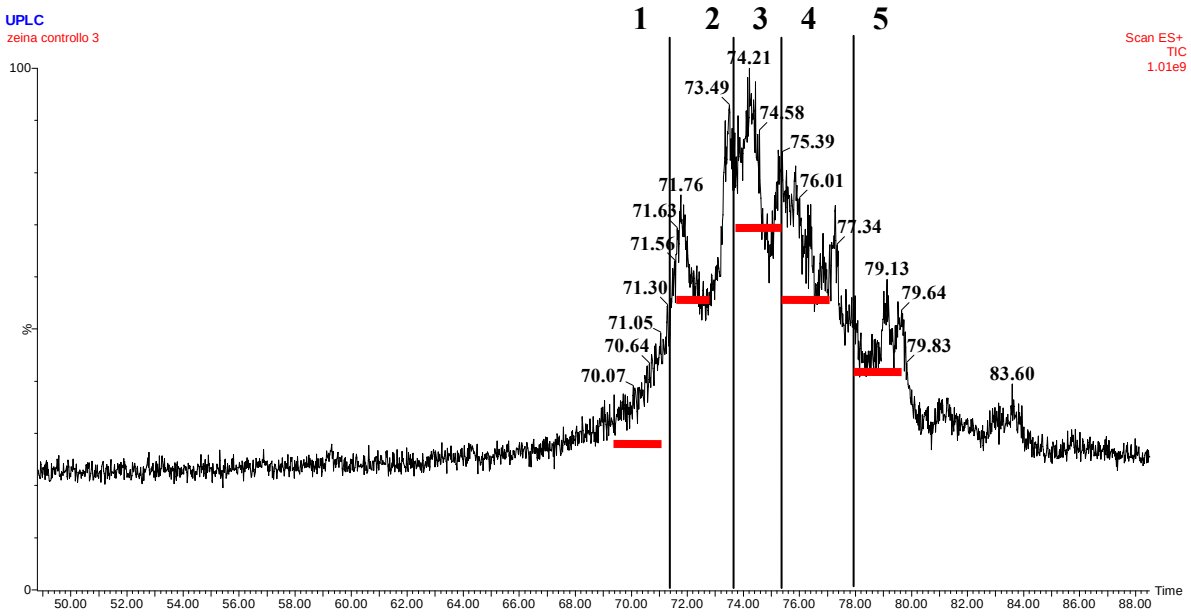


Fig. 51: Zoom of the α-Zein peak in the UPLC-MS chromatogram of a 500 ppm Zein standard in MeOH, 5 µl injection. Peak division in time period (1-5); the red trait show the zone chosen for mass spectrum analysis.

Analysis of the mass spectra in the different part of the α-Zein peak is reported in the following figures, the observed molecular masses are reported in Tab. 38.

Zone 1

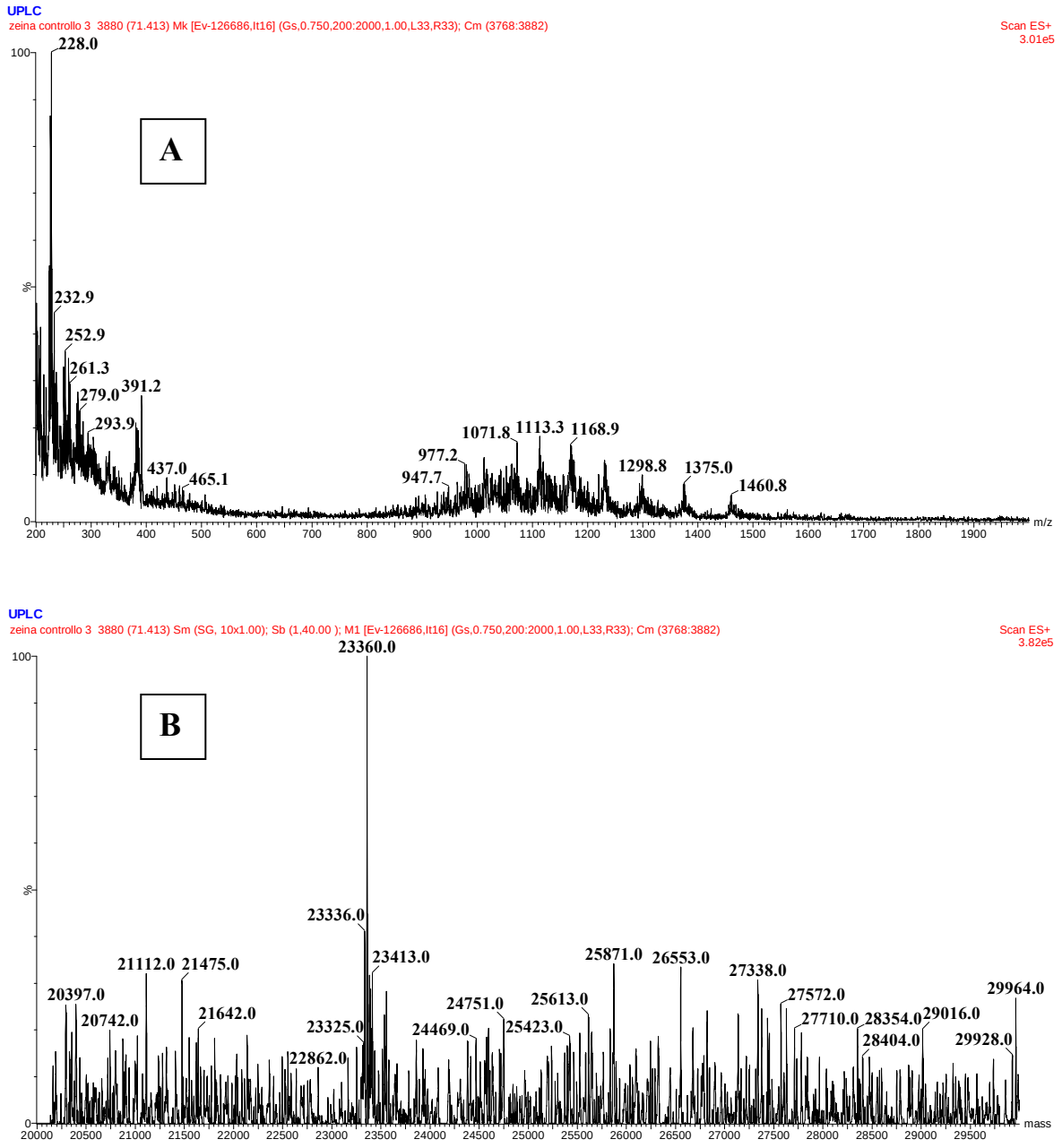


Fig. 52: Mass spectrum analysis of Zone 1 of the α-Zein peak. A: multicharged spectra, B: deconvoluted spectra of Zone 1, showing one predominant mass.

Zone 2

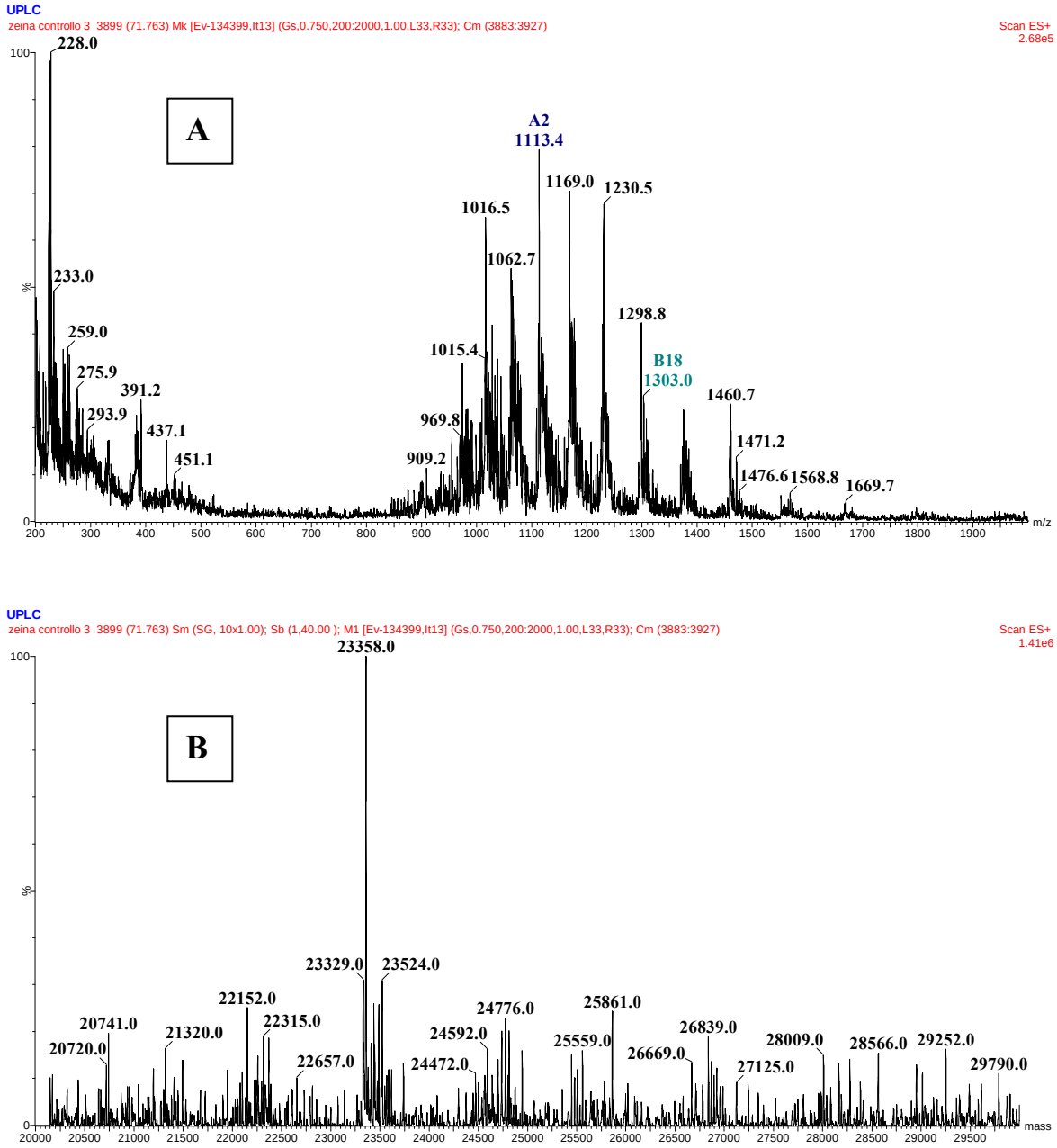


Fig. 53: Mass spectrum analysis of Zone 2 of the α-Zein peak. A: multicharged spectra, B: deconvoluted spectra of Zone 2, showing one predominant mass.

Zone 3

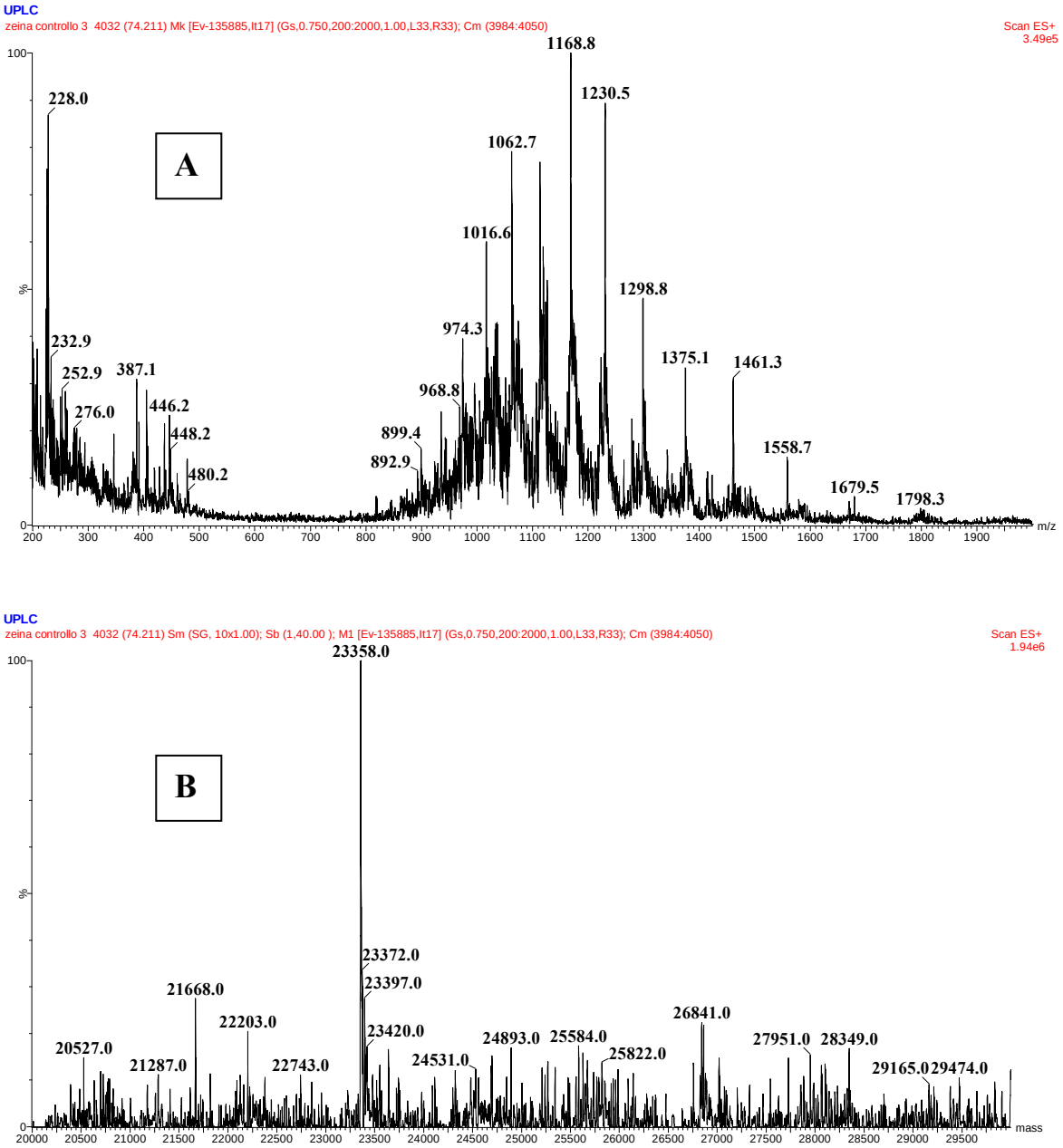


Fig. 54: Mass spectrum analysis of Zone 3 of the α-Zein peak. A: multicharged spectra, B: deconvoluted spectra of Zone 3, showing one predominant mass

Zone 4

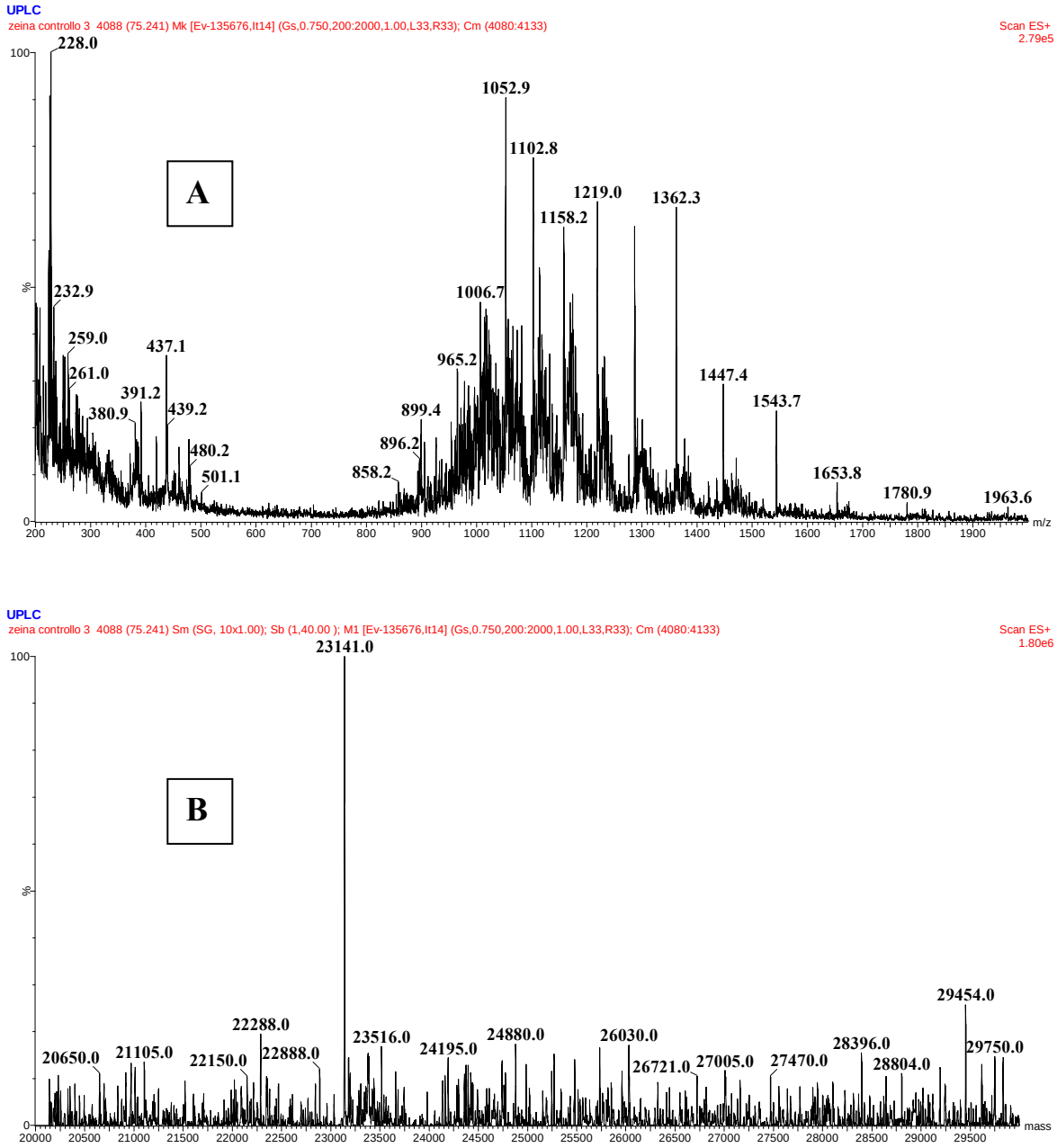


Fig. 55: Mass spectrum analysis of zone 4 of the α-Zein peak. A: multicharged spectra, B: deconvoluted spectra of Zone 4, showing one predominant mass.

Zone 5

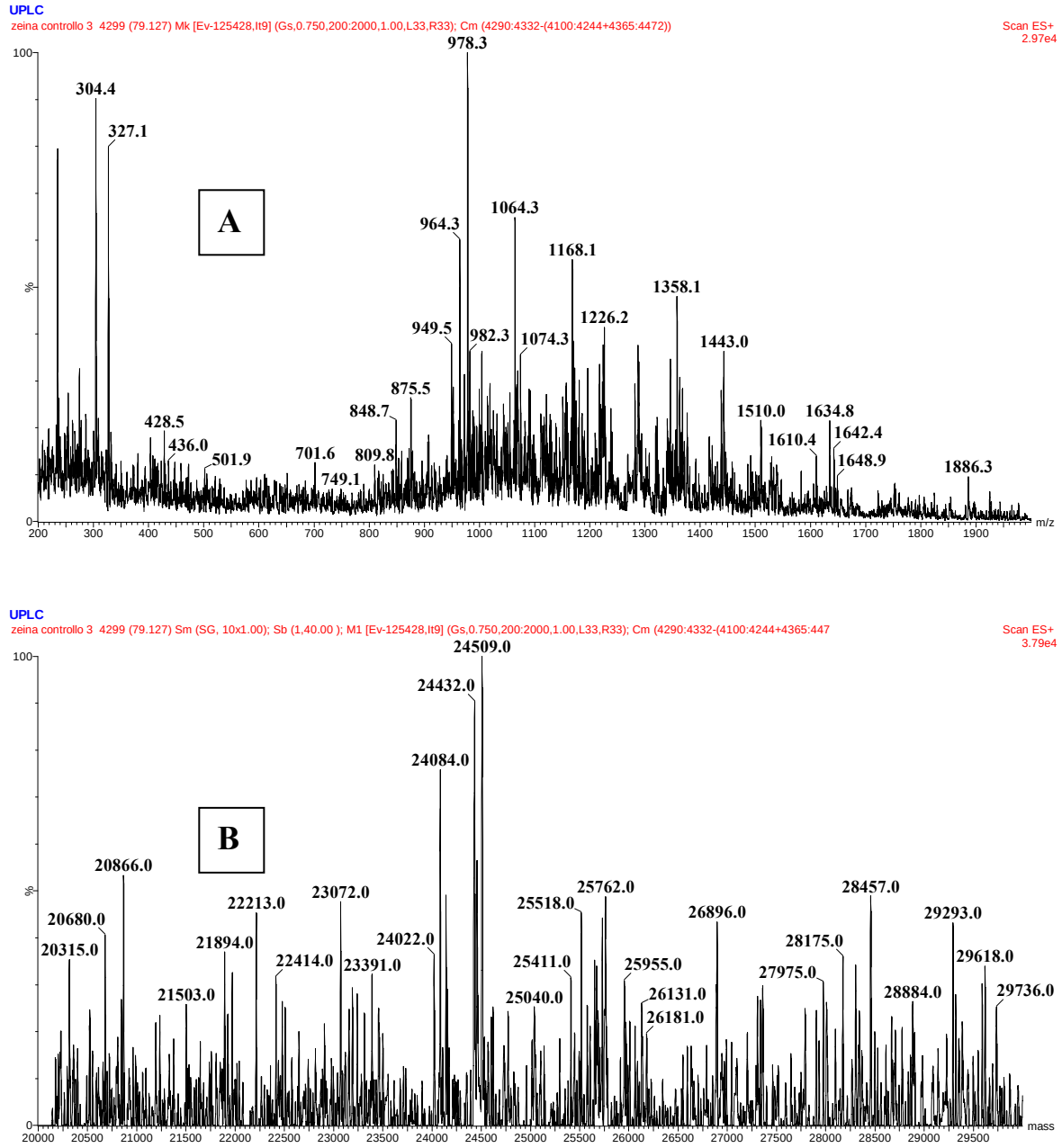


Fig. 56: Mass spectrum analysis of zone 5 of α-Zein peak. A: multicharged spectra, B: deconvoluted spectra of Zone 5, showing 3 predominant mass.

Tab. 58: Observed molecular masses on the different zone of α-Zein peak.

α-Zein peak	*Observed molecular masses*
zone 1	23360 Da
zone 2	23358 Da
zone 3	23358 Da
zone 4	23141 Da
zone 5	24084, 24432, 24509 Da

Although the masses obtained in this way were not accurate, it was possible to compare them with the masses of the α-Zein isoforms present in the literature, in partiuclar those reported by the work by Adams et al. (2004) on MALDI-TOF mass measurement of α-Zein .

Tab. 59: Observed Zein molecular masses in the cited paper by Adams et al. (2004). Dotted red box shows the masses which are in agreement with those observed in the Fluka α-Zein standard.

name (abbreviation)[a]	GenBank accession no.	mol wt (calcd)	no. of cysteines in mature protein	mol wt of underivatized zeins observed in MS	mol wt of derivatized zeins observed in MS
10-kDa δ-zein (dz10)	AF371266	14431	5	14432	14715
15-kDa β-zein (bz15)	AF371264	17458	7	17125	17492
16-kDa γ-zein (gz16)	AF371262	17663	12	17714	18393
18-kDa δ-zein (dz18)[b]	AF371265	21220	3		
27-kDa γ-zein (gz27)	AF371261	21822	15	21793	22581
19-kDa α-zein B1 (az19B1)	AF371269	23359	2	23318	23428
19-kDa α-zein B3 (az19B3)	AF371271	24087	2	24069	24180
19-kDa α-zein D2 (az19D2)	AF371268	24706	1	24515	24564
19-kDa α-zein D1 (az19D1)	AF371267	24818	1	24644	24699
22-kDa α-zein Z1 (az22z1)	AF371274	26359	1	26308	26365
22-kDa α-zein Z5 (az22z5)[b]	AF371277	26701	1		
22-kDa α-zein Z3 (az22z3)	AF371275	26751	1	26741	26794
22-kDa α-zein Z4 (az22z4)[b]	AF371276	26923	1		
19-kDa α-zein B2 (az19B2)[b]	AF371270	27128	2		

Comparison of α-Zein masses, by different sources, is reported in the following table.

Tab. 60: Comparison of α-Zein molecular masses.

MALDI-TOF masses (from Adams)	*Observed molecular masses (α-Zein standard)*	*Calculated From genomic sequence*	*GenBank accession number*
23318 Da	23358-23360 Da	23359 Da	AF371269
24069 Da	24084 Da	24087 Da	AF371271
24515 Da	24509 Da	24706 Da	AF371268

The identified protein sequences were retrieved from Expasy data bank, which have been deduced from the corresponding coding DNA. The signal peptide, a sort of tag able to 'drive' the protein in cellular location where it is needed, present in the databank sequences, was removed from the retrieved sequences. by SignalP (SwissProt) analysis, a software which calculates the probability of cleavage for the signal peptide present in the polipeptidic chain. In fact, the α-Zein standard, as well as that extracted from maize and products, is the mature form of the protein, lacking then of the signal peptide

GenBank accession number: AF371269
TIFPQCSQAPIASLLPPYLSPAVSSVCENPILQPYRIQQAIAAGILPLSPLFLQQSSALLQQLPLVHLL
AQNIRAQQLQQLVLANLAAYSQQQQFLPFNQLAALNSASYLQQQQLPFSQLSAAYPQQFLPFNQ
LTALNSPAYLQQQQLLPFSQLAGVSPATFLTQPQLLPFYQHAAPNAGTLLQLQQLLPFNQLALTN
PTAFYQQPIIGGALF

GenBank accession number: AF371271
TIFPQCSQAPIASLLPPYLPSIIASICENPALQPYRLQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR
AQQLQQLVLPLINQVALANLSPYSQQQQFLPFNQLSTLNPAAYLQQQLLPFSQLATAYSQQQQLL
PFNQLAALNPAAYLQQQILLPFSQLAAANRASFLTQQQLLPFYQQFAANPATLLQLQQLLPFVQL
ALTDPAASYQQHIIGGALF

GenBank accession number: AF371268
TIIPQCSQQYLSPVTAARFEYPTIQSYRLQEAIAASILRSLALTVQQPYALLQQPSLMNLYLQRIAA
QQLQQQLLPIINQVVAANLAAYLQQQQFLPFNQLAGVNPAAYLQAQQLLPFNQLVRSPAAFLLQ
QQLLPFHLQVVANIAAFLQQQQQLLPFYPQVVGNINAFLQQQQLLPFYPQDVANNVAFLQQQQL
LPFNQLALTNPTTLLQQPTIGGAIF

Fig. 57 : Retrieved sequences of mature α-Zein (without the signal peptide) .

The poor accuracy of the mass measured by quadrupole did not allow to be sure that the sequences retrieved on Expasy correspond strictly to the masses observes in the α-Zein standard. A sufficient level of certainty can be achieved by finding other matching properties between sequences and standard. It was thought then to compare the peptides derived from a chymotryptic digestion of the standard with those obtained by the software simulated chymotryptic digestion of the retrieved sequences of α-Zein.

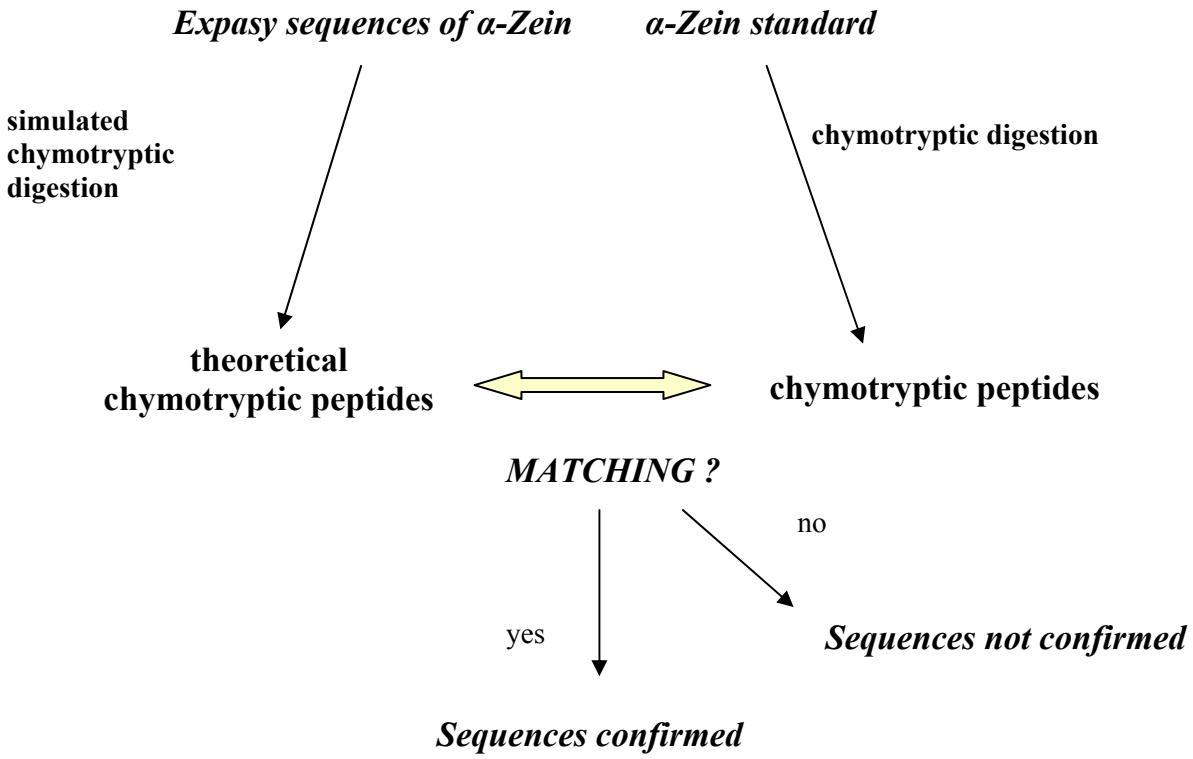


Fig. 58: Process used for confirming the α-Zein sequences.

The simulated digestion was performed by BioLynx (Waters Inc.). This software needs as inputs the sequences, the proteases, and some parameters concerning the ionisation process of the peptides, and the action of the enzyme. Conditions used for simulated digestion are reported in the Experimental Part.

An example of the software output, for a α-Zein sequence, is reported in the following table. The software provide the masses of each potential peptides released from chymotrypsin action on the given sequence. It is also possible to choose the number of missed cleavage sites, and the charge status of the released peptides. All the sequences have been virtually digested with the BioLynx (Waters Inc.) software.

Tab. 61: BioLynx (Waters Inc.) software output for the AF371269 sequence. Simulated chymotryptic digestion with one missed cleavage site. Columns on the right represent the masses for different charge status of the released peptides.

Untitled
Chymotrypsin:/Y-\P /F-\P /W-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]
Y1	1-18	(-)TIFPQCSQAPIASLLPPY(L)	1945.00	1946.01	973.51	649.34	487.26	390.01
Y2	19-35	(Y)LSPAVSSVCENPILQPY(R)	1815.91	1816.92	908.96	606.31	454.98	364.19
Y3	36-52	(Y)RIQQAIAAGILPLSPLF(L)	1807.07	1808.08	904.54	603.37	452.78	362.42
Y4	53-89	(F)LQQSSALLQQLPLVHLLAQNIRAQQLQQLVLANLAAY(S)	4109.83	4110.84	2055.92	1370.95	1028.47	822.97
Y5	90-95	(Y)SQQQQF(L)	764.35	765.35	383.18	255.79	192.09	153.88
Y6	96-98	(F)LPF(N)	375.22	376.22	188.62	126.08	94.81	76.05
Y7	99-109	(F)NQLAALNSASY(L)	1150.56	1151.57	576.29	384.53	288.65	231.12
Y8	110-117	(Y)LQQQQLPF(S)	1000.53	1001.54	501.27	334.52	251.14	201.11
Y9	118-128	(F)SQLSAAYPQQF(L)	1238.59	1239.60	620.30	413.87	310.66	248.73
Y10	129-131	(F)LPF(N)	375.22	376.22	188.62	126.08	94.81	76.05
Y11	132-142	(F)NQLTALNSPAY(L)	1190.59	1191.60	596.30	397.87	298.66	239.13
Y12	143-151	(Y)LQQQQLLPF(S)	1113.62	1114.63	557.82	372.21	279.41	223.73
Y13	152-162	(F)SQLAGVSPATF(L)	1076.55	1077.56	539.28	359.86	270.15	216.32
Y14	163-171	(F)LTQPQLLPF(Y)	1055.60	1056.61	528.81	352.88	264.91	212.13
Y15	172-172	(F)Y(Q)	181.07	182.08	91.54	61.37	46.28	37.22
Y16	173-191	(Y)QHAAPNAGTLLQLQQLLPF(N)	2060.38	2061.39	1031.20	687.80	516.10	413.08
Y17	192-202	(F)NQLALTNPTAF(Y)	1188.61	1189.62	595.31	397.21	298.16	238.73
Y18	203-203	(F)Y(Q)	181.07	182.08	91.54	61.37	46.28	37.22
Y19	204-213	(Y)QQPIIGGALF(-)	1042.58	1043.59	522.30	348.53	261.65	209.52
Y1-2	1-35	(-)TIFPQCSQAPIASLLPPYLSPAVSSVCENPILQPY(R)	3745.38	3746.38	1873.70	1249.47	937.35	750.08
Y2-3	19-52	(Y)LSPAVSSVCENPILQPYRIQQAIAAGILPLSPLF(L)	3607.28	3608.28	1804.65	1203.43	902.83	722.46
Y3-4	36-89	(Y)RIQQAIAAGILPLSPLFLQQSSALLQQLPLVHLLAQNIRAQQLQQLVLANLAAY(S)	5900.01	5901.02	2951.01	1967.68	1476.01	1181.01
Y4-5	53-95	(F)LQQSSALLQQLPLVHLLAQNIRAQQLQQLVLANLAAYSQQQQF(L)	4856.61	4857.62	2429.31	1619.88	1215.16	972.33
Y5-6	90-98	(Y)SQQQQFLPF(N)	1121.55	1122.56	561.78	374.86	281.40	225.32
Y6-7	96-109	(F)LPFNQLAALNSASY(L)	1507.77	1508.77	754.89	503.60	377.95	302.56

The α-Zein standard was digest by chymotrypsin (from Sigma), using the protocol provided with the enzymes, reported in the Experimental Part. The digestion products were analysed by the UPLC-MS method, previously used for the native α-Zein standard.

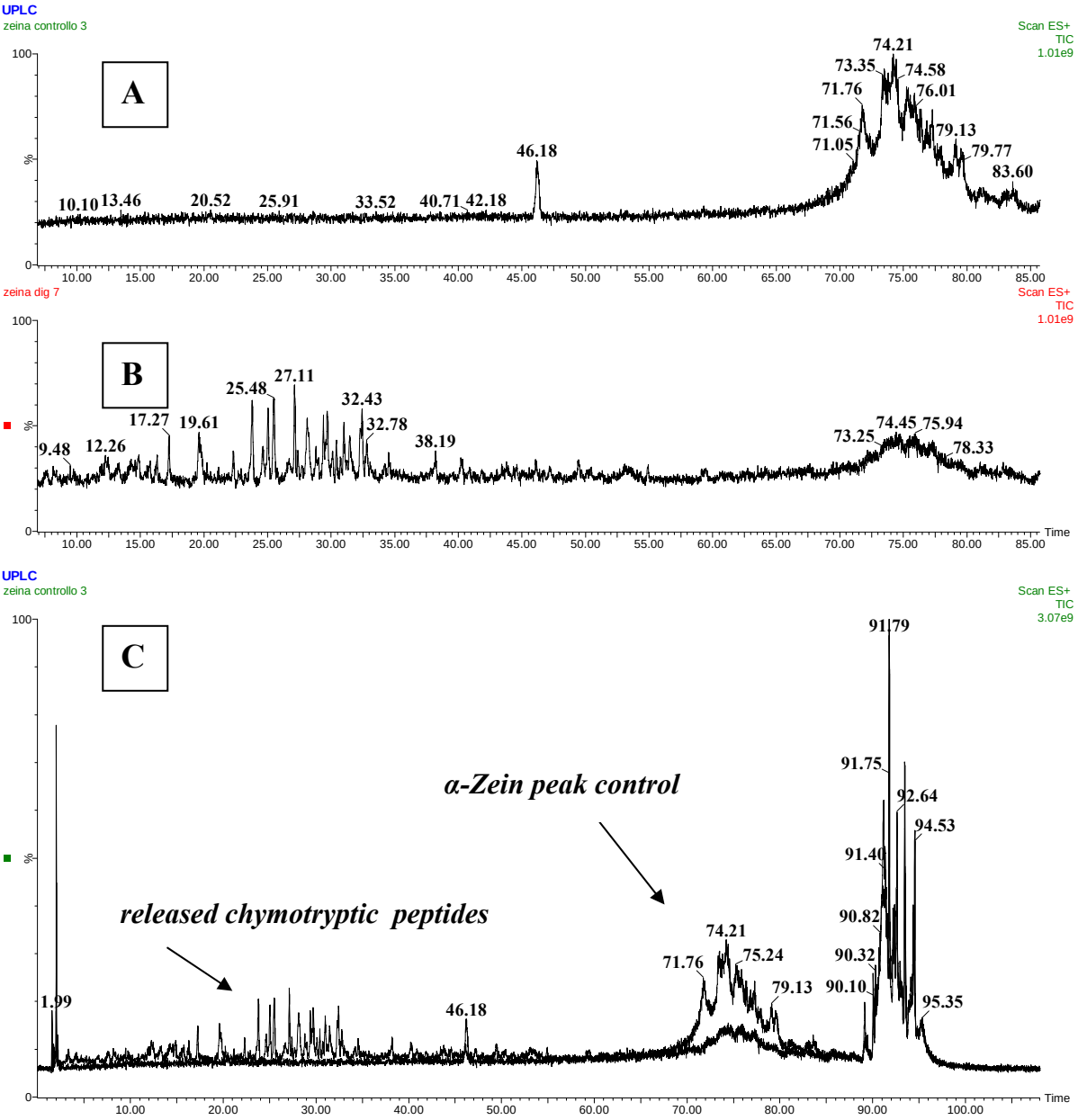


Fig. 59: UPLC-MS chromatograms of the α-Zein standard digestion. A: α-Zein peak zoom in the control experiment, B: α-Zein peak disappearing in the digestion experiment, C: Overlaid chromatograms.

In the following figures the UPLC-MS chromatograms of α-Zein standard digested by chymotrypsin are reported. It is possible to see that after the protease treatment the α-Zein peaks disappear, while several peaks, reasonably released from Zein digestion, appear at a lower retention time. The peaks were then checked for matching with the masses found by the virtually digestion. Example of the process used for peak matching is reported in the next figures. An enlarged portion of the chromatogram is reported in the following figure.

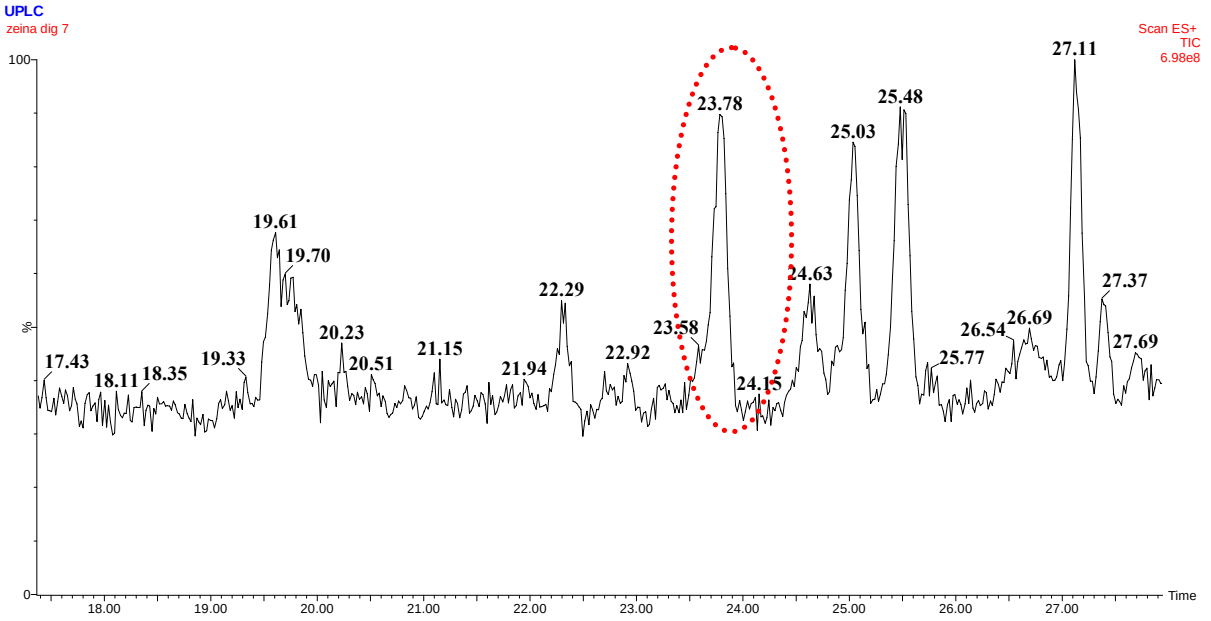


Fig. 60: UPLC-MS chromatogram of the α-Zein standard digestion. Zoom on peptide peaks. Selection of the peak at a retention time 23.78 min.

The mass spectrum, of the selected peak at 23.78 min shows that the molecular ion of the unknown peptide is the 1122.6 m/z ion, while the 562.0 m/z is its doubly charged ion.

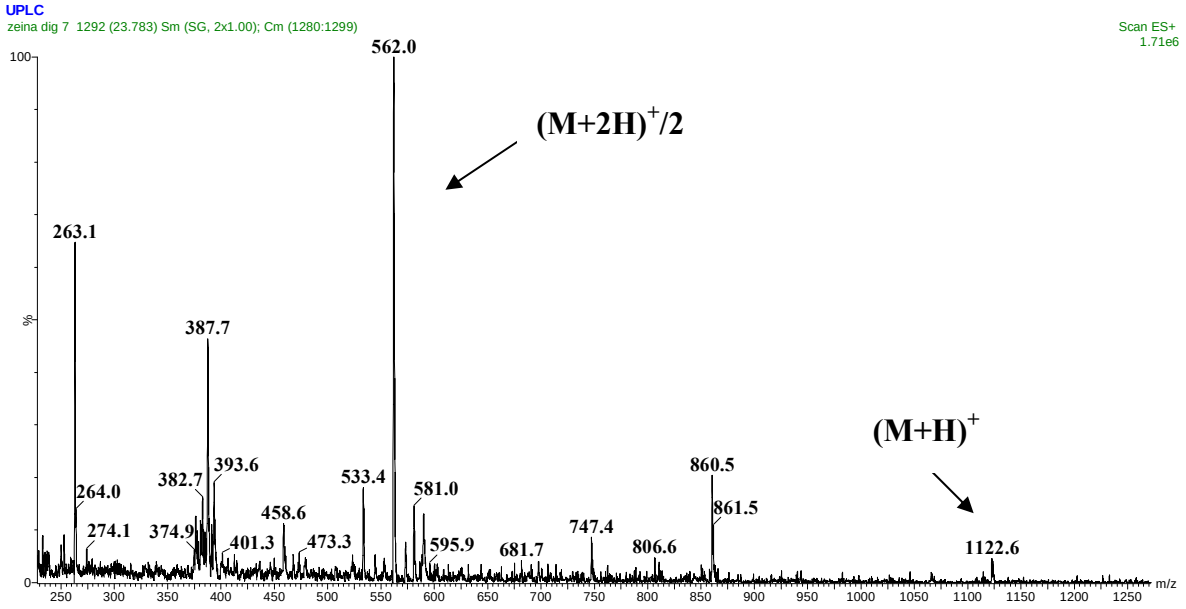


Fig. 61: Mass spectrum of the selected peak at the retention time 23.78 min, showing the molecular ion, m/z 1122.6, and its doubly charged ion 562.0 m/z.

A more detailed analysis of the spectrum shows also that the peptide is fragmented in the ion source, producing the two fragments at 860.5 and 263 m/z. These values can be used now as input for a BioLynx research in the given protein sequences, in order to check the presence of the same peptides. Results for these two values are reported in the next table.

Tab. 62: BioLynx (Waters Inc.) software output for searching of 1122.6 m/z ion $(M+H)^+$, and 562.0 m/z $(M+2H)^+/2$ among the AF371269 sequence.

Untitled
Mass 1 of 2: 1121.98
Mass tolerance: 1.00
Number of hits = 2

Hit#	Res#	Mass	Sequence	C-Term	Total Mass	Dev	S-S
1	90-98	1121.55	(Y)SQQQQFLPF(N)	-OH	1121.55	-0.43	-
2	17-27	1121.54	(P)PYLSPAVSSVC(E)	-OH	1121.54	-0.44	-

The peptide with mw 1122.6 Da is present in the given protein sequence. The matching is then made by checking its presence also in the list of virtual peptides. From the list for AF371269 sequence, pag. 84, is possible to see that peptide #1 is present in the list as fragment 'y 5-6', while the second peptides is not present.
Confirmation of matching for peptide #1 comes from the peptide sequence and the mass spectrum.

Tab. 63: Proposed assignment for spectrum of the selected peak at retention time 23.78 min.

m/z	Ion: sequences
1122.6	$(M + H)^+$: (Y)SQQQQFLPF(N)
860.5	$(M - PF + H)^+$: SQQQQFL
562.0	$(M + 2H)^+/2$: SQQQQFLPF
263.1	$(M + H)^+$: PF

From the matching it is possible to consider the peptides present in the sequence, and in the α-Zein standard. All the peaks released from the α-Zein standard digestion have been matched by the reported procedure. Several examples of the peptides identified after α-Zein standard digestion are reported in the following figures.

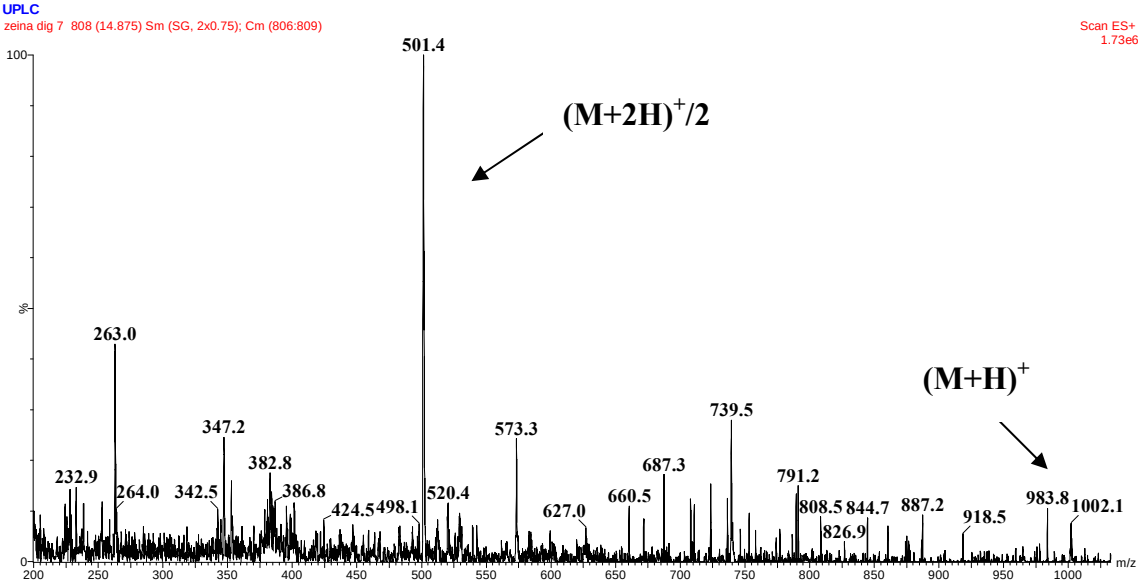


Fig. 62: Mass spectrum of the peak selected at retention time 14.90 min, showing the molecular ion, m/z 1002.1, and its doubly charged ion 501.4 m/z.

Tab. 64: Proposed assignment for spectrum of the selected peak at retention time 14.90 min.

m/z	Ion: sequences
1002.1	$(M + H)^+$: LQQQQLPF
739.5	$(M - PF + H)^+$: LQQQQL
501.4	$(M + 2H)^+/2$: LQQQQLPF
263.0	$(M + H)^+$: PF

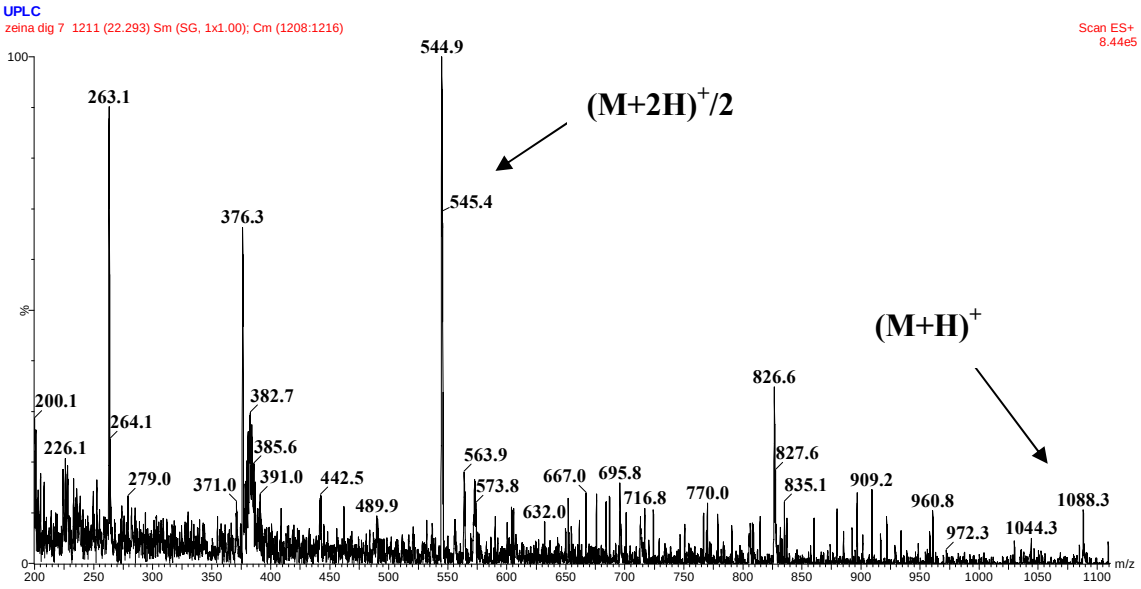


Fig. 63: Mass spectrum of the selected peak at retention time 22.29 min, showing the molecular ion, m/z 1088.3, and its doubly charged ion 544.9 m/z.

Tab. 65: Proposed assignment for spectrum of the selected peak at retention time 22.29 min.

m/z	Ion: sequences
1088.3	$(M + H)^+$: SQQQQLLPF
826.6	$(M - PF + H)^+$: SQQQQLL
544.9	$(M + 2H)^+/2$: SQQQQLLPF
263.1	$(M + H)^+$: PF

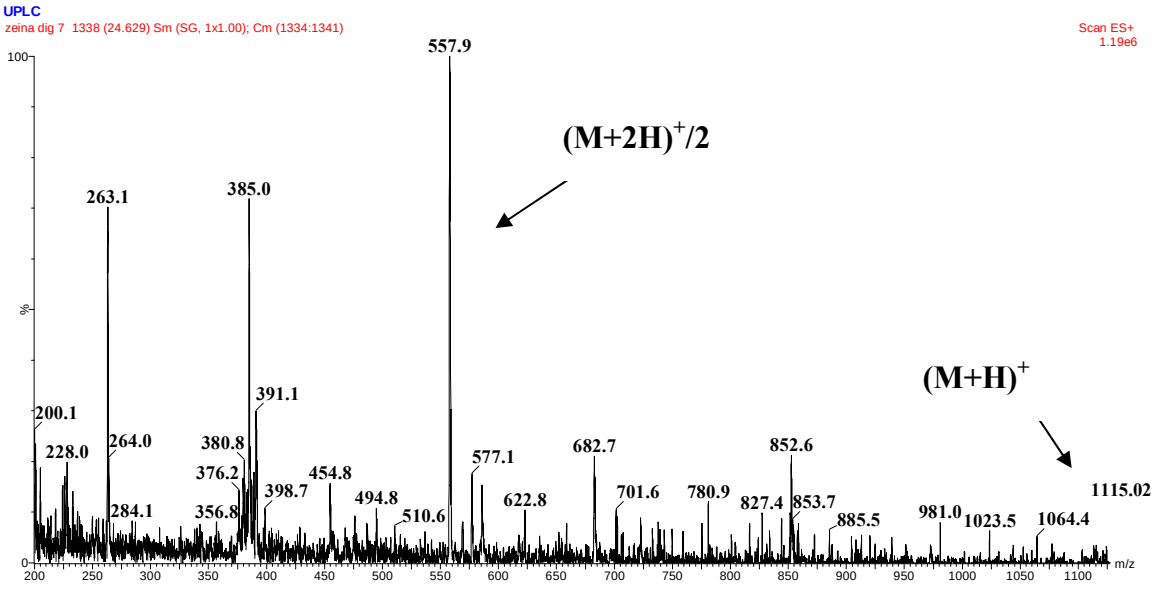


Fig. 64: Mass spectrum of the selected peak at retention time 24.63 min, showing the molecular ion, m/z 1114.9, and its doubly charged ion 557.9 m/z.

Tab. 66: Proposed assignment for spectrum of the selected peak at retention time 24.63 min.

m/z	Ion: sequences
1114.9	$(M + H)^+$: LQQQQLLPF
852.6	$(M - PF + H)^+$: LQQQQLL
557.9	$(M + 2H)^+/2$: SQQQQLLPF
263.1	$(M + H)^+$: PF

Results of the α-Zein peptide matching are reported in the following tables. Ten peptides were identified for both AF371269 and AF371271, thus confirming the sequence of the protein standard. From this first screening, only one peptide was found to match with the AF371268 sequence, which seem to have a low correspondence with the sequence.

Tab. 67: α-Zein peptides identified after chymotryptic digestion.

peptide retention time (min.)	*peptide sequence*	*$(M+H)^+$ (Da)*	*α-Zein sequence*
14.90	(Y)LQQQQLPF(S)	1001.54	AF371269
22.29	(Y)SQQQQLLPF(N)	1088.38	AF371271
23.78	(Y)SQQQQFLPF(N)	1122.55	AF371269, AF371271
24.63	(Y)LQQQQLLPF(S)	1114.63	AF371269
25.03	(Y)QQPIIGGALF(-)	1043.64	AF371269
25.46	(F)LTQQQLLPF(Y)	1087.75	AF371271
27.13	(F)YQQPIIGGALF(-)	1206.78	AF371269
28.07	(F)LTQPQLLPF(Y)	1056.61	AF371269
29.39	(Y)LQQQLLPF(S)	986.57	AF371271
31.00	(F)LLQQQLLPF(H) (Y)LQQQILLPF(S)	1099.55 (isobars)	AF371268, AF371271

Tab. 68: α-Zein sequences found for the protein standard, and matched peptides found after chymotryptic digestion (bold, italic)

GenBank accession number: AF371269
TIFPQCSQAPIASLLPPYLSPAVSSVCENPILQPYRIQQAIAAGILPLSPL***FLQQSSALL***QQLPL
VHLLAQNIRAQQLQQLVLANLAAY***SQQQQFLPF***NQLAALNSASY***LQQQQLPF***SQLSAAYP
QQFLPFNQLTALNSPAY***LQQQQLLPF***SQLAGVSPATF***LTQPQLLPF***YQHAAPNAGTLLQLQ
QLLPFNQLALTNPTAF***YQQPIIGGALF***

GenBank accession number: AF371271
TIFPQCSQAPIASLLPPYLPSIIASICENPALQPYRLQQAIAASNIPLSPLLFQQSPALSLVQSLV
QTIRAQQLQQLVLPLINQVALANLSPY***SQQQQFLPF***NQLSTLNPAAY***LQQQLLPF***SQLATA
Y***SQQQQLLPF***NQLAALNPAAY***LQQQILLPFS***QLAAANRASF***LTQQQLLPF***YQQFAANPATL
LQLQQLLPFVQLALTDPAASYQQHIIGGALF

GenBank accession number: AF371268
TIIPQCSQQYLSPVTAARFEYPTIQSYRLQEAIAASILRSLALTVQQPYALLQQPSLMNLYLQ
RIAAQQLQQQLLPIINQVVAANLAAYLQQQQFLPFNQLAGVNPAAYLQAQQLLPFNQLVR
SPAAF***LLQQQLLPF***HLQVVANIAAFLQQQQQLLPFYPQVVGNINAFLQQQQLLPFYPQDVA
NNVAFLQQQQLLPFNQLALTNPTTLLQQPTIGGAIF

Once that the sequences have been proved, a rapid analysis of their primary structures show s that lysine residues are totally absent in α-Zein. Thus these result do not account for covalent eaction at least at the level of the carballylic esters, and more in favour of the non covalent hypothesis previously discussed.

3.8.3 In vitro reaction between α-Zein and Fumonisin B_1

In order to get further information about the Fumonisin potential reactivity with maize prolamines, an *in vitro* reaction was performed in order to investigate the presence of potential covalent adducts. The *in vitro* reaction was performed by heating FB_1 and the α-Zein standard (5:1 molar ratio), simulating the conditions of high temperature processed products (about 150 °C). The procedure is reported in the Experimental Part. Afterwards, the reaction mixture was suspended in methanol, followed by centrifugation and analysis by the UPLC-MS system. The same reaction was performed also for α-Zein (control solution), in order to check which modification may occur in the protein upon heating.

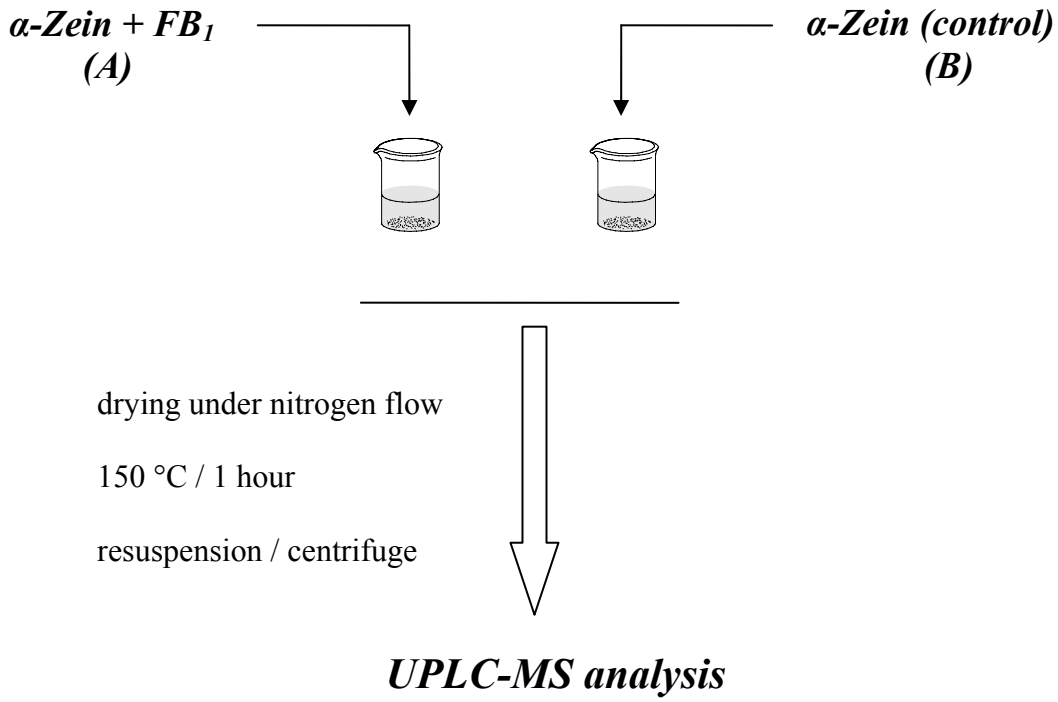


Fig. 65: Schematic procedure of the experiment for covalent reaction evaluation between Fumonisin B_1 and α-Zein after heating.

In the next figures the results of UPLC-MS analysis of the reaction are reported. Significant differences are observed between α-Zein heated alone, or in presence of Fumonisin B_1, which seems to be present, unchanged, after heating.

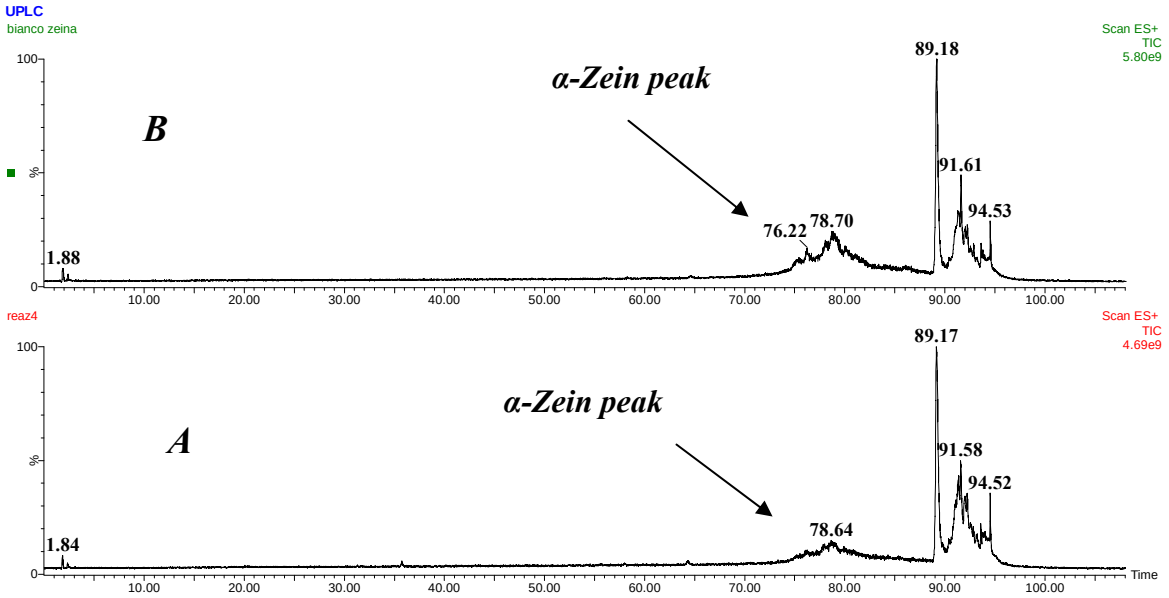


Fig. 66: UPLC-MS chromatogram of the in vitro reaction between Fumonisin B_1 and the Zein standard at 150 °C for 1 hour: (A) reaction mixture;(B) α-Zein standard alone . MS scan (TIC) in the 200-2000 m/z range.

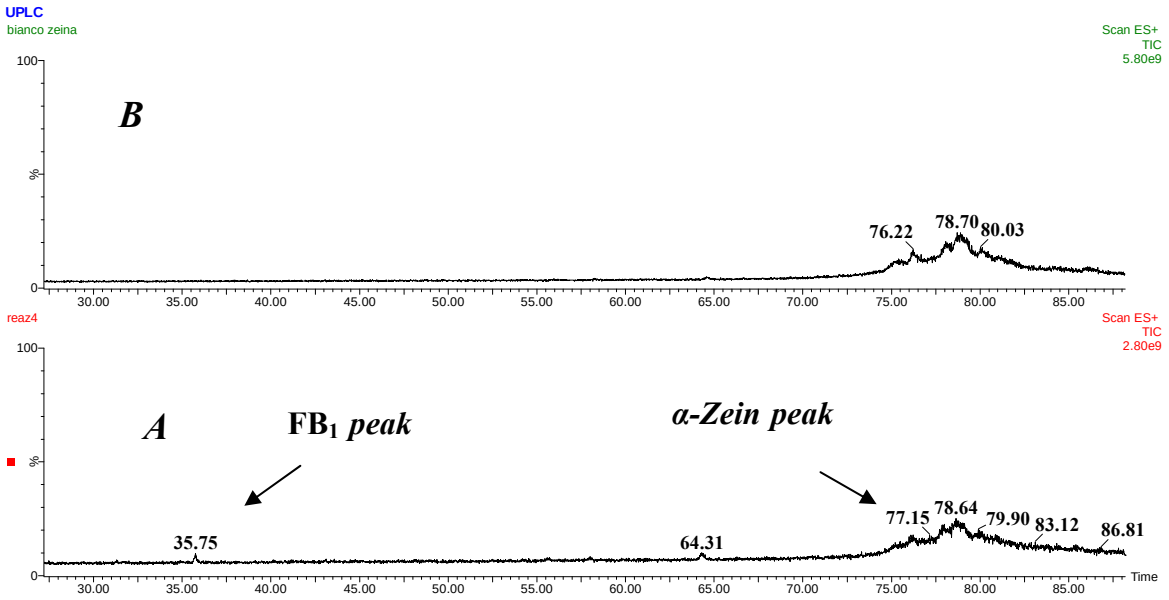


Fig. 67: UPLC-MS chromatogram of the in vitro reaction between Fumonisin B_1 and the Zein standard at 150 °C for 1 hour: (A) reaction mixture; (B) α-Zein standard alone.

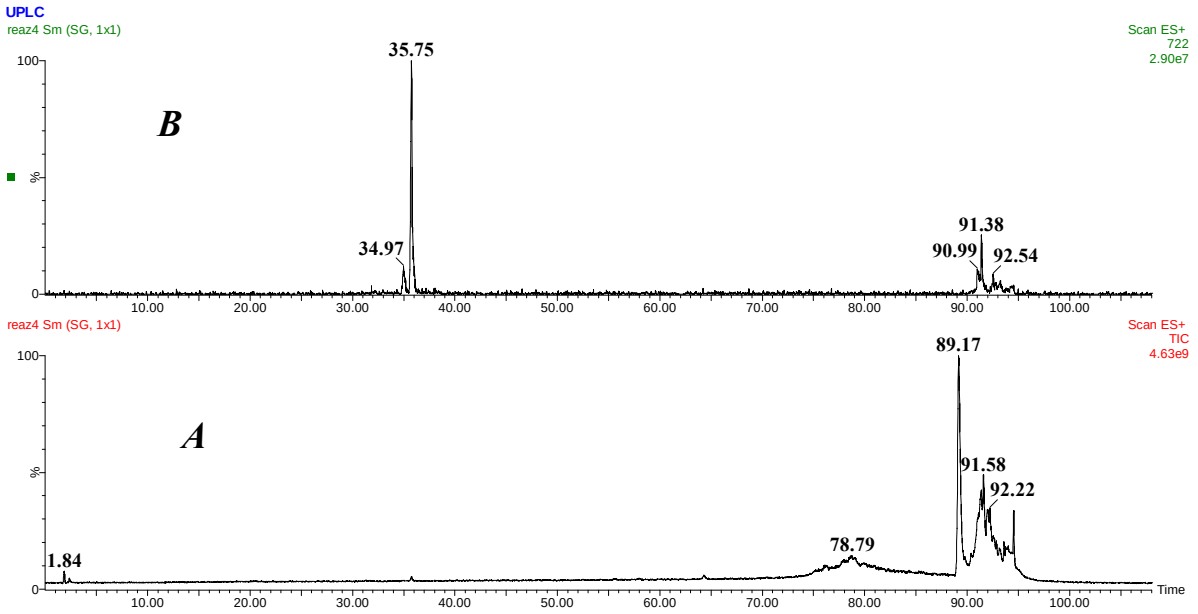


Fig. 68: UPLC-MS chromatograms of in vitro reaction between Fumonisin B_1 and Zein standard at 150 °C for 1 hour. Scan, Total Ion Current (A), extracted ion 722 m/z of FB_1 (B).

The lack of significant differences on the UPLC-MS profiles of the protein suggest that no reaction between FB_1 and α-Zein took place under the tested conditions. Thus this result supports thus the non covalent hypothesis.

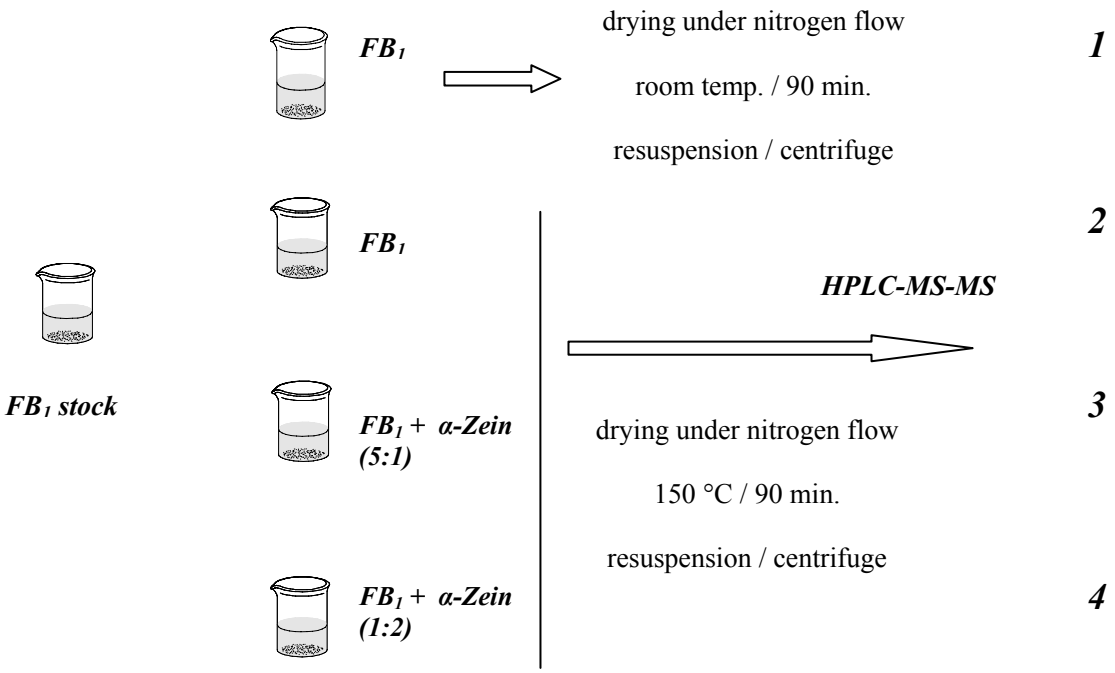


Fig. 69: Schematic procedure of the second experiment for covalent reaction evaluation between Fumonisin B_1 and α-Zein after heating.

The same experiment was repeated, focusing the analysis on FB_1. For this purpose, the solution containing FB_1 and α-Zein and the control solution (FB_1) were analysed by LC-MS/MS after heating.

In the next figures the results of HPLC-MS-MS analysis of FB_1 heated alone and in presence of α-Zein are reported. All the experiments were performed on the same amount of FB_1.

In lane 1 it is possible to see the FB_1 peak in the control experiment (room temperature). In lane 2 the chromatogram obtained for the same amount of FB_1 heated alone at 150 °C: the absence of any peak seems to suggest that the toxin has been destroyed during heating. In lane 3, FB_1 was heated in presence of α-Zein with a molar ratio of 5:1; the absence of peak could suggest that a reaction took place or that the toxin was destroyed as before. In lane 4 the chromatogram obtained for the FB_1 heated in presence of α-Zein with a molar ratio of 1:2 is reported. Surprisingly FB_1 is still present, as proved by its peak.

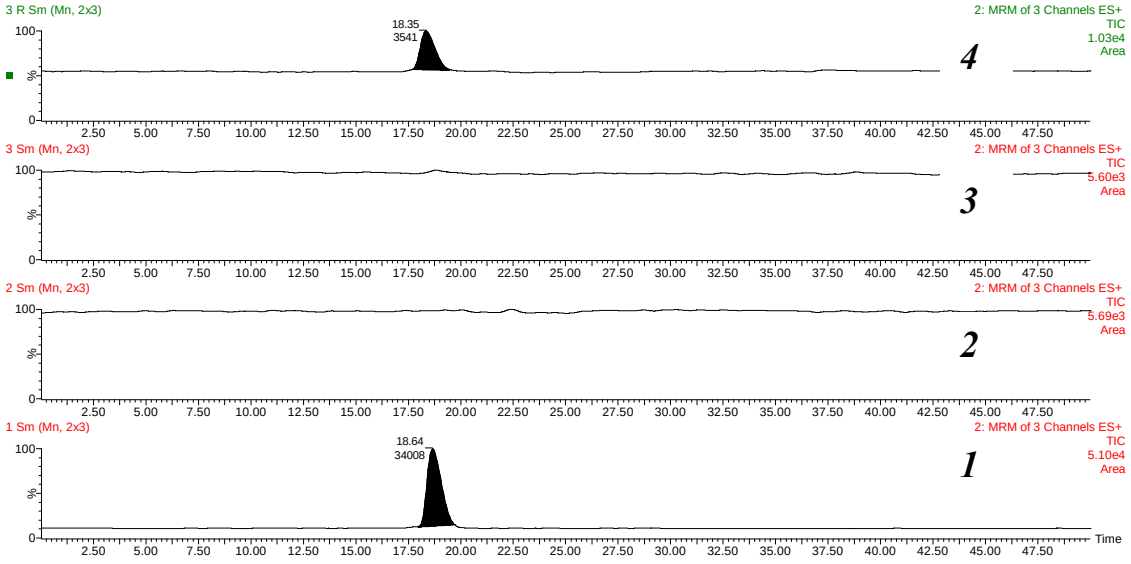


Fig. 70: HPLC-MS-MS, MRM, TIC, chromatogram of the in vitro reaction between Fumonisin B_1 and the Zein standard. (1) Fumonisin B_1 control at room temp. for 1 hour, (2) Fumonisin B_1 heated alone at 150 °C for 90 min; (3) Fumonisin B_1 heated with α-Zein (5:1 molar ratio) at 150 °C for 90 min; (4) Fumonisin B_1 heated with α-Zein (1:2 molar ratio) at 150 °C for 90 min.

The experiment was repeated ones more time, using also an α-Zein digested sample and lower reaction time (60 min.). Results are reported in the next figure. In lane 1 it is possible to see the FB_1 peak in the control experiment, heated alone at 150 °C for 60 min: the lower duration

of treatment didn't induce the complete destruction of the toxin. In lane 2 the chromatogram obtained for the same amount of FB_1 heated at 150 °C for 60 min., in the presence of α-Zein with a molar ratio of 5:1 is reported; The higher FB_1 peak area seems to suggest lower thermal degradation, as if the protein would protect the toxin from degradation.

In lane 3, FB_1 was heated in presence of α-Zein with a molar ratio of 1:2, and ones more it is possible to see the great increase of FB1 peak, suggesting that the toxin is much less destroyed with a protein excess. In lane 4 FB_1 was heated in the presence of α-Zein chymotryptic digestion product, assuming a molar ratio of 1:2 of toxin with the native protein.

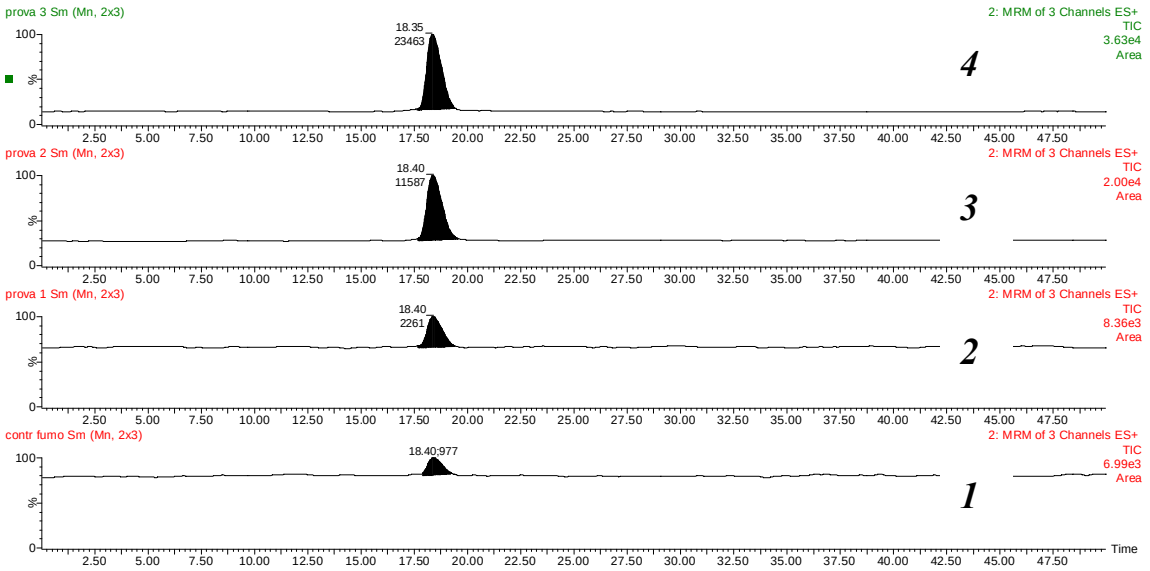


Fig. 71: HPLC-MS-MS, MRM, TIC, chromatograms of the replicate reactions between Fumonisin B_1 and the Zein standard. (1) FB_1 heated alone at 150 °C for 60 min; (2) FB_1 heated with α-Zein (5:1 molar ratio) at 150 °C for 60 min; (3) FB_1 heated with α-Zein (5:1 molar ratio) at 150 °C for 60 min ; (4) Fumonisin B_1 heated with chymotryptic digested α-Zein (1:2 molar ratio) at 150 °C for 60 min.

The replicate seem is in agreement with the previous one, pointing to the new hypothesis for Fumonisin masking. These experiments showed that no significant differences, in the UPLC-MS profile of the protein, exist between the α-Zein standard heated at 150 °C alone, or in presence of a molar excess of FB_1. On the other side, FB_1 is destroyed when heated alone at 150 °C, whereas when the same amount is heated in the presence of a molar excess of protein, it seems to be protected. These results confirm the hypothesis that FB_1 can interact

non-covalently with α-Zein, forming aggregates that prevent the thermal degradation of the toxin.

3.8.4 Preliminary evidence for an inclusion-like mechanism of FB_1 masking

The next step was to find new evidence for substantiating the new hypothesis of FB_1/α-Zein non covalent interaction previously proposed. We thought that, if the new hypothesis is correct, a treatment able to release hidden FBs, without hydrolyzing them must exist. Prolamines extract from highly contaminated maize sample was obtained, and 4 experiments were carried out, as reported in the Experimental Part, with the aim of to investigate which treatment would be able to release the protein bound Fumonisin.

Thus, we investigated the hydrolysis method, the chymotryptic digestion, and urea treatment. It must be noted that only basic hydrolysis can cleave potential covalent bonds between tricarballylic chains of Fumonisins and proteins, whereas the other methods can either cleave the protein itself or disrupt secondary and tertiary protein structures, thus releasing the toxin. In the next figure, the MRM chromatograms of the 4 experiments, made on 4 equal aliquots of maize are reported.

In lane 1 the prolamine fraction was analysed to check the presence of free FBs, which resulted absent or under the limit of detection.

In lane 2 it can be seen that after basic hydrolysis of the prolamine fraction, hydrolysed FB_1 is released. This result is consistent with the first report of hidden FBs in corn-flakes, of Kim et al. (2003). Lane 3 represents the urea treatment, and as it can be seen nothing is released in appreciable amount. Lane 4, representing chymotryptic digestion, surprisingly shows the FB_1 peak. Releasing of the whole toxin after enzymatic digestion of the protein could became very important for several reasons.

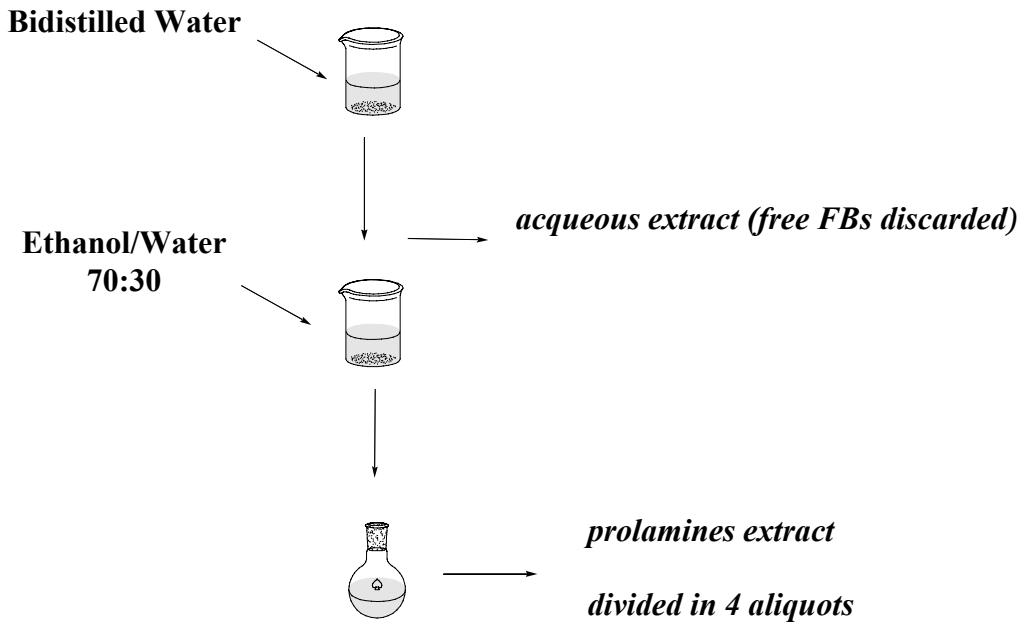


Fig. 72: Schematic procedure of the second experiment for covalent reaction evaluation between Fumonisin B_1 and α-Zein after heating.

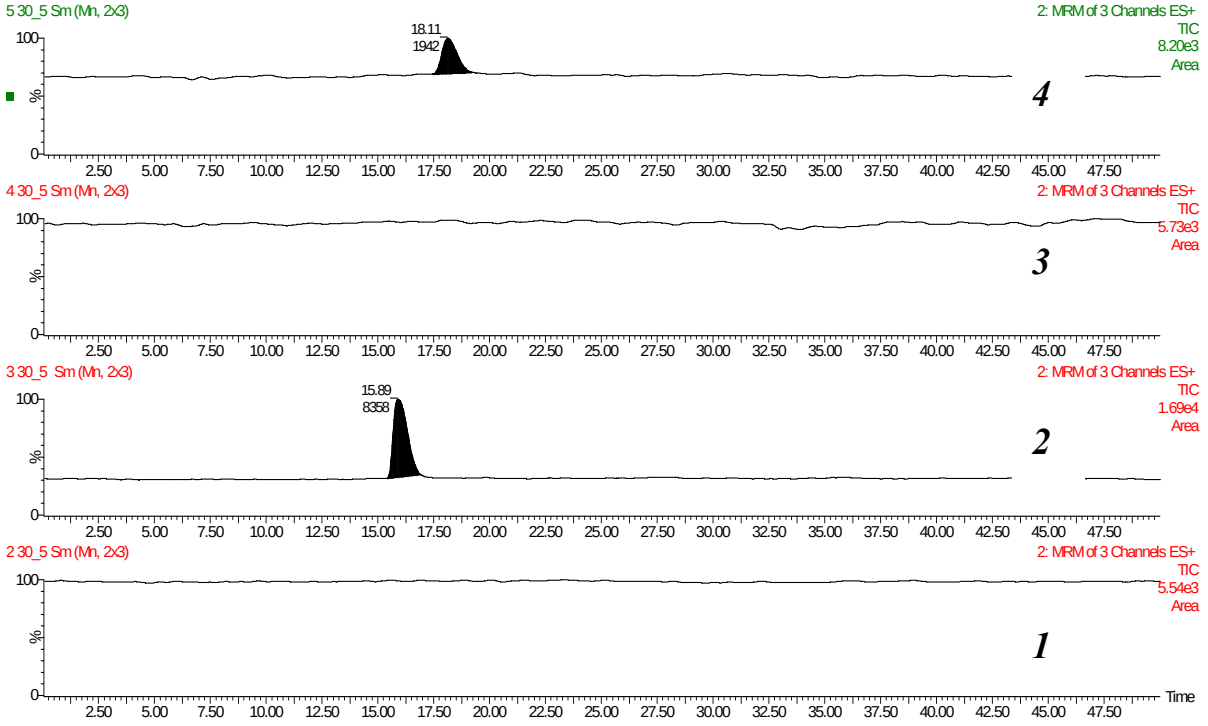


Fig. 73 : HPLC-MS-MS, MRM, TIC, chromatograms of the experiments made on the prolamine fraction aliquots (PFA), extracted from a highly contaminated maize sample. Lane 1: analysis of free FBs in PFA; Lane 2: basic hydrolysis of PFA; Lane 3: urea denaturation of PFA; Lane 4: chymotryptic digestion of PFA.

If these results will be confirmed, the hypothesis of covalent binding of proteins with Fumonisin should be dismissed, leading to account for a more probable non-covalent interaction which is quite strong, since a denaturation with urea is not enough to loose the complex and release the toxin. On the other side, if *in vitro* enzymatic digestion is able to release Fumonisin from the food matrix, it is probable that also durig human or animal digestion, unknown amount of toxins may be released in the gastro-intestinal tract. Moreover, a method able to analyse the real Fumonisin amount in maize and related products is urgently required. In order to explore the possible non covalent association, an ESI-MS titration experiment was performed. Three solutions of Fumonisins B_1 and α-Zein, at different molar ratios in methanol, were infused in the mass spectrometer and the FB_1 molecular ion $(M+H)^+$ monitored. In the following figures the results for the ESI-MS titration experiments are reported.

Tab. 69: Molar ratios of three FB_1-zein mixtures in methanol, used in ESI-MS titration experiments.

FB_1- zein	***molar ratio FB_1 / Zein***	***experiments***
FB_1 10 µM – α-Zein 5 µM	2	A
FB_1 5 µM – α-Zein 5 µM	1	B
FB_1 5 µM – α-Zein 10 µM	0.5	C

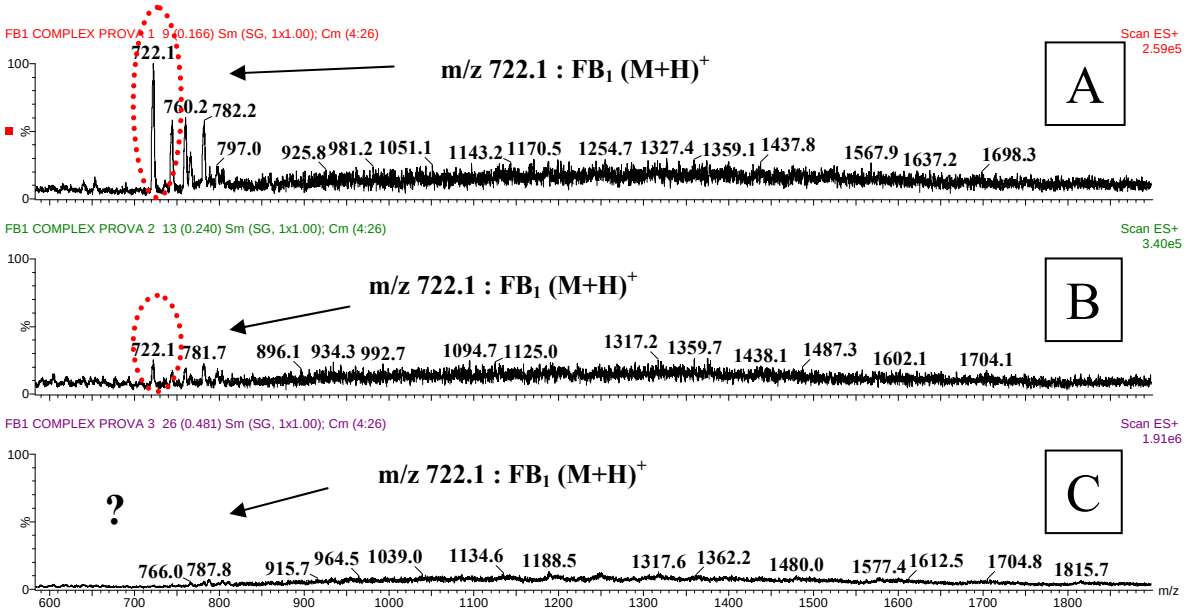


Fig. 74: ESI-MS infusion of three FB_1-Zein mixtures in methanol, at different molar ratios. A:2 (10:5), B:1 (5:5), C:0.5 (5:10) (µM/µM).

As shown, the intensities of the FB_1 molecular ion $(M+H)^+$ are strongly influenced by the presence of α-Zein in solution. This strong effect of ion suppression could be explained in two ways. The former is the general explanation given for ion supression phenomenon, based on the competition for the protons of the analytes. The latter explanation involves the formation of non covalent interactions between FB_1 and Zein in solution. The variation of the FB_1 response seems to be strictly dependent upon the molar ratios of the two compounds (protein and toxin), with a linear or quadratic trend (see the Experimental Part). A small variation of the Zein concentration produces a large decrease of FB_1 ion count, as show in Fig. 74-76. This suggest that different mechanisms might contribute to the decrease, of the FB_1 response in solution, and not only competition for the protons.

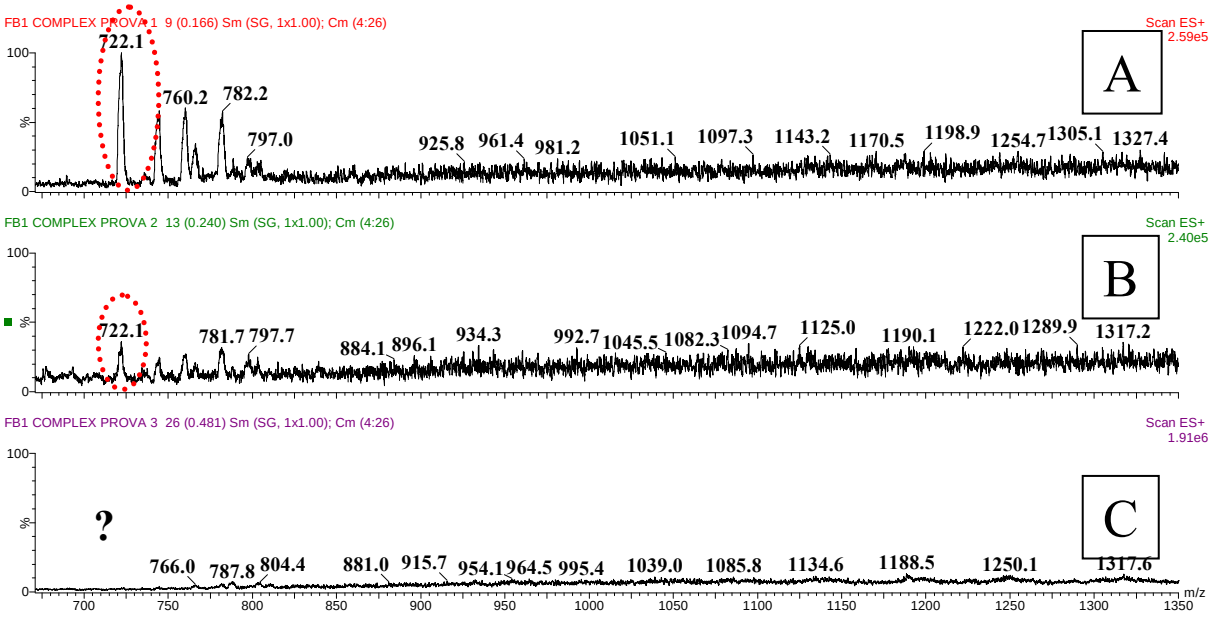


Fig. 75: ESI-MS infusion of three FB_1- α-Zein mixtures in methanol at different molar ratios. A:2 (10:5), B:1 (5:5), C:0.5 (5:10) µM/µM. Zoom on FB_1 molecular ion (722.3 m/z).

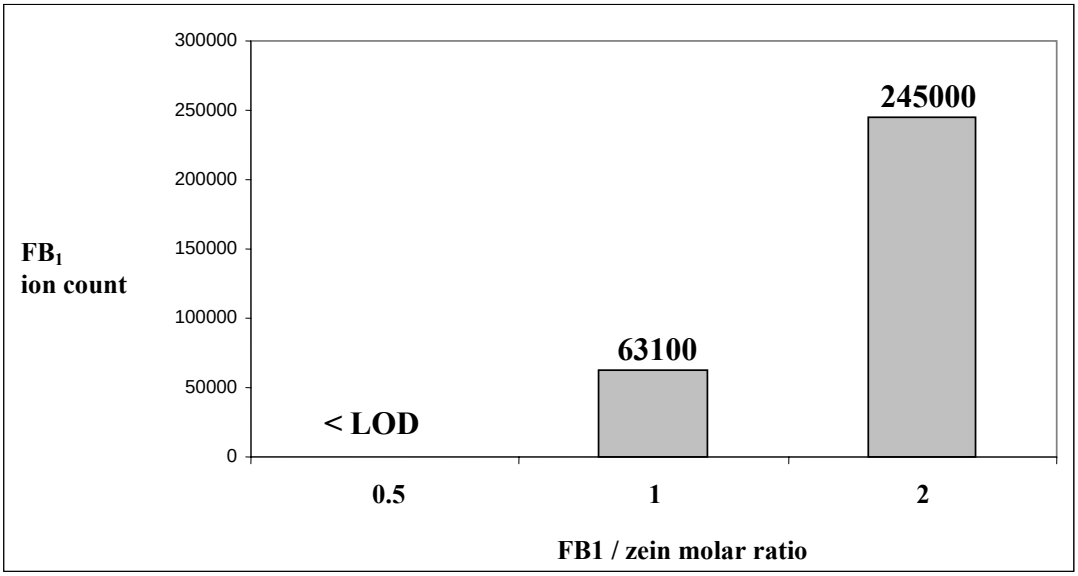


Fig. 76: Ion counts of FB_1 molecular ion (722.3 m/z) is the ESI-MS titration experiments.

Maize prolamines were found to be the Osborne fraction more active in Fumonisin masking. However also other classes of compounds, such as glutelins or starch, could be involved in the masking.

Thus, from these experiments, we were not able to find any modifications of the protein, whereas the mycotoxin appearsto be strongly affected by the presence of the protein. Infact, heating Fumonisin B_1 in the presence of α-Zein standard, no significant changes in the UPLC-MS chromatogram of α-Zein were observed. In contrast, the toxin seems to be less destroyed in the presence of an excess of protein, and the phenomenon seem to be accentuated when the protein is previously digested with chymotrypsin.

Finally, the fact that upon chymotrypsin digestion the native form of FB is release from prolamines fraction, is a strong evidence for a complex formation between FB_1 and α-Zein standard, with an inclusion-like mechanism.

This new hypothesis seems to be consistent with data concerning the widespread presence of hidden Fumonisins along the maize chain.

3.9 Conclusions

In this PhD thesis the presence of masked Fumonisins has been verified not only in finished products, but also in maize, raw materials and even in a standard material. An LC-MS/MS method for indirect evaluation of hidden Fumonisins was developed in our laboratory. The method is based on the comparison of the toxin concentration before and after the basic hydrolysis of the sample. The optimized alkaline hydrolysis of the sample turned out to be a suitable tool to approach the problem of the indirect quantification of hidden FBs.

The application of the method to several batches of gluten free products and raw cereal let to discover that hidden FBs are widespread along the maize chain.

Hidden Fumonisins are present in all type of maize based products and also in the raw cereal. Unexpectedly no significant differences in hidden / free FBs ratio were found between high and low temperature treated food. Significantly lower, but still evident was the same ratio in the raw cereal, suggesting that in the raw cereal the masking mechanism is already active.

While the presence of hidden FBs in maize based products was already reported in the literature, this is the first evidence of the presence of hidden Fumonisins in the raw cereal.

The hypothesis of covalent binding of Fumonisins to the protein in the matrix due to thermal treatments can not explain the presence of hidden Fumonisins in the raw cereal.

Hidden FBs were also found in maize samples used as reference materials for ELISA kit, suggesting that a masking phenomenon is occuring so that antibodies are not able to detect hidden form of Fumonisins. Results of the first part of the work suggest a non-covalent mechanism for FBs masking explanation.

The second part of this work was dedicated to investigate the mechanism of masking.

In agreement with the literature, maize prolamines were found to be the Osborne fraction more active in Fumonisins masking. A commercial standard of α-Zein, the most abundant prolamine and maize protein, was checked for sequencing by UPLC-MS and was found to be completely devoid of lysine.

Moreover, when Fumonisin B_1 is heated in the presence of the α-Zein standard, no covalent modification of the protein seems to occur; on the other hand, the toxin is less destroyed in the presence of an excess of protein. This phenomenon seems to be accentuated when the protein is previously digested with chymotrypsin. Also this facts seems to be consistent with the non covalent hypothesis.

By chymotrypsin digestion the native forms of FBs from the prolamines fraction were released, as if free from binding interactions.

Moreover α-Zein standard was found to be able to strongly suppress the FB_1 molecular ion $(M+H)^+$, in the ESI-MS infusion experiments.

All the experimental data collected in the second part of the work strongly suggest a non-covalent interaction between FB_1 and the α-Zein standard, with an inclusion-like mechanism.

The results of this work can have important consequences in Fumonisin analysis and exposure data. First, the hypothesis of covalent binding of proteins with Fumonisins should be furtherly verified looking for the presence of conjugated products. Most important, if *in vitro* enzymatic digestion is able to release Fumonisins from the food matrix, it is probable that also durig human or animal digestion, unknown amounts of toxins may be released in the gastro-intestinal tract, so that the toxicity may increase dangerously. Moreover, a method able to analyze the real Fumonisin amount in maize and related products should be adopted officially with eventual revision of the legal limits.

A more extended survey will be useful to understand the implications of this phenomenon in Fumonisin analysis and related risk assessment. The study of interaction between mycotoxins and food components.

Finally, a "Pandora vase" has been opened concerning the bioavailability and relative toxicity of masked mycotoxins in food.

4. Experimental Part

4.1 Reagents and solvents

- Fumonisins B_1+B_2 standard solution (Biopure, Austria).
- Fumonisin B_3 standard solution (Biopure, Austria).
- α-Zein standard (powder, Fluka, Germany)
- Bovine chymotrypsin (powder, Sigma, Germany)
- Methanol HPLC grade (Carlo Erba, Italy)
- Acetonitrile HPLC grade (Carlo Erba, Italy)
- Formic acid HPLC grade (Acros Organics)
- Acetic acid HPLC grade (Carlo Erba, Italy)
- Ammonium bicarbonate (Fluka, Germany)
- Calcium chloride (Carlo Erba, Italy)
- Potassium hydroxide (Carlo Erba, Italy)
- Sodium hypochloride
- Buffers (Carlo Erba, Italy)
- Bidistilled water HPLC grade, obtained with Millipore Alpha-Q-System

4.1.1 Precautions

Extreme care is required during organic solvent and mycotoxin standard handling, always using personal protective equipment. Glassware used for contaminated samples or standard solutions requires hypochlorite treatment, in this way mycotoxins are destroyed by oxidation. Glassware is washed with soap, water, and then air dried. During standard preparations, glasses and gloves were worn, and all the operations were conducted under hood, in order to minimyze risk of exposure to mycotoxins.

4.2 Instruments

HPLC system: Alliance Waters 2695 (Automated Rheodyne injector, with 20µl loop)
Triple quadruple detector : 'Micromass Quattro Micro API' (Waters)
UPLC system: Acquity Waters
Single quadruple detector: Acquity SQD (Waters)
Data acquisition and elaboration : MassLynx 4.01
(Water Chromatography Division, Milford, Massachusetts)
pH-meter : InoLab pH Level 1 WTW (Chemifarm s.r.l., Parma, Italy)

4.3 Mobile phase preparation for HPLC and UPLC analysis

The mobile phase, for both HPLC and UPLC, was freshly prepared before each batch of analyses, using graduate cilynders to measure the correct volume of water and organic modifier. For all the analysis, the following eluents were used:

→ Bidistiled water + 0.2% Formic acid

→ Methanol + 0.2% Formic acid

→ Acetonitrile + 0.2% Formic acid

The eluents were filtered with vacuum pump and Millipore 0.45 μm filter.

4.4 Fumonisin analysis

4.4.1 MS/MS detection

In order to develop an MRM detection method, 5 ppm standard solutions of each Fumonsin were prepared. The calculated volumes were taken from stock solutions and placed into vials, dried under nitrogen flow and resuspended in a final volume with methanol in order to obtain the desired concentration. Analytes were infused in the mass spectrometer in order to develop the parameters necessary for the MRM detection method. The operative conditions of the mass spectrometer, used for MRM analysis of Fumonisin, are reported in the next tables.

Tab.70 : Conditions of the ion souce used in the MRM analysis of Fumonisin.

Parameter	***Value***
Polarity	ES+
Capillary	4.00 kV
Cone	50.00V
Extractor	2.00 V
RF Lens	0.2 V
Source Temperature	120 °C
Desolvation Temperature	350 °C
Cone Gas Flow	50 L/hr
Desolvation Gas Flow	700 L/hr

Tab. 71: Mass analyzer conditions used in the MRM analysis of Fumonisin.

Parameter	***Value***
LM 1 Resolution	9.4
HM 1 Resolution	8.5
Ion Energy 1	0.5 eV
Entrance	-2
Collision energy	specific for each transition
Exit	2
LM 2 Resolution	15.0
HM 2 Resolution	15.0
Ion Energy 2	2.0
Multiplier (V)	650
Gas Cell Pirani Pressure	$2.93e^{-3}$ mbar

Once the detection method has been developed, it was necessary to set the parameters for the chromatographic separation of the analytes. A standard solution of the three Fumonisins was prepared and several test were performed in order to achieve the baseline separation of the analytes in HPLC using a 2.5 x 25 mm, 5µ, C_{18} X-Terra column. The flow rate used in the analysis was 0.2 ml/min, with an injection volume of 10 µl. The gradient used for the HPLC analysis of Fumonisin, is reported in the next table.

Tab. 72: Mobile phase gradient used in the MRM analysis of Fumonisin.

Time	*% A* *(H_2O + 0.2 % HCOOH)*	*% B* *(ACN + 0.2 % HCOOH)*
0	70	30
2	70	30
5	55	45
25	10	90
35	10	90
36	70	30
50	70	30

4.4.2 Calibration and matrix effect

Linearity of response was first evaluated by calibration curves. Five standard solutions, each containing all the three FBs, were prepared in a final volume of methanol in order to obtain the desired concentration. Linearity was checked with single injections of standard solutions in the 50-2000 ppb range; curve equations are reported in the next table.

Tab. 73: Standard calibration curves for Fumonisin B_1, B_2 and B_3.

analyte	curve equation	R^2
Fumonisin B_1	y = 2.72x - 42.19	0.9982
Fumonisin B_2	y = 5.68x + 16.70	0.9994
Fumonisin B_3	y = 3.21x + 12.10	0.9985

Once linearity was checked, calibration curves were prepared. Based on the past experience, Fumonisins are known to be affected from matrix effects at least on the instrument used for this work. For this reason we decided to use directly matrix matched calibrations in order to correct the matrix effect and get a more accurate Fumonisin quantification. Matrix matched calibrations were determined by triplicate injection of 6 standards solutions in the range 50-2000 ppb, previously prepared by diluting an opportune stock of the three Fumonisins with an extract of blank maize sample. Calibration curves have been obtained by plotting the signal area of the analyte versus its concentration and are reported in the next table.
Matrix matched calibrations were injected with each batch of samples.

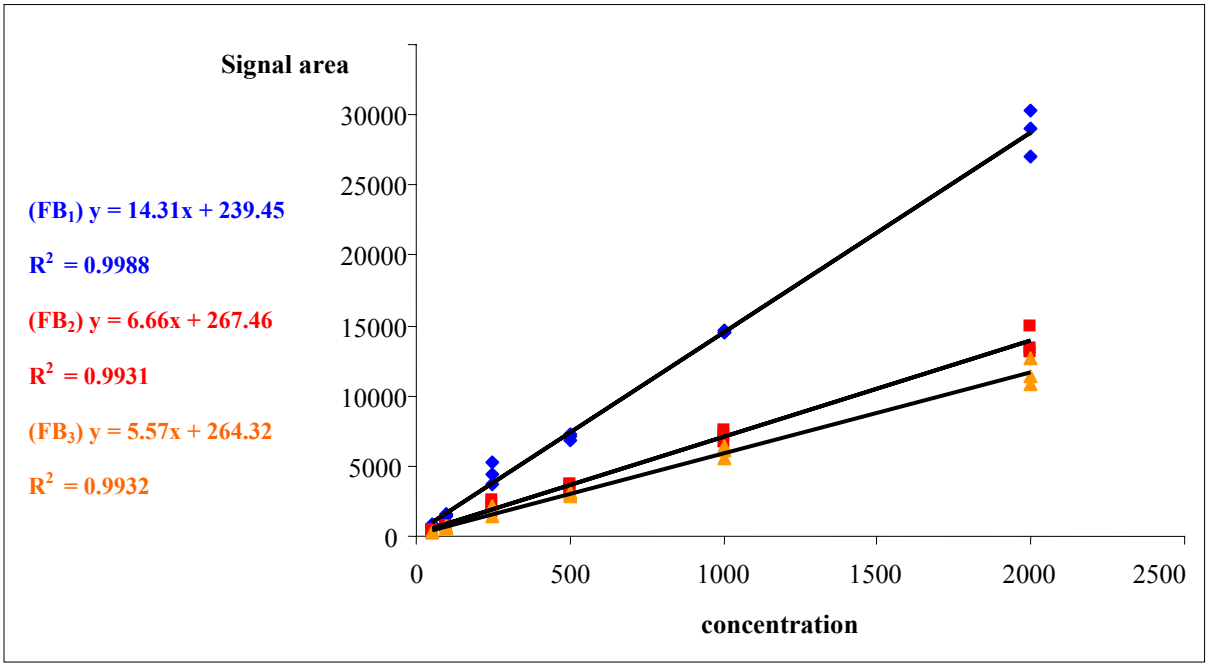


Fig. 77: Matrix matched calibration curves for Fumonisin B_1, B_2 and B_3.

4.4.3 Recovery and Limits of Detection

Fumonisin recovery was first evaluated by spiking procedure at 250 and 1000 ppb levels. Five grams of blank maize were spiked with a calculated volume of FB stock solution and allowed to rest overnight, in order to evaporate the solvent. The day after, the sample was extracted with 50 ml of a MeOH/H_2O 70:30 solvent mixture for 70 minutes (10 min. Turrax + 60 min. magnetic stirring), then the surnatant was recovered by centrifuge.
The surnatant was centrifuged, placed in a vial and directly injected in HPLC; matrix matched calibration solutions were also injected in order to quantify the analytes.

Recovery was calculated by the following formula:

Recovery% = (found concentration / reference concentration) x 100

Recovery values at the 2 levels were used to calculate the average recovery.
Limits of the method were evaluated on matrix matched standards of Fumonisin at 10 and 5 ppb. The standard was prepared by placing an opportune stock of the three FBs in a vial, drying under nitrogen, and resuspending them with an opportune volume of an extract of a blank maize sample.
After the analysis, the signal to noise ratio of the peaks of Fumonisins, at both levels, was evaluated by a special function of MASSLYNX software (Water Micromass Inc.). The software needs inputs as the peak widht at half height and the value of the background noise at the baseline of the peak.
Results from the software were compared with those generally accepted, for limit evaluation:
.S/N = 3, Limit of detection , LOD;
.S/N = 10, Limit of quantification, LOQ;
In the present work, 10 ppb was considered as limit of quantification for Fumonisin B_1 B_2 and B_3.

4.5 Hydrolysed Fumonisin analysis: optimization of the method

4.5.1 Standard preparation

Standards of hydrolysed Fumonisins (HFBs) are not commercially available and were prepared in house. Calculated volumes of FB stock solutions were placed in a 4 ml vial, dried under a nitrogen flow, resuspended in 1ml of KOH, and allowed to react overnight at room temperature. The day after, the solution was extracted twice with 1 ml of acetonitrile.

Two extracts were combined, dried under a nitrogen flow, and then resuspend in the calculated volume of methanol, in order to obtaine the desired standard stock solution of hydrolysed Fumonisins.

Example of a standard preparation:

1 ml of a 5 ppm standard of each hydrolysed Fumonisin were prepared in MeOH starting from 50 ppm commercial Fumonisin stock solutions in ACN/H_2O 1:1 .

1) The volume of Fumonisin stock solution to be hydrolysed was clculated as follows.

5 mg/ml x 1ml = 5 mg of each HFB

5 mg / 405.35 mg/mmol = 0.0123 mmol of HFB_1

5 mg / 389.35 mg/mmol = 0.0128 mmol of HFB_2

5 mg / 389.35 mg/mmol = 0.0128 mmol of HFB_3

by assuming that in the hydrolytic reaction the molar ratio is 1:1 :

0.0123 mmol of HFB_1 = 0.0123 mmol of FB_1

0.0128 mmol of HFB_2 = 0.0128 mmol of FB_2

0.0128 mmol of HFB_3 = 0.0128 mmol of FB_3

0.0123 mmol of FB_1 x 721.39 mg/mmol = 8.87 mg of FB_1

0.0128 mmol of FB_2 x 705.39 mg/mmol = 9.03 mg of FB_2

0.0128 mmol of FB_3 x 705.39 mg/mmol = 9.03 mg of FB_3

8.87 mg of FB_1 / 50 mg/ml = 0.177 ml of FB_1 stock solution 50 ppm

9.03 mg of FB_2 / 50 mg/ml = 0.181 ml of FB_2 stock solution 50 ppm

9.03 mg of FB_3 / 50 mg/ml = 0.181 ml of FB_3 stock solution 50 ppm

2) The calculated volumes of Fumonisin stock solution are placed in a 4 ml vial, dried under nitrogen, resuspended in 1 ml of KOH 2 M and allowed to rest overnight at room temperature.
3) The day after the solution was extracted twice with 1 ml of acetonitrile. Organic phases are collected and dried under nitrogen.
4) Once dried the organic phase was resuspended with 1ml of MeOH to obtain a 1 ml of 5 ppm solution for each hydrolysed Fumonisin.

4.5.2 MS/MS detection

In order to develop an MRM detection method, 5 ppm standard solutions of each hydrolysed Fumonsin were prepared. The calculated volumes were taken from stock solutions and placed into vials, dried under a nitrogen flow and resuspended in a final volume with methanol in order to obtain the desired concentration. Analytes were infused in the mass spectrometer in order to develop the parameters necessary for the MRM detection method. The operative conditions of the mass spectrometer, used for MRM analysis of Fumonisins, are reported in the next tables.

Tab. 74: Conditions of the ion souce used in the MRM analysis of hydrolysed Fumonisin.

Parameter	***Value***
Polarity	ES+
Capillary	4.00 kV
Cone	30.00V
Extractor	2.00 V
RF Lens	0.2 V
Source Temperature	120 °C
Desolvation Temperature	450 °C
Cone Gas Flow	50 L/hr
Desolvation Gas Flow	450 L/hr

Tab. 75: Mass analyzer conditions used in the MRM analysis of hydrolysed Fumonisin.

Parameter	***Value***
LM 1 Resolution	15.9
HM 1 Resolution	9.0
Ion Energy 1	0.5
Entrance	1
Collision energy	specific for each transition
Exit 2	3
LM 2 Resolution	15.0
HM 2 Resolution	15.0
Ion Energy 2	1.0
Multiplier (V)	650
Gas Cell Pirani Pressure	2.99e-3

Once the detection method was developed, it was necessary to set the parameters for the chromatographic separation of the analytes. A standard solution of the three Fumonisins was prepared and several test were performed in order to achieve the baseline separation of the analytes in HPLC using a 2.5 x 25 mm, 5µ, C_{18} X-Terra column. The flow rate used in the analysis was 0.2 ml/min, with an injection volume of 10 µl. The gradient used for the HPLC analysis of HFBs was the same as that used for FBs and is reported in the following Table.

Tab. 76: Mobile phase gradient used in the MRM analysis of Fumonisin.

Time	*% A* *(H_2O + 0.2 % HCOOH)*	*% B* *(ACN + 0.2 % HCOOH)*
0	70	30
2	70	30
5	55	45
25	10	90
35	10	90
36	70	30
50	70	30

4.5.3 Calibration and matrix effect

Linearity of response was first evaluated by calibration curves. Five standard solutions, each containing all the three HFBs, were prepared in a final volume of methanol in order to obtain the desired concentration. Linearity was checked with single injections of standard solutions in the 50-2000 ppb range; curve equations are reported in the next table.

Tab. 77: Standard calibration curves for Fumonisin B_1 B_2 and B_3.

analyte	curve equation	R^2
hydrolysed Fumonisin B_1	y = 1.46x + 7.96	0.9994
hydrolysed Fumonisin B_2	y = 4.27x - 11.27	0.9992
hydrolysed Fumonisin B_3	y = 4.65x - 11.24	0.9997

Once the linearity was checked, calibration curves were prepared. The same approach adopted for the native form was used for hydrolysed Fumonisins, matrix matched calibration was used in order to correct the matrix effect and get more accurate data.

Matrix matched calibration curves were determined by triplicate injections of standard solutions in the range 50-2500 ppb, previously prepared by diluting an opportune stock of the three hydrolysed Fumonisins with an extract of blank maize sample. Calibration curves were obtained by plotting the area of the signal of the analyte versus its concentration.

Matrix matched calibration solutions were injected with each batch of samples.

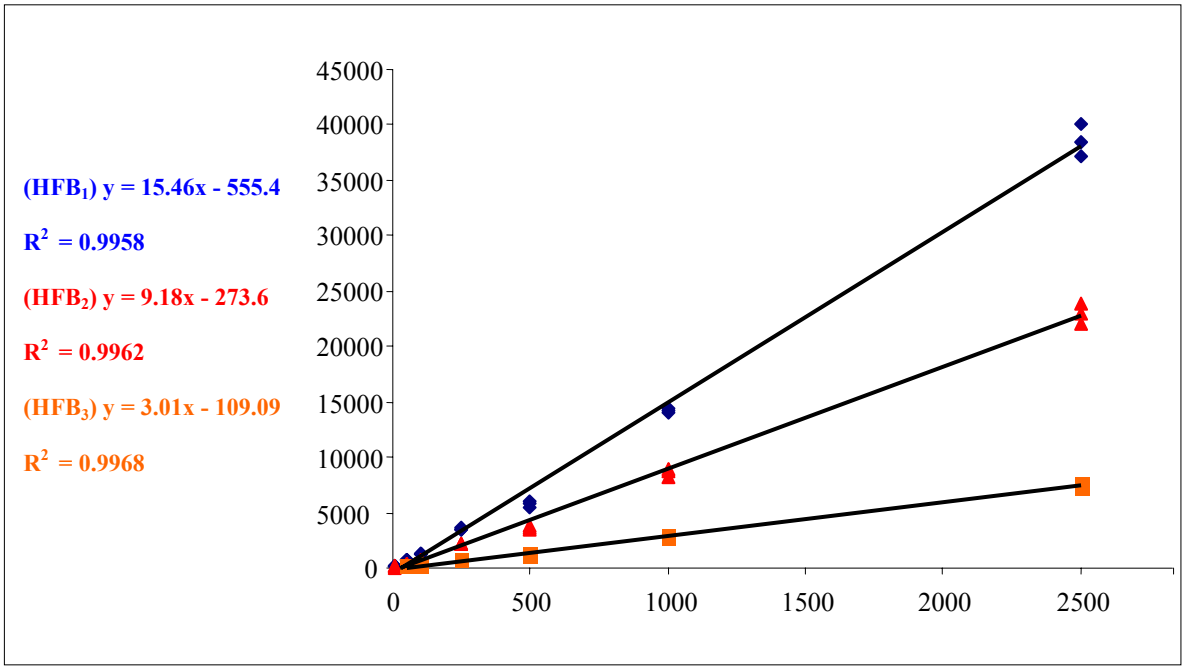


Fig. 78: Matrix matched calibration curves for hydrolysed Fumonisin HFB_1, HFB_2 and HFB_3.

4.5.4 Recovery and Limits of Detection

As already explained in the Discussion chapter, the extraction recovery of hydrolysed Fumonisins can be evaluated only by spiking procedures. However, also this procedure could not be the effective representative of the real samples because the masking mechanism cannot be reproduced in a laboratory, until it remains unknown. Extraction recovery can be evaluated only by spiking native FBs and evaluating the derived HFBs, after sample hydrolysis.

Hydrolyzed Fumonisin recovery was evaluated by spiking procedure at 250 and 1000 ppb levels for HFB_1 and HFB_2, and at 500-2000 ppb levels for HFB_3.

Five grams of blank maize were spiked with a calculated volume of FB stock solution and allowed to rest overnight, in order to evaporate the solvent. The day after, the sample was hydrolysed with 50 ml of 2 M KOH for 60 minutes (10 min. Turrax + 50 min. magnetic stirring) at room temperature, then 50 ml of acetronitrile were added. The immiscible phases were then homogenised by means of Ultra Turrax for 10 minutes, and the organic phase was recovered by centrifuge.

A known volume of the organic phase was dried by rotavapor, resuspended in an equal volume of MeOH/H_2O 70:30 mixture, centrifuged, and injected in HPLC.

Matrix matched calibrations were also injected in order to quantify the analytes.

Recovery of each analyte was calculated by the following formula:

Recovery% = (found concentration / reference concentration) x 100

Recovery values at 2 levels was used to calculate the average recovery.

Limits of the method were evaluated on matrix matched standards of hydrolysed Fumonisins at 10 and 5 ppb. The standard was prepared by placing an opportune stock of the three FBs in a vial, drying under nitrogen, and resuspending them with an opportune volume of an extract of a blank maize sample. After the analysis, the signal to noise ratio of the peaks of Fumonisins, at both levels, was evaluated by a special function of MASSLYNX software (Water Micromass Inc.). The software needs inputs as the peak widht at half height, and the value of the background noise at the baseline of the peak. The results of software have been compared with those generally accepted, for limit evaluation:

.S/N = 3, Limit of detection , LOD;

.S/N = 10, Limit of quantification, LOQ;

In the present work, 10 ppb were considered as limit of quantification for HFB_1 and HFB_2 while 50 ppb was considered for HFB_3.

4.6 The indirect approach

4.6.1 Sample preparation for free Fumonisins evaluation

Sample preparation for the analysis of free Fumonisins, on both maize and gluten-free products, as well as for the recovery evaluation, was performed as follows:

. The sample was first ground by means of a mulinex, (spiked sample were let to rest overnight);

. 5 grams of sample are extracted with 50 ml of MeOH/H_2O 70:30 solvent mixture for 70 minutes (10 min. Turrax + 60 min. magnetic stirring);

. the surnatant was recovered by centrifuge; one aliquots was directly analyzed to evaluate the presence of hydrolysed Fumonisins and to understand if the sample fit in the calibration range;

. highly contaminated samples can be analyzed directly. For low contaminated products a known volume of extract is dried by rotavapor and resuspended in a calculated volume of MeOH/H_2O 70:30 mixture in order to obtain the desired concentration factor;

. each sample was centrifuged at 12000 rpm for 10 minutes before injection in HPLC.

Quantitative determination

Quantification of Fumonisins was performed by applying the following formula:

$$FB_1\ (ng/g) = C \times Solvent \times Res. / W \times Aliquot\ (ng \times ml / g \times ml)$$

$$FB_2\ (ng/g) = C \times Solvent \times Res. / W \times Aliquot\ (ng \times ml / g \times ml)$$

$$FB_3\ (ng/g) = C \times Solvent \times Res. / W \times Aliquot\ (ng \times ml / g \times ml)$$

i.e.:

$$FB_1\ (ng/g) = C \times 0.5 \times H$$

$$FB_2\ (ng/g) = C \times 0.5 \times H$$

$$FB_3\ (ng/g) = C \times 0.5 \times H$$

where:

. C: Concentration found in the injected solution, by calibration curve

. Solvent: Volume of solvent used for the extraction (50 ml)

. Res.: Final volume used to resuspend the sample (2 ml)

. W: Weighted sample material (5 g)

. Aliquot: Volume of extract recovered (40 ml)

. H: Recovery

Highly contaminated samples were analyzed by injecting directly the extracted surnatant, after high speed centrifugation; then the following formula was applied :

$$FB_1\ (ng/g) = C \times Solvent / W\ (ng \times ml / ml \times g)$$

$$FB_2\ (ng/g) = C \times Solvent / W\ (ng \times ml / ml \times g)$$

$$FB_3\ (ng/g) = C \times Solvent / W\ (ng \times ml / ml \times g)$$

i.e.

$$FB_1\ (ng/g) = C \times 10 \times H$$

$$FB_2\ (ng/g) = C \times 10 \times H$$

$$FB_3\ (ng/g) = C \times 10 \times H$$

where:

. C: Concentration found in the injected solution, by calibration curve

. Solvent: Volume of solvent used for the extraction (50 ml)

. W: Weighted sample material (5 g)

. H: Recovery

4.6.2 Sample preparation for total Fumonisins evaluation

Sample preparation for the analysis of total Fumonisins, on both maize and gluten-free products, was performed as follows:

. 5 grams of previously ground sample were hydrolysed with 50 ml of 2 M KOH for 60 minutes (10 min. Turrax + 50 min. magnetic stirring);

. 50 ml of ACN were added and homogenised for 10 minutes by Ultra Turrax;

. the organic phase was recovered by centrifuge; one aliquots was directly analysed, after resuspension in a MeOH/H_2O 70:30 mixture, to understand if the sample fits in the calibration range.

. highly contaminated samples were analyzed directly. For low contaminated products a known volume of organic phase was dried by rotavapor and resuspended in a calculated volume of MeOH/H_2O 70:30 mixture in order to obtain the desired concentration factor,

. each sample was centrifuged at 12000 rpm for 10 minutes before injection in the HPLC.

Quantitative determination:

Quantification of hydrolysed Fumonisins was performed by applying the following formula:

$$HFB_1\ (ng/g) = [C \times Solvent \times Res. / W \times Aliquot]\ (ng \times ml / ml \times g)$$

$$HFB_2\ (ng/g) = [C \times Solvent \times Res. / W \times Aliquot]\ (ng \times ml / ml \times g)$$

$$HFB_3\ (ng/g) = [C \times Solvent \times Res. / W \times Aliquot]\ (ng \times ml / ml \times g)$$

i.e.:

$$HFB_1\ (ng/g) = C \times 0.5 \times H$$

$$HFB_2\ (ng/g) = C \times 0.5 \times H$$

$$HFB_3\ (ng/g) = C \times 0.5 \times H$$

where:

. C: Concentration found in the injected solution, by calibration curve

. Solvent: Volume of solvent used for the hydrolysis/extraction (50 ml)

. Res.: Final volume used to resuspend the sample (2 ml)

. W: Weighted sample material (5 g)

. Aliquot: Volume of extract recovered (40 ml)

. H: Recovery

Highly contaminated samples were analyzed by injecting directly the extracted surnatant resuspended in a calculated volume of MeOH/H_2O 70:30 mixture, after high speed centrifugation.

The formula used are following reported:

$$HFB_1 \text{ (ng/g)} = [C \times Solvent / W \times Aliquot] \times K \text{ (ng x ml / ml x g)}$$

$$HFB_2 \text{ (ng/g)} = [C \times Solvent / W \times Aliquot] \times K \text{ (ng x ml / ml x g)}$$

$$HFB_3 \text{ (ng/g)} = [C \times Solvent / W \times Aliquot] \times K \text{ (ng x ml / ml x g)}$$

i.e.:

$$HFB_1 \text{ (ng/g)} = C \times 10 \times K \times H$$

$$HFB_2 \text{ (ng/g)} = C \times 10 \times K \times H$$

$$HFB_3 \text{ (ng/g)} = C \times 10 \times K \times H$$

where:

. C: Concentration found in the injected solution, by calibration curve

. Solvent: Volume of solvent used for the hydrolysis/extraction (50 ml)

. W: weighted sample material (5 g)

. H: Recovery

Total Fumonisins, or FBs equivalent, can be calculated from HFBs, by the following formula:

$$tot\ FB_1 \text{ (or } FB_1 \text{ equivalents) (ng/g)} = HFB_1 \text{ (ng/g)} \times K$$

$$tot\ FB_2 \text{ (or } FB_2 \text{ equivalents) (ng/g)} = HFB_2 \text{ (ng/g)} \times K$$

$$tot\ FB_3 \text{ (or } FB_3 \text{ equivalents) (ng/g)} = HFB_3 \text{ (ng/g)} \times K$$

where:

. K : Costant derived from the molecular weights ratio between the hydrolysed form and the native form (mw HFB/mw FB).

4.6.3 Sample preparation for total Fumonisins extracted

Evaluation of the total Fumonisin in the extract for free FB evaluation, was performed on maize samples as follows:

. the extract for free FB evaluation was divided in 2 aliquots;

. the aliquots for total FB evaluation was dried by rotavapor, resuspended in 50 ml of 2 M KOH and let to react 1 hour at room temperature with magnetic stirring;

. 50 ml of ACN were added and stirred for 10 minutes;

. the organic phase was recovered by centrifuge and a known volume is directly analysed, after resuspension in a MeOH/H_2O 70:30 mixture.

. Each sample was centrifuged at 12000 rpm for 10 minutes before injection in HPLC.

Quantitative determination:

Quantification of the hydrolysed Fumonisins was performed by applying the following formula:

$$HFB_1\ (ng/g) = [C \times S1 \times S2 \times Res. / W \times S3 \times Aliquot]\ (ng \times ml / ml \times g)$$

$$HFB_2\ (ng/g) = [C \times S1 \times S2 \times Res. / W \times S3 \times Aliquot]\ (ng \times ml / ml \times g)$$

$$HFB_3\ (ng/g) = [C \times S1 \times S2 \times Res. / W \times S3 \times Aliquot]\ (ng \times ml / ml \times g)$$

i.e.:

$$HFB_1\ (ng/g) = C \times 0.8 \times H$$

$$HFB_2\ (ng/g) = C \times 0.8 \times H$$

$$HFB_3\ (ng/g) = C \times 0.8 \times H$$

where:

. C: Concentration found in the injected solution, by calibration curve

. S1: Volume of solvent used for the first extraction (50 ml)

. S2: Volume of solvent used for the hydrolysis/extraction (50 ml)

. S3: Volume of aliquots from the first extraction (20 ml)

. Re+s.: Final volume used to resuspend the sample (2 ml)

. W: weighted sample material (5 g)

. Aliquot: Volume of extract recovered (40 ml)

. H: Recovery

Total Fumonisins, or FBs equivalent, has been calculated from HFBs, by the following formula:

$$tot\ FB_1\ (or\ FB_1\ equivalents)\ (ng/g) = HFB_1\ (ng/g) \times K$$

$$tot\ FB_2\ (or\ FB_2\ equivalents)\ (ng/g) = HFB_2\ (ng/g) \times K$$

$$tot\ FB_3\ (or\ FB_3\ equivalents)\ (ng/g) = HFB_3\ (ng/g) \times K$$

where:

. K : Costant derived from the molecular weights ratio between the hydrolysed form and the native form (mw HFB/mw FB).

4.7 Hidden Fumonisin: understanding the masking mechanism

4.7.1 Association with maize protein

The Osborne fraction were collected as follows:

. 5 grams of previously ground sample were extracted with 50 ml of bistilled water for the globulin fraction, by magnetic stirring for 60 minutes;

. the surnatant was recovered by centrifuge and divided in 2 aliquots;

. the solid residue was recovered and extracted with 50 ml of 0.2 M NaCl for albumins, by magnetic stirring for 60 minutes;

. the surnatant was recovered by centrifuge and divided in 2 aliquots;

. the solid residue was recovered and extracted with 50 ml of EtOH/H_2O 60:40 for prolamines, by magnetic stirring for 60 minutes;

. the surnatant was recovered by centrifuge, and divided in 2 aliquots;

. the solid residue was recovered and extracted with 50 ml of Isopropanol/Water 50:50 + 2% DTT for glutelins, by magnetic stirring for 60 minutes;

. the surnatant was recovered by centrifuge and divided in 2 aliquots;

. the solid residue was recovered and hydrolysed with 50 ml of KOH 2 M for 60 minutes (10 min. Turrax + 50 min. magnetic stirring);

. 50 ml of ACN were added and the mixture was homogenised for 10 minutes by Ultra Turrax;

. a known volume of the organic phase was recovered by centrifuge and resuspended in the same volume of MeOH/H_2O 70:30 mixture.

One aliquot of each Osborne fraction was analyzed for free Fumonisin evaluation by the following procedure:

. a known volume of each fraction was dried by rotavapor and resuspended in a calculated volume of a MeOH/H_2O 70:30 mixture, in order to fit in the calibration range;

. the resuspended sample was centrifuged at 12000 rpm for 10 minutes before injection in HPLC.

Quantitative determination:

Due to the different extraction procedure, data of free FBs in Osborne fraction has been not corrected for the recovery.

Quantification of Fumonisins was performed by applying the following formula:

$$FB_1\ (ng/g) = C \times Solvent \times Res. / W \times Aliquot\ (ng \times ml / ml \times g)$$

$$FB_2\ (ng/g) = C \times Solvent \times Res. / W \times Aliquot\ (ng \times ml / ml \times g)$$

$$FB_3\ (ng/g) = C \times Solvent \times Res. / W \times Aliquot\ (ng \times ml / ml \times g)$$

i.e.:

$$FB_1\ (ng/g) = C$$

$$FB_2\ (ng/g) = C$$

$$FB_3\ (ng/g) = C$$

where:

. C: Concentration found in the injected solution, by calibration curve

. Solvent: Volume of solvent mixture used for the extraction (50 ml)

. Resuspension: Final volume used to resuspend the sample (2 ml)

. W: Weighted sample material (5 g)

. Aliquot: Volume of the aliquot (20 ml)

On the second aliquots of each Osborne fraction total Fumonisins were evaluated by the following procedure:

. the aliquots for total FB evaluation were dried by rotavapor, resuspended in 50 ml of 2 M KOH and let to react 1 hour at room temperature with magnetic stirring;

. 50 ml of ACN were added and stirred for 10 minutes.

. a known volume of the organic phase was recovered by centrifuge, dried by rotavapor and respuspended in a calculated volume of a MeOH/H_2O 70:30 mixture, in order to fit in the calibration range;

. Each sample was centrifuged at 12000 rpm for 10 minutes before injection in HPLC.

Quantitative determination:

Quantification of hydrolysed Fumonisins was performed by applying the following formula :

$$HFB_1 \text{ (ng/g)} = [C \times S1 \times S2 \times Res. / W \times S3 \times Aliquot] \text{ (ng x ml / ml x g)}$$

$$HFB_2 \text{ (ng/g)} = [C \times S1 \times S2 \times Res. / W \times S3 \times Aliquot] \text{ (ng x ml / ml x g)}$$

$$HFB_3 \text{ (ng/g)} = [C \times S1 \times S2 \times Res. / W \times S3 \times Aliquot] \text{ (ng x ml / ml x g)}$$

i.e.:

$$HFB_1 \text{ (ng/g)} = C \times 0.8 \times H$$

$$HFB_2 \text{ (ng/g)} = C \times 0.8 \times H$$

$$HFB_3 \text{ (ng/g)} = C \times 0.8 \times H$$

where:

. C: Concentration found in the injected solution, by calibration curve

. S1: Volume of solvent used for the first extraction (50 ml)

. S2: Volume of solvent used for the hydrolysis/extraction (50 ml)

. S3: Volume of aliquots from the first extraction (20 ml)

. Res.: Final volume used to resuspend the sample (2 ml)

. W: weighted sample material (5 g)

. Aliquot: Volume of extract recovered (40 ml)

. H: Recovery

Total Fumonisins, or FBs equivalent, has been calculated from HFBs, by the following formula:

$$tot\ FB_1 \text{ (or } FB_1 \text{ equivalents) (ng/g)} = HFB_1 \text{ (ng/g) x } K$$

$$tot\ FB_2 \text{ (or } FB_2 \text{ equivalents) (ng/g)} = HFB_2 \text{ (ng/g)x } K$$

$$tot\ FB_3 \text{ (or } FB_3 \text{ equivalents) (ng/g)} = HFB_3 \text{ (ng/g)x } K$$

where:

. K : Costant derived from the molecular weight ratio between the molecular weight of hydrolysed form and that of the native form (MW HFB/MW FB).

4.7.2 The α-Zein model

The operative conditions used for the UPLC-MS analysis of α-Zein are reported in the next tables.

Tab. 78: Conditions of the ion souce used in the UPLC-MS analysis of α-Zein.

Parameter	***Value***
Polarity	ES+
Capillary	3.30 kV
Cone	30.00V
Extractor	3.00 V
RF Lens	0.2 V
Source Temperature	120 °C
Desolvation Temperature	350 °C
Cone Gas Flow	50 L/hr
Desolvation Gas Flow	850 L/hr

Tab. 79: Mass analyzer conditions used in the UPLC-MS analysis of α-Zein.

Parameter	***Value***
LM 1 Resolution 9.4	11.2
HM 1 Resolution 8.5	14.8
Ion Energy 1	0.3 eV
Multiplier (V)	650

Separation was performed using a 2.1 x 15 mm, BEH 300 1.7 µm, C_{18} column. The flow rate used in the analysis was 0.2 ml/min, with an injection volume of 10 µl. The gradient used for the
UPLC-MS analysis of α-Zein, is reported in the next table.

Tab. 80: Mobile phase gradient used in the MRM analysis of Fumonisin.

Time	***% A*** ***(H_2O + 0.2 % HCOOH)***	***% B*** ***(ACN + 0.2 % HCOOH)***
0	85	15
77	50	50
87	50	50
89	0	100
92	0	100
93	85	15
108	85	15

Chymotryptic digestion of the α-Zein standard was performed as follows:
. the chymotrypsin stock solution was prepared dissolving 10 mg of bovine chymotrypsin (from Sigma) in 10 ml of 50 mM CH_3COOH;
. The α-Zein standard stock solution was prepared dissolving 10 mg of α-Zein in 10 ml of MeOH,
. Ammonium bicarbonate buffer 50 mM pH = 7.3 was prepared dissolving the weighted amount of salt in 100 ml of bidistilled water, pH was adjusted with NH_3.
. Calculated volumes of each stock were placed in a 4 ml vial in order to have:

- protein amount = 0.5 mg
- enzyme/protein ratio 1:25
- final volume of 200 µl
- final concentration of methanol = about 30 %

The control experiment was performed without the enzyme. The solutions were let to react for 5 hours at 37 °C in a water bath. The reaction was stopped by lowering temperature in a cold

water bath. Each sample was centrifuged at 12000 rpm for 10 minutes before the UPLC-MS analysis under the conditions previously reported.

4.7.3 In vitro reaction between α-Zein and Fumonisin B_1

The *in vitro* reaction between FB_1 and the α-Zein standard was performed as follows:

. Fumonisin B_1 in stock solution was prepared dissolving 1 mg of standard of FB_1 (from Sigma) in 2 ml of MeOH;

. the α-Zein standard stock solution was prepared dissolving 10 mg of α-Zein in 10 ml of MeOH,

. the calculated volumes of each stock were placed in a 4 ml vial in order to have different molar ratio between FB_1 and α-Zein (the amount of FB_1 was the same in each test):

1) FB_1 alone at room temperture (control)

2) FB_1 alone heated at 150 °C (control)

3) FB_1 + α-Zein molar ratio 5:1

4) FB_1 + α-Zein molar ratio 1:2

Each vial was dried under nitrogen, then the samples 2,3 and 4 were let to react 5 hours at 150 °C for 90 minutes in an oil bath. The reaction was stopped by lowering temperature in a cold water bath. Each sample was resuspended in 1 ml of MeOH and centrifuged at 12000 rpm for 10 minutes before the HPLC-MS/MS for FB_1 analysis with the previous reported conditions.

The experiment was repeated also with an α-Zein chymotryptic digested obtained in the previous experiment, by using the same conditions but a minor time (60 min. instead 90 min.).

4.7.4 Preliminary evidence for inclusion-like mechanism of FB_1 masking

Preliminary experiments on maize prolamine fractions, extracted from highly contaminated maize sample, were performed as follows :

. 20 g of ground maize were extracted twice with 100 ml of bidistilled water, by magnetic stirring for 1 hour,; extracts were discarded, the residue was then air dried over night. The day after it was extracted 4 times with ethanol:water 60:40, by stirring for 1 hour. Extracts were collected, dried with rotavapor, weighted and resuspended in 50 ml of methanol. On 4 aliquots of the prolamines fraction extracted, several treatments able to release the protein bound Fumonisin were performed.

1) The control experiment was carried out by diluting a calculated volume of prolamine fraction to a final volume of 200 µl with methanol.

2) A calculated volume of the resuspended prolamines fraction, corresponding to about 0.5 mg of solid extract, was diluted with 1:5 methanol:ammonium bicarbonate buffer. A calculated volume of chymotrypsin stock solution was added, in order to have 1:25 enzyme/solid extract mass ratio, and allowed to react for 5 hours at 37 °C. The reaction was then stopped by adding methanol to a final volume of 200 µl. Control was made by diluting an equal volume of extract with the same buffer but without enzyme.

3) A calculated volume of resuspended prolamine fraction, corresponding to about 0.5 mg of solid extract, was dried under a nitrogen flow, resuspended in 2 M KOH, then allowed to react 1 hour at room temperature. After that, it was extracted twice with acetonitrile, the extracts were combined, dried under nitrogen flow, then resuspended in 200 µl methanol.

*4)*A calculated volume of resuspended prolamine fraction, corresponding to about 0.5 mg of solid extract, was diluted with 8 M urea and allowed to rest 5 hours at room temperature. Then it was diluted with methanol to a final volume of 200 µl.

All samples were centrifugated 10 minutes at 12000 rpm before LC-MS/MS analysis of Fumonisin B_1 under the condions previously reported.

The preliminary ESI-MS titration experiment was performed as follows :

. Fumonisins B_1 in stock solution was prepared dissolving 1 mg of standard of FB_1 (from Sigma) in 2 ml of MeOH;

. the α-Zein standard solution was prepared dissolving a weighted amounts of α-Zein in 100 ml of MeOH/H_2O 70:30 mixture, in order to have a 250 µM stock; then it was used to prepare a 50 µM dilution;

. the calculated volumes of each standard solution was placed in a 4 ml vial in order to have a different molar ratio between FB_1 and α-Zein

1) FB_1 + α-Zein molar ratio 2 (10:5 µM/µM)

2) FB_1 + α-Zein molar ratio 1 (5:5 µM/µM)

3) FB_1 + α-Zein molar ratio 0.5 (5:10 µM/µM)

Each vial was dried under nitrogen, resuspended with a calculated volume of MeOH, in order to obtain the desired concentrations and centrifugated 10 minutes at 12000 rpm before infusion in ESI-MS, under the conditions reported in the following tables.

Tab. 81: Conditions of the ion souce used in the ESI-MS titration.

Parameter	***Value***
Polarity	ES+
Capillary	3.20 kV
Cone	30.00V
Extractor	4.00 V
RF Lens	0.2 V
Source Temperature	100 °C
Desolvation Temperature	200 °C
Cone Gas Flow	50 L/hr
Desolvation Gas Flow	600 L/hr

Tab. 82: Mass analyzer conditions used in the ESI-MS titration.

Parameter	*Value*
LM 1 Resolution	15
HM 1 Resolution	15
Ion Energy 1	1
Entrance	15
Collision energy	0
Exit 2	15
LM 2 Resolution	15.0
HM 2 Resolution	15.0
Ion Energy 2	2.0
Multiplier (V)	650
Gas Cell Pirani Pressure	< $1e^{-4}$ mbar

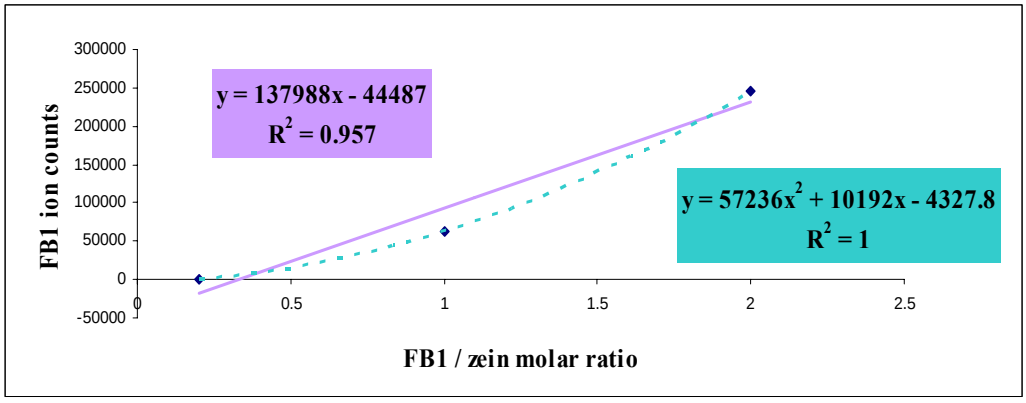


Fig. 79: Correlation curve between FB_1 ion counts in ESI-MS and molar ratio of FB_1 and α-Zein in the infused solutions, violet curve : linear regression, blue curve : quadratic regression.

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Curriculum Vitae

Mattia Mangia born in Parma in 1982 and got the Bachelor Degree in Food Science and Technology (University of Parma, 110/110), in 2005, with a thesis titled "Safety and quality parameters of red wine: Ochratoxin A and Resveratrol". In 2006 he got the Master Degree in Food Science and Technology (University of Parma, 110/110 Honors) with a graduation thesis entitled "Aflatoxin and Ochratoxin production from different fungal biotypes". During the Bachelor Degree (3 years) and the Master Degree (2 years), he received a multisubject education characterized by a "core curriculum" based on Organic and Food Chemistry, Biochemistry and Molecular Biology, Product and Process Planning, Food Microbiology and Human Nutrition. In January 2007 he got the professional abilitation in Food Science and Technology. In 2007 Mattia Mangia start the Ph.D. in Food Science and Technology (University of Parma), under the supervision of prof. Rosangela Marchelli.
The Ph.D. work has been focused on the development of a LC-MS/MS method for the evaluation of hidden Fumonisins. The application of the method to several batches of gluten free products and raw cereal let to discover that hidden FBs are widespread along the maize chain. Moreover, further experimental data collected in the work strongly suggest a non-covalent interaction between FB_1 and the α-Zein (the principal endosperm protein of maize), with an inclusion-like mechanism, to explain the masking mechanism of Fumonisins.

SCIENTIFIC ACTIVITY:

1) **Poster** titled : '*Free and masked Fumonisins in corn and corn-based products*'
M. Mangia, G. Galaverna, C. Dall'Asta, S. Sforza, A. Dossena e R. Marchelli.
Mycotoxins Symposium 25-26 October 2007 Karlsruhe, Germany.

2) **Oral communication** titled : '*Free and masked Fumonisins in corn and corn-based products*'
12° Mold Meeting AGES (Austrian Authority for food safety)
5-6 December 2008, Linz, Austria.

3) **Pubblication** on ISI journal titled :
'Free and bound Fumonisins in gluten-free food products'
C. Dall'Asta, G. Galaverna, M. Mangia., S. Sforza, A. Dossena, R. Marchelli.
MOLECULAR NUTRITION & FOOD RESEARCH, 2009, 53.

4) **Pubblication** on ISI journal titled:

'Masked Mycotoxins: an Emerging Issue for Food Safety'

G. Galaverna, C. Dall'Asta, M. Mangia, A. Dossena, R. Marchelli.

CZECH JOURNAL OF FOOD SCIENCE, 2009, 27 (special issue)

5) **Pubblication** on ISI journal titled:

'Difficulties in fumonisin determination: the issue of hidden Fumonisins'

C. Dall'Asta, M. Mangia, F. Berthiller, A. Molinelli, M. Sulyok, R. Schuhmacher, R. Krska, G. Galaverna, A. Dossena, R. Marchelli.

ANALYTICAL AND BIOANALYTICAL CHEMISTRY, 2009, pubblished on line.

6) **Oral communication** titled : *'Hidden Fumonisins in maize and gluten free food product'*

14° Workshop on the development of the Italian Ph.D. research on Food Science Technology and Biotechnology.

16-18 September 2009, Oristano, Italia.

7) **Poster** titled: *'Maize prolamines: hypothetical role in Fumonisins masking'*

1° MS Food Day - Barilla Inc.

2-3 December 2009, Parma, Italy.