UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA

PhD in AGRI-FOOD SCIENCES, TECHNOLOGIES AND BIO-TECHNOLOGIES - UNIMORE

XXXIV CYCLE

DEVELOPMENT OF NEW ANALYTICAL METHODS FOR DETERMINATION OF KNOWN AND UNKNOWN TOXIC AGENTS IN FOOD CHAIN.

Identification and quantification of Mycotoxins produced by *Alternaria* in food and feed by LC-MS/MS and LC/HRMS

PhD student: Dr **Vita Giaccone**

Tutor(s): **Prof. Andrea Pulvirenti**

Coordinatore del Corso di Dottorato: **Prof. Alessandro Ulrici**

TABLE OF CONTENTS

RIASSUNTO

Le micotossine sono contaminanti significativi negli alimenti e nei mangimi e sono causa di gravi effetti tossici sulla salute umana e animale. Diversi studi hanno dimostrato l'esistenza di una moltitudine di metaboliti fungini che contaminano alimenti e mangimi. Recentemente, le autorità sanitarie internazionali hanno espresso preoccupazione per la presenza di micotossine "emergenti" negli alimenti. Tra le specie fungine responsabili della produzione di tali metaboliti, la specie *Alternaria* ha la capacità di produrre più di 70 tossine, come Alternariol (AOH), Alternariol monomethyl ether (AME), Tenuazonic Acid (TeA), Altenuene (ALT) e Tentoxin (TEN), presenti nei cereali, in prodotti a base di pomodoro, nell'olio d'oliva, nella frutta e nella verdura fresca.

Queste micotossine sono state, recentemente, oggetto di pareri scientifici dell'Autorità Europea per la Sicurezza Alimentare (EFSA), che ha espresso preoccupazione per la scarsa disponibilità di dati sull'incidenza negli alimenti destinati al consumo umano e animale. Esiste, infatti, un alto grado di incertezza legato alla rappresentatività degli alimenti attualmente testati. Inoltre, la mancanza di informazioni sul metodo analitico utilizzato contribuisce ulteriormente a creare incertezza sui livelli di tossine da *Alternaria*.

Sono necessari ulteriori studi per aggiornare la valutazione del rischio da parte dell'EFSA e, pertanto, l'obiettivo specifico di questo progetto era incentrato sullo sviluppo di metodi LC-MS/MS e LC/HRMS accurati e precisi per l'analisi delle tossine da *Alternaria*. Il progetto è stato sviluppato in collaborazione con l'Istituto Zooprofilattico Sperimentale della Sicilia. Il metodo LC-MS/MS è stato utilizzato per l'analisi target delle micotossine emergenti nelle matrici alimentari. In particolare sono state svolte le seguenti attività:

• sviluppo di un metodo analitico multiresiduale per lo screening simultaneo di Aflatossine (AFB1, AFB2, AFG1 e AFG2), Alternariol (AOH), Alternariol monometiletere (AME), Altenuene (AL), Tentoxin (TEN), Acido tenuazonico (TeA), Ocratossina e Zearalenone (ZEA), Fumonisina B1, B2, B3, tossina T-2, tossina HT-2 e deossinivalenolo (DON);

• sviluppo di un protocollo di estrazione e purificazione delle matrici, da applicare a differenti tipi di cereali; questa fase preparativa è stata ottimizzata confrontando diversi protocolli di estrazione e purificazione:

- ✓ *Estrazione con Acetonitrile acidificato;*
- ✓ *Estrazione e successivo clean-up con matrici solide assorbenti;*
- ✓ *Estrazione e purificazione con QUECHERS;*
- ✓ *Purificazione on-line mediante Turboflow™ Technologies;*

• validazione del metodo secondo le linee guida comunitarie (Decisione della Commissione 2002/657/CE); il metodo è stato ottimizzato valutando i seguenti parametri: specificità, precisione, linearità strumentale, recupero, limite di decisione (CCα), capacità di rilevamento (CCβ), effetto matrice e robustezza.

Procedura analoga è stata condotta mediante Cromatografia liquida accoppiata alla Spettrometria di Massa ad Alta Risoluzione (UHPLC-Q-HRMS); è stato sviluppato un metodo per la determinazione, sensibile e specifica, non solo delle tossine da *Alternaria,* ma anche per le restanti molecole oggetto di studio di questo progetto. Le prestazioni ottenute sono state confrontate con quelle ottenute mediante cromatografia liquida e spettrometria di massa a triplo quadrupolo. Tutti e due i metodi hanno mostrato una buona linearità e ripetibilità. E' emerso che lo spettrometro di massa Q-Exactive sia più adatto per il rilevamento in tracce rispetto ai metodi MS/MS basati sul triplo quadrupolo, perché, nonostante abbia prestazioni comparabili, ha una migliore selettività.

Il progetto di ricerca avrebbe dovuto fornire un'ampia indagine sul contenuto emergente di micotossine in vari prodotti alimentari, contribuendo a una stima dell'esposizione al rischio di micotossine. Le difficoltà legate alla pandemia in corso hanno reso difficile la, inizialmente prevista in questo progetto.

Nonostante tutto, lo scopo di questo lavoro era colmare la lacuna di dati e fornire informazioni pertinenti per migliorare la sicurezza dei prodotti locali. Pertanto, il metodo validato è stato applicato per lo screening delle micotossine in 26 campioni di grano duro e 24 campioni di farina bianca, prodotti in Sicilia e raccolti negli ultimi due anni.

ABSTRACT

Mycotoxins are significant contaminants in food and feeds. These molecules have demonstrated serious effects in both human and animal health. Different studies showed the presence of a multitude of fungal metabolites that contaminate food and feed. Recently, the international health authorities have expressed concerns about the presence of "emerging" mycotoxins in foodstuffs. The term “emerging mycotoxins” is commonly referred to those compounds that are currently under the spotlight of the scientific community and the policy-makers, due to their toxicological profile. Among the fungal species responsible for the metabolite production, *Alternaria* species have the ability to produce more than 70 toxins, such as Alternariol (AOH), Alternariol monomethyl ether (AME), Tenuazonic Acid (TeA), Altenuene (ALT) and Tentoxin (TEN), found in cereals, tomato products, olive oil, fresh fruits and vegetables. These mycotoxins have been recently covered by scientific opinions from the European Food Safety Authority (EFSA), who has expressed concern about the low availability of incidence data in food for human and animal consumption. There is, indeed, a high degree of uncertainty related to the representativeness of the food currently tested because it contains too many inaccuracies. Furthermore, the lack of information on the analytical method used further contributes to the uncertainty of the reported *Alternaria* toxin levels.

More comprehensive studies are required to update the risk evaluation by the EFSA and, therefore, the specific aim of this project was focussed on the development of accurate LC-MS/MS and LC/HRMS methods for the analysis of emerging *Alternaria* toxins. The protocol was employed in collaboration with the Istituto Zooprofilattico Sperimentale della Sicilia. LC-MS/MS method was used for the target analysis of emerging mycotoxins in food matrices. In particular, the following activities were carried out:

- development and optimization of the analytical method multi-parameters for simultaneous screening of Alternariol (AOH), Alternariol monomethyl ether (AME), Tenuazonic Acid (TeA) Altenuene (AL), Tentoxin (TEN), Ochratoxins and Zearalenone (ZEA), Fumonisin B1, B2, B3, T-2 toxin, HT-2 toxin, Aflatoxins and deoxynivalenol (DON);
- development of a matrix extraction and purification protocol for different types of cereals; this preparatory phase has been optimized by comparing more extraction and purification protocols:
  - ✓ *Extraction with acidified Acetonitrile;*
  - ✓ *Extraction and clean-up with absorbent solid matrices;*
  - ✓ *Extraction and clean-up with QUECHERS;*
- optimization of an online sample purification using a TurboFlow system with subsequent analysis by LC-HRMS.

- validation of the method according to EU guidelines (Commission Decision 2002/657/EC); the method was performed by evaluating the following parameters: specificity, precision, instrumental linearity, recovery, decision limit (CCα), detection capability (CCβ), matrix effect and ruggedness.

The ultrahigh-performance chromatography coupled to quadrupole-orbitrap high-resolution mass spectrometry (UHPLC-Q-HRMS) was applied to the sensitive and specific determination of the emerging *Alternaria* toxins. Performances were compared to those obtained by high-performance liquid chromatography detection. All two methods showed good linearity and repeatability. The Q-Exactive mass spectrometer is better suitable for trace detection than state-of-the-art MS/MS methods based on the triple quadrupole, because, despite having comparable performance, it has better selectivity.

The research project should have provided a broad investigation of the emerging mycotoxin content in various food products, contributing to an estimate of mycotoxin risk exposure. The difficulties associated with the ongoing pandemic have made it difficult to analyse a larger number of foods, initially planned within this project. Despite everything, the purpose of this work was to fill the data gap and provide relevant information to improve the safety of local products. Therefore, the validated method was applied to screen mycotoxins in 26 samples of durum wheat grain and 24 samples of white flour, produced in Sicily and collected in the last two years.

1. INTRODUCTION

Mycotoxins are low-molecular-weight secondary fungal metabolites that can contaminate various food and feed commodities, including but not limited to grain and grain-based products, vegetables, fruits and fruit juices, oil seeds and oils, spices, coffee and wine. They may enter the food chain worldwide because of the ability of mycotoxin-producing molds to infect a wide number of crops and food commodities. They are produced by fungal infection of agricultural crops while in the field or during harvest, drying or subsequent storage. These compounds are very stable and cannot be readily destroyed by heating or food processing, although some processes, such as the milling of grains, can reduce the level in the product. Food crops susceptible to mycotoxin contamination include corn, wheat, barley, rye, rice, nuts, dried fruit, vegetables, and their derivatives. It is remarkable that cereals and cereal by-products that are often unfit for human consumption are frequently used, in feed formulations and act as excellent substrates for the fungal proliferation and production of mycotoxins. Recent surveys indicate that 70% of raw materials are contaminated with these toxins Good Agricultural Practices (GAP) are important to ensure that fungal infection is minimized and food and feed are produced with the lowest levels of mycotoxins achievable.

From about 200 identified filamentous fungi, the most prevalent toxigenic species belong to the genera *Aspergillus, Fusarium, Penicillium*, and *Alternaria*. *Fusarium* and *Alternaria* usually represent a high mycotoxicological risk at pre-harvest level or in freshly harvested products on drying, whereas *Aspergillus* and *Penicillium* toxigenic species pose a higher risk for stored food and feed products or other sorts of processing. Approximately 400 mycotoxins are known, but only a few are regulated by legislation.

The reason for mycotoxins production is not yet known since they seem not to be necessary for growth nor the development of fungi. Moreover, it is genotypically specific but not limited to one species or one toxin per species. Several factors such as environmental and ecological conditions, temperature, relative humidity, substrate and use of fungicides, contribute to mycotoxin presence or production in food and feed, however, the interrelations between all these factors are not well understood and toxin production cannot reasonably be predicted. Since temperature and humidity are important parameters for the growth of fungi climate change is anticipated to impact on the presence of mycotoxins. In particular, mycotoxins may pose a toxicological concern for humans and animals since they may exert a wide number of effects including acute toxic, mutagenic, carcinogenic, teratogenic, estrogenic and immunotoxic actions.

Due to their diverse chemical structures and biosynthetic origins, their myriad biological effects, and their production by a wide number of different fungal species, mycotoxins are not only hard to define, they are also challenging to classify.

For their clinic effects, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, but also, such as teratogens, mutagens, carcinogens, and allergens. Organic chemists have attempted to classify them by their chemical structures (e.g., lactones, coumarins); biochemists according to their biosynthetic origins (polyketides, amino acid-derived, etc.); mycologists by the fungi that produce them (e.g., *Aspergillus* toxins, *Penicillium* toxins). None of these classifications is entirely satisfactory.

Mycotoxin occurrence in food and feed is either consequence of direct contamination of plant materials or products thereof, or by carry-over of mycotoxins and their metabolites into animal tissues, milk, and eggs after contaminated feed intake. The term carry-over is often used to describe mycotoxin transfer from feed to edible tissues in order to enable a risk evaluation for the consumer arising from feeding mycotoxin-contaminated diets to food producing animals.

Mycotoxins pose a challenge to food safety as they are unavoidable and unpredictable contaminants in crops. In fact, the Food and Agriculture Organization (FAO) estimated that over one-quarter of the world's food crop are contaminated with mycotoxins. The mycotoxins with greatest agro-economic and health impact are aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), fumonisins (FBs), and trichothecenes (T-2 toxin, HT-2 toxin, deoxynivalenol and nivalenol). In the last decade, attention to the risk posed to human and animal health has also been extended to the so-called emerging *Fusarium* mycotoxins as well as the *Alternaria* toxins.

The term emerging mycotoxins is commonly referred to those compounds that, although still not covered by legislation, are currently under the spotlight of the scientific community and the policy-makers, due to their toxicological profile. However, lack of knowledge in terms of occurrence and toxicity data collection are hindering an accurate risk assessment and, therefore, the derivation of proper health based guidance values. The international health authorities have recently expressed concerns about the presence of "emerging" mycotoxins in cereal-based foods and feeds. Few published data are available on the occurrence of these toxins in commodities and the European Commission has not set maximum permitted levels in foods and feeds.

For some mycotoxins without any ML, the European Food Safety Authority (EFSA) has published opinions, often indicating the need of harmonized and suitable methods to make sound exposure assessments.

The assumption, on which the project is based, is the necessity to better assess the risk of the emerging mycotoxins. Any exposure assessment and any risk characterisation needs accurate data related to occurrence, contamination level and toxicity. Intense investigations have been conducted to study the traditional mycotoxins. However, one of the most know genus of fungi, *Alternaria* spp, is also capable of to produce other toxic metabolites showed to occur frequently in agricultural products. Studies,

that are more comprehensive, are required to update the risk evaluation by the EFSA. The main objective of this research project is to give to the scientific community more data on the chemistry, conditions of production and occurrence of the emerging mycotoxins, to understand deeply the role of these compounds in the human and animal food chain. The aim of this work is to improve the laboratory tests necessary to analyze mycotoxins contaminated products.

1.1 MAJOR MYCOTOXINS

1.1.1 Aflatoxins

Aflatoxins (B1, B2, G1 and G2) are considered the group of mycotoxins of greatest concern from a global perspective. Aflatoxins are mycotoxins produced by two species of *Aspergillus*, *A. flavus*, *A. parasiticus*, and in rare cases, by *A. nomius*, a fungus found especially in areas with hot and humid climates. Climate change is expected to have an impact on the presence of aflatoxins in food in Europe. As aflatoxins are known to be genotoxic and carcinogenic, exposure through food should be kept as low as possible. Aflatoxins can occur in foods such as groundnuts, tree nuts, maize, rice, figs and other dried foods, spices, crude vegetable oils and cocoa beans, because of fungal contamination before and after harvest. Several types of aflatoxins are produced naturally. In addition to the above-mentioned four aflatoxins, these fungi also form other substances such as aflatoxicol and sterigmatocystin. Aflatoxin B1 is the most common in food and among the most potent genotoxic and carcinogenic aflatoxins. It is produced by both A. *flavus* and A. *parasiticus*. Aflatoxin M1 is a major metabolite of aflatoxin B1 in humans and animals, which may be present in milk from animals fed with aflatoxin B1 contaminated feed. Lactating animals fed aflatoxin B1contaminated diets will produce milk contaminated with its mono-hydroxylated derivative aflatoxin M1, classified as 2B, possibly carcinogenic to humans.

Aflatoxin production occurs primarily in regions with tropical or subtropical climates. Hence, from a European perspective, imported cereals is considered the most common source of exposure.

The main target organ of aflatoxin toxicity is the liver. Long-term exposure of animals to subacutely toxic levels of AFs is associated with liver lesions and/or tumours, inferior eggshell and carcass quality, increased disease susceptibility, reduced feed efficiency, and teratogenicity. Aflatoxin B1, aflatoxin G1 and aflatoxin M1 are carcinogenic when delivered orally via the diet or by gavage. There is limited evidence for the carcinogenicity of aflatoxin B2 and inadequate evidence for carcinogenicity of aflatoxin G2. Aflatoxin B1 is more potent than aflatoxin G1 with respect to liver carcinogenicity but aflatoxin G1 induced a higher incidence of kidney tumours than aflatoxin B1. Aflatoxin B1 is also more potent than aflatoxin M1 with respect to liver carcinogenicity by approximately 10-fold.

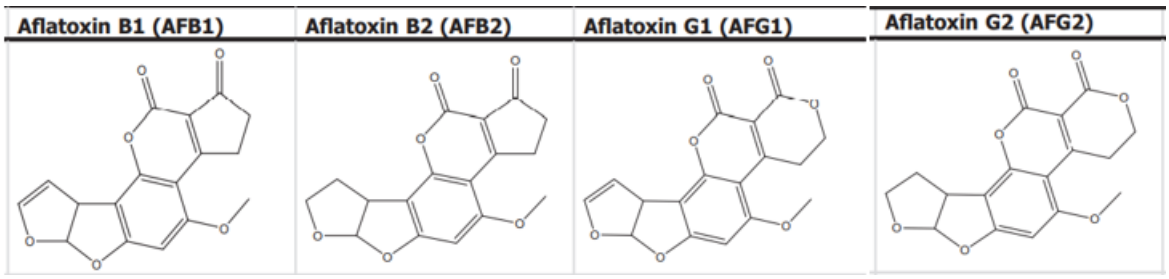


Figure 1 *Chemical structures of aflatoxins B1, B2, G1 and G2*

In order to protect public health, maximum levels (MLs) for aflatoxins and other mycotoxins in various foodstuffs were laid down in the Annex, Section 2 of Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting MLs for certain contaminants in foodstuffs. The MLs for aflatoxins are set following the principle of 'as low as reasonably achievable', derived from the frequency distribution of the respective food classes (usually at the 90–95th percentile), taking into account the outcome of the risk assessment and the analytical capabilities.

Maximum levels are set for aflatoxin B1 and the sum of aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 in tree nuts, apricot kernels, groundnuts (peanuts) and other oilseeds, dried fruit, cereals, and some species of spices as well as processed products thereof. For all cereals and their derivatives, including products processed from cereals, ML for aflatoxin B1 is **2,0 µg/kg** and for the sum of aflatoxins is **4,0 µg/kg.**

For aflatoxin B1, MLs are set also for baby food and processed cereal based food for infants and young children as well as in dietary foods for special medical purposes intended specially for infants. For this products, ML for aflatoxin B1 is **0,10 µg/kg.**

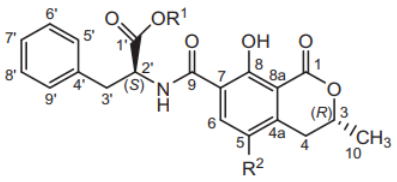
1.1.2 Ochratoxin A

Ochratoxins are mycotoxins produced by various fungi of the genus *Aspergillus* and *Penicillium*, e.g. *A. ochraceus*, *A. carbonarius* and *P. verrucosum*. Ochratoxins are stable to moderate heating, but losses ranging up to 90% are observed at temperatures above 180°C. Their chemical structures contain the amino acid L-phenylalanine linked via an amide bond to a substituted dihydroisocoumaric acid (Figure 2). The most prevalent and toxic ochratoxin is ochratoxin A (OTA), which is the subject of this study. A notable feature of the OTA structure is the chlorine substituent, which appears to be important for the toxicity of OTA. Ochratoxin B (OTB) is the non-chlorinated form of OTA, whereas ochratoxin C represents the ethyl ester of OTA (Figure 2). Other related mycotoxins formed at low levels by OTA-producing fungi are methyl esters of OTA and OTB, free dihydroisocoumaric acid and amides of OTalpha with serine, hydroxyproline and lysine. They are rapidly absorbed and distributed but slowly eliminated and excreted leading to potential accumulation in the body, which is due mainly to binding to plasma proteins and a low rate of metabolism. Plasma half-lifes range

from several days in rodents and pigs to several weeks in nonhuman primates and humans. The major metabolic pathway of OTA is hydrolysis to OTalpha, followed by conjugation with glucuronic acid. Formation of DNA-reactive metabolites is either minor or absent under physiological conditions. Based on efficient degradation in the rumen, OTA levels in cow milk are low. In several studies, relatively high OTA concentrations have been found in human milk in comparison with those found in cow milk. OTA exerts a variety of adverse effects in repeated dose studies in mice, rats, rabbits and pigs including immunotoxicity, neurotoxicity, developmental effects at maternally toxic doses. The critical effects occur in the kidney, the pig being the most sensitive animal species. Upon chronic oral exposure to OTA, increased incidences of kidney tumours have been observed in mice and rats, the rat being the most sensitive species.

The European Commission has established maximum levels for OTA, for unprocessed cereals, dried vine fruits (currants, raisins and sultanas), green coffee, roasted coffee beans, ground roasted coffee, Soluble coffee, wine and grape juice; these MLs are in a range from **0.5** to **10 µg/kg**. For all cereals and their derivatives, including products processed from cereals, ML for OTA is **3.0 µg/kg**, for baby food and processed cereal based food for infants, it is **0.5 µg/kg.**

Furthermore, Commission Recommendation (EC) 2006/576/EC5 on the presence of deoxynivalenol, zearalenone, OTA, T-2 and HT-2 toxin and fumonisins in products intended for animal feeding provides guidance levels for OTA for feed materials and complementary and complete feedingstuffs ranging from **0.05** to **0.25 mg/kg**.



Ochratoxin	Substituents	Molecular formula	Molar mass ($g \cdot mol^{-1}$)	CAS number
OTA	$R^1 = H$; $R^2 = Cl$	$C_{20}H_{18}ClNO_6$	403.8	303-47-9
OTB	$R^1 = H$; $R^2 = H$	$C_{20}H_{19}NO_6$	369.4	4825-86-9
OTC	$R^1 = C_2H_5$; $R^2 = Cl$	$C_{22}H_{22}ClNO_6$	431.9	4865-85-4

Figure 2: *Chemical structure of ochratoxin A, B and C*

1.1.3 Fusarium Toxins

From about 200 identified filamentous fungi, the most prevalent toxigenic species belong to the genera *Aspergillus, Fusarium, Penicillium*, and *Alternaria.*

Fusarium and *Alternaria* usually represent a high mycotoxicological risk at pre-harvest level or in freshly harvested products on drying, whereas *Aspergillus* and *Penicillium* toxigenic species pose a higher risk for stored food and feed products or other sorts of processing.

Fusarium genus includes over 90 described species and produces three of the most important classes of mycotoxins with respect to animal health and production; trichothecenes (TCTs), fumonisins (FBs), and zearalenones (ZEAs), and the less studied emerging mycotoxins; fusaproliferin (FUS), beauvericin (BEA), enniatins (ENs), and moniliformin (MON).

Trichothecenes toxins (TCTs) are produced by several fungal genera; however, most of them have been isolated from *Fusarium* spp. TCTs have been found to contaminate wheat, barley, corn, rice, rye, oats, and other crops. The effect of TCTs has been extensively studied on poultry and farm animals as TCTs contaminate feed largely. Epoxides are found in all TCTs at the C_{12} and C_{13} positions, which is responsible for its toxic activity. The widest variety of different trichothecene compounds includes the A-trichothecenes, T-2 toxin, HT-2 toxin, and the B-trichothecenes, deoxynivalenol (DON) and nivalenol (NIV). They are the most widespread and/or toxic compounds isolated from natural sources. DON inhibits the synthesis of DNA and RNA and protein synthesis at the ribosomal level. The toxin has a haemolytic effect on erythrocytes. Because of its strong emetic effects in both humans and animals, the US Department of Agriculture originally referred to it as "vomitoxin" but its diverse, toxic effects also include diarrhoea, anorexia, immunotoxicity, as well as impaired maternal reproduction and foetal development.

Furthermore, DON ingestion may also cause neuroendocrine changes, leukocytosis, haemorrhage, circulatory shock, and ultimately death. An acute dose of DON can induce vomiting (emesis) in pigs, whereas at lower concentrations in the diet it reduces growth and feed consumption (anorexia). Both effects, which are also seen with other trichothecene toxins, are thought to be mediated by affecting the serotonergic activity in the CNS or via peripheral actions on serotonin receptors. DON is a heat-resistant compound; thus, its deleterious effects should even be considered after culinary treatment of foods and feeds.

T-2 toxin and HT-2 toxin (Figure 3a) belong to a large group of approximately 180 trichothecenes discovered so far which are produced by *Fusarium* species. Trichothecenes have a common tetracyclic, sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system and are divided into four groups (A-D) according to their different chemical functionalities. The stable epoxide group between C_{12} and C_{13} seems to account for many of the typical toxic effects of trichothecenes.

T-2 toxin is readily metabolised to HT-2 toxin (and other substances) by various animals but can also be metabolised by plants and fungi. The structure of HT-2 toxin differs from T-2 toxin only in the functional group at the C4-position.

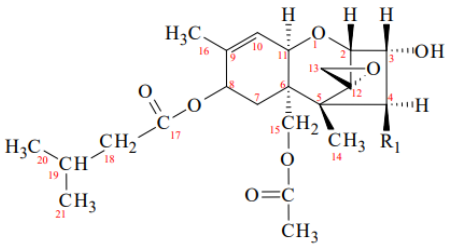


Figure 3a: *Chemical structure of T-2 toxin (R1 = OAc) and HT-2 toxin (R1 = OH).*

The available information on the toxicokinetics of T-2 and HT-2 toxins is incomplete. T-2 toxin is rapidly metabolised to a large number of products, HT-2 toxin being a major metabolite. The metabolic pathways include hydrolysis, hydroxylation, de-epoxidation, glucuronidation and acetylation. Distribution and excretion of T-2 toxin and its metabolites are rapid. There are no significant data available on the toxicity of most metabolites. De-epoxidation is believed to be a detoxification process. Grains and grain-based foods, in particular bread, fine bakery wares, grain milling products, and breakfast cereals, made the largest contribution to the sum of T-2 and HT-2 toxin exposure. For infants, the highest contributors were in the food group "Foods for infants and small children", mainly cereal-based foods. No significant difference in the dietary exposure to the sum of T-2 and HT-2 toxins was found between vegetarians and the general population, although the data were limited. The European Commission (EC) has asked the European Food Safety Authority (EFSA) for a scientific opinion on the risk to human and animal health related to the presence of T-2 and HT-2 toxin in food and feed. Various countries from all over the world have set MLs for T-2 toxin in feed products, ranging from 25 to 1000 µg/kg. MLs are laid down in Regulation (EC) No 1881/2006 of 19 December 2006, in which legal levels for T-2 and HT-2 toxins in unprocessed cereals and cereal products for human consumption are envisaged but are not established yet.

ZEN is a phenolic resorcylic acid lactone mycotoxin produced by several *Fusarium* species, particularly *F. graminearum*. ZEN can be modified in plants, fungi and animals by phase I and phase II metabolism. Modified forms of ZEN occurring in feed include its reduced phase I metabolites, i.e. a-zearalenol and b-zearalenol (a-ZEL and b-ZEL), a-zearalanol and b-zearalanol (a-ZAL and b-ZAL), zearalanone (ZAN) and its phase II derivatives, such as those conjugated with glucose, sulfate and glucuronic acid. a-ZAL, one of the phase I metabolites of ZEN, is used as a growth promoter in non-European Union (EU) countries under the name of Zeranol. It is banned in Europe, and therefore, it is included in official control plans. ZEA is a nonsteroidal estrogenic mycotoxin and it has been implicated in numerous mycotoxicoses in farm animals. It is a stable compound, both during storage/milling and the processing/cooking of food and it does not degrade at high temperatures. Studies of pharmacokinetics and metabolism indicate that ZEA is rapidly absorbed following oral administration and can be metabolised by intestinal tissue in pigs and possibly in humans during its

absorption, with the formation of α- and β-zearalenol and α- and β-zearalanol, which are subsequently conjugated with glucuronic acid. ZEA acute toxicity is relatively low but it strongly interferes with estrogen receptors and, consequently, affects the reproductive tract. Moreover, ZEA leads to decreased fertility, precocious puberty, changes in weight of the thyroid, adrenal, and pituitary glands; alteration of progesterone and estradiol levels in serum, fibrosis and hyperplasia in the uterus, breast cancer, endometrial carcinoma, and liver damages that may lead to liver cancer.

Currently, the Commission Regulation (EC) No 1881/2006 of 19 December 2006 lays down MLs for zearalenone. The MLs apply to the edible part of the foodstuffs unless it is otherwise specified. The MLs set for unprocessed cereals and unprocessed maize apply to unprocessed cereals placed on the market for first-stage processing. For unprocessed cereals other than maize, the ML is **100 µg/kg**, for Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption the ML is **75 µg/kg**, for processed cereal-based foods and baby foods for infants and young children, the ML is **20 µg/kg**.

Directive 2002/32/EC on undesirable substances in animal feed stipulates that rules on feedingstuffs are needed to ensure agricultural productivity and sustainability and to make it possible to ensure public and animal health, animal welfare and the environment. Annex I of this Directive contains maximum levels of a number of undesirable substances (chemical contaminants) that may be tolerated in products intended for animal feed. ZEN is not regulated under this Directive. Guidance values for ZEN have been recommended under Commission Recommendation 2016/1319/EC. The guidance values, for cereals and cereal products, are 2 mg/kg and 3mg/kg for maize by-products. Currently, modified forms of ZEN are not considered in the legislation.

The most abundantly produced member of the FBs is fumonisin B1 (Figure 3b). They are thought to be synthesized by condensation of the amino acid alanine into an acetate-derived precursor.

FBs toxicity is mainly due to their capacity of inhibiting ceramide synthase leading to sphingolipid biosynthesis disruption with disturbances of cellular processes, such as cell growth, differentiation, morphology, permeability, and apoptosis. In addition, FB1 promotes the development of cancer in animals and seems to increase the incidence of esophageal and hepatic cancer in humans, neural tube defects, as well as, multiple diseases in experimental animals such as leukoencephalomalacia in horses and pulmonary edema syndrome in pigs.

To limit the exposure to these mycotoxins, the European Union established, in the Commission Decision 2006/576/EC guidance, values regarding presence of DON, ZEN, FBs, and OTA in products used as animal feeding. For FBs, this value is 60 mg/kg as summa FB1 and FB2 in maize and derived product.

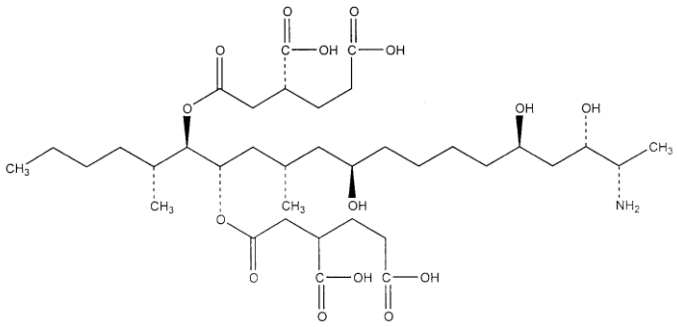


Figure 3b: *Chemical structure of Fumonisin B1*

1.1.4 Alternaria Toxins

Among the various categories of mycotoxins, those produced by the genus *Alternaria* are gaining increasing interest due to their frequent occurrence in food, the recent insights on their genotoxic potential and mechanisms of action, and their consequent possible effects on human health. *Alternaria* toxins are secondary metabolites produced by *Alternaria* fungal species, most commonly *Alternaria alternata* but also *Alternaria tenuissima* and *Alternaria infectoria*. As pathogens, they affect many crops including grains oil seeds, spices, and various fruits and vegetables, and may thus enter the food chain. Due to their growth even at low temperature, these fungal species are also responsible for spoilage of commodities during refrigerated transport and storage. *Alternaria* fungal species produce more than 70 toxins, from which the five most important ones are tenuazonic acid (TEA), altenuene (ALT), alternariol (AOH), tentoxin (TEN), and alternariol monomethyl ether (AME) (Figure 4a,b).

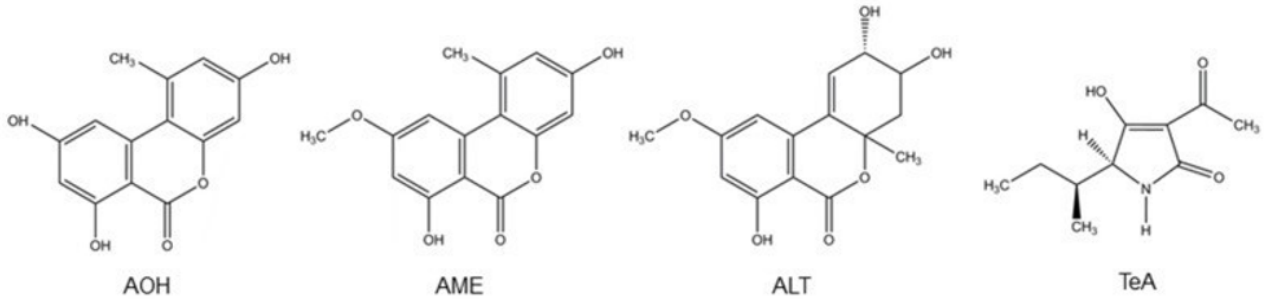


Figure 4a: *Chemical structure of Alternaria toxins*

They exhibit broad structure divergence and are commonly divided into five different classes, but only a small number of them have been chemically characterized so far.

Some toxins such as AOH, AME, TeA and altertoxins were described to induce harmful effects in animals, including fetotoxic and teratogenic effects. The acute toxicity of AOH, AME, ALT, and TEN is low, although there are several reports on the mutagenic and genotoxic activities mainly of AOH and AME. These two mycotoxins are teratogenic and fetotoxic, they seem to be mutagenic, and to have estrogenic activity. Culture extracts of A. *alternata* but also AOH and AME were found

mutagenic and clastogenic in various in vitro systems. Considering their possible harmful effects, presence of these toxins in food may be considered as a serious threat to public health. Toxicological data are limited to the above-mentioned major mycotoxins, and even these data are incomplete, with neither good bioavailability studies nor long-term clinical studies available.

No regulation applies for *Alternaria* toxins in foodstuffs yet, but maximum levels are currently under consideration by the European Commission. In June 2019, a draft EU Commission Recommendation on the monitoring of three *Alternaria* toxins (AOH, AME, TeA) in food was issued which included reference values above which investigations by Food Business Operators would be appropriate to identify the factors resulting in high levels in certain foods. Reference values in processed tomato products, sesame seeds, sunflower seeds, sunflower oil, but also in cereal-based foods for infants and young children, were set at levels ranging from 5 to 30 µg/kg (AOH, AME) and 100–500 µg/kg (TeA). Benchmark levels were set as well for TeA in tree nuts (100 µg/kg), dried fruits (1000 µg/kg), and paprika powder (10,000 µg/kg). As stated in the draft document, these reference values do not represent safe levels in food.

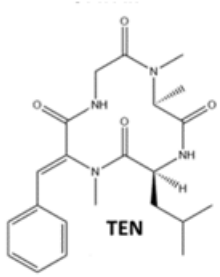


Figure 4b

The *Alternaria* toxins belong to the group of the so-called “emerging” mycotoxins. They are compounds of possible concern due to their abundance, occurrence or toxicity, but the limited available data do not allow a comprehensive risk assessment with an acceptable degree of certainty.

The European Commission (EC), in order to enable it to consider the need for possible follow up actions, including filling of the knowledge gaps, asked the European Food Safety Authority (EFSA) to provide a scientific opinion on the risks for public health related to the presence of Alternaria toxins in feed and food. Currently there are no regulations on Alternaria toxins in food and feed in Europe or in other regions of the world. The development of a standard method is imperative. A few control laboratories have analyzed them on a regular basis using LC-MS/MS technique, which is the most suitable technique according to the EFSA.

1.2 MASS SPECTROMETRY

Mass Spectrometry (MS) is an analytical chemistry technique that helps identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions. In this instrumental technique, the sample, which may be solid, liquid, or gaseous, is ionized, for example, by bombarding it with a beam of electrons and charged particles are separated according to their masses. By accelerating them and subjecting them to an electric or magnetic field, ions of the same mass-to-charge ratio will undergo the same amount of deflection (Figure 5). For most ions, the charge is one, and thus, the m/z ratio is simply the molecular mass of the ion. The ions pass through magnetic and electric fields to reach the detector where they are detected and signals are recorded to give mass spectra. A mass spectrum is a plot of relative abundance against the ratio of mass/charge (m/z). The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules and other chemical compounds. MS is often coupled with other separation techniques, such as GC-MS and LC-MS and it is used in many different fields and is applied to pure samples as well as complex mixtures. The atoms or molecules in the sample can be identified by correlating known masses (e.g. an entire molecule) to the identified masses or through a characteristic fragmentation pattern.

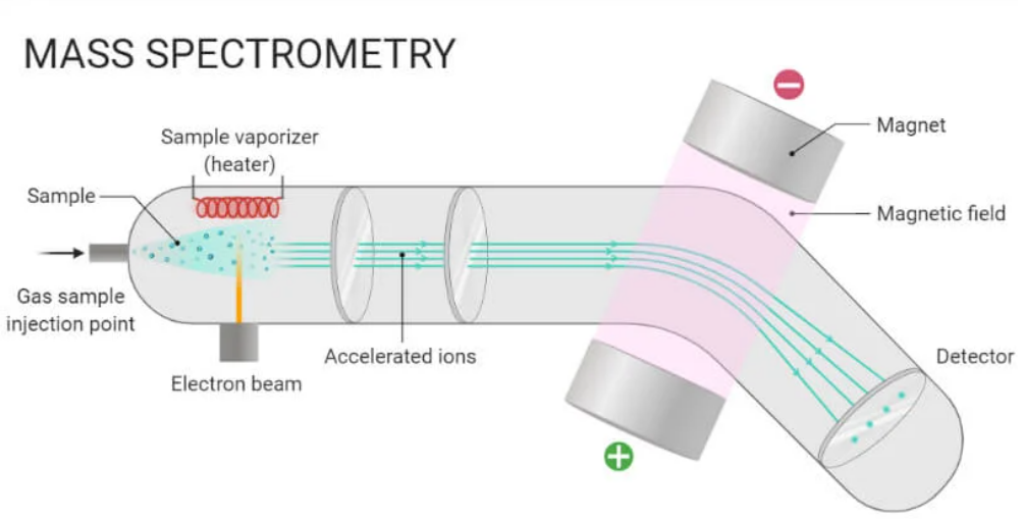


Figure 5 *Scheme of mass Spectrometry*

1.3 TRIPLE-QUADRUPOLE MASS SPECTROMETER

A triple-quadrupole mass spectrometer, also known as QqQ, is a tandem MS method in which the first and third quadrupoles act as mass filters and the second causes fragmentation of the analyte

through interaction with a collision gas—it is a radiofrequency-only quadrupole, and can be used in either SIM or scan mode.

A tandem mass spectrometer involves two stages of mass analysis (MS1 and MS2) separated in time or space by a mass or charge-changing reaction, most often ion dissociation. The MS2 mass spectrum of the reaction products can provide a variety of information about the ions selected by MS1. Early MS/MS instruments were based on magnetic and electric sector mass analyzers with collisional dissociation energies in the kV range. Later MS/MS instruments used two quadrupole mass analyzers separated by a radio frequency-only quadrupole reaction chamber. The method can be used to obtain structural information or for quantitation. For structural mass spec, a common sequence is product ion scan,

Figure 6 *Triple-quadrupole Mass Spectrometer – IZS Sicilia*

precursor ion scan, and neutral loss scan, followed by selected reaction monitoring (SRM) or multiple reaction monitoring (MRM).

1.4 ORBITRAP MASS SPECTROMETRY

LC-high resolution (HR)-MS has become more and more important in bioanalysis of small molecules over the last few years. Its high selectivity and specificity provide best prerequisites for its use in broad screening approaches.

The Orbitrap mass analyzer has become a powerful addition to the arsenal of mass spectrometric Techniques for probing biological systems as well as increasing selectivity and confidence of routine analyses. Analytical performance of the trap can support a wide range of applications from routine compound identification to the analysis of trace-level components in complex mixtures, for example, in proteomics, drug metabolism, doping control, and detection of contaminants in food and feed.

Mass spectrometry (MS) is a key technology for the identification and structural elucidation of small molecules. The molecular formulas of small molecules can be determined from accurate mass values measured by high-resolution (HR) MS because relative atomic masses other than carbon are not strictly integer quantities. Furthermore, there is a close relationship between molecular structure and reactivity of ions in MS, so information on molecular structure can be obtained by interpretation of their fragment ions in the mass spectra. HRMS can make interpretation of mass spectra fairly certain due to its high accuracy and precision. It uses mass spectrometers capable of high resolution, as well as high mass accuracy measurements. These instruments can be used to distinguish between compounds with the same nominal mass, determine elemental compositions, and identify unknowns.

1.5 TURBO FLOW

Turbulent flow is a an online, automated sample cleanup and pre-concentration technique that, combining diffusion, chemistry and size exclusion, performs selective sample clean up, prior to HPLC separation and MS analysis, offering more efficient removal of potential interferences and allowing direct injection of biological fluids.

All to happenens in two steps: Loading and Eluting.

In the first step of the method, sample is loaded onto the TurboFlow column. After sample loading, analytes are eluted onto the analytical column. Following elution, there is a series of forward and reverse time for wash steps that help to minimize carryover. The final step in LC method re-equilibrates both columns.

The interference molecules from the matrix are unretained and are moved to waste during the loading step of the TurboFlow column, while the analyte of interest is retained on the extraction column (Figure 7).

This is followed by organic elution of the analytes to the analytical column and gradient elution to the MS for detection (Figure 8).

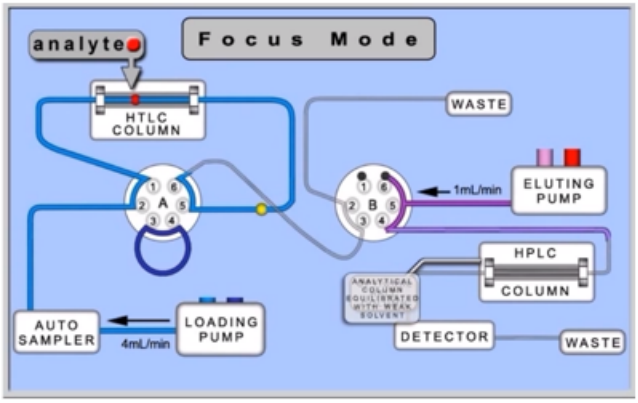


Figure 7 - *Loading*

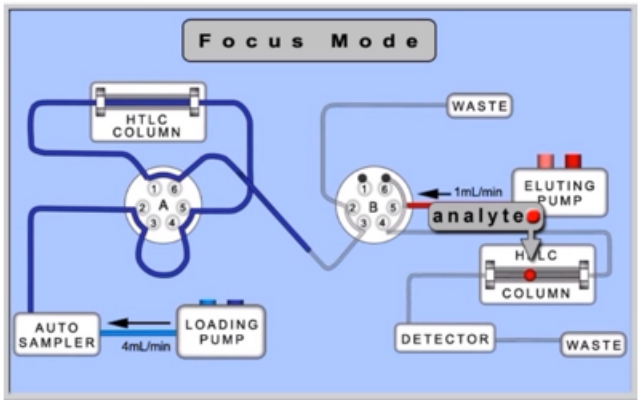


Figure 8 - *Eluding*

1.6 METHOD VALIDATION

Validation of the analytical method is required for every method used for the official control of foodstuffs. The Commission Decision of 12 August 2002 implements Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. The European Decision 2002/657/EC imposes that each analytical method used for official control of foodstuffs is validated and specifies common criteria for the interpretation of analytical results of official control laboratories for such samples. The methodologies described in the ISO 5725 and the ISO 11843 standards are used to set up an experimental validation plan that minimises the number of samples without increasing the uncertainty of the performance characteristics. Recovery, repeatability, within-lab reproducibility, decision limit (CCα), detection capability (CCβ) are determined simultaneously. The uncertainty of each performance characteristic is not increased since the degrees of freedom are equal or higher compared to the conventional validation plan. The validation procedure and the performance characteristics for the analytical methods are defined in the European Decision 2002/657/EC. A performance characteristic is a quality measure of the analytical method. The performance characteristics specificity, accuracy, trueness, precision, repeatability, reproducibility, recovery, detection capability and ruggedness are required by the latter decision. To obtain these characteristics a lot of analytical work has to be performed in the laboratory. It is generally known that validation of analytical methods is a time consuming activity. An experimental plan has to be designed depending on the number of different species and different factors under investigation. Hence, the first step of the entire validation procedure shall consider the sample populations that will be analysed in the laboratory in the future in order to select the most important species and those factors, which may influence the measurement results. Subsequently, the concentration range shall be chosen in a purpose-adapted way according to the level of interest. The validation effort can easily be reduced by introducing a smaller number of samples. However, this will enlarge the uncertainty associated with the performance characteristic. The increase in uncertainty of the performance characteristics can be avoided by combining the validation experiments. A maximum of performance characteristics should be obtained with a minimum of samples without an increase of the uncertainty.

SPECIFICITY

Specificity is the ability of a method to distinguish between the analyte being measured and other substances. This characteristic is predominantly a function of the measuring technique described, but can vary according to class of compound or matrix. To test the selectivity/specificity of the method, 20 representative blank samples of different origin could be analysed to verify the absence of potential interfering compounds at analyte retention time. A method shall be able to distinguish between the analyte and the other substances under the experimental conditions. An estimate to which extent this

is possible has to be provided. Strategies to overcome any foreseeable interference with substances when the described measuring technique is used, e.g. homologues, analogues, metabolic products of the residue of interest have to be employed. It is of prime importance that interference, which might arise from matrix components, is investigated.

RECOVERY

Recovery means the percentage of the true concentration of a substance recovered during the analytical procedure. It is determined during validation, if no certified reference material is available. During the analysis of samples, the recovery shall be determined in each batch of samples, if a fixed recovery correction factor is used. If the recovery is within limits, the fixed correction factor may then be used. Otherwise, the recovery factor obtained for that specific batch shall be used, unless the specific recovery factor of the analyte in the sample is to be applied in which case the standard addition procedure or an internal standard shall be used for the quantitative determination of an analyte in a sample.

LINEARITY

The linearity test can be used to check the concentration range of the analyte in which there is a linear response for the instrument system used in the determination; in this case, the solvent curves are used. Through the acquisition of the curves in the matrix, it is possible to evaluate overall the linearity range of the method (instrument, sample treatment, recoveries, matrix effects etc ...). The curves are intended concentration *vs* signal ratio of the analyte / signal of the IS if an internal quantitative reference standard is used or concentration *vs* analyte signal in other cases.

REPEATABILITY and REPRODUCIBILITY

Repeatability is precision under repeatability conditions. These are conditions where the same operator using the same equipment obtains independent test results with the same method on identical test items in the same laboratory. The test results should be obtained also under reproducibility conditions, in the same method on identical test items in different laboratories with different operators using different equipment.

The European Decision 2002/657/EC introduces the distinction between screening methods and confirmation methods:

SCREENING METHODS

Screening methods mean methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are a process that extracts, isolates and identifies a compound or group of components in a sample with the minimum number of steps and the least manipulation of the sample. Basically, a screening method is

a simple measurement providing a “yes/no” response. They are specifically designed to avoid false compliant results.

It is used only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated; 5 % (â-error) at the level of interest shall be used for screening purposes in conformity with Directive 96/23/EC. In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.

CONFIRMATORY METHODS

Confirmatory methods provide complete or complementary information to identify substances unambiguously and, if necessary, quantify them at the level of interest. Confirmatory methods shall provide information on the chemical structure of the analyte. Consequently, methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods. However, if a single technique lacks sufficient specificity, the desired specificity shall be achieved by analytical procedures consisting of suitable combinations of clean-up, chromatographic separation(s) and spectrometric detection. When more than one compound gives the same response, then the method cannot discriminate between these compounds. Methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods. Where used in the method, a suitable internal standard shall be added to the test portion at the beginning of the extraction procedure. Depending on availability, either stable isotope-labelled forms of the analyte, which are particularly suited for mass-spectrometric detection, or compounds that are structurally related to the analyte shall be used. When no suitable internal standard can be used, the identification of the analyte shall be confirmed by co-chromatography. In this case only one peak shall be obtained, the enhanced peak height (or area) being equivalent to the amount of added analyte. With gas chromatography (GC) or liquid chromatography (LC), the peak width at half-maximum height shall be within the 90-110 % range of the original width, and the retention times shall be identical within a margin of 5 %. Reference or fortified material containing known amounts of analyte, at or near either the permitted limit or the decision limit (non-compliant control sample) as well as compliant control materials and reagent blanks should preferably be carried through the entire procedure simultaneously with each batch of test samples analysed. The order for injecting the extracts into the analytical instrument is as follows: reagent blank, compliant control sample, sample(s) to be confirmed, compliant control sample again and finally non-compliant control sample. Any variation from this sequence shall be justified.

TRUENESS OF QUANTITATIVE METHODS

The ISO standard 5725, under the title “Accuracy (trueness and precision) of measurement methods and results”, uses the combination of two terms, “trueness” and “precision”, to describe the accuracy

of a measurement method. According to ISO 5725, “Trueness” refers to the closeness of agreement between the arithmetic mean of a large number of test results and the true or accepted reference value. In this work, It was expressed in terms of recovery rates and precision as relative standard deviation. “Precision” refers to the closeness of agreement between different test results and it is related to how close several measurements of the same quantity are to each other.

However, the International Organization for Standardization (ISO) uses “trueness” for the above definition while keeping the word “accuracy” to refer to the combination of trueness and precision. On the other hand, precision in the field of statistics it is rather common to use the terms “bias” and “variability” to refer to the lack of “trueness” and the lack of “precision” respectively.

DECISION LIMIT AND DETECTION CAPABILITY

CCα is defined as the concentration level, as determined by the method, at which there is probability α (usually defined as 0.05 or 5%) that a blank sample will give a signal at this level or higher. CCβ is defined as the concentration level of the analyte in sample at which there is a probability β (again usually defined as 0.05 or 5%) that the method will give a result lower than CCα, meaning that the analyte will be declared as undetected. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 - β. In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1 - β.

For substances for which no permitted limit has been established, CCα can be established:

- either by the calibration curve procedure according to ISO 11843. In this case, blank material shall be used, which is fortified at and above the minimum required performance level in equidistant steps. Analyse the samples. After identification, plot the signal against the added concentration. The corresponding concentration at the y-intercept plus 2,33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit. This is applicable to quantitative assays only (α = 1 %);
- or by analysing at least 20 blank materials per matrix to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise ratio can be used as a decision limit. This is applicable to quantitative and qualitative assays.

In the case of substances with established permitted limit, CCα can be established:

- either by the calibration curve procedure according to ISO 11843. In this case, blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the samples. After identification, plot the signal against the added concentration. The corresponding concentration at the

permitted limit plus 1,64 times the standard deviation of the within-laboratory reproducibility equals the decision limit (α = 5 %);

- or by analysing at least 20 blank materials per matrix fortified with the analyte(s) at the permitted limit. The concentration at the permitted limit plus 1,64 times the corresponding standard deviation equals the decision limit (α = 5 %).

For substances for which no permitted limit has been established, CCβ can be established by:

- the calibration curve procedure according to ISO 11843. In this case, representative blank material shall be used, which is fortified at and below the minimum required performance level in equidistant steps. Analyse the samples. After identification, plot the signal against the added concentration. The corresponding concentration at the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability (β = 5 %),
- analysing at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. Analyse the samples and identify the analytes. The value of the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility of the measured content equals the detection capability (β = 5 %),
- where no quantitative results are available, the detection capability can be determined by the investigation of fortified blank material at and above the decision limit. In this case the concentration level, where only 5 % false compliant results remain, equals the detection capability of the method. Therefore, at least 20 investigations for at least one concentration level have to be carried out in order to ensure a reliable basis for this determination.

In the case of substances for which a permitted limit has been established, CCβ can be established:

- either by the calibration curve procedure according to ISO 11843. In this case representative blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the samples and identify the analyte(s). Calculate the standard deviation of the mean measured content at the decision limit. The corresponding concentration at the value of the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility equals the detection capability (â = 5 %),
- or by analysing at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. The value of the decision limit plus 1,64 times the corresponding standard deviation equals the detection capability (β = 5 %).

RUGGEDNESS

Ruggedness is the susceptibility of an analytical method to changes in experimental conditions, which can be expressed as a list of the sample materials, analytes, storage conditions, environmental and/or

sample preparation conditions under which the method can be applied as presented or with specified minor modifications. For all experimental conditions which could in practice be subject to fluctuation (e.g. stability of reagents, composition of the sample, pH, temperature) any variations which could affect the analytical result should be indicated. Such studies use the deliberate introduction of minor reasonable variations by the laboratory and the observation of their consequences. The pre-investigative studies have to be carried out by selecting factors of the sample pre-treatment, clean up and analysis, which may influence the measurement results. Such factors may include the analyst, the source and the age of reagents, solvents, standards and sample extracts, the rate of heating, the temperature, the pH-value as well as many other factors that may occur in the laboratory. These factors should be modified in an order of magnitude that matches the deviations usually encountered among laboratories.

- Identify possible factors that could influence the results.
- Vary each factor slightly.

Conduct a ruggedness test using the approach of Youden. The Youden approach is a fractional factorial design. Interactions between the different factors cannot be detected.

- Where a factor is found to influence the measurement results significantly, conduct further experiments to decide on the acceptability limits of this factor.
- Factors that significantly influence the results should be identified clearly in the method protocol.

STABILITY

It has been observed that insufficient stability of the analyte or matrix constituents in the sample during storage or analysis may give rise to significant deviations in the outcome of the result of analysis. Furthermore, the stability of the calibration standard in solution should be checked. Usually analyte stability is well characterised under various storage conditions. Monitoring of the storage condition will form part of the normal laboratory accreditation system.

The European Decision 2002/657/EC establishes the performance criteria and other requirements for mass spectrometric detection.

Mass spectrometric methods are suitable for consideration as confirmatory methods only following either an on-line or an off-line chromatographic separation. For LC-MS procedures, the chromatographic separation shall be carried out using suitable LC columns. In any case, the minimum acceptable retention time for the analyte under examination is twice the retention time corresponding to the void volume of the column. The retention time (or relative retention time) of the analyte in the test portion shall match that of the calibration standard within a specified retention time window. The retention time window shall be commensurate with the resolving power of the chromatographic system. The ratio of the chromatographic retention time of the analyte to that of the internal standard,

i.e. the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ± 2,5 %. Mass-spectrometric detection shall be carried out by employing MS-techniques such as recording of full mass spectra (full scans) or selected ion monitoring (SIM), as well as MS-MSn techniques such as Selected Reaction Monitoring (SRM), or other suitable MS or MS-MSn techniques in combination with appropriate ionisation modes. In high-resolution mass spectrometry (HRMS), the resolution shall typically be greater than 10.000 for the entire mass range at 10 % valley. The **Full scan** is obtained when mass spectrometric determination is performed by the recording of spectra in a mass range that includes all the masses of the analysed analytes. The presence of all measured diagnostic ions (the molecular ion, characteristic adducts of the molecular ion, characteristic fragment ions and isotope ions) with a relative intensity of more than 10 % in the reference spectrum of the calibration standard is obligatory. The **SIM** mass spectrometric determination is performed by fragmentography. The molecular ion shall preferably be one of the selected diagnostic ions (the molecular ion, characteristic adducts of the molecular ion, characteristic fragment ions and all their isotope ions). The selected diagnostic ions should not exclusively originate from the same part of the molecule.

2. PURPOSE OF THE WORK

More comprehensive studies are required to update the risk evaluation by the EFSA and, therefore, the specific aim of this project is focussed on the development of accurate LC-MS/MS and LC/HRMS methods for the analysis of emerging Alternaria toxins (AOH, AME, TeA, TEN).

LC-MS/MS method will be used for the target analysis of emerging mycotoxins in food matrices. In particular, the following activities will be carried out: (1) develop of a multiresidual analytical method with enhanced performance characteristics; (2) development of sample preparation protocols, suitable for various foodstuffs; (3) validation of the methods according to EU guidelines; (4) monitoring of raw materials, food and feed.

Concerning LC-HRMS analysis, advanced mass spectrometry will be compared with the LC-MS/MS method, to obtain mass spectrometers capable of high resolution, as well as high mass accuracy measurements.

The research project will provide a detailed survey of emerging mycotoxins content in various food products contributing to an estimate of mycotoxin risk exposure of consumers. The wide survey planned within this study will allow us to fill the data gap, and to provide relevant information for improving the safety of local products. Particular attention will be paid to organic farming and local agricultural varieties, with the overarching goal of protecting and valorizing the local high quality primary production. The need to have more sensitive and specific analytical methods and the lack of incidence studies is an innovative contribution to the risk characterization associated with the presence of mycotoxins from Alternaria. Any exposure assessment and any risk characterisation needs accurate data related to occurrence, contamination level and toxicity.

3. MATERIAL AND METHODS

3.1. *Chemicals and Solvents*

Organic solvents, HPLC gradient grade water, sodium acetate anhydrous, glacial acetic acid, ammonium acetate, anhydrous magnesium sulphate, sodium acetate, Z-Sep+ (zirconium oxide) formic acid and ammonium formate (HPLC grade) were purchased from Sigma-Aldrich – Merck (Merck Life Science S.r.l., Milan, Italy).

For an accurate mass calibration, a mixture of acetic acid, caffeine, Mete-Arge-Phee-Alae-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulphate, taurocholic acid sodium salt hydrate and Ultramark 1621 (fluorinated phosphazines) (Proteo-Mass LTQ/ FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) were employed in the Orbitrap analyzer. The cleanup PSA tubes 55228-U, containing 150 mg of Supelclean PSA and 900 mg of MgSO4, were purchased from Supelco (Bellefonte, PA, USA).

3.2. Certified reference materials and working solutions

The following certified reference materials of Mycotoxins and isotopically labelled analogues, used as internal standard (ILIS), were purchased from Sigma-Aldrich – Merck (Merck Life Science S.r.l., Milan, Italy): AFG_1, 5 mg; AFG_2, 5 mg; AFB_1, 5 mg; AFB_2, 5 mg; OTA, 5 mg; ZEN, 5 mg; DON, 5 mg; T-2 toxin, 5 mg; HT-2 toxin, 5 mg; Fumonisin mixture, 50 µg/mL (each of FB_1 and FB_2) in acetonitrile:water; Fumonisin B_3, 50 µg/mL; Alternariol, 0,1 mg; Alternariol monomethyl ether, 0,1 mg; Tenuazonic Acid, 0,1 mg; Tentoxin, 0,1 mg; OTA-d5, 10 µg/mL; AFG_1-$^{13}C_{17}$, 0.5 µg/mL; DON-$^{13}C_{15}$, 25 µg/mL in acetonitrile, ZEN-$^{13}C_{18}$, 25 µg/mL in acetonitrile. The Altenuene, 1 mg, Alternariol-d2, 10mg, and Tentoxin-d3, 0,25 mg were purchased from LGC Standards.

Standard stock solutions of individual analytes were prepared in methanol at the concentration of 1000 µg/mL, except for Fumonisin mixture, *Alternaria* toxins and for ILIS.

The intermediate work solutions (10 µg/mL) were made by dissolving the standard in methanol. These solutions were stored in dark glass bottles at −20°C up to 12 months and 6 months, respectively.

The standard stock solutions and the intermediate work solutions were used for spiking, as different spiking levels are required for method validation of each mycotoxin.

Multicompound working standards solution was prepared daily, diluting with methanol the intermediate working solutions up to a concentration of 0.1 µg/mL.

A working mixed solution of internal standards (0.1 µg/mL) was similarly prepared by dilution of intermediate work mixed solution with methanol.

3.3. Sample collection and storage.

A total of 26 samples of durum wheat grain and 24 samples of white flour, produced in Sicily in the years 2019-2021, were collected. All the samples were stored at room temperature until the LC-MS/MS analysis.

Twenty-five samples of cereals for feed were tested in the survey. Samples were collected directly from farms of Sicily (Italy) from April 2019 to October 2020.

A sample of durum wheat grain and a sample of white flour were obtained from a local store. After checking that no mycotoxins were detected, they were used as blanks, for preparing fortified samples for recovery assays and matrix-matched standards for calibration purposes.

3.4. Extraction procedure

3.4.1 Protocol 1: *acidified acetonitrile*

2.0 g of the finely ground and homogenous sample was weighed into a 50 mL centrifuge tube. The sample was spiked with 200µl of working mixed solution at 0.1 µg/mL to achieve a final concentration of 10 µg/kg of the target compounds and IS. The sample was mixed by vortex for 30 sec. and left at room temperature for 60 min. Eight mL of acidified acetonitrile (acetonitrile/water/formic acid, 80:19.9:0.1, v/v/v) was added and the tube was shaken for one minute. Then, the sample was centrifuged at 5000 rpm for 10 min. and the supernatant was filtered with a 0.2µm nylon filter into an LC-vial.

3.4.2 Protocol 2: *extraction and clean-up*

Applying Protocol 1, after centrifugation, 1.5 mL of the extract was transferred to a clean-up micro tube containing 150 mg of Supelclean PSA and 900 mg of $MgSO_4$ or 100 mg of Z-Sep+ , respectively and shake it by vortex for 1 min. Then, the tube was centrifuged at 5000 rpm for 10 min and 1 mL of the organic phase was transferred to a vial for chromatographic analysis.

3.4.3 Protocol 3: *QuEChERS*

Acetate QuEChERS was applied as follow: 2 g of sample were weighed in a 50 mL centrifuge tube. The sample was fortified with 200µl of working mixed solution at 0.1 µg/mL to achieve a final concentration of 10 µg/kg of the target compounds and IS and let stand it for 30 min. Eight mL of water were added to the mixture, which was shaken vigorously for 30 s and let stand it for 15 min. The mixture of acetonitrile and acetic acid at 1% (v/v) was added to the sample and shaken vigorously for 1 min. Then, 4 g of magnesium sulphate and 1 g of sodium acetate were added to the mixture and shaken vigorously for 1 min. Finally, the mixture was centrifuged at 3700 rpm during 10 min and 1 mL of the organic phase was transfer to a vial for chromatographic analysis.

3.5. Chromatographic conditions

LC analysis was carried out through a Thermo Fischer UHPLC system (Thermo Fisher Scientific, California, USA) constituted of an ACCELA 1250 quaternary pump and autosampler. Chromatographic separation was obtained using a Thermo Scientific Hypersil Gold reversed-phase UHPLC column (100 mm, 2.1 mm ID, 1.9μm). The LC eluents were water (A) and methanol (B), both containing 0.1% (v/v) formic acid and ammonium formate 5mM. The gradient was initiated with 90% eluent A and 10% eluent B for 1.0 min, continued with linear variation to 10% A and 90% B in 4.0 min. This condition was maintained for 1.0 min. The system returned to 90% A and 10% B in 0.5 min and was re-equilibrated for 3 min. A multiple heater controller Multisleeve™ equipped with the HotSleeve Plus™ was used to maintain the columns at 30°C and the sample temperature was kept at 6°C. The flow rate was 0.4 mL min^{-1} and the injection volume 10 μL. The LC conditions are given in Table 1.

Time (min)	%A	%B
0	90	10
1.0	90	10
5.0	10	90
5.5	10	90
6.0	90	10
9.0	90	10

Table 1 *LC conditions*

3.6. Mass spectrometry: MS-MS conditions

The mass spectrometer was a triple quadrupole TSQ Vantage (Thermo Fisher Scientific, California, USA) in positive and negative electrospray ionisation mode (ESI). Product ion scans of each analyte were performed by direct infusion (10 μL min^{-1}) of 1 mg/L individual standard solutions with the built-in syringe pump through a T-junction, mixing with the blank column eluate (200 μL min^{-1}). ESI parameters optimised were as follows: capillary voltage 3.8 kV; capillary temperature 310° C; Vaporiser temperature 340°C; sheath and auxiliary gas pressure were fixed at 35 and 15 (arbitrary unit), respectively. The collision gas was argon at 1.5 m torr and peak resolution of 0.7 FWHM was used on Q1 and Q3. The scan time for each monitored transition was 0.01 s and the scan width was 0.01 m/z. The two most intense ion transitions of target compounds used for the MS/MS detection are detailed in Table 2. In addition, the collision energy parameters associated with the precursor and the product ions are given in Table 2. Acquisition data were recorded and elaborated using XcaliburTM version 2.1.0.1139 software from Thermo Fisher Inc. The instrument was calibrated using a Thermo Scientific™ Pierce Triple Quadrupole Calibration Solution, according to the manufacturer's instructions.

Analyte	m/z precursor ion	m/z product ion 1	CE (V)	m/z product ion **2**	CE (V)	Ion mode
Ochratoxin A	404.1	221.1	37	239.2	27	$[M+H]^+$
D5-Ochratoxin A	409.1	226.2	37	-	-	$[M+H]^+$
Zearalenone	321.2	187.0	22	283.2	20	$[M+H]^+$
$^{13}C_{18}$ Zearalenone	337.3	301.4	15	-	-	$[M+H]^+$
Aflatoxin B1	313.1	241.0	38	285.1	25	$[M+H]^+$
Aflatoxin B2	315.1	259.0	30	287.1	27	$[M+H]^+$
Aflatoxin G1	329.1	243.0	27	311.0	25	$[M+H]^+$
$^{13}C_{17}$ Aflatoxin G1	346.1	212	40	-	-	$[M+H]^+$
Aflatoxin G2	331.1	245.0	30	313.0	30	$[M+H]^+$
Deoxynivalenol	297.1	248.9	15	203	30	$[M+H]^+$
$^{13}C_{15}$ Deoxynivalenol	312.1	262.9	10	-	-	$[M+H]^+$
Fumonisin B1	722.4	352.3	40	334.2	40	$[M+H]^+$
Fumonisin B2	706.4	336.3	40	318.3	40	$[M+H]^+$
Fumonisin B3	706.4	336.3	40	318.3	40	$[M+H]^+$
HT-2 toxin	425.2	263.1	15	215	20	$[M+H]^+$
T-2 toxin	467.2	305	15	215.2	20	$[M+H]^+$
Alternariol	257.1	147.0	20	213.0	10	$[M-H]^-$
D2-Alternariol	259.1	149.0	20			$[M-H]^-$
Alternariol monomethyl ether	271.1	256.0	10	228.0	10	$[M-H]^-$
Tenuazonic Acid	196.1	139.1	15	86.0	26	$[M-H]^-$
Tentoxin	413.2	141.1	20	271.1	20	$[M-H]^-$
D3-Tentoxin	416.2	143.1	20	-	-	$[M-H]^-$
Altenuene	291.1	203.0	40	161.0	40	$[M-H]^-$

Table 2 *MRM monitoring. Precursor and product ions and their collision energy and ion mode.*

3.7. Sample preparation for TurboFlow™ purification

To finely ground and homogenous samples (5.0 g) were added 50µl of working mixed solution at 1.0 µg/mL to achieve a final concentration of 10 µg/kg of the target compounds and IS. After 10min, they were extracted, by vortex, for 45 minutes with a mixture of water containing 0.1% formic acid (FA)/acetonitrile (ACN) (43:57). After filtration with a 0.2 µm nylon filter into an LC-vial, 100 µL of the sample was directly injected into the TurboFlow system, coupled with high-resolution mass spectrometric (HRMS) detection.

3.8. Online TurboFlow™ purification and LC separation

A Thermo Scientific Transcend™ II system (Thermo Fisher Scientific, CA, USA) was used for online cleanup and LC separation. This system combines TurboFlow™ online sample preparation technology and a UHPLC separation technique. The whole system was fitted with Accela Open AS autosampler maintained at 6°C, a mixing quaternary loading pump, for online clean-up, and a mixing

quaternary eluting pump, for analytical separation, UltiMate™ 3000 with a valve interface module for switching four port valve. The system was controlled by Aria software. The TurboFlow™ column (TX) was a Thermo Scientific™ Cyclone P 50x0.5mm. Chromatographic separation was obtained using a Thermo Scientific™ Hypersil GOLD™ reversed-phase UHPLC column (LX) (100 mm, 2.1 mm ID, 1.9 μm). A multiple heater controller Multisleeve™ equipped with the HotSleeve Plus™ was used to maintain the columns at 30°C.

The sample extract clean-up was performed in Focus mode which consists of the four general steps: 1-Loading, 2-Transfer, 3-Cleaning and 4-Equilibration. The mobile phases were as follows: 0.1% formic acid in water (eluent A), MeOH (eluent B), ACN/acetone/2-propanol (4/2/4, v/v/v) (eluent C) and ammonium acetate 10mM pH 9.0 (eluent D). Sample supernatant (20μL) was injected under turbulent flow (1.5mL/min.) of eluent D and eluent B (90/10, v/v) and passed through the TX column for 60sec.; during this Loading step, TX and LX columns were not connected. Retained analytes were back-flashing from TX column using eluent B (100%) stored in a holding loop and focused onto the LX column. For the entire Transfer step (90sec.), the TX column was online with LX column and the flow was reduced to 0.05mL min-1. While the gradient elution was running, the loop was re-filled with solvent B and the TX column was washed with eluent B, eluent A and eluent C. The system was then re-equilibrated prior to next injection.

The LC eluents were water (E) and Methanol (F), each containing 0.1% (v/v) formic acid. The flow rate was 0.3 mL min-1 and the gradient was initiated with 90% eluent A and 10% eluent B for 2.5 min., continued with linear variation to 10% A and 90% B in 1.5min. Eluent A has been reduced at 20% in 4.0min and this condition was maintained for 1.0 min. The system returned 90% eluent A and 10% eluent B in 0.5 min. and was re-equilibrated for 3.0min. The final run time of the method with automated online sample cleanup and analytical separation was 12.5 min. To avoid possible carryover and cross contamination, the injector and the needle was washed with 10% methanol in water and ACN/acetone/2-propanol (4/2/4, v/v/v).

The TLX and LC conditions are given in Table 3.

Step	Time	TX system (Loading pump)[a]					Valves		LX system (Eluting pump)[b]				Comment
	Start (min)	Flow (mL/min)	A%	B%	C %	D%	Tee	Loop	Flow (mL/min)	Gradient mode	E%	F%	
1	0.00	1.50	-	10.0	-	90.0	-	Out	0.30	Step	90.0	10.0	Sample loading onto TX column
2	1.00	0.05	-	-	-	100.0	T	In	0.30	Step	90.0	10.0	Sample transfer onto LX column
3	2.50	1.50	20.0	80.0	-	-	-	In	0.30	Ramp	60.0	40.0	Rinsing TX column Elution LX column
4	4.00	1.00	20.0	-	80.0	-	-	In	0.30	Ramp	10.0	90.0	Rinsing TX column Elution LX column
5	8.00	1.50	-	100.0	-	-	-	In	0.30	Step	10.0	90.0	Filling the loop Elution LX column
6	9.00	1.00	-	10.0	-	90.0	-	Out	0.30	Ramp	90.0	10.0	Equilibrating TX and LX column
7	9.50	1.50	-	10.0	-	90.0	-	Out	0.30	Step	90.0	10.0	Equilibrating TX and LX column

Table 3 *TLX and LC conditions*

3.9. Mass spectrometry: Orbitrap-HRMS analysis

The mass spectrometer was operated in both positive and negative ion mode using fast polarity switching by setting two scan events. All data were acquired using the full MS scan/dd-MS2 mode. The ESI source was operated in positive mode (ESI+) under the following specific conditions: capillary voltage, 4.2 kV; capillary temperature, 310 °C; and vaporizer temperature, 100 °C. Nitrogen (>99.98%) was employed as sheath and auxiliary gas. These were fixed at 20 and 5 (arbitrary unit), respectively. The S-lens radio frequency (RF) level was 55.

Ion source parameters in negative (ESI−) mode were: spray voltage −3.8 kV; capillary temperature 310 °C; S-lens RF level 50; sheath gas pressure 35, auxiliary gas 10, and auxiliary gas heater temperature 100 °C. MS spectra were acquired over an m/z range of 150–750. In full MS scan, without fragmentation, Orbitrap resolution was set to 70,000 FWHM (m/z 200). The automatic gain control (AGC) was set at 1×10^6 ions for a maximum injection time of 100 ms. In dd-MS2 mode, a resolution of 17,500 FWHM (m/z 200) was used. The AGC target was set at 1×10^5 ions for a maximum injection time of 100 ms. To filter the product ions, the quadrupole operated at an isolation window of 2 m/z. An inclusion list was created and the acquisition parameters are listed in Table 4. All the analyses were performed without lock mass. The exact mass of the compounds was calculated

using the Qual browser in Xcalibur 4.0. Mass accuracy was daily calibrated by the use of multi-compound standards, previously indicated.

Precursor ion scans of each analyte were performed by direct infusion (10 µL min^{-1}) of 1 µg mL^{-1} individual standard solutions with the syringe pump through a T-junction, mixing with the blank column (200 µL min^{-1}). The most intense and signal stable adducts were selected for each analyte. The product ions were found by increasing the normalised collision energy (NCE) using the Q-Exactive Tune 4.0 software from Thermo. Genesis peak detection was applied.

Analyte	Retention time (min)	Molecular formula	Adduct	Monoisotopic exact mass		Error (ppm)
				Theoretical	Experimental	
Ochratoxin A	6.50	$C_{20}H_{18}NO_6Cl$	$[M+H]^+$	404,08954	404,08973	-0,47
D5-Ochratoxin A	6.5	$C_{20}H_{13}D_5NO_6Cl$	$[M+H]^+$	409,12920	409,12874	1,12
Zearalenone	5.0	$C_{18}H_{24}O_5$	$[M+H]^+$	321,16965	321,17046	-2,52
$^{13}C_{18}$ Zearalenone	5.0	$^{13}C_{18}H_{24}O_5$	$[M+H]^+$	337,2351	337,23518	-0,24
Aflatoxin B1	4.64	$C_{17}H_{12}O_6$	$[M+H]^+$	313,07066	313,07090	-0,77
Aflatoxin B2	4.98	$C_{17}H_{14}O_6$	$[M+H]^+$	315,08631	315,08715	-2,67
Aflatoxin G1	4.79	$C_{17}H_{12}O_7$	$[M+H]^+$	329,06558	329,06500	1,76
$^{13}C_{17}$ Aflatoxin G1	4.79	$^{13}C_{17}H_{12}O_7$	$[M+H]^+$	346,15280	346,15354	-2,14
Aflatoxin G2	4.61	$C_{17}H_{14}O_7$	$[M+H]^+$	331,08123	331,08129	-0,18
Deoxynivalenol	4.20	$C_{15}H_{20}O_6$	$[M+H]^+$	297,13326	297,13377	-1,72
$^{13}C_{15}$ Deoxynivalenol	4.20	$^{13}C_{15}H_{20}O_6$	$[M+H]^+$	312,21635	312,21719	-2,70
Fumonisin B1	6.0	$C_{34}H_{59}NO_{15}$	$[M+H]^+$	722,39575	722,39514	0,84
Fumonisin B2	6.8	$C_{34}H_{59}NO_{14}$	$[M+H]^+$	706,40083	706,40048	0,50
Fumonisin B3	6.8	$C_{34}H_{59}NO_{14}$	$[M+H]^+$	706,40083	706,40048	0,50
HT-2 toxin	5.60	$C_{22}H_{32}O_8$	$[M+H]^+$	425,21699	425,21795	-2,26
T-2 toxin	6.10	$C_{24}H_{34}O_9$	$[M+H]^+$	467,22756	467,22857	-2,16
Alternariol	5.90	$C_{14}H_{10}O_5$	$[M-H]^-$	257,04555	257,04518	1,44
D2-Alternariol	5.90	$C_{14}H_8D_2O_5$	$[M-H]^-$	259,05810	259,05827	-0,66
Alternariol monomethyl ether	6.80	$C_{15}H_{12}O_5$	$[M-H]^-$	271,06119	271,06155	-1,33
Tenuazonic Acid	6.2	$C_{10}H_{15}NO_3$	$[M-H]^-$	196,09791	196,09815	-1,22
Tentoxin	6.8	$C_{22}H_{30}N_4O_4$	$[M-H]^-$	413,21942	413,21875	1,62
D3-Tentoxin	6.8	$C_{22}H_{27}D_3N_4O_4$	$[M-H]^-$	416,23825	416,23762	1,51
Altenuene	6.9	$C_{15}H_{16}O6$	$[M-H]^-$	291,08741	291,08723	0,62

Table 4. UHPLC-HRMS parameters for the determination of mycotoxins included in this study.

3.10. Validation procedure

The method was validated in order to ensure reliable results according to the criteria specified in Commission Decision 2002/657/EC for a quantitative confirmatory method. The extraction protocol 3 was studied for the validation. The optimized method (Section 3.4.3) was using to spike and analyse Blank samples.

The performance of the method was assessed regarding instrumental linearity, specificity, precision, recovery, decision limit (CCα), detection capability (CCβ), matrix effect and ruggedness.

3.10.1. Selectivity/Specificity

To test the selectivity/specificity of the method, 20 representative blank samples of different origin (12 durum wheat grain, 5 white flour, 3 feed) were analysed to verify the absence of potential interfering compounds at analyte retention time.

3.10.2. Linearity

The linearity of the calibration curves was checked in standard solutions by monitoring the molecular ion of each mycotoxin analyzed (Table 5). Calibration curves were prepared in triplicate at 6 concentration levels. The solutions were prepared in a mixture of acetonitrile /water containing 0.1% formic acid (FA) (80:20). The curves were constructed including zero, plotting the ratio analyte area/internal standard area (=y) versus analyte concentration (=x) for all analytes, for which Internal standards were available, for the other, curves were constructed plotting the peak area of analyte versus the corresponding concentration was plotted. Then, a regression model was applied to the calibration data set. In all cases, the correlation coefficients of linear functions were >0.998.

For the estimation of the validation parameters, the blank samples of the durum wheat grain, white flour and animal feed were spiked with daily working mixed solution of analytes and ISs and processing as the samples normally. The linearity was studied in different range as reported in Table 5.

Analyte	Linearity Range (µg/kg)				
	Methanol		**Wheat**	**Flour**	**Feed**
Ochratoxin A	0.5-5	0.5-5	0.5-5	0.5-5	0.5-5
Zearalenone	5-250	50-2500	5-250	5-250	50-2500
Aflatoxin B1	0.5-5	0.5-5	0.5-5	0.5-5	0.5-5
Aflatoxin B2	0.5-5	0.5-5	0.5-5	0.5-5	0.5-5
Aflatoxin G1	0.5-5	0.5-5	0.5-5	0.5-5	0.5-5
Aflatoxin G2	0.5-5	0.5-5	0.5-5	0.5-5	0.5-5
Deoxynivalenol	125-2000	250-2500	125-2000	125-2000	250-2500
Fumonisin B1	50-500	500-5000	50-500	50-500	500-5000
Fumonisin B2	50-500	500-5000	50-500	50-500	500-5000
Fumonisin B3	50-500	500-5000	50-500	50-500	500-5000
HT-2 toxin	10-200	250-2500	10-200	10-200	250-2500
T-2 toxin	10-200	250-2500	10-200	10-200	250-2500
Alternariol	2-20	2-20	2-20	2-20	2-20
Alternariol monomethyl ether	2-20	2-20	2-20	2-20	2-20
Tenuazonic Acid	2-20	2-20	2-20	2-20	2-20
Tentoxin	2-20	2-20	2-20	2-20	2-20

Table 5 *Linearity range of mycotoxins in solvent, wheat, white flour and animal feed.*

3.10.3. Precision, Trueness, CCα, and CCβ

Method trueness and precision were determined by spiking blank samples with analytes, resulting in three analytical series, each with three concentration levels and six samples for concentration level. Three different concentrations was studied: 1.0, 2.0 and 3.0 µg/kg for OTA; 0.5, 1.0 and 2 µg/kg for AFs, 250, 500 and 1000 µg/kg for DON; 25, 50 and 100 µg/kg for ZEN; 50, 100 and 250 µg/kg for FBs; 50, 100 and 150 µg/kg for T-2 and HT-2 toxins; 5.0, 10.0 and 15.0 µg/kg for Alternaria toxins. The 30 replicate analysis (ten for each level) were repeated in three separate days giving 90 independent determinations.

Blank samples were spiked at the beginning of the extraction procedure with the solution of analytes and ISs. Six replicates (n = 6) were carried out for each concentration level and repeated on three separate days (p = 3) giving 54 replicates, varying time, operator, and calibration status of instrument**.** Trueness was expressed in terms of recovery rates and precision as relative standard deviation. Decision limit (CCα) and detection capability (CCβ) were calculated by applying the procedure described in Commission Decision 2002/657/EC. CCα was expressed as the concentration corresponding to the y intercept plus 2.33-fold the within-laboratory standard deviation of the lowest calibration level. CCβ was calculated as CCα + 1.64-fold the standard deviation of the within-laboratory reproducibility. The detection limit (LOD) was based on the results for six replicates of blank sample spiked at the lowest calibration level. The average percent recovery was estimated using these matrix results.

3.10.4. Matrix Effect

In order to evaluate the matrix effect for different species of cereal with various origins two aliquots for each species of blank samples previously analyzed were spiked after the extraction, at the concentration level of 10 µg/kg. At the same time, a solution of the detected analytes was prepared at the concentration level of 10 µg/kg.

3.10.5. Ruggedness (Minor Changes)

Method ruggedness was estimated by means of the Youden robustness test. This study was performed at 10 µg/kg and seven operating factors were chosen: vortex shaking time (s), Time to left the sample at room temperature (min), Volume of extraction solution (ml), Volume of extract to clean-up (ml), Time of centrifuge (min), Speed of centrifuge (rpm) and Shaking time for clean-up (s). The different

factors and their levels were mixed in the Youden experimental plan. In Table 6, the factors and the levels of variation studied are reported.

Selected variable	*High level*	*Low level*
Vortex shaking time (s)	*33*	*27*
Time to left at room temperature (min)	*66*	*54*
Volume of extraction solution (ml)	*8.8*	*7.2*
Volume of extract to clean-up (ml)	*1.65*	*1.35*
Time of centrifuge (min)	*11*	*9*
Speed of centrifuge (rpm)	*5500*	*4500*
Shaking time for clean-up (s)	*66*	*54*

Table 6. *Factors and level mixed in the Youden experimental plan*

3.10.6. Analyte Stability

According to the Commission Decision 2002/657/EC, stability of the analytes in solution was studied at the concentration of 10 and 0.1 µg/mL. Different conservation methods were evaluated every week in a period of 24 weeks; methanol solutions were maintained at −20 °C away from light, at 4 °C away from light, and at room temperature in light. In order to evaluate the stability, these solutions were compared with fresh made solutions. Furthermore, due to the low stability of the instrumental response for the low concentration solutions of the calibration curve, different solvents were evaluated to increase the stability of the signal: acetonitrile, acetonitrile/water with 0.1% FA (80:20), methanol/ water with 0.1% FA (80:20). These solutions were injected with an interval of a week and their stabilities of the instrumental response were compared.

4. RESULTS AND DISCUSSION

A sensitive, accurate, precise and robust UHPLC–MS/MS method for determination of Mycotoxins in cereals and feed was developed. Three different extraction protocols were compered to obtain the better results for the method performances.

In addition, an analytical tool based on a UHPLC-Q-Orbitrap HRMS method that combines quantitative target analysis with the determination of accurate masses with < 3ppm mass accuracy, was developed. TurboFlow method based on online sample clean-up was developed to replace clean-up techniques involving numerous manual sample preparation steps.

Isotope labeled internal standards were included to correct for variations in sample preparation recovery and for possible ion suppression/enhancement effects.

4.1 Optimization of UHPLC–MS/MS Conditions

The scope of this work was the development of a sensitive, specific but also robust analytical tool that would enable to provide solid data from "difficult" samples and quantify trace amounts of mycotoxins. Due to the nature of the samples and the "gravity" of the anticipated result, detection and identification of toxins should be impeccable. Finally, analysis should not be lengthy and should not cause system degradation such as column or source capillary blockage. It can be understood that meeting such requirements necessitates the optimization of the LC–MS analysis in both ends: LC and MS.

Chromatographic separation was optimised by injecting a pure standard solution onto a Hypersil Gold (HG) reversed-phase UHPLC column. The selection of this column was adopted from a previous LC/MSMS method developed in the laboratory of Istituto Zooprofilattico.

In the terms of gradient elution, optimization included the selection of the starting composition of the mobile phase, the number of the steps, the slope of the gradient steps and the total run time. Water-methanol, from70:30 v/v to 90:10 v/v, were tested as starting points. Multi-step linear gradients ranged from 16 min to 9 min. The selected gradient was initiated with 90% eluent A and 10% eluent B for 1.0 min, continued with linear variation to 10% A and 90% B in 4.0 min. This condition was maintained for 1.0 min. The system returned to 90% A and 10% B in 0.5 min and was re-equilibrated for 3 min. This program gave the best chromatographic separation with analyte peaks spread along the run time. Because the majority of the compound in this study possess a basic group, satisfactory results were obtained using 0.1% formic acid as an aqueous component of the mobile phase, favouring the formation of the protonated molecule. Ammonium formate was added to assist with the separation of the analytes. This chromatographic optimisation allowed for the separation and detection of different mycotoxins within 9 min, including a 3 min column flush and equilibration.

After optimisation of the gradient, retention times of the analytes were estimated from six replicates at one concentration level. The stability of the LC method was evaluated by calculating retention time repeatability. Because this repeatability always exhibited a RDS% of < 1%, separation of the mycotoxins was found to be satisfactory with the HG column and gradient elution.

MS/MS was optimized by direct infusion of standard solutions of each analyte separately. The cone voltage for the precursor ion and the collision energy of the product ions were investigated. The latter were selected as the ions that provided the highest abundance; in case where certain analytes had common or neighbouring precursor ions, selectivity was the second selection criterion, so unique product ions were selected. All values were optimised to give the highest response of each analyte.

Detection was performed in Multiple Reaction Monitoring (MRM) mode in specific time windows with two or in some cases three different transitions. Details of precursor ion selected, product ions monitored and collision energy applied for each analyte are shown in Table 2. The system operated in electrospray ionization in polarity switching mode and all analytes exhibited higher sensitivity in positive mode, except for *Alternaria* mycotoxins, which formed adduct in negative mode. A low injection volume of only 10 μL was chosen for the developed method as a compromise between introducing low amounts of matrix components into the system and to inject enough to get satisfactory sensitivity of the different compounds.

4.2 LC-High Resolution Mass Spectrometry

At a nominal mass resolving power of 70,000 FWHM, the analytes were detected using the accurate mass measurements of the MH^+ ions. The Orbitrap analyser provided excellent accuracy (below 3 ppm for all the compounds) in the determination of the masses, without the use of lock mass, as reported in Table 4. Fragments determination was achieved processing the data obtained from Orbitrap with the Xcalibur™ Qual Browser program. Firstly, the chemical formula for each compound was processed with the software to obtain the theoretical mass and molecular ion. Then, characteristic fragments were obtained using all ion fragmentation (AIF). About the monitored ions, positive ionization was applied for all of the studied compounds and all of the spectra showed the $[M+H]^+$ adduct, except for *Alternaria* mycotoxins, which formed adduct in negative mode. Despite the high selectivity and specificity of HRMS, isomers with same accurate mass cannot be discriminate by HRMS. Because reference standards were available and chromatographic separation was satisfactory, discrimination of the analytes could be made from their retention time. This consideration indicates that LC separation is indispensable for accurate mass measurements, especially regarding structural isomers that have the same calculated exact mass.

To provide additional information for identification and to improve specificity, fragmentation of the protonated molecules was used. Because the mass spectrometer was operated in a data-dependent-acquisition (DDA) mode, which means that the full-scan analysis with the Orbitrap analyser was followed by a MS-MS scan, shorter detection times were preferred. Considering the correlation between the acquisition time and mass resolution, a nominal resolution power of 17,500 FWHM was employed in this study. Regarding chromatographic resolution, it is important to notice than two compounds were isomers and coeluted. Therefore, they were not being able to be distinguished by chromatographic step and/or mass determination when this gradient was used. These compounds were Fumonisine B2 and B3 (m/z 706.40083, retention time window (RTW): 6.70–6.9 min). Therefore, these compounds were quantified as the sum of the two coeluted compounds.

4.3 Study of Sample Preparation

Various procedures have been tested during the optimization phase of the extractive step. In order to decide which protocol is the most efficient, different factors were evaluated and compared. More specifically the selection criteria investigated were the following: number of analytes and recovery % of each analyte. In the first place, we choosed to use acidified acetonitrile (acetonitrile/water/formic acid, 80:19.9:0.1, v/v/v) and the shake for one minute, followed by centrifugation and filtration. However, during preliminary experiments, a strong matrix effect was observed and by duplicate tests of this procedure, we did not obtain repeatable results. Therefore, an insoluble residue in the ACN phase was observed and the recovery values were between 20% and 50% for all the molecules. Then, we choosed to process the sample through 30 min of automatic shacking after adding the ACN; in this conditions we improved the recoveries (30%-70%), but not the repeatability, suggesting that the cause of the problem could have been tracked down in the residual matrix of the extracts. In order to remove this interference, a clean-up step was added and two comparison tests were performed. In the first test, different sorbents were tested and the extract was treated with Supelclean PSA and MgSO4, C18 and Z-Sep+; in the second, we applied the QuEChERS clean-up technique using the dispersive SPE (dSPE) primary secondary amine (PSA), as reported in a study about determination of pesticides in food. Better results were obtained when Supelclean PSA and MgSO4 was used for durum wheat grain and Z-Sep+ for white flour. When this clean-up step was used, suitable recoveries (between 70% and 90%) for the target compounds were obtained. These results indicate the relevance of the clean-up steps when this kind of matrices are analysed, observing that the extracted compounds increased from 30% to 70% when this clean-up step was included in the method. However, it was observed, that depending on the type of matrix, a different sorbent is needed, and it is difficult to find a sorbent that can efficiently work for all matrices. Matrix suppression was shown for all the studied

compounds when the QuEChERS clean-up was applied. Therefore, matrix-matched calibration standards were used in order to avoid the above mentioned matrix effects. Least-squares regression of peak areas versus concentration of the calibration standards in the whole range was applied for linearity evaluation. The calculated determination coefficients (r^2) were always higher than 0.99 in all the studied samples and the deviation of each individual level from the calibration curve was always lower than 20%. By comparing the test results, we obtained good repeatability and satisfying recovery values (78% - 98%) in the case of the QuEChERS clean-up. This procedure was used to validated the method. The other protocol did not extract adequately all selected mycotoxins and recoveries were not suitable enough.

4.4 Turboflow™ Purification

The general approach to the analysis of mycotoxins involves liquid extraction, solid-phase extraction (SPE) or immunoaffinity-column (IAC) clean-up followed by HPLC with fluorescence detection or LC-MS/MS.

The requirement for high sensitivity and the problems of matrix interferences either necessitate a lengthy clean-up process or require the use of high-specificity detection such as LC-MS/MS.

However, even though direct analysis is possible without clean-up, dirty extracts can result in ion suppression and the need for frequent cleaning of the instrument source in LC-MS/MS.

The TurboFlow method based on online sample clean-up and HRMS detection replaces clean-up techniques involving numerous manual sample preparation steps, such as the purification of sample extracts using immunoaffinity cartridges.

For effective clean-up without losing any of the target compounds present in the cereals, it was necessary to select the optimum TurboFlow™ column and to establish loading and elution conditions. The capability of the Cyclone-P column to retain mycotoxins was tested. According to manufacturer, this kind of column is recommended for the determination of a wide range of analytes in complex matrices, being a good option for the retention of mycotoxins, taking into account the wide variability of structures and polarities of the compounds present in natural matrices.

In order to get the optimum conditions for the on-line TurboFlow™-LC, several parameters were modified, using the focus mode in the TurboFlow™ system. Samples fortified to 10 µg/kg were used during method development, and total ion current intensity was used as analytical signal.

First, the loading step (sample clean up) should be evaluated and the selection of a suitable solvent is very important to eliminate matrix components. Thus, ammonium acetate 10mM pH 9.0 and ammonium acetate 10mM pH 5.6 were compared, and the maximum peak intensity was obtained when ammonium acetate 10mM pH 9.0 was used as solvent A.

In order to increase the intensity of the peak, several loading times (1.0, 1.5 and 2.0 min) were also tested. It can be observed that the lower loading time, the higher peak intensity. Therefore, 1.0 min was selected as loading time.

Then, the loading flow rate was evaluated, bearing in mind that it can determine the efficiency of pre-concentration and matrix elimination. Two flow rates (1.5 and 2 mL/min) were compared. The results showed a decrease of peak intensity at 2 mL/min. Therefore, a flow rate of 1.5 mL/min was selected, allowing the retention of small molecules with appropriate chemistry into the pores of the TurboFlow column, whereas the rest of matrix components will be removed to the waste.

After that step, analytes were eluted from TurboFlow column to the analytical column (transfer step) and some parameters as mobile phase, transfer time and flow rate were optimized. Several organic solvents were evaluated as mobile phases during the transfer step such as a mixture of ACN:MeOH, 50:50 (v/v), a mixture of MeOH and an aqueous solution of formic acid (0.1%) at several ratios (50:50, 60:40, 70:30, 80:20 and 90:10), and MeOH (100%). The responses of the compounds increased when MeOH and an aqueous solution of formic acid (0.1%) at 90:10 was used. Then, the effect of elution time was tested at 3, 4 and 5 min. Peak intensity decreased when elution times greater than 4 min were used. Therefore, an elution time of 4 min was employed. Finally, two elution flow rates (0.3 and 0.4 mL/min) were evaluated. When 0.4 mL/min was used, the compounds were not eluted from the TurboFlow column to the analytical column in the transfer step and they were transferred in later stages. This indicates that when high flow rates were used, compounds were not completely eluted from the column and they were retained in the column till wash step was performed. However, when 0.3 mL/min was used, the compounds were eluted during the transfer step.

In order to eliminate carryover, mobile phase and flow rate were optimized during the cleaning step. Thus, different mobile phases with different polarity were evaluated. Better results were obtained when ACN/acetone/2-propanol (40:30:30, v / v / v), were used in different parts of this step, to ensure the elimination of analytes or matrix components with different polarities retained in the TurboFlow column. The flow was optimized as well, being increased in some parts of this stage from 1.5 mL/min to 2 mL/min in order to ensure the cleaning of TurboFlow column, but there were not significant differences between these two flows.

Cereals contains lots of matrix compounds as water, protein, carbohydrates and lipids, free amino acids and minerals which may affect the quantification of toxin compounds. Therefore, the results obtained when TurboFlow was used for the clean-up of the cereal samples where compared with those obtained when direct injection of the matrix into the chromatographic system was performed. For that purpose, a sample of white flour was analysed with and without the addition of the TurboFlow step. It was observed that all compounds were detected in the sample, obtaining in all cases the highest

intensities when TurboFlow system was used, Therefore, TurboFlow method was selected for the analysis of mycotoxins, reducing matrix effect and increasing the lifetime of the LC column. Besides, this method allowed the combination of the extraction and purification process in one single step.

4.5 Identification criteria

Identification of mycotoxins was indicated by the presence of accurate mass ions obtained at a resolving power of 70,000 FWHM at m/z 200 and a mass accuracy window below 3 ppm, corresponding to the retention times of appropriate standards. Additional mass confirmation was given by the simultaneous detection of HCD fragments. According to EU Commission Decision 2002/657/EC, that implemented the use of "identification point", for a precursor ion, measured accurate mass in accordance with expected accurate mass with a mass tolerance of 5 ppm, and retention time were used. Theoretical monoisotopic exact mass of protonated compounds based on their molecular formulas were calculated using Xcalibur software. Theoretical masses and detected masses in standards in methanol are listed in Table 4. Accurate mass deviation was determined to be below 3 ppm.

4.6 Method Performance

4.6.1 Selectivity/Specificity

Results obtained from the analysis of 20 batches of blank and comparison to samples spiked at the lower validation level indicated the absence of some interference at the retention times of the studied analytes and IS. This high selectivity is confirmed by experiments conducted in dd-MS^2 mode with the concomitant acquisition of accurate mass.

4.6.2 Linearity

The instrumental linearity has been evaluated in different range, as reported in Table 5, using the average of three determinations for each level. The calibration curves have shown values of the linear correlation coefficient very satisfying for each analyte. Coefficients of determination obtained were in the range of 0.9988-0.9997. The axis origin was included in the calculation.

4.6.3 Matrix effect

The matrix effect was investigated as described in order to reveal possible ionization suppression or enhancement caused by matrix components. It was evaluated on different species cereals. Two aliquots of each sample were extracted as previously described and the extracts were spiked of analytes and IS. At the same time, a solution of the detected analytes was prepared at the same concentration level. Matrix effect (% ME) was calculated as following:

$$\%\mathrm{ME} = \frac{\left(\frac{analyte\ peak\ area}{IS\ peak\ area}\right)_{(post-extraction\ sample)}}{\left(\frac{analyte\ peak\ area}{IS\ peak\ area}\right)_{(solution)}} \times 100$$

A % ME value less than 100 indicates an ion suppression effect; if it is grater then 100 indicates an absolute matrix effect. No significant ion enhancement was found for each analyte and the values of mean percentage recovery were in the range of 98.6–102.4%.

Furthermore, the comparison of the individual responses for each type of cereals, has allowed us to study the relative matrix effect; no change was observed for the typologies concerned.

4.6.4 Precision, trueness, CCα and CCβ

CCα and CCβ values were calculated by extracting six known blank samples spiked at three concentration levels in three different days. CCα values were in the range of 0.55 – 120 µg/Kg and CCβ values were in the range of 0.25 – 60 µg/Kg for all the analytes. To ensure greater food security, LOD and LOQ were also calculated; were estimated based on the results of six replicates of samples spiked at 0.50 µg/Kg level; Limits of detection (LODs) obtained were between 0.25 and 0.40 µg/Kg; LOQs were calculated from 0.40 and 0.50 µg/Kg.

Trueness and precision data were also calculated from results obtained during the CCα and CCβ measurements. Precision was expressed as relative standard deviation (RSD). Intra-day and inter-day relative standard deviations (RSDs) showed reliable repeatability (RSD < 11%) and within-laboratory repeatability (RSD < 19%) of the developed method. Trueness was expressed in terms of recovery rates; the recovery data for all studied mycotoxins at the fortification levels assayed, low, medium and high, ranged from 78% to 98% with lower recoveries for T2, HT2 and Fumonisins. The carry-over was evaluated by injecting a blank sample after the highest calibration point. No carry-over was present since no peaks were detected at retention time of all studied mycotoxins. Those results highlighted that the proposed methodology is accurate enough for the quantitative determination of the target mycotoxins.

4.6.5 Ruggedness

The ruggedness was performed using the Youden approach. This experimental design resulted the conduction of eight experiments relative at the selection of seven variables, chosen in the sample preparation and analysis. The application of this test consisted in the introduction of minor simultaneous changes in these parameters according to an established experimental design, with the

aim identifying the critical factors that have to be controlled in order to obtain accurate assay results. This study confirmed that the tested factors are not critical.

4.7. Application of the method to real samples

In this study, both methods, QuEChERS extraction connected with UHPLC-MS/MS and TurboFlow with Orbitrap-HRMS analysis, were applied to monitoring mycotoxins in real cereal samples for food and feed. A total of 26 samples of durum wheat grain and 24 samples of white flour, produced in Sicily in the years 2019-2021, were collected. Twenty-five samples of cereals for feed were tested in the survey. Samples were collected directly from farms of Sicily (Italy) from April 2019 to October 2020.

Results showed that 72 out of 75 total samples (96%) were contaminated by at least one mycotoxin. AOH, AME, TeA, and TEN were detected in more samples, while ALT was not detected in any of the samples. AME, TeA, and TEN were found in 65% of the samples at levels up to 1.2 (AME), 9.0 (TEN), and 57 µg/kg (TeA). *Alternaria* toxins occurred regularly in white flour. *Fusarium* toxins representatives were detected in 54% of samples and the mycotoxins with the highest incidence were ZEN and DON. Twenty-four samples of durum wheat grain were found to be contaminated with ZEA in the range of 7.5 – 12.8 µg/Kg. Deoxynivalenol, in unprocessed cereals, was detected within the range of 11.5–525.3 µg/kg. Detectable presence of OTA was found in 5 samples of white flour with a range of 0.7-1.1 µg/Kg.

All the samples examined revealed mycotoxin levels under the limit imposed by the Commission Regulation 1881/2006.

5. CONCLUSION

This work gives an important contribution in terms of analysis and occurrence of both traditional and emerging mycotoxins, in cereals for food and feed, for the final consumers of durum wheat and maize derived products.

A Ultraperformance liquid chromatography tandem mass spectrometry and Quick, Easy, Cheap, Effective, Rugged, and Safe method, based analytical methodologie to quantitate Alternaria toxins in cereals for food and feed were developed and validated.

The method resulted accurate, precise and suitable for the simultaneous determination of common mycotoxins and "emerging" mycotoxins. The extractive process has been proven rapid and efficient. The chromatographic method allowed an optimal separation of the analytes furthermore the MS/MS detection ensured a univocal identification and an excellent sensitivity.

The work was continued to extend the LC-MS/MS method and to develop a LC/HRMS method for determination of other emerging *Alternaria* toxins, suitable for various food and feedstuffs. The need to have more sensitive and specific analytical methods and the lack of incidence studies is an innovative contribution to the risk characterization associated with the presence of mycotoxins from *Alternaria*.

With regard to the widespread occurrence of *Alternaria* mycotoxins in various food and feedstuffs intended for human or animal consumption and their high toxicity, more toxicological studies are needed on the field, transport, storage, and processing stages. Maximum levels admitted for these toxins should be released by EFSA in the near future. As these contaminants could be found in a wide range of food products, their current and future determination is essential for regulatory bodies with the purpose of improving the quality of products and preserving consumers' health.

The difficulties associated with the ongoing pandemic have made it difficult to analyze a larger number of foods, initially planned within this project. Despite everything, the purpose of this work was to fill the data gap and provide relevant information to improve the safety of local products. Therefore, the validated method was applied to screen mycotoxins in 26 samples of durum wheat grain and 24 samples of white flour, produced in Sicily and collected in the last two years.

NORMATIVE REQUIREMENTS

1. Comission Regulation 1126/2007 of September 2007 on amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants foodstuffs as regards Fusarium toxins in maize and maize products. Official Journal of the European Union. L 255, 14-17, 2007.
2. Commission Regulation 1881/2006 on setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union. L 364, 5-24, 20.12.2006.
3. Commission Regulation 401/2006 of February 2006 on laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuff. Official Journal of the European Communities. L70, 12-34, 2006.
4. Commission Decision 2002/657/EC of 12 August 2002, Off. J. Eur. Commun. L221 (2002) 8 Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.
5. DIRECTIVE 2002/32/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 7 May 2002 on undesirable substances in animal feed (OJ L 140, 30.5.2002, p. 10).
6. CEN/TR 16059:2016 Food Analysis. Performance Criteria for Single Laboratory Validated Methods of analysis for the Determination of Mycotoxins. Available online: infostore.saiglobal.com/preview/98697665123.pdf? sku=874908_saig_nsai_nsai_2079941.

REFERENCES

1. Turner, N.W.; Bramhmbhatt, H.; Szabo-Vezse, M.; Poma, A.; Coker, R.; Piletsky, S.A. Analytical methods for determination of mycotoxins: An update (2009–2014). Anal. Chim. Acta 2015, 9011, 2–33.
2. Tsitsigiannis, I.; Antoniou, P.; Tjamos, C. Biological control strategies of mycotoxigenic fungi and associated mycotoxins in Mediterranean basin crops. Phytopathol. Mediterr. 2012, 51, 158–174.
3. Xu, L.; Zhang, Z.; Zhang, Q.; Li, P. Mycotoxin determination in foods using advanced sensors based on antibodies or aptamers. Toxins 2016, 8, 239.
4. Yang, J.; Li, J.; Jiang, Y.; Duan, X.; Qu, H.; Yang, B.; Chen, F.; Sivakumar, D. Natural Occurrence, Analysis, and Prevention of Mycotoxins in Fruits and their Processed Products. Crit. Rev. Food Sci. Nutr. 2014, 54, 64–83.
5. Zollner, P.; Mayer-Helm, B. Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. J. Chromatogr. A 2006, 1136, 123–169.
6. Iqbal, S.Z.; Selamat, J.; Ariño, A. Mycotoxins in food and food products: Current status. In Food Safety; Springer: Berlin, Germany, 2016; pp. 113–123.
7. Chhonker, S.; Rawat, D.; Naik, R.; Koiri, R. An Overview of Mycotoxins in Human Health with Emphasis on Development and Progression of Liver Cancer. Clin. Oncol. 2018, 3, 1408.
8. Streit, E.; Schatzmayr, G.; Tassis, P.; Tzika, E.; Marin, D.; Taranu, I.; Tabuc, C.; Nicolau, A.; Aprodu, I.; Puel, O. Current situation of mycotoxin contamination and co-occurrence in animal feed—Focus on Europe. Toxins 2012, 4, 788–809
9. Escrivá L., Font G., Manyes L. and Berrada H. Studies on the Presence of Mycotoxins in Biological Samples: An Overview. Toxins 2017, 9, 251; doi:10.3390/toxins9080251
10. EFSA. Scientific Opinion Risk assessment of aflatoxins in food. EFSA Journal 2020;18(3):6040.
11. EFSA. Scientific Opinion Risk assessment of ochratoxin A in food. EFSA Journal 2020;18(5):6113 doi: 10.2903/j.efsa.2020.6113
12. COT (Committee on Toxicity of Chemicals in Food Consumer Products and the Environment), 2018. Review of potential risks from ochratoxin A (OTA) in the diet of infants aged 0 to 12 months and children

aged 1 to 5 years. Available online: https://cot.food.gov.uk/committee/committee-on-toxicity/cotstatements/cotstatementsyrs/cot-statements-2018/cot-statement-on-potential-risks-from-ochratoxin-a-ota-in-the-diet-of-infants-aged-0-to-12- months-and-children-aged-1-to-5-year.

13. F. Crudo, E.Varga, G. Aichinger, G. Galaverna, D. Marko, C. Dall'Asta and L. Dellafiora. Co-Occurrence and Combinatory Effects of Alternaria Mycotoxins and Other Xenobiotics of Food Origin: Current Scenario and Future Perspectives. Toxins 2019, 11, 640; doi:10.3390/toxins11110640
14. EFSA. Scientific Opinion. Dietary exposure assessment to Alternaria toxins in the European population. EFSA Journal 2016;14(12):4654
15. EFSA. Scientific Opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food. EFSA J. 2011, 9, 2407
16. Verstraete, F. Analytical Challenges for an Effective EU Policy on Contaminants in Food and Feed to Ensure a High Level of Animal and Human Health Protection. In Proceedings of the Recent Advances in Food Analysis 2019, Prague, Czech Republic, 5–8 November 2019.
17. Food Chemistry Institute of the Association of the German Confectionery Industry. Alternaria Toxins: Occurrence, Toxicity, Analytical Methods, Maximum Levels. Available online: https://www.lci-koeln.de/deutsch/veroeffentlichungen/lci-focus/alternaria-toxins-occurrence-toxicity-analytical-methods-maximumlevels (accessed on 11 August 2020).
18. Ádám Tölgyesi 1,*, Luca Kozma 1 and Virender K. Sharma. Determination of Alternaria Toxins in Sunflower Oil by Liquid Chromatography Isotope Dilution Tandem Mass Spectrometry. Molecules 2020, 25, 1685; doi:10.3390/molecules25071685
19. Tölgyesi, Á.; Stroka, J. Collaborative Study Report: Determination of Alternaria toxins in Cereals, Tomato Juice and Sunflower Seeds by Liquid Chromatography Tandem Mass Spectrometry. 2016. Available online: https://ec.europa.eu/jrc/en/publication/collaborative-study-report-determination-alternaria-toxins-cereals-tomato-juice-and-sunflower-seeds (accessed on 26 July 2019).
20. Walravens, J.; Mikula, H.; Rychlik, M.; Asam, S.; Devos, T.; Ediage, E.N.; Di Mavungu, J.D.; Jacxsens, L.; Van Landschoot, A.; Vanhaecke, L.; et al. Validated UPLC-MS/MS Methods to Quantitate Free and Conjugated Alternaria Toxins in Commercially Available Tomato Products and Fruit and Vegetable Juices in Belgium. J. Agric. Food Chem. 2016, 64, 5101–5109.
21. L. Escrivá, S. Oueslati, G. Font, and L. Manyes. Alternaria Mycotoxins in Food and Feed: An Overview. Hindawi Journal of Food Quality Volume 2017, Article ID 1569748, 20 pages https://doi.org/10.1155/2017/1569748
22. L. Castaldo, G. Graziani, A. Gaspari, L. Izzo, J.Tolosa, Y.o Rodríguez-Carrasco, and A. Ritieni. Target Analysis and Retrospective Screening of Multiple Mycotoxins in Pet Food Using UHPLC-Q-Orbitrap HRMS. Toxins 2019, 11, 434; doi:10.3390/toxins11080434
23. Jürg Noser & Patrick Schneider & Martin Rother & Hansruedi Schmutz. Determination of six Alternaria toxins with UPLC-MS/MS and their occurrence in tomatoes and tomato products from the Swiss market. Mycotox Res DOI 10.1007/s12550-011-0103-x
24. S. Fraeyman, S. Croubels, M. Devreese and G. Antonissen. Emerging Fusarium and Alternaria Mycotoxins: Occurrence, Toxicity and Toxicokinetics. Toxins 2017, 9, 228; doi:10.3390/toxins9070228
25. Zinedine, A.; Fernandez-Franzon, M.; Manes, J.; Manyes, L. Multi-mycotoxin contamination of couscous semolina commercialized in Morocco. Food Chem. 2017, 214, 440–446.
26. Bunmi K. Olopade, Solomon U. Oranusi, Obinna C. Nwinyi, Sefater Gbashi, Patrick B. Njobeh. Occurrences of Deoxynivalenol, Zearalenone and some of their masked forms in selected cereals from Southwest Nigeria. https://doi.org/10.1016/j.nfs.2021.03.001
27. Rychlik, M.; Lepper, H.; Weidner, C.; Asam, S. Risk evaluation of the Alternaria mycotoxin tenuazonic acid in foods for adults and infants and subsequent risk management. Food Control 2016, 68, 181–185
28. Solhaug, A.; Eriksen, G.S.; Holme, J.A. Mechanisms of action and toxicity of the mycotoxin alternariol: A review. Basic Clin. Pharmacol. Toxicol. 2016, 119, 533–539.
29. Anita Solhaug1, Gunnar S. Eriksen1and Jørn A. Holme. Mechanisms of Action and Toxicity of the

MycotoxinAlternariol: A Review Basic & Clinical Pharmacology & Toxicology, 2016,119, 533–539 Doi: 10.1111/bcpt.12635
30. European Commission Health & Consumer Protection Directorate-General. Opinion of the Scientific Committee on Food on Fusarium Toxins. Part 1: Deoxynivalenol. 02 December 1999
31. European Commission Health & Consumer Protection Directorate-General. Opinion of the Scientific Committee on Food on Fusarium Toxins. Part 2: ZEARALENONE. 22 June 2000
32. European Commission Health & Consumer Protection Directorate-General. Opinion of the Scientific Committee on Food on Fusarium Toxins. Part 3: Fumonisin B1. 17 October 2000
33. European Commission Health & Consumer Protection Directorate-General. Opinion of the Scientific Committee on Food on Fusarium Toxins. Part 5: T-2 Toxin and HT-2Toxin. 30 May 2001
34. EFSA. Scientific Opinion. Risks for animal health related to the presence of zearalenone and its modified forms in feed. EFSA Journal 2017;15(7):48
35. EFSA. Scientific Opinion. Risks for animal health related to the presence of fumonisins, their modified forms and hidden forms in feedEFSA Journal 2018;16(5):5242
36. Scientific Report of EFSA Deoxynivalenol in food and feed: occurrence and exposure. EFSA Journal 2013;11(10):3379
37. EFSA Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feedEFSA Journal 2011;9(12):2481
38. EFSA Scientific Opinion on the risks for public health related to the presence of zearalenone in food EFSA Journal 2011;9(6):2197
39. EFSA Scientific Opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food. EFSA Journal 2011;9(10):2407
40. C. Gruber-Dorninger, B. Novak, V. Nagl, and F. Berthiller. Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants. J. Agric. Food Chem. 2017, 65, 33, 7052–7070