Method development using LC-MS/MS and LC-IMS-QTOF MS to determine mycotoxins in fish feed samples

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Ying Yi

Master degree thesis majored in Chemistry

Bergen, Norway

University of Bergen
Bergen, Norway

Institute of Marine Research
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Supervisors

Rita Hannisdal, PhD

Sylvain Merel, PhD

Scientist, Department of Seafood and Nutrition, Institute of Marine Research

Senior Scientist, Department of Feed Safety, Institute of Marine Research

Monica Jordheim, PhD

Associate Professor, Department of Chemistry, University of Bergen

Bergen, Norway

May 2020

ACKNOWLEDGMENTS

This master thesis was carried out at the Institute of Marine Research (HI) and the Chemistry Department of the University of Bergen (UIB). I am very grateful for the contributions from both parties makes my master project feasible.

I want to express my deepest appreciation and gratitude to my supervisor at HI, Dr. Rita Hannisdal, for her sincere help throughout my study. I appreciate so much for her great efforts in guiding me step by step. She is always inspiring and encouraging me in this period. She provided constructive suggestions whenever I need and spent much time to revise my thesis. I also want to thank my co-supervisor, Dr. Sylvain Merel, for always being patient to explain the principles and steps on how to use the instruments for the experiments, which is an essential part for my project. I appreciate his time for trouble-shooting my puzzles and providing practical comments for my thesis.

I want also to express my sincere thanks to my supervisor at UiB, Prof. Monica Jordheim for always being supportive for my curriculums, my thesis and other learning opportunities. I deeply appreciate her coordination during my master project and her constructive feedbacks on my thesis. I want to use this chance to thank all the teachers and administrations at Chemistry Department for sharing their knowledge and experiences through different courses and activities and helping me out whenever I need.

I want to extend my appreciation to Annette Bjordal for teaching me on the standard preparation, Marianne Hauglid Flågeng for teaching me on fish feed extraction and the following up instrument guiding, Felicia Dawn Couillard for the HMS training and to all staff at Seafood and Nutrition Department for making me feel at home during my study at HI.

My sincere thanks go to my dear master friends Valérie Suzanne Vugteveen, Usanee Kongjampee, Kari Nesse Johnsen, Lene Revheim Sandøy, Wiktoria Karolina Szapoczka, Øystein Opheim, Rolf Jacob Vågen and Thomas Næsheim for all the friendly assistance and encouragement during my study.

A special thanks goes to my family for their unconditional supports, both financially and emotionally. Your love always drives me forward.

Ying Yi

Bergen, May 2020

LIST OF ABBREVIATIONS

Abbreviation	Full name
ug	microgram
ml	Micro liter
3D	Three-dimensional
3-ADON	3-Acetyldeoxynivalenol
15-ADON	15-Acetyldeoxynivalenol
αZEL	alpha-zearalenol
βZEL	beta-zearalenol
ACN	Acetonitrile
AFB1- $^{13}C_{17}$	Aflatoxin B1-$^{13}C_{17}$
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFs	Aflatoxins
AmAc	Ammonium acetate
APCI	Atmospheric Pressure Chemical Ionization
BEA	Beauvericin
CCS	Collision cross section
CE	Capillary Electrophoretic
CI	Chemical ionization
CIT	Citrinin
CV	Coefficient of variation
DAS	Diacetoxyscirpenol
DOM-1	Deepoxy deoxynivalenol
DON	Deoxynivalenol
D3G	Deoxynivalenol-3-glucoside
EFSA	European Food and Authority
EI	Electron ionization

ENA	Enniatin A
ENA1	Enniatin A1
ENB	Enniatin B
ENB1	Enniatin B1
ESI	Electrospray ionization
FA	Formic acid
FAO	Food and Agriculture Organization
FB1	Fumonisin B1
FB2	Fumonisin B2
FB1- 13C34	Fumonisin B1-13C34
FB3	Fumonisin B3
FDA	Food and Drug Administration
FLD	Fluorescence Detection
FUMs	Fumonisins
FWHM	full-width half-maximum
H2O2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HPLC	High Pressure/ Performance Liquid Chromatography
HPLC-FLD	High Performance Liquid Chromatography coupled with Fluorescence Detection
HRMS	High Resolution Mass Spectrometry
HT2	HT-2 Toxin
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LPLC	Low Pressure Liquid Chromatography
MeOH	Methanol

mg	Mini gram
ml	Mini liter
mM	Mini mole
MRM	Multiple Reaction Monitoring
m/z	Mass to Charge ratio
MON	Moniliformin
MPA	Mycophenolic acid
NEO	Neosolaniol
NIV- 13C15	Nivalenol-13C15
No.	Number
O3	Ozone
Orbi-MS	Orbitrap mass spectrometry
OTA	Ochratoxin A
OTA- 13C20	Ochratoxin A-13C20
OTB	Ochratoxin B
OTC	Ochratoxin C
PAT	Patulin
ppb	Part per billion
ppm	Part per million
QTOF	Quadrupole Time of flight
R2	Correlation coefficient
RALs	Resorcyclic
RCF	Relative centrifugal force
RPM	Revolutions per minute
RSD	relative standard deviation
SD	Standard deviation
SRAC	Southern Regional Aquaculture Center
SRM	Selected reaction monitoring
ssp.	Several species
T2	T2-Toxin

TPs	Transformation Products
TLC	Thin Layer Chromatography
TWIMS	Traveling-wave Ion Mobility Spectrometry
UHPLC	Ultrahigh-performance Liquid Chromatography
UV	Ultraviolet
v:v	Volume: Volume
ZEA	Zearalenone
ZEL	Zearalenol

List of Tables

List of Figures

Abstract

Mycotoxin contamination is an increasing concern in aquafeed industry due to the replacement of marine-based ingredients to plant-based ingredients and climate changes associated with growing mycotoxin contamination in plant ingredients. Consequently, the health and growth of aqua animals could be affected by contaminated fish feeds, and finally influence human health. Therefore, it is necessary to develop an effective screening method for mycotoxin determination in fish feed. This master project develops two screening methods (using low resolution UHPLC-QqQ-MS/MS and high resolution UHPLC-IMS-QTOF MS instruments) for the determination of 18 mycotoxins: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), beauvericin (BEA), deoxynivalenol (DON), diacetoxyscirpenol (DAS), enniatin A (ENA), enniatin A1 (ENA1), enniatin B (ENB), enniatin B1 (ENB1), fumonisin B1 (FB1), fumonisin B3 (FB3), moniliformin (MON), ochratoxin A (OTA), T2-Toxin (T2), HT-2 Toxin (HT2) and neosolaniol (NEO) and 6 metabolites: 15-Acetyldeoxynivalenol (15-ADON), 3-Acetyldeoxynivalenol (3-ADON), alpha-zearalenol (αZEL), beta-zearalenol (βZEL), deoxynivalenol-3-glucoside (D3G) and deepoxy deoxynivalenol (DOM-1).

In addition to screening method, a quantitative method was developed by UHPLC-QqQ-MS/MS for all compounds except AFs, MON and DAS. The developed method was validated in terms of linearity. ENA, ENA1, ENB, ENB1, BEA, NEO, HT-2, T-2 and αZEL had a $R^2 > 0.99$; DON, 3-ADON, OTA and βZE had a $R^2 > 0.95$, showing good linearity performance. Recovery (95%-111%) and intra-day precision (RSD of 4%-18%) for ENA, ENA1, ENB, ENB1 and BEA using T-2 as IS, demonstrated excellent validation performances. Both isotopic IS and structural analogue IS were used during the validation. T-2 was confirmed as a good alternation to substitute expensive isotopic IS for the validation of ENA, ENA1, ENB, ENB1 and BEA. Ten fish feeds including one fish feed ingredient collected in the market were analyzed using the screening method. Results show considerable mycotoxin contamination for all samples. The developed method by UHPLC-QqQ has shown great promise in quantification of mycotoxins.

The other method was developed by UHPLC-IMS-QTOF MS for all compounds with CCS value as extra identification point. The developed method was applied to ten commercial fish feeds including one fish feed ingredient and results reveal considerable mycotoxin contamination for almost all samples. Mycotoxins detected in the fish feed samples were ENNs (including ENA, ENA1, ENB and ENB1) and BEA, while FUMs (FB1 and FB3) were detected in the fish feed ingredient sample.

A chemical degradation experiment for two frequently occurred mycotoxins (BEA and ENB) was also performed. The transformation products (TPs) of both compounds were tentatively predicted.

Key Words: Mycotoxin, metabolite, transformation products, chemical degradation, fish feed, Triple Quadrupole LC-MS/MS, ion mobility spectrometry, quadrupole time of flight, LC- IMS-QTOF

1 Introduction

1.1 Mycotoxin and mycotoxicosis

Mycotoxins are toxic secondary metabolites produced by fungi organisms and are able to cause illness or even death in animals and human beings (Bennett & Klich, 2003). The poisoning caused by exposure to mycotoxins is called mycotoxicosis (CAST, 2003). Since the first detection of aflatoxins in 1960s, more than 400 mycotoxins have been identified in the last sixty years (Cinar & Onbaşı, 2019). Only a few mycotoxins are regularly found in agriculture crops, and even less mycotoxins have been extensively researched with modern analytical methods (Deligöz & Bilge, 2017). The classification of mycotoxins is complicated due to their diverse chemical structures, biosynthetic origins, biologic effects and different production origins (Bennett & Klich, 2003).

Table 1 lists some major groups of mycotoxins and metabolites classified by their producer fungus and molecular structure, as well as vulnerable infected agriculture species and corresponding mycotoxicosis symptoms (Jennifer, 2019; Kralj Cigić & Prosen, 2009; Morgavi & Riley, 2007; Nazari et al., 2015; Richard, 2007; Speijers & Speijers, 2004). Among all mycotoxins, aflatoxins (AFs) consisting of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) have been found mostly occurring in agriculture crops which are aimed for human and animal consumption. AFs have been proven to be carcinogenic and mutagenic to humans and have been strictly regulated by the Food and Agriculture Organization (FAO) of the United Nations (Jonker & Egmond, 2004). Fumonisins (FUMs) which include fumonisin B1 (FB1), fumonisin B2 (FB2) and fumonisin B3 (FB3) are a type of commonly found mycotoxins in corn and other small grains. A major disease caused by FUMs are liver and kidney tumors (Richard, 2007). Trichothecenes, which are large groups of mycotoxins including type A: T-2, HT-2, neosolaniol (NEO), diacetoxyscirpenol (DAS) and type B: deoxynivalenol (DON) and nivalenol (NIV), occur in general crops and are considered with weight loss or poor weight gain, diarrhea and vomiting effects. Resorcylic acid lactones, including zearalenone (ZEA) and several metabolites, could affect the re-productivity ability to the consumer due to their estrogenic effects. Ochratoxins, with the main metabolites ochratoxin A (OTA), have been associated with kidney and liver damage. Emerging mycotoxins, such as enniatin A (ENA), enniatin A1 (ENA1), enniatin B (ENB), enniatin B1 (ENB1) and beauvericin (BEA), which could occur in various crops, have only recently been studied (AB Serrano et al., 2013), while they have demonstrated a potential risk for human health due to their toxic effects in cell lines (Prosperini et al., 2012). Moniliformin (MON) usually exists as a sodium or potassium salt in agriculture crops (C. W. Lim et al., 2015). The risk for MON is related with cardiac impairment. Most of these mycotoxins are generated by fusarium, except aflatoxins and ochratoxins, which are mainly produced

by aspergillus. There are other toxicological important mycotoxins not listed in Table 1 since they are not included in this study.

The potential risk listed in Table 1 for each mycotoxin are most frequently linked with the exposure of a certain mycotoxin. In addition, exposure to multi-mycotoxins could aggravate the negative consequences even with low contamination levels, with suspicion of synergistic effects (Creppy et al., 2004; Speijers & Speijers, 2004). Ingesting is the main source for mycotoxin entering human and animal system, despite some other ways like through dermal (when skin is exposure to contaminated source) and inhalation (when inhale mycotoxin contaminated air) approaches (Jennifer, 2019).

A variety of agriculture commodities are at risk of mycotoxin contamination such as cereals, grains, nuts, fruits, dried fruits, vegetables, cocoa and coffee beans, wine, herbs and spices, where most of them are intended for human consumption (Cinar & Onbaşı, 2019). The vulnerable agriculture crops listed in Table 1 could be consumed both by human and animal.

Table 1 Major mycotoxin groups, types, fungal origins, vulnerable agriculture species and mycotoxicosis symptom

Mycotoxins	Fungal species	Major types	Vulnerable agriculture species	Potential hazard
Aflatoxins	Aspergillus ssp.	Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2	Maize, Groundnut, Oilseed, Cotton seed	carcinogenic, teratogenic, mutagenic, hepatotoxic, immunosuppressive
Fumonisins	Fusarium ssp.	Fumonisin B1, Fumonisin B2, Fumonisin B3,	Corn, wheat, oats, barley	Liver and kidney, tumors, oesophagal cancer, lung oedema
Trichothecenes	Fusarium ssp.	Type A: T-2, HT-2, Neosolaniol, DiacetoxyscirpenolType B: Deoxynivalenol, Nivalenol	Wheat, Oats, Barley, corn	weight loss,diarrhea,dermal necrosis, food refusal, vomiting
Resorcyclic acid lactones	Fusarium ssp.	Zearalenone, Zearalanone,α-zearalenol, β-zearalenol	Maize, Wheat, Barley, Sorghum	estrogenic effects, reproductive toxicity
Ochratoxins	Aspergillus, Penicillium ssp.	Ochratoxin A, Ochratoxin B, Ochratoxin C, Ochratoxin α	Barley, Maize, Sorghum	kidney and liver toxin,carcinogen, chronic toxicity as accumulates in body

Enniatins	Fusarium, Beauveria ssp.	Enniatin A, Enniatin A1, Enniatin B, Enniatin B1, Beauvericin	Flour, Barley, Maize, Wheat, Rice	acutely toxic, cardiac symptoms, herbicidal, insecticidal
Moniliformin	Fusarium, Penicillium, Aspergillus ssp.	Sodium adduct, Potassium adduct	Maize	acutely toxic, cardiac impairment

1.2 Fungal classification and reason for increasing mycotoxin contamination in agriculture

Fungi can grow and generate mycotoxins pre-harvest or during storage, transport, processing and feeding as seen in Fig.1 (Cinar & Onbaşı, 2019). Accordingly, those that invade before harvest, called field fungi; while those that occur only after harvest, called storage fungi (Kovalsky et al., 2016; Tola & Kebede, 2016). The species of field fungi and storage fungi may differ from places to places depending on local geographical and climate conditions. A research by the Norwegian Scientific Committee for Food Safety (VKM) has investigated (Table 2) that the most important mycotoxin-producing field fungi in Norway belong to fusarium genus, while the most important mycotoxin-producing storage fungi are species of aspergillus and penicillium (Bernhoft et al., 2013). Main corresponding mycotoxins that generated by these field and storage fungi are also listed.

Table 2 Important field- and storage- fungi and corresponding main mycotoxins in Norway

Species	Genus	Mycotoxins
Field fungi	Fusarium	BEA, DON, ENNs, FUMs, HT-2, MON, NIV, T-2, ZEA
Storage fungi	Aspergillus	AFs, OTA
	Penicillium	OTA

As seen in Fig. 1, before harvest, biological factors such as crops sensitivity and mycotoxin genic fungi species could affect mycotoxin occurrence; while after harvest, environmental factors such as temperature, humidity and atmospheric conditions are more related with the mycotoxin occurrence. Compared with the host crops, some toxigenic fungi may become more adapted to changing environmental conditions, for example changes in temperature and precipitation patterns, and therefore produce more toxic mycotoxins and other metabolites (Magan et al., 2011). Studies have already shown evidence that several species could become more actively and grow effectively at challenging environments, especially under drought stress (Chin et al., 2010; Manning, 2010; Williams & Hallsworth, 2009). In addition, the earth climate is turning to warmer and wetter by the global warming effect, which is also favorable for mycotoxin formation and propagation. Consequently, climate change could be one of the most important factors contributing to increased

problems with fungus growth and mycotoxin contamination world widely (BIOMIN, 2019; Magan et al., 2011).

Miraglia et al. (2009) ascribes the occurrence of mycotoxins in food and feed as an emerging food safety issue that impacted by climate change. This view is based on the evaluation of how global climate change including temperature increase, variation in precipitation, drought and atmospheric carbon dioxide influence fungi to produce mycotoxins.

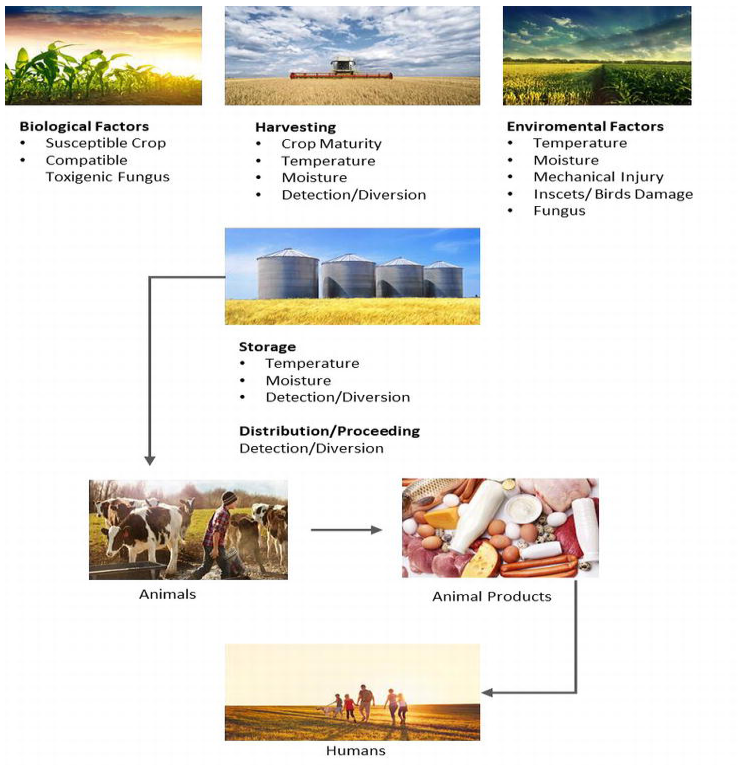


Fig. 1 Factors affecting mycotoxin occurrence in the food and feed chain. Courtesy Cinar & Onbaşı, (2019).

A previous study by Miller (1998) shows approximately 25% of the world's food crops are affected by mycotoxin contamination in different levels. A new study by BIOMIN investigated the occurrence of some main mycotoxins (AFs, ZEA, DON, T-2, FUM and OTA) in 18424 raw ingredients and finished feed samples from 79 countries, including corn (maize), wheat, barley, rice, soybean meal, corn gluten meal, dried distillers' grains and silage (BIOMIN, 2019). The results revealed that exposure to mycotoxin contamination could be a real issue for almost all plant-based feeds in the globe although with regional differences in the occurrence and species of mycotoxins due to different regional climates.

1.3 Mycotoxin contamination in fish feed and mycotoxicosis in aqua-animals

Fish feed is a kind of nutritious materials for fish consumption. It provides balanced nutrition such as protein, carbohydrate, fat, vitamins and minerals to ensure aquaculture growing and surviving. During the relatively short history of intensive fish farming, commercial fish feed composition has changed greatly. According to a research focused on the Atlantic salmon fish feed (Ytrestøyl et al., 2015), almost 90% of the feed came from marine origin at the year of 1990 or earlier, whereas this value decreased to 30% or less by the year of 2013 (Fig. 2). The shortened supply of fish feed from expensive and limited marine origin (primarily from marine oil and marine protein) is substituted by more economical and plentiful plant-based ingredients (mainly from plant oil, plant protein, starch and micro ingredients) (Hooft et al., 2011). The new fish feed option based on plant-sources has the advantage of both satisfying the increasing amount of fish feed requirement and decreasing the cost

of feed prices. However, it could also bring new contaminants that were not earlier associated with fish farming, like mycotoxins. A study by analyzing 175 fish and shrimp feeds (plant-based meal and finished feed) which were sourced from southeast Asia, reveals that 4% of the samples were free of detectable mycotoxin, 8% had one mycotoxin and 88% were contaminated with more than one mycotoxin (R.A. Gonçalves et al., 2018). Therefore, the awareness of mycotoxin related issues should be a concern in the aquaculture industry.

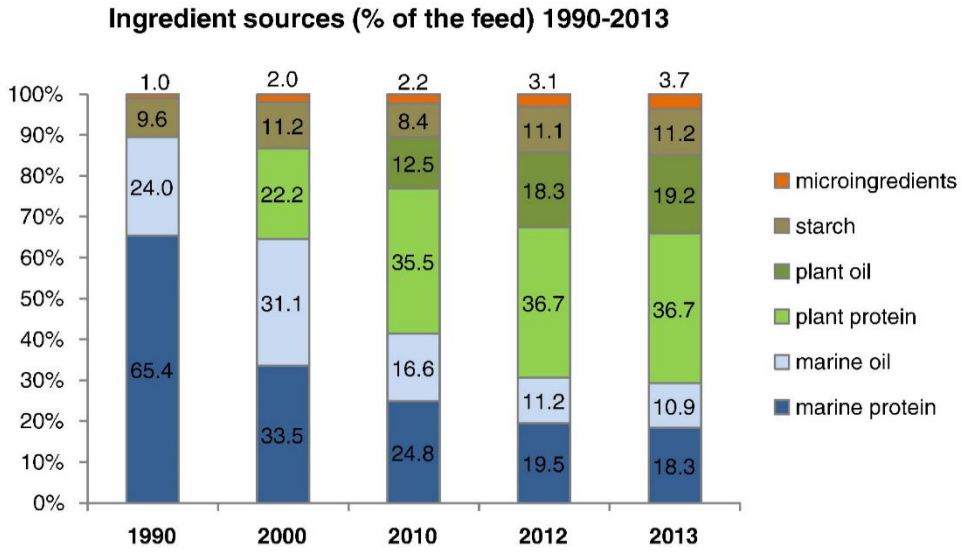


Fig. 2 Nutrient sources in Norwegian salmon farming from 1990 to 2013. (Each ingredient type is shown as its percentage of the total diet). Courtesy, Ytrestøyl et al., (2015).

Damage by mycotoxins in aqua-animals depends on the aquaculture species, mycotoxin species and the exposure extent. Among all the mycotoxins, AFs are the most researched mycotoxins with respect to fish health and well-being due to their wide existence, highly toxic and carcinogenic property (C. Lim & Webster, 2001; Sahoo & Mukherjee, 2001).

In an aquarium study giving by the Southern regional aquaculture center (SRAC) (Manning, 2010), rainbow trout is very sensitive to the presence of aflatoxin in the diet, with a low concentration at 0.4 ppb (µg/kg) could lead hepatocellular carcinoma (HCC) in 14 percent of trout fish over a period of 15 months. While channel catfish appears to be more tolerable to aflatoxin presented diet, feeding by moldy corn containing up to 275 ppb aflatoxins showed no reductions in weight gain or survival. An experiment carried on tilapia when fed a diet with 1880 ppb aflatoxin for 25 days showed apparently reduced growth rates, but not when fed a diet with 940 ppb aflatoxin.

Generally, most of the mycotoxins that produced by aspergillus, penicillium and fusarium species have the potential to reduce growth and impair health conditions of aqua-farmed animals (Rui A. Gonçalves et al., 2016). Specifically, the health issues could be either carcinogenic (e.g. AFB1, OTA, FB1), oestrogenic (e.g. ZON), neurotoxic (FB1), nephrotoxic (OTA), dermatotoxic (several mycotoxins belong to trichothecenes) or immuno-suppressive (AFB1, OTA and T-2) (Rui A.

Gonçalves et al., 2016). In addition, co-contamination by more than one mycotoxin could produce greater toxicity to aqua animals, but very few studies have evaluated the effects of multi-mycotoxin contamination in aquatic species. Manning (2010) has evidenced lower weight gains could be found when feeding with two mycotoxins contaminated feed than only one mycotoxin.

1.4 Mycotoxin regulations

The high risk of exposure to mycotoxins has resulted in the establishment of mycotoxin regulation in more than 99 countries (Jonker & Egmond, 2004). National regulations have been established for mycotoxins such as AFs, DON, DAS, T-2, HT-2, FUMs (B1, B2, B3), OTA, ZEA and some other mycotoxins (agaric acid, argot alkaloids, patulin and phomopsins) (Jonker & Egmond, 2004). European Commission Regulation (EC) No. 1881/2006 and its amendments has specified maximum levels in foodstuffs for AFs, DON, FUM, OTA, PAT and ZEA (European Commission, 2006). According to the regulation listed in Table 3 (adapted from European Commission (2006)), the maximum level for total AFs is ranging from 4ppb (ug/kg) to 15 ppb in most variety of food (moreover the separate limit for AFB1 is also listed); for OTA is ranging from 0.5ppb to 10ppb; for DON is between 200ppb and 1750ppb; for ZEA is between 20ppb and 100ppb; for FUMs is between 200ppb and 2000ppb. Besides, Commission Recommendation 2013/165/EU provides indicative levels, above which investigations should be performed on the factors leading to the presence of T-2 and HT-2 mycotoxins (European Commission, 2013). These compounds are included in routine monitoring programs from national food authorities and data has also been collected for the occurrence of these mycotoxins in different foodstuffs.

Table 3 Maximum levels for mycotoxins in foodstuffs

Mycotoxin	Food commodities	EU (EC 2006) ppb (ug/kg)	
		B1	Sum of B1, B2, G1 and
Aflatoxins (AFs)	Groundnuts (peanuts) before direct consumption	8.0	15.0
	Nuts, dried fruit, spices and maize before direct	5.0	10.0
	Groundnuts, dried fruit and cereals intend for direct	2.0	4.0
	Several kinds of baby food and food for special medical	0.1	-
Ochratoxin A (OTA)	Unprocessed cereals, Roasted coffee beans and coffee	5.0	
	Products derived from unprocessed cereals and normal cereals intended for direct consumption	3.0	
	Dried vine fruit and soluble coffee	10.0	

Table 3 Maximum levels for mycotoxins in foodstuffs

	Several kinds of baby food and food for special medical	0.5
Dioxynivalenol (DON)	Unprocessed durum wheat, oats and maize	1750
	Unprocessed cereals	1250
	Cereals intended for direct consumption and pasta	750
	Bread	500
	Baby food from cereals	200
Zearalenone (ZEA)	Unprocessed maize and maize intended for direct	200
	Unprocessed cereals other than maize	100
	Other cereals intended for direct consumption	75
	Bread, maize snacks and maize based breakfast cereals	50
	Maize and maize based-food intended for direct	100
	Several types of baby foods	20
	Processed maize based-foods	20
Fumonisins (FUMs)	Unprocessed maize	2000
	Maize and maize based-food intended for direct	1000
	Maize-based foods for direct consumption	400
	Processed maize-based foods and baby foods	200
Patulin, citrinin and ergot alkaloids are excluded in this table because they are out of the study		

So far, regulations for mycotoxins (except AFs) exist mostly for human foods and more incidentally for animal feeds. AFs have been regulated in feed industry among many countries. From a guidance by EC specified for AFB1 (European Commission, 2002c), the maximum level in feeding stuffs is legislated ranging from 0.005 mg/kg (ppm) for dairy animals to 2 ppm for pet animals (Table 3). While the maximum regulatory levels for many other mycotoxins and important metabolites such as, enniatins, beauvericin, 3-ADON, 15-ADON, NEO are still not available until now, even though their potential risk has been confirmed in various studies. Therefore, it is to be expected that more regulations for mycotoxins and metabolites will increase in the near future, for both food and feed.

Table 4 Maximum content for Aflatoxin B1 in animal feed

Undesirable substances	Products intended for animal feed	Maximum content in ug/kg (ppb) relative to a feeding stuff with a moisture content of 12%

Aflatoxin B1	All feed materials	20
	Complete feeding stuff for cattle, sheep and	20
	- Complete feeding stuff for dairy animals	5
	- Complete feeding stuff for calves and lambs	10
	Complete feeding stuff for pigs and poultry	20
	Other complete feeding stuff	10
	Complementary feedings stuff for cattle, sheep	20
	Complementary feeding stuff for pigs and	20
	Other complementary feeding stuff	5

1.5 Mycotoxin metabolites and transformation products

The topic of mycotoxin (or fungus) metabolites came out in the middle of 1980s, because in some cases of mycotoxicosis, the high toxicity came from undetected metabolites rather than the parent mycotoxins (Binder, 2007). Now the metabolites (also known as masked mycotoxins) receive the same attention as mycotoxins in the study of mycotoxin and mycotoxicosis.

The metabolization process of mycotoxins occurs in three phases in the cell, depending on the structure of the precursor (De Boevre et al., 2012). Phase I is a transformation phase where reactive groups are generated on the mycotoxins by reduction, oxidation or acetylation reaction (De Boevre et al., 2012). For example, DON could transform to different chemical-end products like 3-ADON and 15-ADON by acetylation (Crippin et al., 2019), while AFB1 could transform to AFM1 by hydroxylation in animal tissues and fluids (milk and urine) (Richard, 2007). Phase II is a conjugation phase facilitated by enzymes, where mycotoxins are conjugate with polar compounds such as sugars, amino acids or sulphates. It is shown, that ZEA could transform to αZEL and βZEL by plant enzymes in maize cell suspension (Engelhardt et al., 1988). Phase III is a transportation process where the conjugated mycotoxins are transported out of the cell (De Boevre et al., 2012). Except of 3-ADON and 15-ADON, DOM-1 and D3G are two other common metabolites from DON and exists widely in feed ingredients. Pierron (2016) found that D3G contributes to up to 10% of the overall DON contamination in wheat and maize, even exceeding the level of 3-ADON and 15-ADON.

The exist of variety metabolites, besides free mycotoxins, may also pose a potential risk to the consumers after digestion, as they can be toxic to target animals and/or may augment the toxicity of the known mycotoxins (Bullerman & Bianchini, 2007; Xu et al., 2006). Mycotoxins may also transform to other products outside the cell through diverse chemical transformation pathways. Research on biological pathological changes of mycotoxins is now increasing dramatically (Berthiller et al., 2006; Böswald et al., 1995; Engelhardt et al., 1988; Zöllner & Mayer-Helm, 2006). However, there is very limit study on chemical transition of mycotoxins outside of cell.

Meca et al. (2012) performs the reduction experiment for BEA using allyl isothiocyanate as a reactant. Results show that BEA degraded from 20% to 100% in the solution, compared with from 10% to 65% in a comparison. The research also identifies two degradation products. Young et al. (2006) use aqueous ozone to oxidant ten trichothecene mycotoxins. The start site of reaction in mycotoxins with the impact of different PH values has been evaluated and the structure of some transformation products (TPs) have also been proposed. No TPs are included in mycotoxin routine monitoring programs because of the lack of knowledge on them, but they could contribute to the total mycotoxin content. Therefore, there is a huge potential demand for the study of TPs of mycotoxin by chemical approaches.

1.6 Mycotoxin determination

Determination of mycotoxins in different matrix includes generally three steps: sampling, sample preparation and final analysis. Sampling includes selecting a certain size of samples from a bulk lot, grinding, homogenization and taking a representative sub-sample of the homogenized materials (Prichard & Barwick, 2007). It can be presumed that mycotoxin distribution in raw fish feed batches is inhomogeneous. Therefore, a suitable sampling procedure is required.

Besides sampling, a proper sample preparation method to extract mycotoxins from the complicated matrices is another crucial factor for the determination. The selection of extraction methods depends on the chemical properties of the mycotoxins, the food matrix and the detection methods which are going to be implied (Alshannaq & Yu, 2017). Solid matrices, like fish feed, could use a solid-liquid extraction method whereby a mixture of organic solvent with addition of water and acidic buffer is used as solvent. Reason is that most mycotoxins are highly soluble in organic solvents, except FB1, which is soluble in water. After extraction, filtration and centrifugation are important steps to remove other interfering compounds before further clean-up steps.

Since the maximum permissible levels of mycotoxins are quite low, analytical methods for mycotoxin determination have to be both sensitive and specific. Different methods used for mycotoxin determination in various matrices have been reviewed by Kralj Cigić & Prosen (2009). A variety of immunological methods has been detected and widely applied, which require no further purification process. This technique provides a rapid, specific and easy-to-use method for mycotoxin analysis, but also with a disadvantage of only one or one type of mycotoxin could be determined by each test. For multi-mycotoxin determination purpose, chromatographic methods are the most commonly used method. Due to the low volatility and high polarity of most mycotoxins, GC analysis, which suitable for volatile compounds, is less often used than LC analysis in the mycotoxin field. Liquid chromatography techniques, especially the introducing of high-performance liquid chromatography

(HPLC), coupled with mass spectrometer (e.g. (HP)LC-MS, (HP)LC-MS/MS) are routinely used for analysis of mycotoxins in foodstuffs (Pereira et al., 2014).

HPLC separates compounds due to their relative differences in travel through the column by applying high pressure during process (Snyder et al., 2011). The different travel rates come from their relatively affinities with mobile phase (solvent) and stationary phase. HPLC could improve analysis efficiency by allowing the separation of a wide range of compounds with even low concentrations at fast rates (Snyder et al., 2011). Unlike other detectors (e.g. fluorescence), good chromatographic separation of analytes is not mandatory and overlapping is allowed when HPLC is in combination with MS (Kralj Cigić & Prosen, 2009).

Apart from the great separation performance by HPLC, MS as a powerful confirmatory technique, offers higher sensitivity and selectivity, as well as chemical structural information from the mass spectrum. Basically, MS works by ionizing the molecules, sort and identify them based on their mass-to-charge ratio (m/z) (Downard, 2004). Different ionization mode could be used in MS, such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). In addition, different types of analyzers could be used in MS, such as quadrupole, time-of-flight (TOF) and ion-trap. Among various ionization and detection techniques, ESI, triple quadrupole and TOF are the most extensively used for mycotoxin analysis (Krska et al., 2008; Turner et al., 2009). Usually applying one MS spectrometer is not specific enough because several molecules may have the same m/z. In order to improve the sensitivity and specificity, tandem MS spectrometry (see 1.7.1) is more often applied.

A lot of LC-MS based methods have been published for the determination of mycotoxin in different matrices with tandem MS (De Baere et al., 2011; García-Moraleja et al., 2015; Kovalsky et al., 2016; C. W. Lim et al., 2015; Monbaliu et al., 2010; Spanjer et al., 2008; Vendl et al., 2009). Monbaliu et al. (2010) in his research evaluates 23 mycotoxins in three different animal feed matrices (sow feed, maize and wheat) by LC-MS/MS, and reveals 82% of animal feed measured were contaminated with mycotoxins. García-Moraleja et al. (2015) using LC-MS/MS quantitatively measures mycotoxins including NIV, T-2, HT-2, DAS, AFs, FUMs, OTA, ZEA, ENA, ENA1, ENB and ENB1 in commercial coffee and successively detects the exact concentration range for existed mycotoxin in coffee samples. C. W. Lim et al. (2015) using LC-QTOF quantitatively measures MON in cereals and performed degradation experiment for MON over two weeks to test its stability.

1.7 LC-QqQ-MS/MS and LC-IMS-QTOF MS

1.7.1 Tandem mass spectrometry

Tandem mass spectrometry is a technique where two or more mass analyzers are employed to increase the measuring ability (IUPAC, 1997). Ionized molecules were separated by the first spectrometer according their m/z ratio, let ions with a certain m/z fragmented and introduced into the second spectrometer, which in turn separates the ions by their m/z ratio and detect them ("Tandem Mass Spectrometry," 2020). Tandem MS includes Triple quadrupole (QqQ), Quadrupole time of flight (QTOF) and quadrupole orbitrap (Q-Orbi)-MS. QqQ MS is a low-resolution MS technique while the other two are high resolution MS.

Typically, MS resolution (R) is defined as R= m/Δm FWHM, where m and Δm corresponds to the theoretical m/z and the distribution of m/z at full-width half-maximum of the peak height (Δm FWHM), respectively as shown in Fig. 3 (Rochat, 2018). The units of Δm is Da, u or Th.

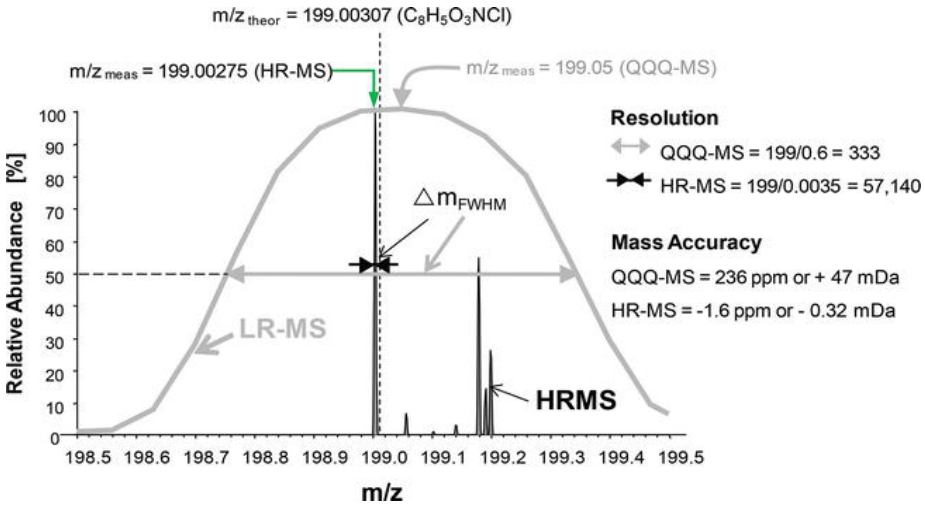


Fig.3 Example of typical mass resolution and mass accuracy (Rochat, 2018)

MS accuracy is defined as the delta between theoretical and measured m/z and is given in mDa, u, mTh or ppm (Rochat, 2018). Low- and high-resolution MS are defined with an R value below or above 10000, respectively (Rochat, 2018). That is why QqQ-MS is classified as low-resolution MS while QTOF is classified as high-resolution MS.

Liquid Chromatography coupled with tandem mass spectrometry combines the nice separation ability of LC to separate the compounds physically and the good detection power of MS to ionize, separate and identify ions. Ultra-high-resolution liquid chromatography (UHPLC) coupled with low resolution QqQ-MS and high resolution QTOF-MS are used in this study and a brief introduction is shown below.

1.7.2 UHPLC-QqQ-MS/MS

UHPLC-QqQ-MS/MS consists a UHPLC system, an electrospray ion source and triple quadrupole (QqQ) mass analyzer. Compounds separated by UHPLC enters the electrospray ion source where an electric field (2000-3000V) and thermal heat are employed. The analyte droplets become charged and split into smaller ones before entering the QqQ-MS (Faktor et al., 2012). In QQQ-MS (Fig.4), the first quadrupole (Q1) is used to remove ions that are not relevant, and allow ions which are relevant to pass into the second quadrupole (Q2). The fragmentation happens in Q2 by applying a collision energy that fragments precursor ions to product ions. The product ions then pass through a third quadrupole (Q3) and selected ions are separated there before passing through the detector (Faktor et al., 2012).

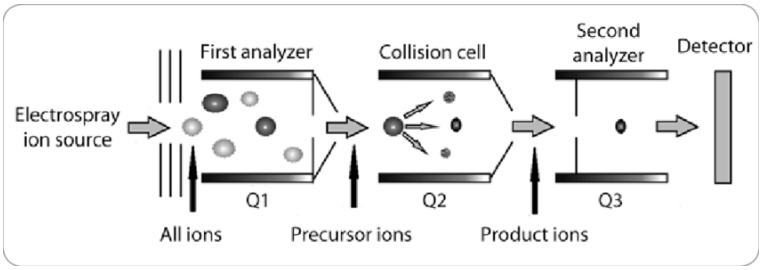


Fig. 4: Triple quadrupole working mechanism. Courtesy Faktor et al.,(2012)

QqQ-MS is slow and insensitive when applying full scan mode. Therefore, the selected reaction mode (SRM) / multiple reaction mode (MRM) is usually chosen in order to be selective and sensitive (Rochat, 2018). The applying of MRM could give excellent quantitative capabilities and low limit of detection (LOD) because only selected precursor ion with specific m/z value is fragmented into product ions and only these targeted product ions are measured. Chemical noise from other ions could be reduced and thus increase detector sensitivity. However, it also means that all necessary information such as the precursor ion, product ion and collision energy have to be known before the analysis. This requiring optimization of each analyte, which in turn restricts the applicability to those interested compounds and excludes other unknown compounds (Regueiro et al., 2016). In addition, the maximum number of analytes in one run is limited due to the dwell time of MRM transitions. Generally, QqQ-MS is a targeted approach for routine and quantitative analysis.

1.7.3 UHPLC-IMS-QTOF MS

UHPLC-IMS-QTOF instrument includes a UHPLC system, an electrospray ionization, an Ion-mobility spectrometry (IMS) and tandem mass analyzer QTOF.

IMS is a gas phase separation technique that separates ions in the gas phase based on their different mobility in the presence of electrical field (Fang, 2017; Regueiro et al., 2017). The ion mobility of a compound depends on its size, charge and shape, which could be characterized by collision cross section (CCS) value (Fang, 2017). CCS is unique for each compound and independent of instrument conditions, therefor it could be used as an additional identification point for compound

characterization (Regueiro et al., 2016). Besides of the good identification purpose, CCS could also be used to confirm the structure of expected unknowns by matching the theoretical and the experimental CCS values (Peter, 2019). In the IMS-QTOF spectrometer, the mobility cell sits between ion source and the quadrupole because IMS separates ions in milliseconds (Fang, 2017). Therefore, this sensitive technique is orthogonal to the HPLC separation that occurs in minutes. The addition of IMS increases peak capability and the resolving power for complex mixtures (Fang, 2017). Laganà (2018) evaluates the capability of using CCS as an extra dimension for mycotoxin identification and the benefits of using CCS to separate isomeric mycotoxins.

QTOF is actually a variation of QqQ MS with the final quadrupole replaced by a time of flight device as seen in Fig.5. TOF separates ions based on the kinetic energy and velocity of ions, specifically, on the different time that accelerated ions need to pass the flight path (Chernushevich et al., 1999). The time spent in the flight path depends on velocity, while the velocity of ions depends on their m/z (basically heavier ions of the same charge reach lower speeds) (Chernushevich et al., 1999). As discussed above, TOF, as a high-resolution MS can differentiate ions that differs in mass within 250 ppm (Steen et al., 2001). QTOF is sensitive and selective in the full scan mode. With high resolution full scan mode, generic parameters could be applied which allows all ions within large mass range being measured. So, QTOF measures both target and non-targeted compounds through targeted and full-scan.

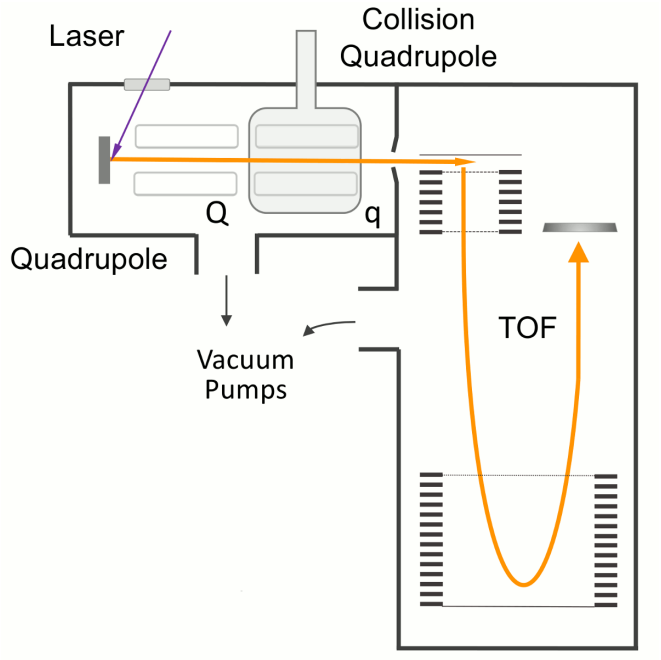


Fig. 5 Quadrupole time of flight schematic. Courtesy (Chernushevich, 1999)

In advanced LC-IMS-QTOF, the IMS determining CCS value offers another identification parameter for the accurate separation and assignment. Besides, QTOF, as a high-resolution MS provides greater selectivity and resolving power. The combination of LC-IMS-QTOF could be used for extensive analytical research with both targeted and untargeted purpose.

1.8 Method validation

After a method has been developed by LC-MS/MS, a validation of the method reliability is required. Method validation is the process used to confirm that the developed analytical method for a specific test is suitable for intended use (Ludwig Huber, 1998). It is aimed at demonstrating that when the

procedure is correctly applied, results produced are fit for purpose (ICH, 1995). The main parameters evaluated in method validation could be divided into four section showing below:

- applicability, fitness for purpose and acceptability limits.
- specificity and selectivity
- calibration study including sensitivity, limit of detection (LOD), limit of quantification (LOQ) as well as matrix effect assessment.
- accuracy study involving accuracy/trueness, precision, measurement uncertainty and robustness (Gustavo González & Ángeles Herrador, 2007).

Some important parameters that could be considered with method validation of the current work are given below.

1.8.1 Selectivity and specificity

Selectivity of an analytical method is its ability to measure accurately the analyte to be determined from other substances present in the same matrix (Prichard & Barwick, 2007). Selectivity is checked by examining chromatographic blanks (from a sample that has been confirmed to contain no analyte) in the expected time interval of the analyte peak (Rao, 2018). Specificity is the ultimate of selectivity, which refers 100% selectivity or 0% interference.

1.8.2 Precision

Precision is the closeness of agreement between independent test results obtained under specific conditions (Prichard & Barwick, 2007). Generally, precision can be determined by running at least three concentrations in triplicate. The concentration for the analyte should cover a low concentration, a medium concentration and a high concentration. When measuring three sample concentrations, precision could be determined, and measuring five sample concentrations, the standard curve could draw. The test of precision could be performed in the same day, using intra-day differences, or in different days, using inter-day differences. Precision could be calculated by standard deviation (SD) or relative standard deviation (RSD) as equations showing below (European Commission, 2002c; Stöckl et al., 2009)

$$SD = \sqrt{\frac{\sum_0^n (xi - \bar{x})^2}{n-1}} \quad \cdots\cdots (1)$$

$$\bar{x} = \frac{\sum_0^n xi}{n} \quad \cdots\cdots (2)$$

$$RSD = \frac{SD}{\bar{x}} \times 100 \quad \cdots\cdots (3)$$

1.8.3 Trueness

Trueness is the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (European Commission, 2002). If certified reference materials (CRMs) are available, trueness could be measured by comparing the result from analytical method with a certified reference value. If no CRMs are available, the measurement of trueness could be calculated by recovery of known analyte into blank matrices as calculated by equation showing below:

$$\text{Recovery} = \frac{C_{calculated}}{C_{theoretical}} \times 100 \cdots\cdots (4)$$

1.8.4 Limit of detection (LOD) and Limit of Quantification (LOQ)

LOD is the lowest amount of an analyte that can be measured with reasonable statistical certainty; while LOQ is the lowest amount of an analyte that can be quantitatively determined with a closely defined confidence. There are several approaches to measure LOD and LOQ, e.g. the noise to signal ratio approach and the standard deviation of the response and the slope approach (Rao, 2018).

Based on signal to noise ratio, the signal to noise ratio for LOD should be 1:3 and for LOQ should be 1:10 by taking signal to noise ratio of a lowest /known concentration of linearity samples (Rao, 2018)

$$\text{LOD} = 3 \times \frac{\text{Signal}}{\text{Noise}} \times \text{lowest concentration of the linearity sample} \cdots\cdots (5)$$

$$\text{LOQ} = 10 \times \frac{\text{Signal}}{\text{Noise}} \times \text{lowest concentration of the linearity sample} \cdots\cdots (6)$$

Based on the standard deviation of the response and the slope, the LOD and LOQ may be expressed as:

$$\text{LOD} = 3 \times \frac{\text{Standard deviation of low concentration}}{\text{Slope of the calibration line}} \cdots\cdots (7)$$

$$\text{LOQ} = 10 \times \frac{\text{Standard deviation of low concentration}}{\text{Slope of the calibration line}} \cdots\cdots (8)$$

1.8.5 Linearity and range

Linearity is assessment of the range over which there is a proportional relationship between analyte concentration and signal response. Linearity is determined by spiking the samples with analyte at a minimum of five different concentrations in the range of 50% -150% of the expected working range (Rao, 2018). The range of an analytical method is the interval between the upper and lower level that the method has been demonstrated to determine with precision, accuracy and linearity. This range correspond to the concentration range in which the linearity test is done.

1.9 Internal standards

Internal standard (IS) is the compound added in a constant amount to samples, blanks and calibration standards during quantification analysis (Nič et al., 2009). The chosen IS should behave similar as the analyte but to provide a signal that could easily be differentiate from the analyte (Nič et al., 2009). Usually an IS is added to the sample immediately after the aliquoting and then follows the analyte through the same preparation and instrument analysis. This implies that IS experiences equal sample preparation and analysis as the analyte. In this way, the IS will adjust for the effect of any random and systematic errors (e.g. insufficient extraction, instrument fluctuations or matrix effects). An IS could be a structure analogue or a staple isotope labeled compound. By using the similar structure as IS, the signal from analyte and IS should be maximally similar. During the detection, they experience similar ion suppression from matrix effect. If the analyte and IS are not structurally similar, the ratio of detector responses for them may vary due to different degrees of ion suppression, thus leading bad quantification. IS with very similar structure could best cover up errors mentioned before.

In chromatography, the use of IS to calculate the concentration of the analyte is through the response factor (RF). Usually a known concentration of analyte ([A]) and IS ([IS]) run first (calibration curve), followed by running of an unknown of analyte (SA) and a known IS (SIS). The RF is calculated by the first run, the unknown concentration could be calculated based on the RF, and concentration of the second IS.

$$RF = \frac{[IS]}{[A]} \times \left(\frac{S_A}{S_{IS}}\right) \quad (9)$$

1.10 Aim of the study

The aim of this study is to develop sensitive and reliable methods for the screening of multi-mycotoxins and metabolites in fish feeds and fish feed ingredients using UHPLC-QqQ-MS/MS and UHPLC-IMS-QTOF MS instruments. These methods can be used to monitor fish feed and fish feed ingredients to ensure fish feed safety. In addition, a quantification method should be developed for ENA, ENA1, ENB, ENB1 and BEA to be able to measure the level of these compounds in fish feed and fish, to study the transfer from feed to fish.

A chemical degradation experiment using H2O2 for the most commonly appeared mycotoxins in fish feed, BEA and ENB, should be performed. The Purpose of this approach is to measure and identify possible transformation products.

2 Materials and methods

2.1 Materials

2.1.1 General reagents and equipment

General reagents in this experiment are listed below in Table 5:

Table 5 General chemicals and supplier

Chemicals	Supplier
HPLC grade acetonitrile	Sigma Aldrich (St. Louis, MO, USA)
LC-MS grade methanol	Merck (Darmstadt, Germany)
LC-MS grade acetonitrile	Merck (Darmstadt, Germany)
LC-MS grade ammonium acetate	VWR International (Oslo, Norway)
Formic acid	Fluka Chemie (Basel, Switzerland)
Formic acid (98%-100%)	Merck (Darmstadt, Germany)
Formic acid for extraction	VWR International (Oslo, Norway)
Deionized water	MILLI-Q system (Millipore, Milford, MA, USA)
Leucine-enkephalin	Waters (Manchester, U.K.)

General equipment used in the experiment are listed below in Table 6:

Table 6 General equipment and supplier

Equipment	Supplier
Column for LC	Agilent Technologies, Santa Clara, CA
Pipette for titration	Eppendorf AG, Hamburg, Germany
Pipette for titration	VWR International, Germany
Balance	Sartorius, Germany
Multi- tube vortexer (Bench Mixer XL)	Benchmark Scientific, NJ, USA
Blender (GRINDOMIX GM 300)	Retsch, Haan, Germany
Eppendorf 5810R centrifuge	Eppendorf, Hamburg, Germany
Eppendorf 5427R centrifuge	Eppendorf, Hamburg, Germany
Cellulose syringe filter	Sigma Aldrich (St. Louis, MO, USA)

2.1.2 Mycotoxin standards studied in this project

Eighteen mycotoxin standards, six mycotoxin metabolites and five 13C isotopically labeled standards were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The amount, concentration and CAS numbers of all standards are shown in Table 8. The use of mycotoxins and metabolite standards was to establish screening methods for determination purpose, while isotopically labeled standards were used as internal standards for mass spectrometry-based methods. Among all the standards, 15-ADON, 3-ADON, αZEA, BEA, βZEA, DON, ENA, ENA1, ENB, ENB1, FB1, MON and OTA were solid powder, and they were prepared in methanol (MeOH) for both stock solutions and test solutions. While Aflatoxin Mix (B1, B2, G1, G2), DOM1, D3G, FB3, DON-13C15, AFB1- 13C17, OTA-13C20, OTA- 13C20, FB1- 13C34, NIV- 13C15, T2, HT2, HT2 were liquid standards which had already diluted in acetonitrile (ACN) from purchase, so they were further diluted in ACN for the stock solutions and working solutions. Stock solutions for solid standards were obtained by diluting the original standards and for liquid standards were obtained by directly transfer the original standards to amber glass bottles; corresponding working solutions were prepared by further appropriate dilution of stock solutions. They were stored in glass-stoppered bottles and darkness in security conditions. All stock and working solutions stayed in freezer at -20 °C except BEA, FB1, OTA and MON which were stored in fridge at 4°C, followed the recommended storage temperature until the instrument analysis.

Table 7 Individual standard solutions

Compounds	CAS Number	Amount
Solid Standards		
15-acetyldeoxynivalenol	88337-96-6	5mg
3-acetyldeoxynivalenol	50722-38-8	5mg
Beauvericin	26048-05-5	5mg
Alpha-Zearalenol	36455-72-8	5mg
Beta-Zearalenol	71030-11-0	5mg
Deoxynivalenol	51481-10-8	5mg
Ochratoxin A	303-47-9	5mg
Enniatin A	2503-13-1	1mg
Enniatin A1	4530-21-6	1mg
Enniatin B	917-13-5	1mg
Enniatin B1	19914-20-6	1mg
Fumonisin B1	116355-83-0	1mg
Moniliformin	71376-34-6	1mg
Liquid Standards (diluted in acetonitrile)		
Aflatoxin B1	1162-65-8	2ml;0.5 μg/ml for B2 and G2
Aflatoxin B2	7220-81-7	
Aflatoxin G1	1165-39-5	

Aflatoxin G2	7241-98-7	2 µg/ml for B1 and G1
Deepoxy deoxynivalenol	88054-24-4	2ml; 50 µg/ml
Deoxynivalenol-3-glucoside	131180-21-7	1ml; 50 µg/mL
Diacetoxyscirpenol	2270-40-8	2ml; 100 µg/ml
Fumonisin B3	136379-59-4	1ml; 50 µg/ml
T2-Toxin	21259-20-1	2ml; 100 µg/ml
HT-2 Toxin	26934-87-2	2ml; 100 µg/ml
Neosolaniol	36519-25-2	2ml; 100 µg/ml
Internal Standards (diluted in acetonitrile)		
Aflatoxin B1-13C17	1217449-45-0	1ml; 0.5 µg /mL
Ochratoxin A-13C20	911392-42-2	1ml; 10 µg /mL
Fumonisin B1-13C34	1217458-62-2	1ml; 25 µg /mL
Nivalenol-13C15	911392-40-0	1.2ml; 25 µg /mL

Fig. 6 to Fig. 7 show the chemical structural of the mycotoxins included in this study..

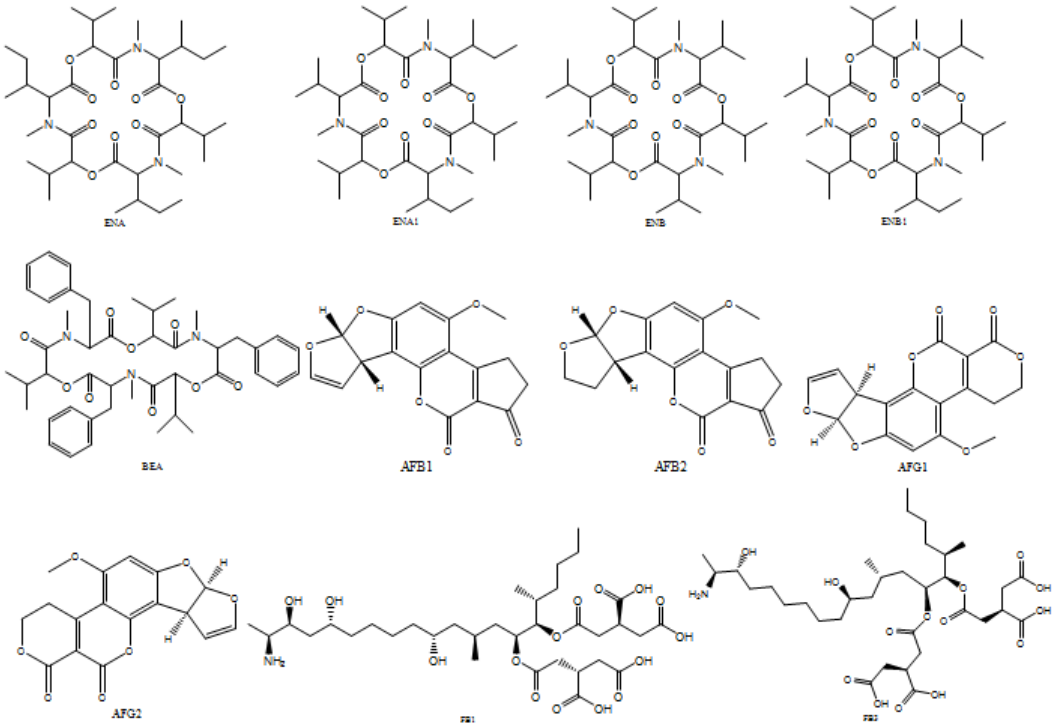


Fig. 6: Structures for ENA, ENA1, ENB, ENB1, BEA, AFB1, AFB2, AFG1, AFG2, FM1 and FM3

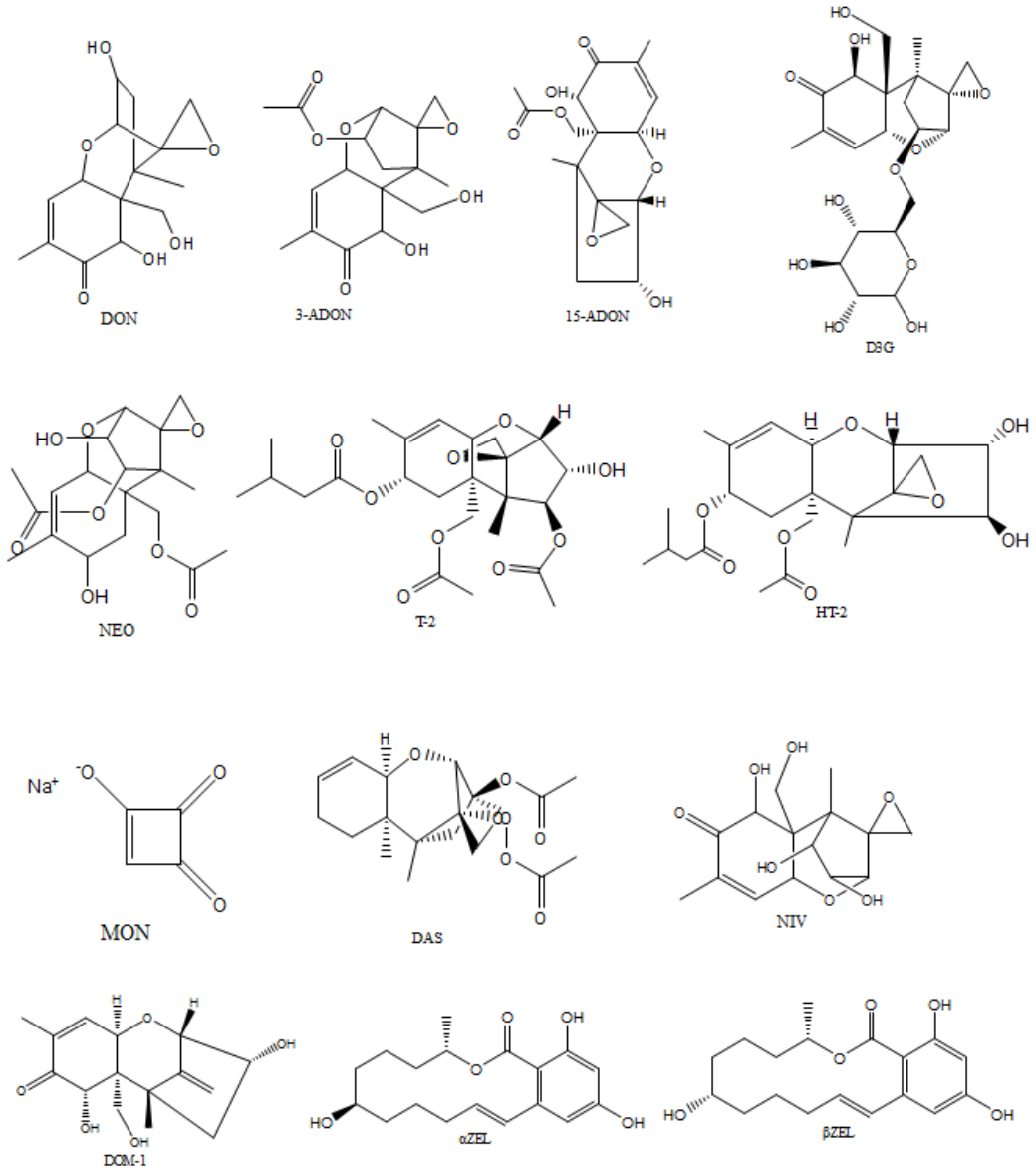


Fig. 7 Structures for DON, 3-ADON, 15-ADON, D3G, NEO, T-2, HT-2, MON, DAS, NIV, DOM-1, αZEL and, βZEL

2.1.2 Fish feed samples

Ten commercial fish feed samples were chosen randomly, involving one fish feed ingredient from maize flour and nine general fish feed samples consisting of different proportions of marine ingredients and plant ingredients. They came from different manufacturers in Norway through the Norwegian national surveillance program conducted by the Institute of Marine Research (IMR) on behalf of the Norwegian Food Safety Authorities. All fish feed samples were grinded into powder, homogenized thoroughly at 2000 RPM using a stainless-steel blender GRINDOMIX GM 300 from Retsch and then stored in polypropylene containers at -25°C before use.

2.1.3 Instruments and software

The UHPLC-QqQ-MS/MS (Agilent Technologies, Santa Clara, CA) system includes several parts showing below:

- Agilent 1290 Infinity UHPLC system consisting of
  - Agilent 1290 Infinity Binary Pump
  - Agilent 1290 High performance Autosampler
  - Agilent 1290 Infinity Thermostatic Column Compartment
- Agilent G6460A Triple Quadrupole Mass Spectrometer
- Agilent Jet Stream electrospray ionization source
- Agilent Mass Hunter Workstation Software

The UHPLC-IMS-QTOF MS (Waters, Manchester, UK) system includes several parts showing below:

- Waters ACQUITY UHPLC I-Class system consisting of
  - Waters Binary Pump
  - Waters vacuum degasser
  - Waters autosampler
  - Waters column oven
- Waters Vion IMS QTOF hybrid mass spectrometry
- Waters Lock Spray ion source
- Waters UNIFI Software

Chemical draw (v. 19.0 / July 16, 2019 PerkinElmer, America) was used for drawing and characterizing chemical structures.

Zeneth software is used for TPs prediction, it works by following the principles shown in Fig.8.

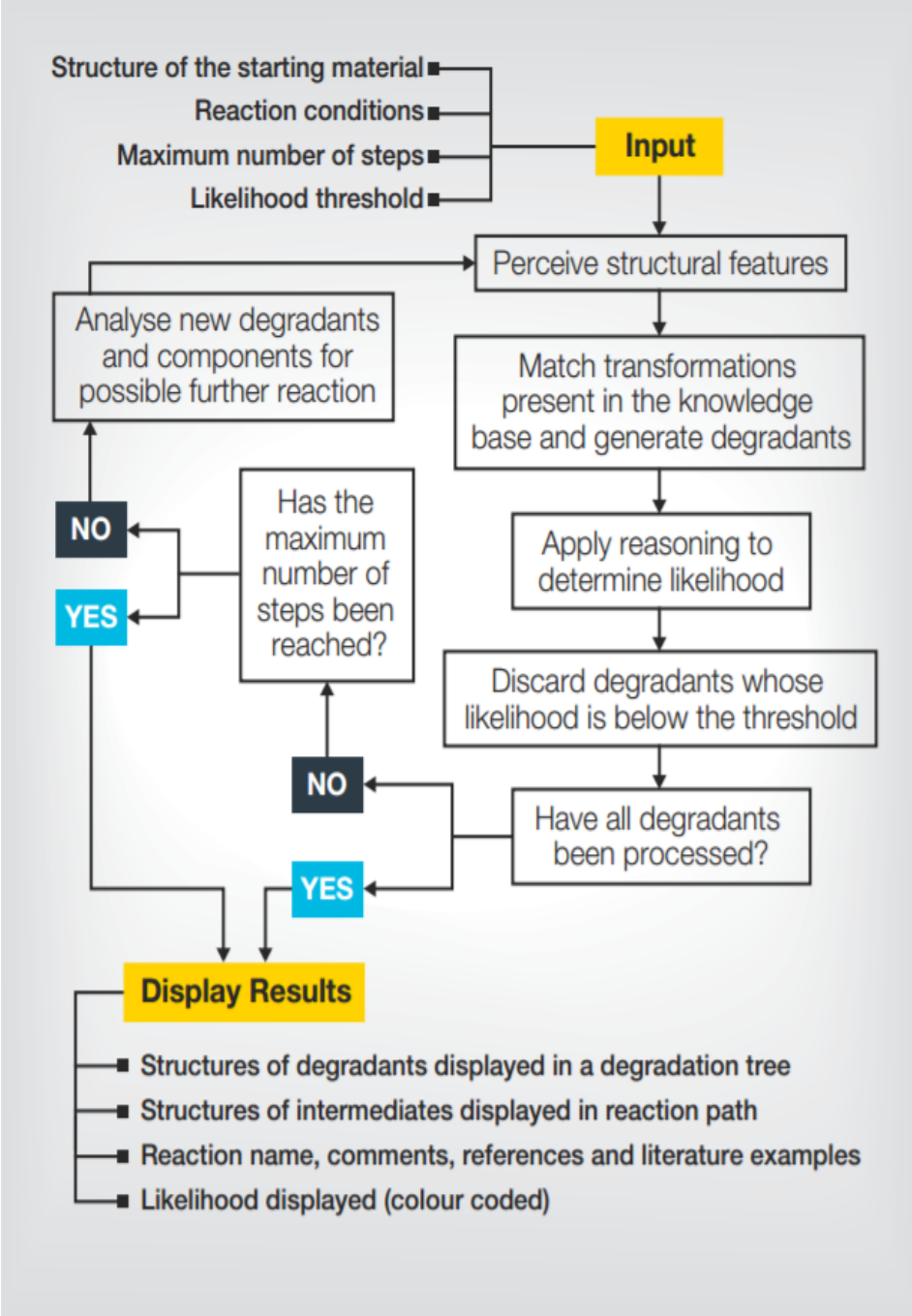


Fig. 8 Working principles for Zeneth software ((Parenty et al., 2013).

2.2 Method development

2.2.1 Fish feed extraction

All fish feed samples were thawed at room temperature (20 ± 2 ºC). Sample extraction follows protocol showing below:

Fish feed (2.5 g) were put into a 15 ml polypropylene centrifuge tube. 5ml of acetonitrile/water/formic acid (75:24:1, v/v/v) were added and the tube was shaken for 1h at 2500RPM in a multi-tube vortexer Bench Mixer XL. After shaking, the tube was centrifuged at 3000 RCF by an Eppendorf 5810R

centrifuge for 10 min, the supernatant was collected and stored in a 15 ml polypropylene centrifuge tube at -25 °C for 2-3 h (in order to precipitate lipids and other macromolecules). After freezing, 0.5 ml of the upper phase was transferred into a 2 ml polypropylene micro-centrifuge tube. The sample was diluted with 0.5 ml of 0.1% formic acid in water (v/v) and votex shaked for 1 min to yield a cloudy suspension. The suspension was centrifuged at 18000 RCF for 5 min at 10 oC by Eppendorf 5427R. The supernatant was filtered through a 0.20 μm regenerated cellulose syringe filter from Sigma into an autosampler vial. Some sample states are shown in Figure 13. Blank samples were prepared followed the same preparation process.

Fig. 9 Sample state during preparation

2.2.2 Degradation experiment

Degradation experiments were performed on BEA and ENB using H2O2. Stock solutions of BEA and ENB was used in the study, the experiment was performed on BEA and ENB separately. Two aliquots, 50 ml each, of the standard (50ug/ml) were taken. Each aliquot contained 50 ml. H2O2 (30%, 50 ml), was added to one of the samples, while 50 ml deionized water was added to the other sample (control sample). The samples were vortexed before they were analysed with the UHPLC-IMS-QTOF instrument.

The whole treatment lasted up to 15 days to obtain proper transportation products, as demonstrated in Appendix Table 1. Degradation kinetics as well as structures of the main TPs were measured by high resolution LC-IMS-QTOF MS. Prediction was carried out using Zeneth software. For both BEA and ENB, the constraints used for prediction was followed by Table 9. Identification of these TPs is based on comparison of the detected peaks for the standards that have been treated by H2O2 with data generated from prediction software.

Table 8 Prediction constraints by Zeneth software

Menu	Descriptions
Reasoning	Absolute reasoning: likely Relative reasoning: grow from first n (n=1) level Pathway likelihood: lowest likelihood

Chemical	Reaction types: allow the query compound to react on its own; allow the query compound to react with itself and with degradants; allow a degradant to react with itself; Maximum monomer count: 2 Hydrogen: perceive implicit and explicit hydrogens
Numerical	Maximum total number of degradants: 500 Maximum number of steps in a pathway: 2
Transformations	All transformations
Physical state	Solution
Condition	temperature: 20 °C; water; oxygen; radical initiator; peroxide

2.2.3 Chromatographic conditions and Mass spectrometry conditions for UHPLC-QqQ-MS/MS analysis

For UHPLC-QqQ-MS/MS instrument, a summary of the 1290 Infinity UHPLC conditions is shown in Table 9, and Table 10 summarizes the 6460 QqQ-MS parameters. Identification of polarity, precursor and product ions, as well as optimization of fragment voltages and collision energies (CE) were first done using the Mass Hunter Optimizer Software without a UHPLC column. Both positive and negative ionization were tested. The optimizer selects precursor ions, product ions, and the optimum CE based on the most abundant signal from the entire data collection window. Optimization was performed by different mobile phases to test how it impact the ionization. Six different solutions were tested; 50% 2 mM ammonium acetate (AmAc) in water and 50% MeOH; 50% 2 mM AmAc in water and 50% ACN; 50% 10 mM AmAc in water and 50% MeOH; 50% 10 mM AmAc in water and 50% ACN 50% of a solution of 0.1% formic acid (FA) in water and 50% MeOH; and 50% of a solution of 0.1% FA in water and 50% ACN. After optimization of precursor ions, product ions and MS parameters, the LC conditions were optimized. All standard solutions were analyzed by applying a UHPLC column. A gradient of 2mM AmAc in water and MeOH were applied. In addition, gradient of 10 mM AmAc in water and MeOH and a gradient of 0.1% FA in water and methanol were tested.

Analyses of mycotoxin standards was carried out with both positive and negative electrospray ionization (ESI) in triggered multiple reaction monitoring (MRM). The final method was built by choosing the optimized parameters under either positive or negative mode and corresponding retention time for minimizing potential analyte misidentification. Fish feed extractions were measured by applying the method developed from mycotoxin standards.

Data were evaluated using the Mass Hunter Quantitative Analysis Software.

Table 9 Agilent 1290 UHPLC Parameters

UHPLC column	Agilent ZORBAX RRHD eclipse plus C18 2.1 × 150 mm, 1.8 µm (P/N 959759-902) at 25 °C	
Mobile phase	A: 100% MeOH B: 2mM ammonium acetate in water, 10mM	
Gradient program	Time (min)	B%
	0.00	95.00
	0.20	95.00
	10.00	2.00
	11.00	2.00
	11.30	95.00
	14.00	95.00
Stop time	14 min	
Flow rate	0.4ml/min	
Injection	2 µl	

Table 10 Agilent 6460 QqQ Parameters

Ionization mode	Positive or negative ESI with Agilent Jet Stream
Scan type	Dynamic MRM
Gas temperature	250 °C
Gas flow	10 l/min
Nebulizer pressure	20 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 l/min
Capillary voltage	positive: 3500V
Nozzle voltage	positive: 1100V
Dwell time	70 ms
Resolution	0.5 Da or 0.7 Da

2.2.4 Method validation, LC-QqQ-MS/MS

Validation followed method development; performance characteristics including linearity, recovery, and precision (intra-day precision) are evaluated to determine the reliability of the developed method.

Linearity was evaluated by using two fish feed samples spiked with mycotoxin mix standard (including all mycotoxins except MON, DAS and AFs) at different concentrations and stable isotope labelled mix IS (13C labeled DON, OTA and FB1) at a constant concentration. The concentration for mycotoxin-mix standard was ranging from 0-1000 ng/g with eight different concentration levels (0, 0.1, 1, 5, 50, 100, 500 and 1000 ng/g) as seen in Table 11. The concentration for mix IS was 50 ng/g.

Because MON and DAS gave no peaks during the MRM transitions, they were not included in the validation. The purchased aflatoxin standard was mix standards with total amount of 2ul and

concentration at 2ug/ml for AFB1 and AFB2, 0.5 ug/ml for AFG1 and AFG2. Due to a high cost, the small amount and low concentration were not included in the linearity test. The original concentration for DOM-1, D3G and FB3 was 50 ug/ml, with total amount at 2ml, 1ml and 1ml, respectively. To avoid using all the amount available of these compounds, they were not included in the high concentration levels at 500ng/g and 1000 ng/g. In addition to the spiking of the feeds, two blank samples, empty vial without any matrix, were also spiked. One of the blanks was added mix IS only with concentration at 50 ng/g; the other blank was added mix mycotoxin standard and mix IS, both at 50ng/g.

Table 11 Level of mycotoxin standards and IS for linearity test

Mycotoxin included in the test	Level	Concentration for mycotoxin standard (ng/g)	Concentration for mycotoxin IS (ng/g)
ENA, ENA1, ENB, ENB1, BEA, DON, 3-ADON, 15-ADON, αZEL, βZEL, OTA, T-2, HT-2, NEO, DOM-1, D3G and FB3	Blank level 0	0	50
	Blank level 5	50	50
	Feed Level 0	0	50
	Feed Level 1	0.1	50
	Feed Level 2	1.0	50
	Feed Level 3	5.0	50
	Feed Level 4	50	50
	Feed Level 5	100	50
ENA, ENA1, ENB, ENB1, BEA, DON, 3-ADON, 15-ADON, αZEL, βZEL, OTA, T-2, HT-2, NEO	Feed Level 6	500	50
	Feed Level 7	1000	50

Precision and recovery were performed for ENA, ENA1, ENB, ENB1 and BEA because they were the most interested mycotoxins to have a quantification method for. The validation was carried out using six fish feeds spiked with mycotoxin standard (ENA, ENA1, ENB, ENB1 and BEA) at three concentration levels and IS at constant concentration (Table 12). Three levels included a low concentration level (10 ng/g), a medium level (100 ng/g) and a high concentration level (500 ng/g). Due to the high cost of stable isotopic labeled IS, structural analogues were used to test if they could be a good alternative. The IS used was a mix of six mycotoxin standards (αZEL, βZEL, OTA, T-2, HT-2 and NEO). These compounds had shown good linearity performance and they were added in each sample at 100 ng/g. A blank level with no mycotoxin standard but IS added was also performed for these 6 fish feeds to measure the mycotoxin level existed in unspiked fish feed. Besides, the

measuring of standard curve was also carried out by using one fish feed spiked with mycotoxin standard at 6 different concentration levels (0, 20, 50, 100, 300 and 500 ng/g) and IS added in at 100ng/g. Sample extraction procedure follows the same protocol as presented in 2.2.1.

Table 12 Levels of mycotoxin standards and IS during precision and recovery test

Purpose	Feed	Level	Concentration for mycotoxin standard (ng/g)	Concentration for mycotoxin IS (ng/g)
Standard curve	Feed 1	Level 0	0	100
		Level 1	20	100
		Level 2	50	100
		Level 3	100	100
		Level 4	300	100
		Level 5	500	100
Precision and recovery	Feed 2 - 7	Blank level	0	100
		Low level	10	100
		Medium level	100	100
		High level	500	100

2.2.5 Chromatographic conditions and Mass spectrometry conditions for UHPLC-IMS-QTOF MS analysis

Chromatographic conditions for Waters ACQUITY UHPLC were summarized in Table 13, ionization conditions were summarized in Table 14 following by Table 15 shown operation conditions for further IMS separation.

Data were acquired by performing the high-definition (HD) MSE scan mode over the range 50—1000 m/z without precursor ion selected. Two independent scans with different collision energies were alternately acquired during the run. First, low energy scan with collision energy at 4 V was performed to monitor the protonated molecules and other potential adducts; then high energy scan with collision energy between 8-45V was performed to fragment the ions which travel through the collision cell.

The TOF analyzer was used in the sensitivity mode, providing high resolution at approximately 40000. Leucine-enkephalin (m/z at 556.2771 in positive and 554.2615 in negative ionization modes respectively) was used as lock Spray™ interface (mass standard) to ensure mass accuracy by QTOF analyzer. It was infused at a concentration of 200 ug/µl in Acetonitrile/water/formic acid (50:49.9:0.1, v/v/v) mixed solvent at a flow rate of 30 µl/min. The choose of mobile phase and gradient program followed the same option previously used for the analysis of ethoxyquin in fish feed (Merel et al., 2019).

Data acquisition and processing were performed using UNIFI (version 1.8) software supplied by Waters, with the minimum intensity threshold at 20 counts and the background noise at low level so that compounds with low intensity level could be measured.

The measuring of fish feed samples and TPs followed the instrument conditions described in table 13-15 .

Table 13 Waters ACQUITY UHPLC chromatographic conditions

UHPLC column	Waters ACQUITY UPLC BEH C18 100 × 2.1 mm, 1.7 µm at 45 °C	
Mobile phase	A: Water, Ammonium acetate (10 mM, PH 5.0) B: MeOH, Ammonium Acetate (10 mM, PH 5.0)	
Gradient program	Time (min)	B%
	0.00	2.00
	0.25	2.00
	12.25	99.00
	13.00	99.00
	13.01	2.00
	17.00	2.00
Stop time	17 min	
Flow rate	0.45ml/min	
Injection volume	5 ul	

Table 14 Waters TWIMS-QTOF ionization conditions

Ionization mode	**Positive ESI with Lock Spray ion source**
Desolvation gas	Nitrogen (>99.5%)
Desolvation gas temperature	450 °C
Desolvation gas flow	900 l/h
Nebulizer pressure	20 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 l/min
Capillary voltage	450V
Reference capillary voltage	30000V
Cone voltage	10V
Cone gas	Nitrogen (>99.5%)
Cone gas flow	40 l/h
Source offset	80V
Source temperature	110°C
Resolution	40000

Table 15 IMS separation settings

Trap bias	40V
Stopper height	40V
Gate height	40V
Trap wave velocity	100 m/s
Trap pulse height	A: 20V
IMS wave velocity	250 m/s
IMS wave height	45 V
Gate release	2 m/s
Trap gas	Nitrogen (>99.5%) 1.6 l/min
IMS buffer gas	Nitrogen (>99.5%) 25 ml/min

2.2.6 Method validation with UHPLC-IMS-QTOF analysis

In order to be able to use CCS value as an additional point for the identification of mycotoxins, it is necessary to demonstrate the precision of CCS measurement. Thus, one approach for method validation is to evaluate intraday or interday precision of CCS measurement. This can be achieved by analysis of several replicates of a standard solution in one day or over several days. The reproducibility study on CCS is not conducted in this study because it has been well demonstrated that it is stable and usually within less than 2% deviation (Fiebig & Laux, 2016; Paglia et al., 2015; Regueiro et al., 2016, 2017).

3 Results and discussion

3.1 Results for mycotoxins and metabolites determined by UHPLC-QqQ-MS/MS

3.1.1 Method development by UHPLC-QqQ-MS/MS

A multi-target method for the screening and identification of 24 mycotoxins and metabolites was developed using UHPLC-QqQ-MS/MS. Out of the 22 compounds analyzed in the present work, 17 were characterized by the corresponding protonated precursor ions, whereas 5 were selected by sodium or acetate adducts when protonated precursor ions were either absent or present in very low abundance. Two compounds- MON and DAS were skipped from the method since no precursor ion could be detected during the optimization. A list of the optimized parameters including all transitions and conditions, as well as retention times for these analytes (22) is shown in table 17. From the table, most analytes (except NEO) had two or more product ions been monitored, giving increased identification confidence, which is also in accordance with identification criteria specified in Commission Decision 2002/657/EC (European Commission, 2002).

Table 16 Optimized parameters for each compound by LC-QqQ-MS/MS

Compounds	Retention time (min)	precursor ion (m/z)	Fragmentor voltage (v)	Ion name	product ion (m/z)	Collision energy (V)
BEA	9.8	784.4	270	[M+H] +	244/134	30/70
ENA	10.1	682.5	265	[M+H] +	210/100	26/74
ENA1	10.0	668.5	255	[M+H] +	210/86	22/74
ENB	9.6	640.4	250	[M+H] +	196/86	22/70
ENB1	9.8	654.4	260	[M+H] +	100/196	70/30
DON	2.6	355.1	120	[M+CH3COO	295/265/59	10/10/18
αZEL	7.5	319.2	200	[M-H]-	275/160	18/30
βZEL	7.0	319.2	200	[M-H]-	275/160	18/30
3-ADON	4.3	397.1	120	[M+CH3COO	307/59	10/10
15-ADON	4.3	337.1	110	[M-H]-	150/59	10/10
FB1	5.6	722.4	230	[M+H] +	95/81/74	72/80/54
OTA	5.0	404.1	140	[M+H] +	358/239	10/22
DOM-1	3.4	281.4	120	[M+H] +	109/91/77	18/54/70
FB3	6.9	706.4	245	[M+H] +	336/95	40/62
D3G	2.7	457.2	215	[M-H]-	427/247	10/18
NEO	3.5	405.2	200	[M+Na] +	345	18
T2	6.9	489.2	205	[M+Na] +	387/327/24	18/22/26
HT2	6.6	447.2	170	[M+Na] +	285/345	18/18
AFB1	5.9	313	135	[M+H] +	285/241	25/40

AFB2	5.7	315	135	[M+H] +	287/259	30/20
AFG1	5.4	329	135	[M+H] +	283/243	18/25
AFG2	5.1	331	135	[M+H] +	285/245	25/25

Several mycotoxins experience the problem with different adducts for precursor ion. As seen in Fig.10, when HT-2 (neutral mass at 424) runs with MeOH-FA as mobile phase, peaks for precursor ion are sodium adduct (m/z: 447) and proton adduct (m/z: 425); when it is run with MeOH-AmAc as mobile phase, peaks for precursor ion are sodium adduct (m/z: 447), ammonium adduct (m/z: 442) and proton adduct (m/z: 425). Changing of mobile phase from FA to AmAc could transform part of sodium adducts to ammonium adducts, but still could not resolve the problem of multi-precursor ions. The formation of several precursor ions from the same molecule will lead to lower intensity. In addition, it could also affect the precision if the ratio between the precursor ion varies.

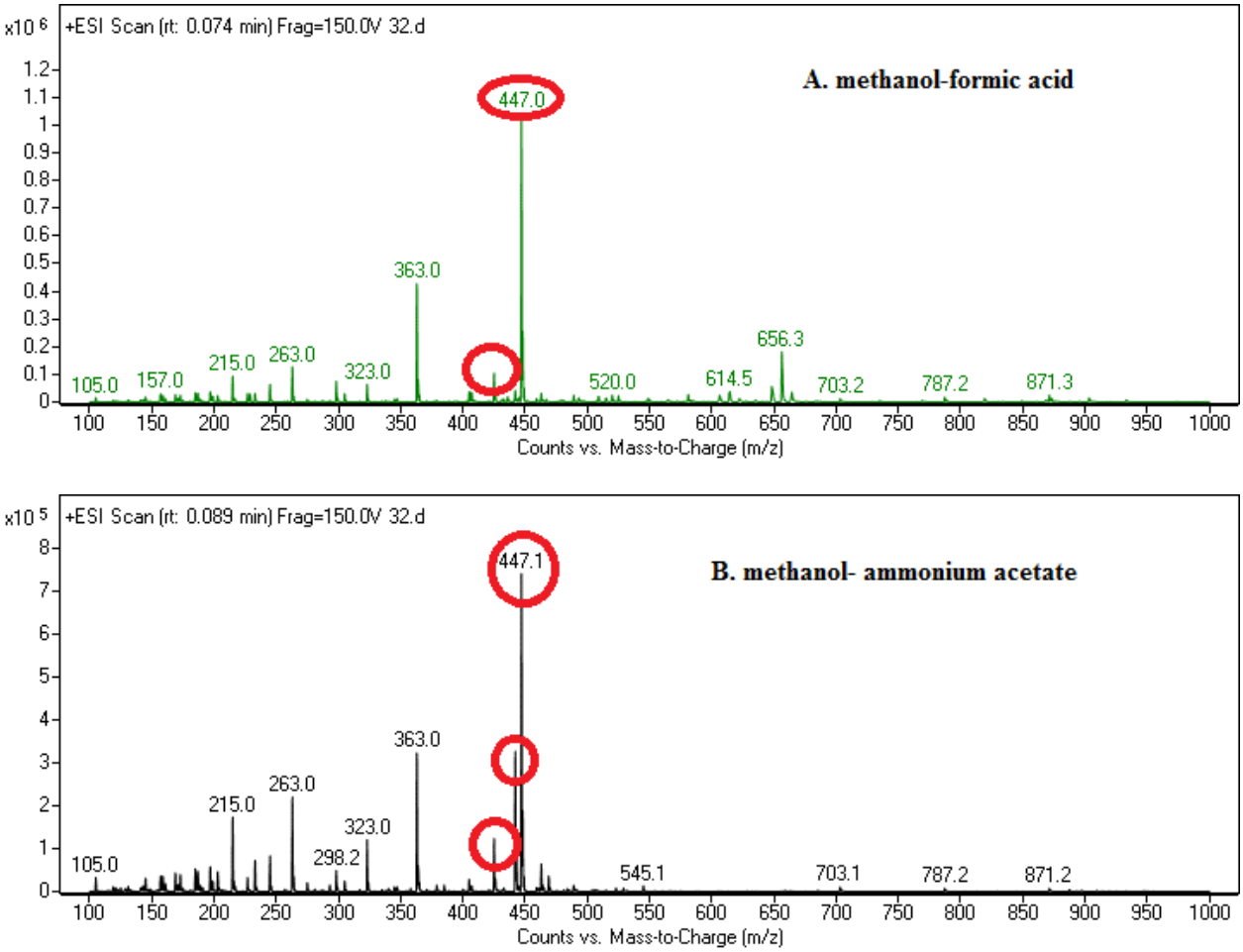


Fig. 10 MS for HT-2 when using different mobile phase

As seen in Fig.11, RT for ENA, ENA1, ENB, ENB1 and BEA are not the same. Besides, they have different masses and their MRM transitions vary. Therefore, these mycotoxins could be separated either by different precursor ions, product ions or by different RT.

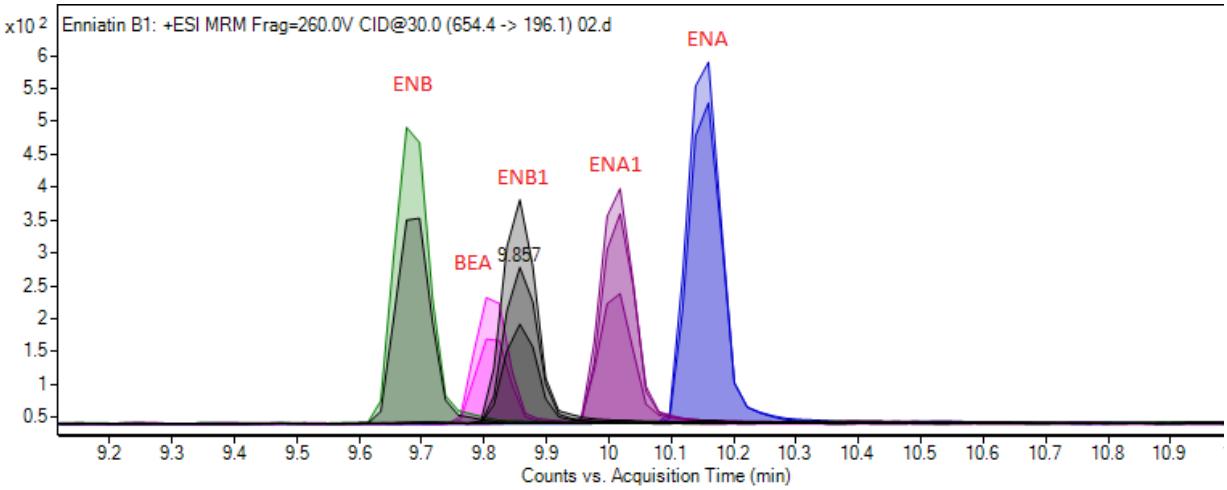


Fig. 11 Chromatograms for ENA, ENA1, ENB, ENB1 and BEA

A special notice should pay attention to is the separation of αZEL and βZEL, 3-ADON and 15-ADON by low resolution UHPLC-QqQ-MS/MS. Since they are isomeric, they have the same mass. The αZEL and βZEL had the same precursor ion and the same product ions. Therefore, they could not be separated by MRM scan. By injecting pure standard solutions separately, αZEL elutes later at 7.5 min, compared with βZEL at 7.0 min. Therefore, retention time could be used for the separation and identification of αZEL and βZEL. However, 3-ADON and 15-ADON elute at very close time (around 4.3 min), so it was not possible to separate them by retention time. They were also fragmented into the same product ions, however, at different abundances, as seen in Fig.12. Therefore, the intensity differences at the same MRM transition could be used for the separation of these two compounds. Sample 3 represents 3-ADON and sample 4 represents 15-ADON. At the same transition which has precursor ion at 337 and product ion at 150, the peak abundance for 3-ADON is at 10^1 level, much lower than the peak abundance for 15-ADON which is at 10^3 level. However, for the next two transitions with both precursor ion at 397 and product ions at 59 and 307, the peak abundances for 3-ADON are higher than that for 15-ADON. Therefore, using the peak abundance differences between the transitions, we could roughly decide which compound it is. To accurately separate these two compounds, optimization of chromatographic conditions could be performed, e.g. optimize column, mobile phase and gradient.

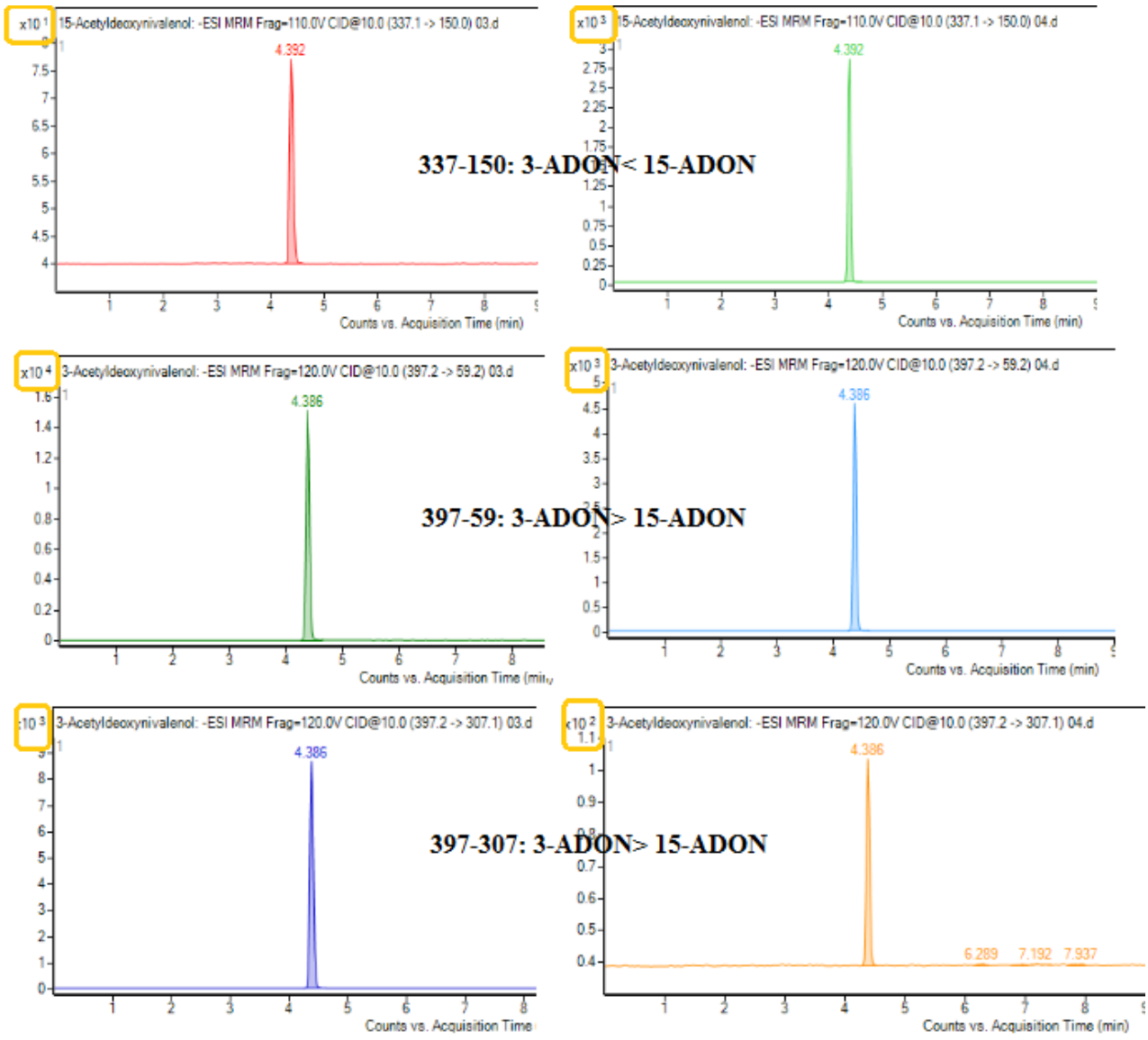


Fig. 12 MRM transitions for 3- and 15-ADON in their respective retention windows

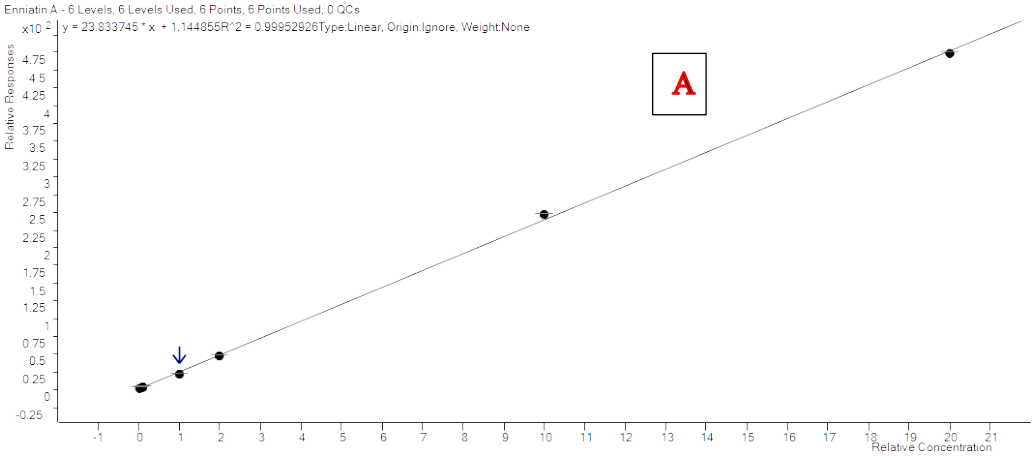
3.1.2 Method validation by UHPLC-QqQ-MS/MS

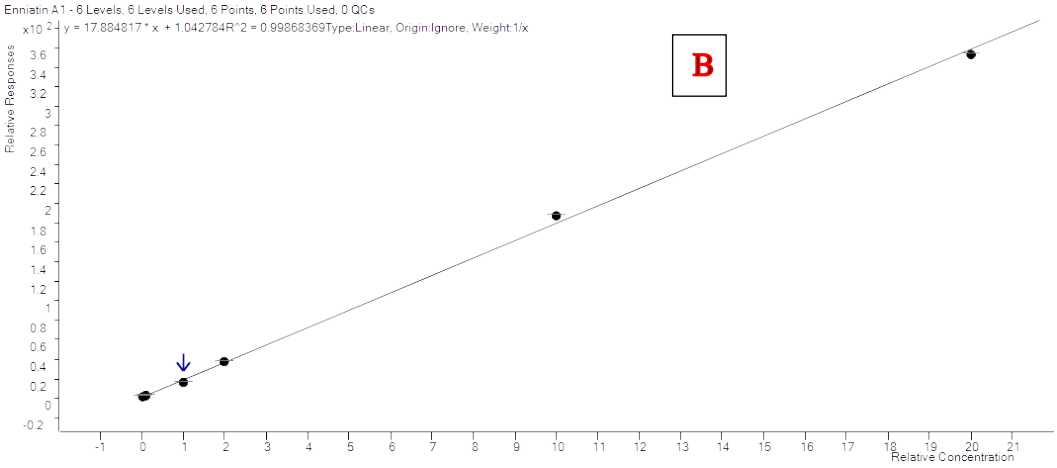
Linearity evaluation was performed for ENA, ENA1, ENB, ENB1, BEA, NEO, HT-2, T-2, DON, 15-ADON, 3-ADON, OTA, αZEL, βZEL DOM-1, FB1, FB3 and D3G. The correlation coefficient (R^2) were checked for each compound. A $R^2 > 0.95$ were considered acceptable linearity for the method. When applying 13C labeled OTA as IS, ENA, ENA1, ENB, ENB1, BEA, NEO, HT-2, T-2 and αZEL had a $R^2 > 0.99$, DON, 3-ADON, OTA and βZEL had a $R^2 > 0.95$. Therefore, the method is linear over the studied concentration range for these 13 compounds.

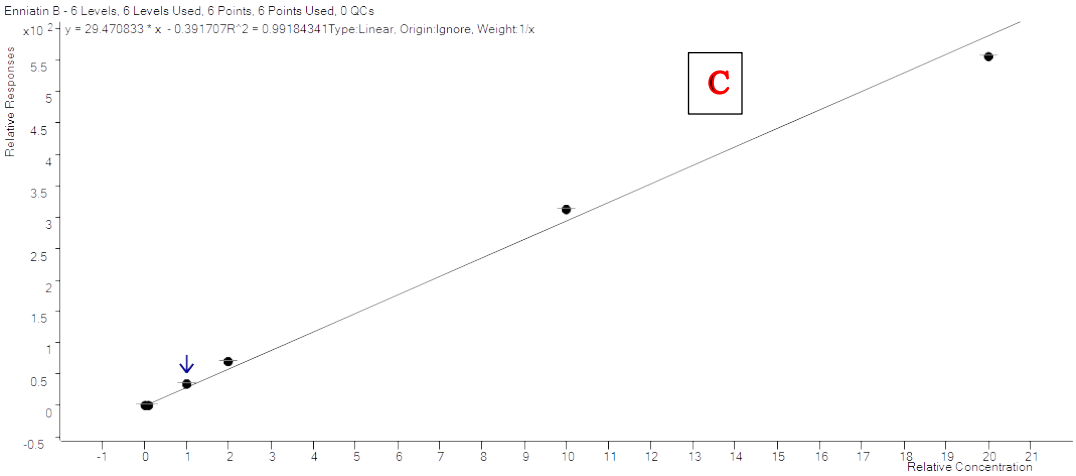
R^2 for 15-ADON, DOM-1, FB1, FB3 and D3G were below 0.95 and improvements have to be performed before a quantitative method can be established for these compounds. The peaks were low and R^2 were low when using 13C-DON and 13C-FB1 as IS. Therefore, these two isotopic labeled IS could not use for quantification.

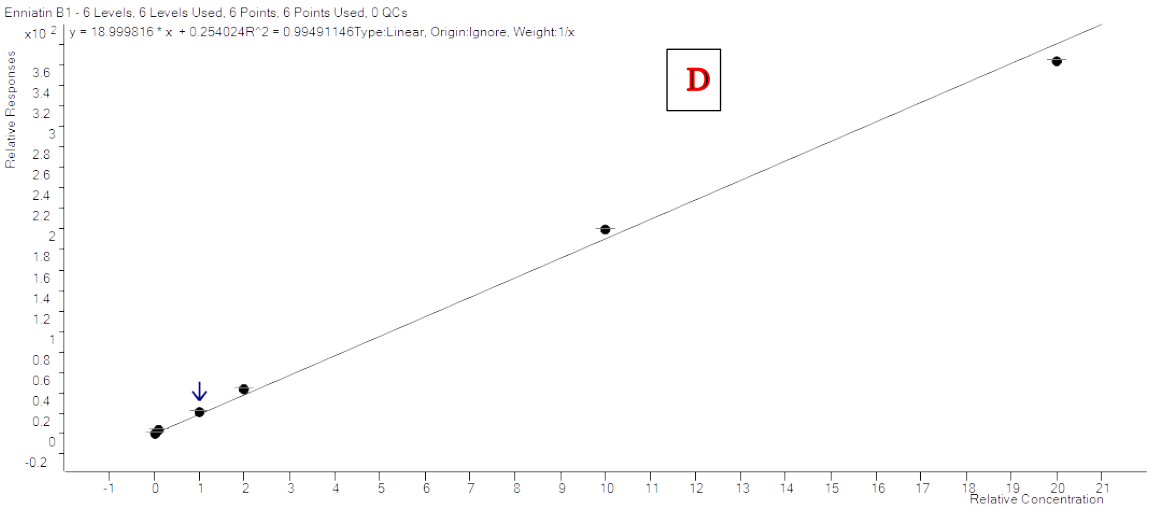
Based on signal to noise from the linearity test, preliminary LOQs were established. The LOQ for BEA, ENA, ENA1, ENB, ENB1 and DOM-1 was 1.0 ng/g. The LOQ for OTA and T-2 was 5.0 ng/g, while the LOQ was 10 ng/g for αZAL, βZAL and HT-2. For NEO, FB3, 3-ADON, 15-ADON the LOQ was 50 ng/g, while the LOQ for DON was 100 ng/g. D3G and FB1 did not give any proper peaks in this run, therefore no LOQ was established. These LOQs should be verified by more analyses, since the sensitivity could vary with matrix, the status of the LC-column, day-to-day variation for the MS, how clean the ion source is and other parameters. The LOD will be around 0.3*LOQ.

Linear regression graphs for ENA, ENA1, ENB, ENB1 and BEA are given below.




Enniatin A1 - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 0 QCs
y = 17.884817 * x + 1.042784R^2 = 0.99868369Type:Linear, Origin:Ignore, Weight:1/x
x10 2
Relative Responses
Relative Concentration
B


Enniatin B - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 0 QCs
y = 29.470833 * x - 0.391707R^2 = 0.99184341Type:Linear, Origin:Ignore, Weight:1/x
x10 2
Relative Responses
Relative Concentration
C


Enniatin B1 - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 0 QCs
y = 18.999816 * x + 0.254024R^2 = 0.99491146Type:Linear, Origin:Ignore, Weight:1/x
x10 2
Relative Responses
Relative Concentration
D

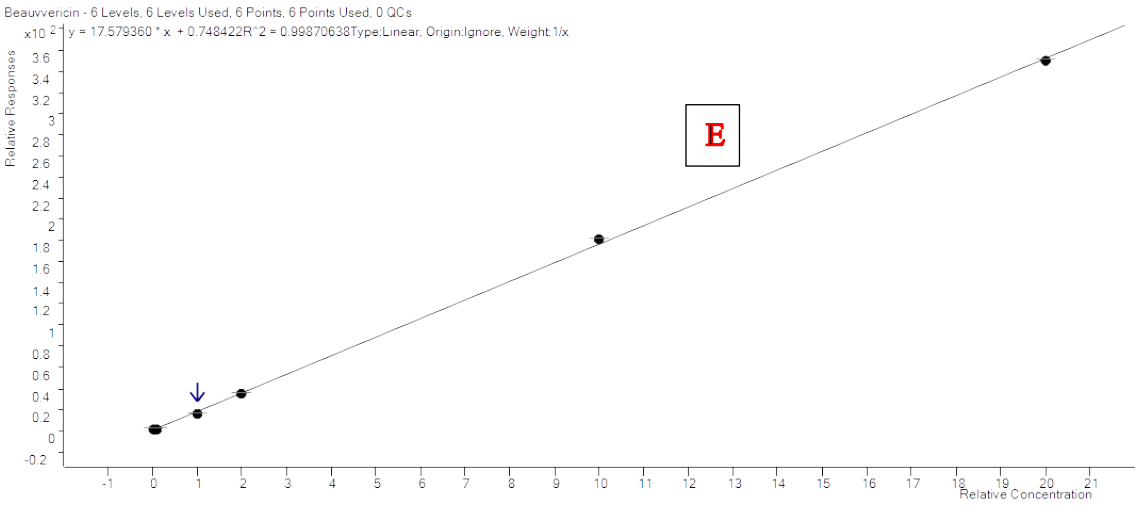


Fig. 13 Linear regression graph for ENA (A), ENA1 (B), ENB (C), ENB1 (D) and BEA (E)

Further method validation determining the recovery and precision were only performed for ENA, ENA1, ENB, ENB1 and BEA. No stable isotope labelled ENN or BEA were available, and since the available isotope labelled compounds were very expensive and available only in a very low amount, it was tested if any of the mycotoxins that were excluded from the quantitative method was suitable as IS. T-2, HT-2, OTA, NEO and αZEL were tested as IS. As seen in Table 18, the recovery is between 96%-111%, 92%-113%, 86%-106%,76%-87% and 71%-88%, respectively for ENA, ENA1, ENB, ENB1 and BEA. RSD range is 4%-18%, 10%-22%, 8%-22%, 14%-32% and 13%-24% respectively, for these five compounds.

According to criteria specified in Commission Decision 2002/657/EC (European Commission, 2002). the recovery should be between -80% to +110% in the range that were evaluated, and the criteria for the RSD should follow the Horwitz Equation. However, for levels lower than 10 ng/g, it is stated that the application of the Horwitz Equation gives unacceptable high values so the RSD shall be as low as possible. The acceptable RSD at 100 ng/g should be less than 23%, and at 500 ng/g RSD should be less than 16%. From table 18, most recoveries are in accordance with the method criteria when using T-2 (except ENB at low concentration), HT-2 (except ENB at medium concentration) and OTA as IS. Several results exceed the recovery criteria range when using NEO, αZEL as IS. All calculated RSD satisfy the criteria when using T-2 as IS. Most RSD fulfill the criteria when using HT-2 as IS. OTA, NEO and αZEL gave much worse precision performance as IS, compared with T-2 and HT-2.

Table 17 Precision, recovery and correlation coefficient for ENA, ENA1, ENB, ENB1 and BEA when using T-2, HT-2, OTA, NEO, αZEL and βZEL as IS

Mycotoxin	IS	10 ng\g		100 ng\g		500 ng\g		Correlation coefficient
		RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	
ENA	T-2	13	97	11	96	10	96	0.9994
	HT-2	18	95	18	101	16	92	0.9975
	OTA	22	91	16	89	18	86	0.9993
	NEO	26	78	25	77	21	76	0.9973
	αZEL	16	75	18	80	20	71	0.9959
ENA1	T-2	9	104	10	102	7	100	0.9992
	HT-2	14	100	17	107	14	96	0.9975
	OTA	20	96	15	94	15	90	0.9989
	NEO	26	85	25	82	18	79	0.9968
	αZEL	13	79	17	84	17	14	0.9951
ENB	T-2	16	111	7	109	7	108	0.9990
	HT-2	18	96	14	113	10	102	0.9961
	OTA	29	107	8	100	9	96	0.9989
	NEO	63	86	21	87	14	86	0.9930
	αZEL	21	71	14	88	11	77	0.9949
ENB1	T-2	4	110	14	104	6	106	0.9989
	HT-2	14	102	20	108	11	100	0.9973
	OTA	19	102	17	96	12	94	0.9990
	NEO	35	83	24	85	18	83	0.9968
	αZEL	15	77	16	87	13	77	0.9955
BEA	T-2	18	101	17	98	14	95	0.9993
	HT-2	22	99	21	103	19	92	0.9984
	OTA	27	106	22	101	22	96	0.8923
	NEO	32	81	31	79	26	76	0.9980
	αZEL	20	78	24	82	23	81	0.9969

Besides the precision and recovery, the linearity is also obtained by the standard curve as given in Table 18. All the correlation coefficients are >0.99, except for BEA using OTA as IS. There are no apparent differences between correlation coefficient obtained from isotopic IS, in the previous described linearity experiment, and from the structural analogues, this was expected since none of the isotopic IS were isotope labelled ENN or BEA. Based on the results from the recovery, precision and linearity T-2 seems to be the best choice to use as IS. Combine the validation parameters by using different IS, T-2 is the most promising IS used for the method validation for ENA, ENA1, ENB, ENB1 and BEA. Feed is a very complex matrix to analyze, however, the method demonstrates excellent linearity, precision and recovery performance for the analyses of ENA, ENA1, ENB, ENB1 and BEA. Besides, the robustness of the method must be tested more thoroughly over a longer time.

The matrix effects have not been thoroughly investigated. However, the good results in the precision experiment, performed with six different feeds, indicate that any variation in matrix effects between feeds seems to be adjusted by the internal standard for BEA, ENA, ENA1, ENB, ENB1.

3.1.3 Method application by LC-QqQ-MS/MS method

The developed method was applied for the screening of mycotoxins in 9 fish feed samples and one fish feed ingredient. The qualitative analyses (as seen in Table 18) reveals that almost all samples are contaminated with mycotoxins, and most predominant mycotoxins are ENNs and BEA. Besides, FUMs occurs in the fish feed ingredient, while not in fish feeds.

Table 18 Mycotoxin measured for fish feeds by UHPLC-QqQ-MS/MS

Sample	Mycotoxins
1	ENB, ENB1
2	ENB, ENB1, ENA, ENA1
3	BEA, FB1, FB3
4	ENB, ENB1
5	ENB, ENB1
6	BEA, ENB, ENB1
7	BEA, ENB, ENB1
8	BEA, ENB, ENB1
9	BEA, ENB, ENB1
10	BEA, ENB, ENB1

3.2 Results for mycotoxins and metabolites determined by UHPLC-IMS-QTOF MS

3.2.1 Method development by UHPLC-IMS-QTOF MS

A method for the screening and identification of 24 mycotoxins and metabolites was developed using UHPLC-IMS-QTOF MS instrument. A database/library, including accurate mass, observed m/z, retention time, drift time, CCS values, response and different adducts were generated. Mass error, observed m/z, RT, drift time and CCS for the peak with highest intensity of each mycotoxin are included in Table 17. For FB3, AFB1, AFB2, AFG1, AFG2 and OTA, CCS values for the protonated ions (H+) were used. Ammonium adducts (NH4+) was used for BEA, ENA, ENA1, ENB and ENB1, while the CCS for NEO, DAS, HT-2, D3G, FB1, DOM-1, DON, T-2, 3-ADON, 15-ADON, αZAL, βZAL and MON were characterized by their sodium adduct. Most of the compounds experience several precursor adducts, similar as with the UHPLC-QqQ-MS/MS. Sodium adduct had the highest intensities for majority of the compounds.

As seen from the table, mass error for most mycotoxins are below 5ppm, which demonstrates excellent mass accuracy. For ENA1 and ENB1, the mass error is above 5ppm, but still below 10 ppm, which is acceptable. Since all mycotoxin standards were injected at 1ng/ml into the instrument, this concentration could be OK for some compounds, while for other compounds, like ENA1 and ENB1, could be too high. When samples were injected at high concentration, the ions could saturate the detector and cause mass accuracy drifting, leading to high mass error. Therefore, optimization the concentration could decreases mass error for these two compounds.

Besides, unlike other compounds that have only one adduct with highest intensity, αZEL has two peaks with same mass error, observed m/z, RT and adduct but different drift time and CCS value, showing equal high abundance. Apparently, these two peaks were same ion (one chromatogram peak) before it entered into ion mobility cell and experienced different separation (two CCS drift time).

Table 19 Mass error, observed m/z, RT, drift time and CCS for the main adduct of each mycotoxin by UHPLC-IMS-QTOF

Compound	Exact mass (Da)	Observed mass (Da)	Mass error (ppm)	Observed m/z	Retention time (min)	Drift time (ms)	CCS (Å)	Adduct
BEA	783.40948	783.4113	2.2	801.4451	11.12	9.57	291.90	+NH4
ENA	681.45643	681.4569	0.7	699.4907	11.41	8.69	263.88	+NH4
ENA1	667.44078	667.4465	8.3	685.4803	11.24	8.52	258.61	+NH4
ENB	639.40948	639.4103	1.2	657.4441	10.81	8.22	249.28	+NH4
ENB1	653.42513	653.4306	8.2	671.4645	11.04	8.40	254.70	+NH4
NEO	382.16277	382.1620	-1.8	405.1513	4.26	5.77	181.97	+Na
DAS	366.16785	366.1687	2.3	389.1580	6.6	5.68	179.91	+Na
HT-2	424.20972	424.2107	2.3	447.2000	7.64	6.55	202.41	+Na
D3G	58.17881	458.1780	-1.7	481.1672	2.8	6.66	205.16	+Na
FB1	721.38847	721.3895	1.5	722.3968	7.67	8.58	260.39	+Na
FB3	705.39356	705.3915	-2.8	706.3988	8.40	8.53	258.73	+H
AFB1	312.06339	312.0637	0.9	313.0709	6.36	4.98	163.35	+H
AFB2	314.07904	314.0794	1.3	315.0867	6.08	5.05	164.98	+H
AFG1	328.05830	328.0589	1.8	329.0662	5.78	5.10	166.03	+H
AFG2	30.07395	330.0748	2.7	330.0748	5.48	5.15	167.17	+H
DOM-1	280.13107	280.1308	-0.8	303.1201	3.95	5.16	167.95	+Na
DON	296.12599	296.1252	-2.4	319.1145	3.00	5.32	171.53	+Na
T-2	466.22028	466.2197	-1.1	489.2089	8.31	6.87	210.90	+Na
3-ADON	338.13655	338.1361	-1.2	361.1254	4.84	5.84	184.14	+Na
15-ADON	338.13655	338.1363	-0.8	361.1255	4.88	5.53	176.28	+Na
αZEL	320.16237	320.1622	-0.6	343.1514	8.43	5.62	178.89	+Na

						7.72	236.48	
βZEL	320.16237	320.1621	-0.8	343.1513	7.85	7.69	235.40	+Na
OTA	403.08227	403.0823	0.02	404.0896	7.63	6.00	188.10	+H
MON	119.98234	119.9820	-2.3	142.9712	0.58	5.64	186.35	+Na

The chromatogram and spectra for BEA in both low and high-energy scan mode is given in Fig. 14 and Fig. 15. Low energy scan shows the mass of the precursor ions, including ammonium adduct, proton adduct and sodium adduct. High-energy scan includes the mass of the product ions.

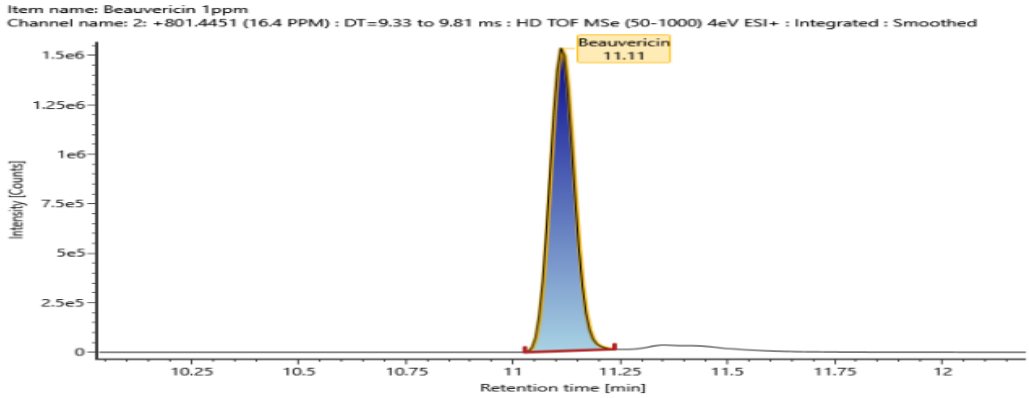


Fig. 14 Chromatogram for BEA measured

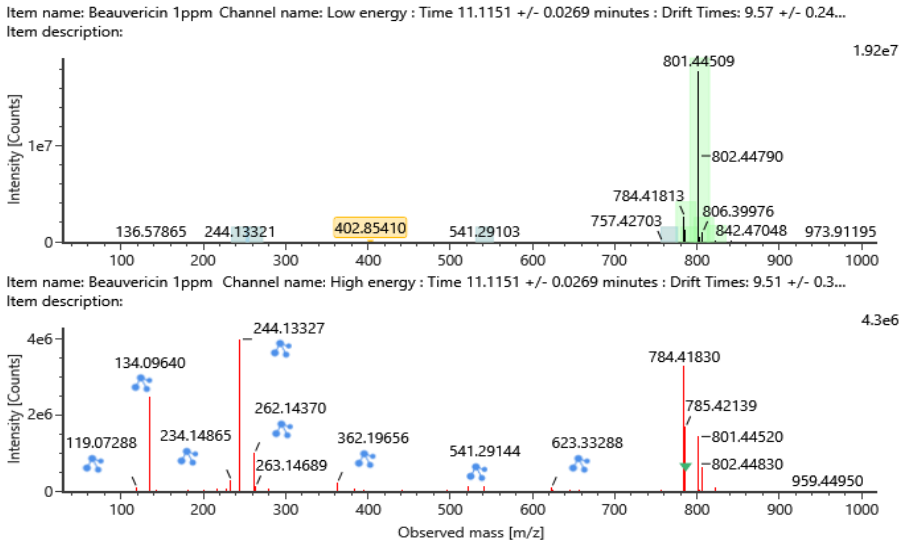


Fig. 15 Low energy and high-energy spectra of BEA

A typical ion mobility 3D-view for BEA is shown in Fig.16, where the retention time is 11.06 min, m/z at 801.4475 and drift time at around 9.5 ms. The plot demonstrates the orthogonal relationship between drift time for the ion to pass through the mobility cell and RT for a HPLC separation. The combination of drift time and RT increases the method sensitivity by increasing peak capability and the resolving power for complex mixtures. Besides, the chromatogram, spectrum and mobility trace

are all interactive with the ion mobility 3D view, which also make it possible for the investigation of all ions in a 4D view (m/z, intensity, mobility drift time plus the retention time from LC)

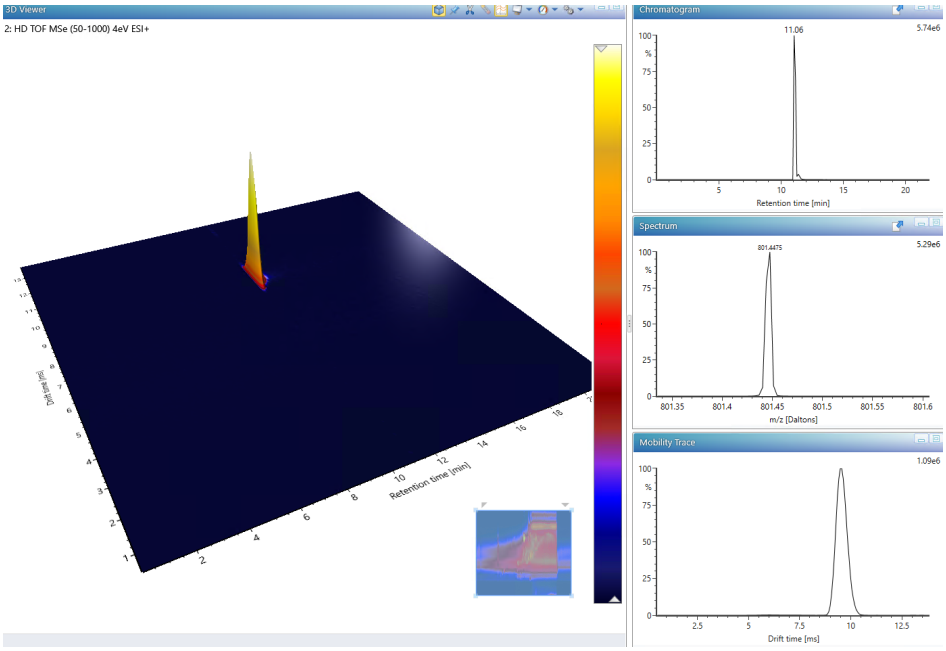


Fig. 16 Ion mobility 3D view for BEA

The method also demonstrates the advantage of IMS in separating and distinguishing isomers. Similar to the RTs obtained by UHPLC-QqQ-MS/MS, αZEL and βZEL could be separated from each other by their different RTs (8.43 and 7.85) using UHPLC-IMS-QTOF MS, while 3-ADON and 15-ADON have very close RT (4.84 and 4.88, respectively). However, their CCS values, 184.14 for 3-ADON and 176.28 for 15-ADON, differ greatly. Therefore, these two compounds could be separated and identified using CCS values.

3.2.2 Method application by LC-IMS-QTOF MS method

The developed method was applied for the screening of mycotoxins in 10 fish feed samples. The criteria for identification of a compound followed these conditions: the retention time error was less than 0.1 min, the mass difference was lower than 5 ppm, the error on the CCS value was lower than 2% and the expected fragments found was at least one. Each fish feed sample was measured in two parallels to further increase measurement confidence. The results for the fish feed samples are given in Table 20.

Table 20 Mycotoxins detected by UHPLC-IMS-QTOF MS in fish feeds

Sample No.	Parallel 1			Parallel 2		
	Mycotoxin name	Response	Adducts	Mycotoxin name	Response	Adducts

Sample 1	ENB ENB1	28949 8314	+NH4, +Na, +H +NH4, +H	ENB ENB1	29450 8024	+NH4, +Na, +H +NH4, +Na, +H
Sample 2	ENA ENA1 ENB ENB1	1421 4138 161496 87912	+NH4, +H +NH4, +Na, +H +NH4, +Na, +H +NH4, +Na, +H	ENA ENA1 ENB ENB1	1532 10443 101090 51615	+NH4 +NH4, +Na, +H +NH4, +Na, +H +NH4, +Na, +H
Sample 3	BEA ENB FB1 FB3	667425 9597 193707 94501	+NH4, +Na, +H +NH4, +Na, +H +H, +Na +H, +Na	BEA ENB FB1 FB3	446391 7275 153166 78369	+NH4, +Na, +H+NH4, +H +H, +Na +H, +Na
Sample 4	ENB ENB1	60398 23067	+NH4, +Na, +H +NH4, +Na, +H	ENB ENB1	60565 24209	+NH4, +Na, +H +NH4, +Na, +H
Sample 5	ENB ENB1	50741 16130	+NH4, +Na, +H +NH4, +Na, +H	ENB ENB1	51657 17995	+NH4, +Na, +H +NH4, +Na, +H
Sample 6	BEA ENB ENB1	14587 28192 8654	+NH4, +Na +NH4, +Na, +H +NH4, +H	BEA ENB	6606 10426	+NH4 +NH4, +Na, +H
Sample 7	BEA ENB ENB1	2669 30397 9268	+NH4 +NH4, +Na, +H +NH4, +Na, +H	BEA ENB ENB1	3043 29887 9639	+NH4 +NH4, +Na, +H +NH4, +Na, +H
Sample 8	BEA ENB ENB1	32097 78355 23820	+NH4, +Na, +H +NH4, +Na, +H +NH4, +Na, +H	BEA ENB ENB1	30781 74590 23479	+NH4, +Na, +H +NH4, +Na, +H +NH4, +Na, +H
Sample 9	ENB	78536	+NH4, +Na, +H	ENB	79630	+NH4, +Na, +H
Sample 10				BEA ENB ENB1	20539 42486 14906	+NH4, +Na, +H +NH4, +Na, +H +NH4, +Na, +H

Generally, results obtained between parallels are similar with minor intensity differences except for sample 10 where nothing was detected in the first parallel. This might be due to fluctuations in recovery or response (Regueiro et al., 2017). When the sample was re-run on the instrument, similar result was obtained as the second parallel showed.

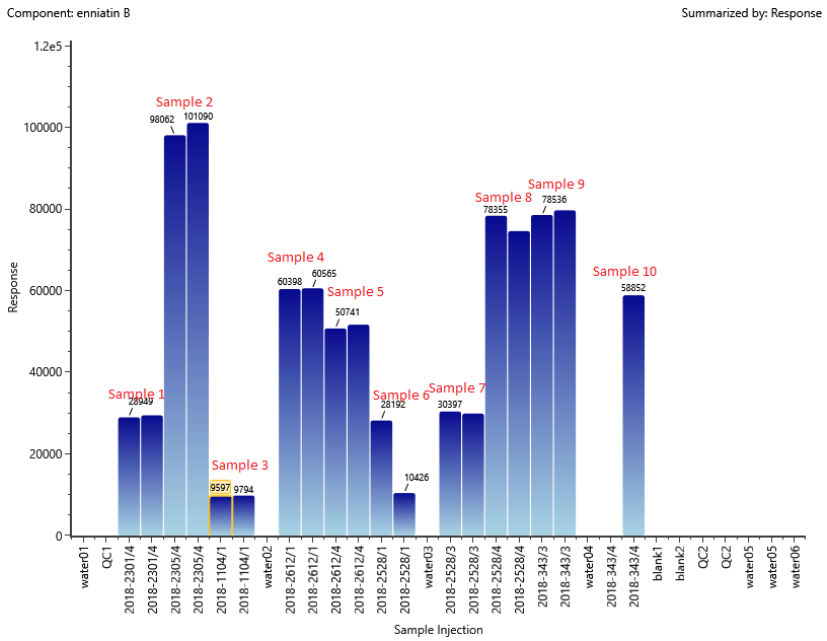


Fig. 17E NB occurrence in fish feed samples

In total, all samples were contaminated with at least one mycotoxin, including ENB, ENB1, BEA, ENA, ENA1, FB1 and FB3. ENB was the most detected mycotoxins (as seen in Fig.15), which occurred in almost all samples with high responses. Followed by ENB1, BEA, ENA and ENA1. FB1 and FB3 were only detected in sample 3 with high responses (FB3 occurrence shown in Fig.18). Sample 3 is the only fish feed ingredient while other samples are finished fish feeds.

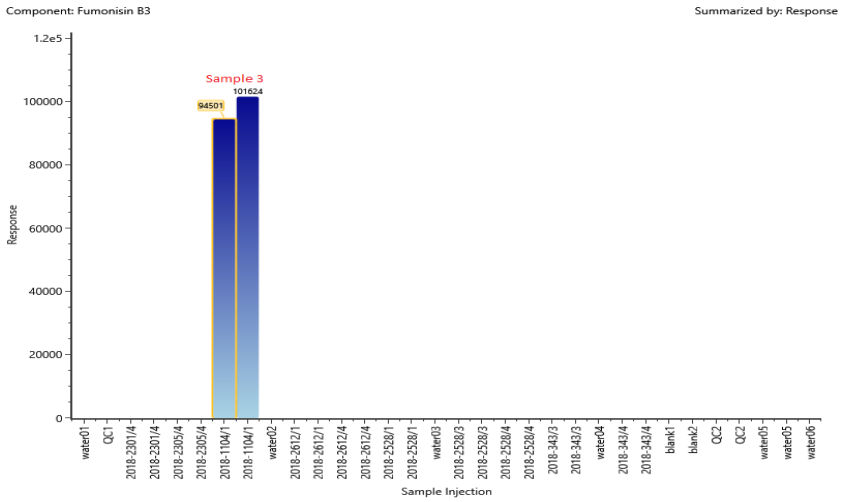


Fig. 18 FB3 occurrence in fish feed samples

3.3 Degradation experiment for BEA and ENB and identification of unknown TPs

When considering the existence of TPs it is commonly agreed that if the mycotoxin itself is not found in fish feed samples, it is very little chance to find the TPs of this parent mycotoxin. Based on the obtained results from fish feed samples, BEA and ENB are two of the most abundant mycotoxins detected in fish feeds. Therefore, BEA and ENB were chosen to perform degradation experiment.

Oxidant chosen in this degradation experiment was hydrogen peroxide (H2O2). Other studies, e.g. (Young et al.) 2006, have used ozone as a stronger oxidant is used as treatment. However, H2O2 has the advantage of giving more gentle, feasible and controllable degradation conditions. Besides, as has been discussed in the introduction, TPs occurred in nature implies a weak oxidation process caused by exposure to air, which will be a more similar to oxidation byH2O2 than ozone.

3.3.1 Prediction of TPs for BEA and ENB

The prediction from Zeneth software revealed 64 structures for BEA (including BEA) and 46 structures for ENB (including ENB), as seen from Table 21 and Table 22, respectively. Some chemicals have the same molecular formula, but the alteration of the molecular occurs on different location, so they are not duplicates. Data generated from the prediction was used for transformation products identification.

Table 21 TPs predicted for BEA

Degradant	Formula	Exact Mass	Degradant	Formula	Exact Mass
Beauverici	C45H57N3O9	783.40948	D43	C44H57N3O10	787.4044
D1	C45H59N3O1	801.42005	D49	C45H53N3O11	811.36801
D2	C45H57N3O1	799.4044	D51	C44H55N3O10	785.38874
D3	C45H55N3O1	797.38874	D52	C44H55N3O10	785.38874
D4	C44H57N3O9	771.40948	D53	C44H55N3O10	785.38874
D5	C44H57N3O9	771.40948	D54	C44H55N3O10	785.38874
D6	C15H21NO4	279.14706	D55	C44H55N3O10	785.38874
D7	C30H40N2O7	540.28355	D56	C44H55N3O10	785.38874
D10	C45H59N3O1	817.41496	D57	C79H103N5O1	1393.7349
D11	C45H59N3O1	817.41496	D58	C52H63N3O10	889.45135
D12	C45H59N3O1	817.41496	D60	C9H11NO	149.08406
D13	C45H57N3O1	815.39931	D61	C36H51N3O8	653.36762
D14	C45H57N3O1	815.39931	D69	C8H8O2	136.05243
D15	C45H57N3O1	815.39931	D70	C35H48N2O9	640.33598
D16	C15H19NO3	261.13649	D79	C44H55N3O9	769.39383
D18	C45H57N3O1	799.4044	D80	C44H55N3O10	785.38874
D19	C45H57N3O1	815.39931	D88	C24H30N2O4	410.22056
D21	C14H19NO3	249.13649	D89	C40H51N3O8	701.36762
D22	C29H38N2O6	510.27299	D90	C4H8O2	88.05243
D25	C19H27NO5	349.18892	D93	C84H106N6O1	1454.7665
D26	C34H46N2O8	610.32542	D94	C84H106N6O1	1454.7665
D27	C25H32N2O5	440.23112	D95	C48H63N3O10	841.45135
D28	C10H13NO2	179.09463	D96	C48H63N3O10	841.45135
D32	C45H57N3O1	815.39931	D105	C44H57N3O10	787.4044
D34	C45H55N3O1	813.38366	D108	C44H55N3O10	785.38874

D35	C45H55N3O1	813.38366	D109	C44H55N3O9	769.39383
D36	C45H55N3O1	797.38874	D110	C44H55N3O10	785.38874
D37	C45H57N3O1	799.4044	D112	C39H49N3O7	671.35705
D38	C44H57N3O1	787.4044	D115	C14H19NO3	249.13649
D39	C44H57N3O1	787.4044	D116	C29H38N2O6	510.27299
D40	C44H57N3O1	787.4044	D119	C89H114N6O1	1554.8189
D41	C44H57N3O1	787.4044	D120	C89H114N6O1	1554.8189
D42	C44H57N3O1	787.4044			

Table 22 TPs predicted for ENB

Degradant	Formula	Exact Mass	Degradant	Formula	Exact Mass
Enniatin	C33H57N3O9	639.40948	TP33	C4H8O2	88.05243
TP1	C33H59N3O10	657.42005	TP34	C11H19NO3	213.13649
TP2	C32H57N3O9	627.40948	TP36	C32H55N3O9	625.39383
TP3	C32H57N3O9	627.40948	TP37	C32H55N3O10	641.38874
TP4	C32H57N3O9	627.40948	TP38	C10H19NO3	201.13649
TP5	C11H21NO4	231.14706	TP39	C21H38N2O6	414.27299
TP6	C22H40N2O7	444.28355	TP42	C26H46N2O8	514.32542
TP9	C11H19NO3	213.13649	TP43	C15H27NO5	301.18892
TP11	C33H57N3O10	655.4044	TP45	C16H30N2O4	314.22056
TP12	C33H57N3O11	671.39931	TP46	C36H63N3O10	697.45135
TP14	C10H19NO3	201.13649	TP47	C59H103N5O1	1153.7349
TP15	C21H38N2O6	414.27299	TP48	C28H51N3O8	557.36762
TP18	C15H27NO5	301.18892	TP56	C27H48N2O9	544.33598
TP19	C26H46N2O8	514.32542	TP70	C28H51N3O8	557.36762
TP20	C17H32N2O5	344.23112	TP74	C60H106N6O1	1166.7665
TP21	C6H13NO2	131.09463	TP76	C36H63N3O10	697.45135
TP22	C36H63N3O10	697.45135	TP82	C32H55N3O9	625.39383
TP23	C59H103N5O1	1153.7349	TP83	C32H55N3O10	641.38874
TP24	C28H51N3O8	557.36762	TP85	C27H49N3O7	527.35705
TP25	C5H11NO	101.08406	TP88	C10H19NO3	201.13649
TP26	C22H40N2O7	444.28355	TP89	C21H38N2O6	414.27299
TP27	C11H21NO4	231.14706	TP92	C65H114N6O1	1266.8189
TP32	C27H48N2O9	544.33598	TP93	C65H114N6O1	1266.8189

3.3.2 Identification of TPs

The identification of TPs was carried out by looking at compounds measured from instrument with the library generated by the prediction software. By applying a mass error within 5 ppm, fifteen compounds experimentally detected for BEA match the predicted TPs from prediction database and twenty compounds detected for ENB match with database. They are given in Table 23 and Table 24,

with corresponding mass error as well as RT and CCS value for adduct with highest intensities measured from instrument.

Table 23 Peaks experimentally determined that match the prediction for BEA

Component name	Neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Observed drift time (ms)	Observed CCS (Å²)	Response	Adduct
BEA	783.40948	801.4454	2.6	11.05	9.6	292.59	2390714	+NH4
D1	801.42005	802.4274	0.1	10.39	9.81	299.76	25254	+H
D2, D18, D37	799.4044	817.4393	1.3	10.62	9.75	297.79	208317	+NH4
		817.4381	-0.2	10.51	9.76	298	195455	+NH4
		817.4397	1.8	10.88	9.67	295.16	184810	+NH4
		817.4360	-2.7	10.38	9.68	295.30	2883	+NH4
D3,D36	797.38874	815.4243	2.1	11.23	9.7	295.88	7705	+NH4
		815.4234	1	10.93	9.74	297.28	3947	+NH4
D13, D14, D15, D19, D32	815.3993	833.4329	-0.3	10.47	9.78	298.74	95016	+NH4
		833.4339	1	10.91	9.83	300.43	18372	+NH4
		833.4344	1.6	10.67	9.79	299.07	14977	+NH4
		833.4324	-0.9	10.15	9.66	294.63	12203	+NH4
		833.4331	0	10.33	9.85	300.91	9169	+NH4
		833.4323	-1	10.02	9.91	302.95	6233	+NH4
		833.4325	-0.8	9.87	9.93	303.59	3346	+NH4
D61	653.36762	654.3748	-0.2	10.27	8.38	254.3	1251	+H

Table 24 Six peaks experimentally determined that match the prediction for ENB

Component name	Neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Observed CCS (Å²)	Response	Adducts
enniatin B	639.40948	657.4442	1.3	10.77	8.25	250.42	4333064	+NH4
TP12	671.39931	689.4343	1.8	10.11	8.4	254.87	2021869	+NH4
		689.4343	1.7	10	8.4	254.98	1422518	+NH4
		689.4336	0.7	10.31	8.35	253.44	553402	+NH4
		689.4338	1	10.85	8.37	253.85	25664	+NH4
		689.4330	-0.3	10.96	8.22	249.33	7676	+NH4
		689.4319	-1.8	9.75	8.46	256.76	1628	+NH4
TP11	655.4044	656.4123	1	10.03	8.09	245.66	18758	+H
		656.4121	0.6	10.52	8.4	254.8	4381	+H

		656.4111	-0.8	9.86	8.42	255.47	4072	+H
TP15, TP39, TP1	414.27299	415.2803	0	9.95	7.91	241.05	1839	+H
	657.42005	658.4271	-0.3	9.6	8.14	246.91	4526	+H
TP2, TP3, TP4	627.40948	628.4169	0.3	9.95	7.86	238.8	57650	+H
		628.4175	1.1	10.12	7.92	240.63	2647	+H
TP24, TP48, TP70	557.36762	558.3753	0.8	8.61	7.37	224.54	34708	+H
		558.3748	-0.2	9.79	7.42	226.05	16865	+H
TP22, TP46,	697.45135	715.4859	1.1	10.92	8.85	268.6	709	+NH4
TP34, TP9	213.13649	214.1441	1.3	10.09	8.29	257.73	3034	+H
		214.1439	0.7	9.96	8	248.8	2913	+H
TP36, TP82	625.39383	643.428	0.5	10.53	8.22	249.48	17535	+NH4
TP32, TP56	544.33598	545.3434	0.3	9.01	7.38	225.08	2148	+H

As seen from the table 23, four compounds that matched D2, D18 and D37 were detected, the mass error was 1.3, -0.2, 1.8 and -2.7, respectively. One peak detected matched D1 and the mass error was 0.1; two peaks detected matched D3 and D36 and the mass error was 2.1 and 1, respectively. Seven peaks measured matched D13, D14, D15, D19 and D32, with maximum mass error of 1.6. One peak matched D 61. The structures of all TPs tentatively predicted during the degradation are given in Appendix.

As seen from Table 24, six compounds observed that matched predicted TP12 and the maximum mass error was 1.8. Three compounds observed matched predicted TP11 and their mass errors were all below 1. One compound detected matched predicted TP15, TP39 and TP89 and one match predicted TP1; two compounds observed matched TP2, TP3, TP4 and two matched TP24, TP48 and TP70; one matched TP22, TP46 and TP76; one matched TP34 and TP9; one matched TP36 and TP82 and one matched TP32 and TP56.

Here several of the peaks detected might correspond to one predicted TP. The reason for this could be that one TP could have several isomers which represent different peaks in the chromatogram. This happens frequently when the TP is formed from a hydroxylation reaction occurred at different spots on the parent molecule. Besides, when compound is measured by UHPLC-IMS-QTOF, after chromatography, the compound is ionized into the source and the mass spectrometer detects the charged ion, e.g. protonated ions. Here several protomers could form due to the position of the proton. These protomers could not be separated by chromatogram and spectra because they have same RT and mass. However, with the employment of IMS, their different shapes could be detected and as a result, lead to several peaks with same RT and mass, but withdifferent CCS values.

In addition, one peak could also be associated to several potential TPs. As seen from the predicted library in Table 21 and 22, many TPs are isomers with the same mass and therefore they could be assigned for one peak.

The tentative prediction could be improved by looking at the spectra of low and high-energy scan for each detected compound and compare with the predicted structure. Moreover, the CCS measured could also be used to compare with theoretical value from the predicted compounds. Then we can more accurately assign the peak with corresponding structure.

4 Summary and conclusion

One objective of this study was to develop two LC-MS-MS based methods for mycotoxin screening in fish feed. The two screening methods by UHPLC-QqQ-MS/MS and UHPLC-IMS-QTOF MS work well for the determination of most of the mycotoxins and have been used to measure fish feed and fish feed ingredients. αZEL and βZEL, which are isomers, could be identified through retention time by both methods. The other pair of isomers: 3-ADON and 15-ADON, which have similar retention time by both instruments, could not be totally distinguish by UHPLC-QqQ-MS/MS. However, by employing the IMS and the measuring of CCS value, these two compounds could be separated using UHPLC-IMS-QTOF.

The measuring of commercial fish feed samples demonstrates the presence and co-occurrence of mycotoxins. To our surprise, AFs as a kind of ubiquitous mycotoxins which exists widely in a diversity of agriculture commodities, didn't exists in any of the fish feed samples; FUMs, which is a type of legislated mycotoxins, exist in fish ingredient but not in the fish feeds; while BEA and ENNs which were not considered previously and even not legislated in fish feed control, did exist in most of the samples.

The method validation from UHPLC-QqQ-MS/MS demonstrates excellent linearity performance for most of the mycotoxins, good precision and recovery performances for ENA, NEA1, ENB, ENB1and BEA, which are of most interest. T-2 is confirmed as an excellent alternation to replace the expensive isotope labeled IS during method validation aimed for ENA, ENA1, ENB, ENB1 and BEA.

Degradation experiment for BEA and ENB is carried out using H2O2. Some TPs have tentatively been identified with the help of Zeneth prediction software. This novel degradation approach provides the possibility to identify the TPs for more mycotoxins. The identified TPs could make it possible to measure total mycotoxins and assessment of total mycotoxin risk. Besides, degradation experiment for BEA and ENB also reveals poor stability property of both compounds.

The determination of mycotoxins in fish feeds gives the possibility to investigate the introduction of mycotoxins in fish feed, provide the possibility for the study of these mycotoxin group connections (e.g. which types of mycotoxins usually occur together), and the potential effects consumption of these mycotoxin contaminated fish feeds could have on fish health.

5 Future perspective

The screening method developed for the LC-QqQ MS/MS should be tested for false negatives and false positives. This can be done by analyzing 20 samples spiked at the LOQ, and 20 blank samples. A screening detection limit can then be established at the LOQ if the spiked mycotoxins could be detected in at least 95% of the spiked samples (Sante 2017). There are no regulations for how many false positives there could be, as long as a positive result have to be verified by a quantitative method. However, false positives will lead to extra work, and should be avoided if possible. Similar experiment should be performed by the UHPLC-IMS-QTOF.

For the quantitative method using LC-QqQ MS/MS the method validation has shown promising results. However, further validation has to be performed in order to have a fully validated method.

For both the screening and quantitative method it is a goal to expand the matrix to fish muscle.

Several transformation products from chemical degradation for BEA and ENB has been tentatively identified. Further accurately assigning each detected TP with the exact predicted library could be carried out.

The pathway of TPs formation could also be important to figure out. For example, if the TPs come from a cleavage of the initial mycotoxin, then it is unlikely that they will form back to the parent mycotoxin. The toxicity could be decreased after degradation. While if the TPs come from an oxidation of the initial mycotoxin, these TPs might undergo a reduction process when the environment is suitable and form the initial mycotoxin again. In this situation, the degradation might not decrease the initial toxicity form the parent mycotoxin.

Appendix

Appendix Table 1Injection on the QTOF (oxidation started on 2019/09/25 19:15:00)

Name	Acquisition Start Time	Name	Acquisition Start Time
Blank initial	2019/09/25 19:13:45	BEA Peroxide E	2019/09/27 01:05:15
Blank initial	2019/09/25 19:37:25	ENB Peroxide E	2019/09/27 01:28:18
QC	2019/09/25 20:00:21	Blank	2019/09/27 01:52:12
BEA Ref A	2019/09/25 20:24:07	QC	2019/09/27 06:53:48
ENB Ref A	2019/09/25 20:47:01	BEA Ref F	2019/09/27 07:18:23
BEA Peroxide A	2019/09/25 21:10:48	ENB Ref F	2019/09/27 07:41:43
ENB Peroxide A	2019/09/25 21:33:41	BEA Peroxide F	2019/09/27 08:05:44
Blank	2019/09/25 21:57:22	ENB Peroxide F	2019/09/27 08:28:42
QC	2019/09/26 02:58:16	Blank	2019/09/27 08:52:28
BEA Ref B	2019/09/26 03:22:27	QC	2019/09/27 13:53:24
ENB Ref B	2019/09/26 03:45:40	BEA Ref G	2019/09/27 14:17:05
BEA Peroxide B	2019/09/26 04:10:19	ENB Ref G	2019/09/27 14:40:18
ENB Peroxide B	2019/09/26 04:33:44	BEA Peroxide G	2019/09/27 15:04:01
Blank	2019/09/26 04:57:57	ENB Peroxide G	2019/09/27 15:26:56
QC	2019/09/26 09:58:55	Blank	2019/09/30 10:54:22
BEA Ref C	2019/09/26 10:22:34	QC	2019/09/30 11:19:12
ENB Ref C	2019/09/26 10:45:28	BEA Ref G	2019/09/30 11:42:08
BEA Peroxide C	2019/09/26 11:09:12	ENB Ref G	2019/09/30 12:07:51
ENB Peroxide C	2019/09/26 11:32:07	BEA Peroxide G	2019/09/30 12:33:37
Blank	2019/09/26 11:55:56	ENB Peroxide G	2019/09/30 12:59:21
QC	2019/09/26 16:56:54	QC	2019/10/04 16:59:15
BEA Ref D	2019/09/26 17:20:32	BEA Peroxide I	2019/10/04 18:08:59
ENB Ref D	2019/09/26 17:43:32	ENB Ref I	2019/10/04 17:46:07
BEA Peroxide D	2019/09/26 18:07:14	BEA Ref I	2019/10/04 17:22:08
ENB Peroxide D	2019/09/26 18:30:09	ENB Peroxide I	2019/10/04 18:32:58
Blank	2019/09/26 18:53:53	BEA Ref J	2019/10/09 22:39:58
QC	2019/09/26 23:54:50	ENB Ref J	2019/10/09 23:05:42
BEARef E	2019/09/27 00:18:35	ENB Peroxide J	2019/10/09 23:54:26
ENB Ref E	2019/09/27 00:41:29	BEA Peroxide J	2019/10/09 23:29:12

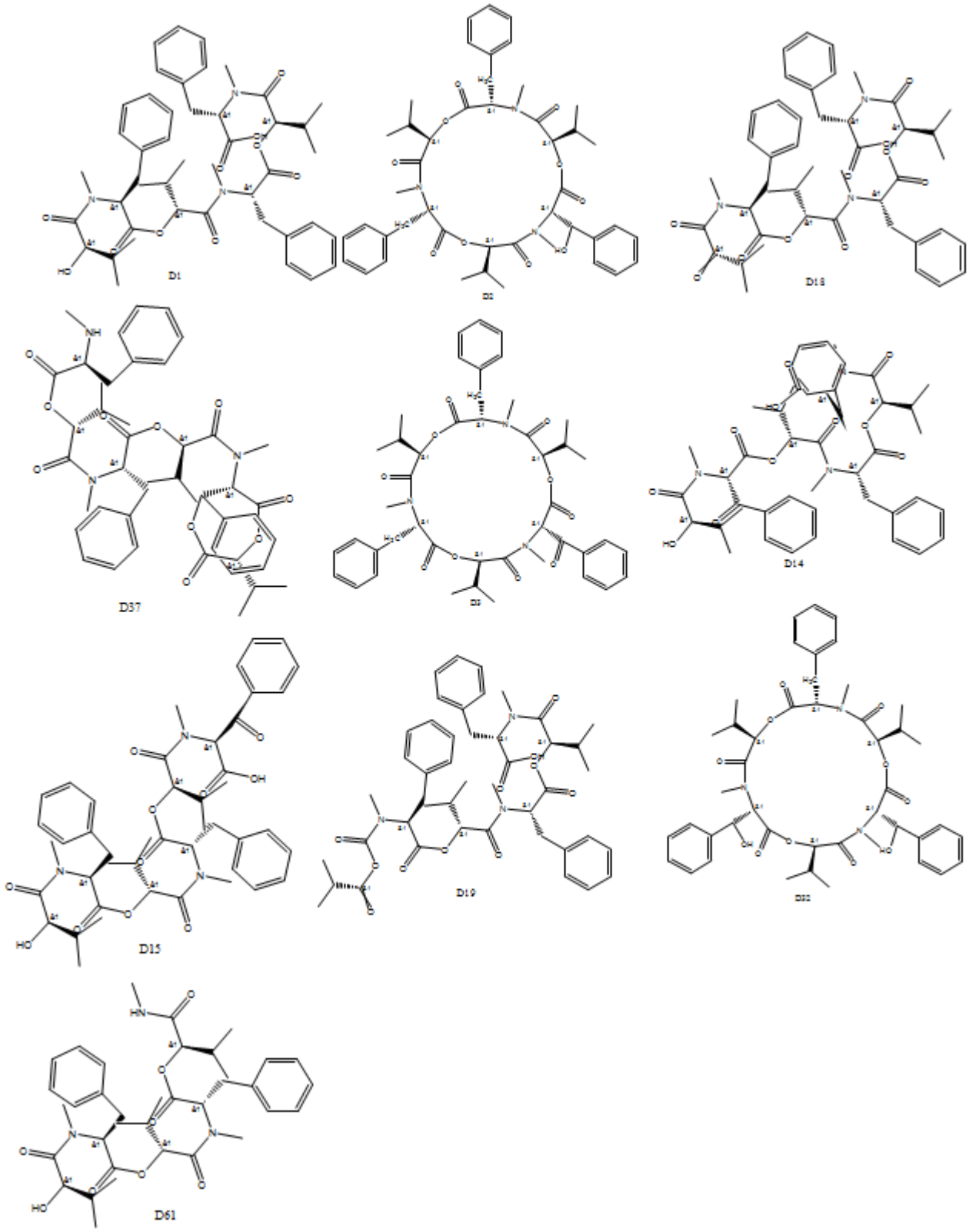
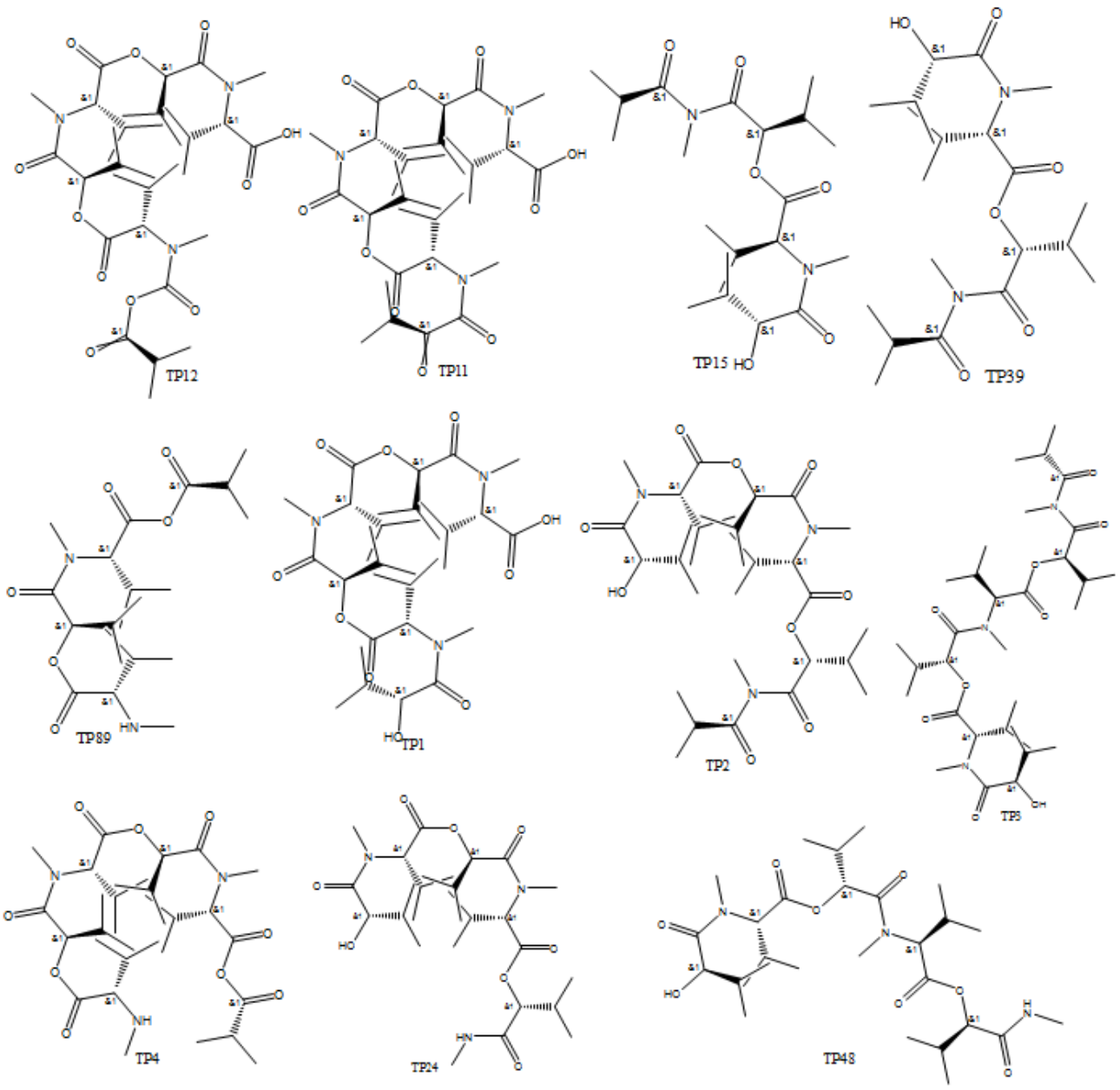


Fig. 19: Structures of the TPs that were predicted tentatively observed for BEA



A

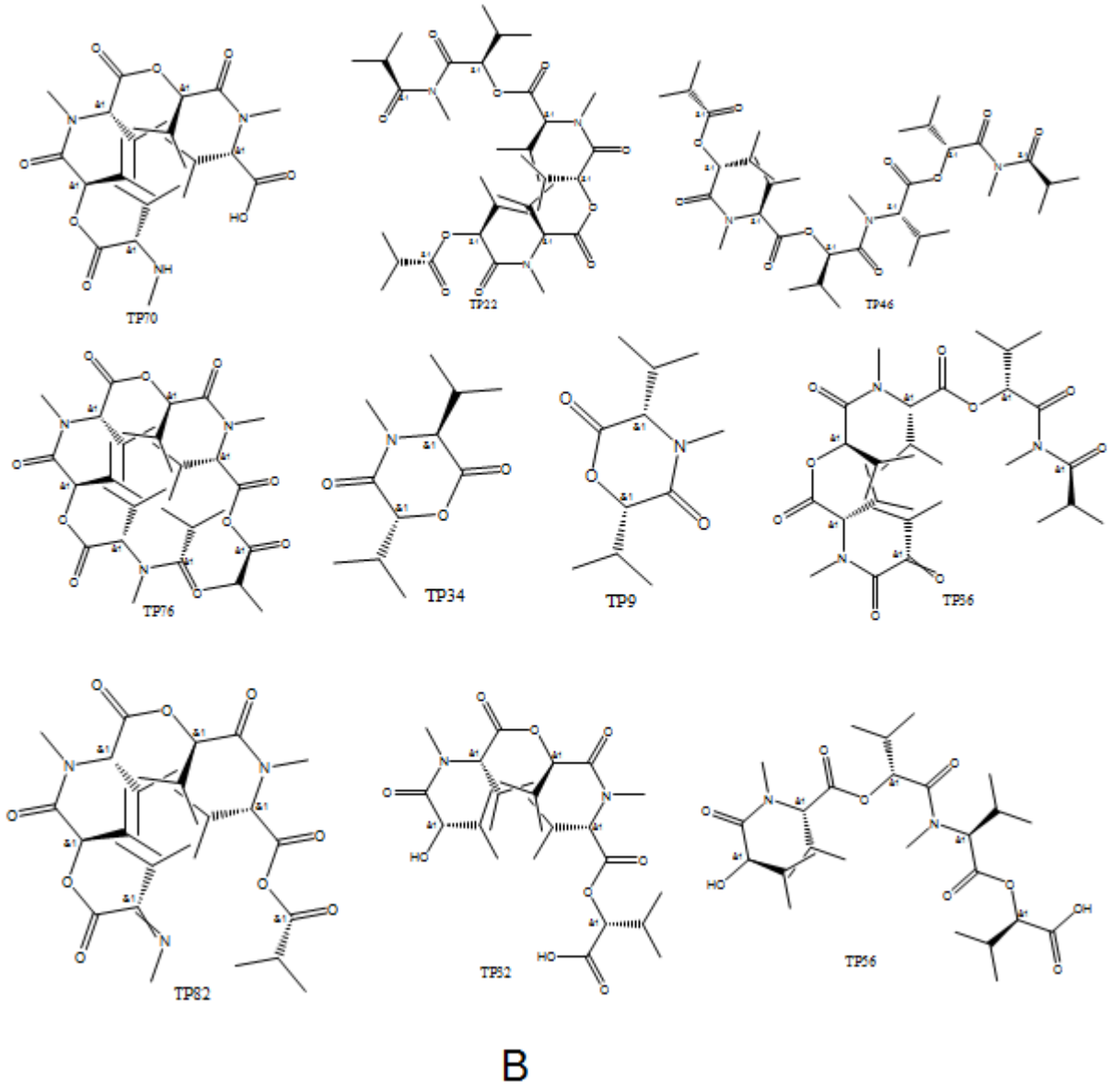


Fig. 20 (A and B) Structures of the TPs that were predicted tentatively observed for ENB

References

lshannaq, A., & Yu, J.-H. (2017). Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. International Journal of Environmental Research and Public Health, 14(6). https://doi.org/10.3390/ijerph14060632

Bennett, J. W., & Klich, M. (2003). Mycotoxins. Clinical Microbiology Reviews, 16(3), 497–516. https://doi.org/10.1128/cmr.16.3.497-516.2003

Bernhoft, A., Eriksen, G. S., Sundheim, L., Berntssen, M., Brants, A. L., & Brodal, G. (2013). Risk assessment of mycotoxins in cereal grain in Norway. 287.

Berthiller, F., Werner, U., Sulyok, M., Krska, R., Hauser, M.-T., & Schuhmacher, R. (2006). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant Arabidopsis thaliana. Food Additives & Contaminants, 23(11), 1194–1200. https://doi.org/10.1080/02652030600778728

Binder, E. M. (2007). Managing the risk of mycotoxins in modern feed production. Animal Feed Science and Technology, 133(1), 149–166. https://doi.org/10.1016/j.anifeedsci.2006.08.008

BIOMIN. (2019, March 5). 2018 BIOMIN Mycotoxin Survey Results. https://www2.biomin.net/fr/blog-posts/2018-biomin-mycotoxin-survey-results/

Böswald, C., Engelhardt, G., Vogel, H., & Wallnöfer, P. R. (1995). Metabolism of the Fusarium mycotoxins zearalenone and deoxynivalenol by yeast strains of technological relevance. Natural Toxins, 3(3), 138–144. https://doi.org/10.1002/nt.2620030304

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. International Journal of Food Microbiology, 119(1), 140–146. https://doi.org/10.1016/j.ijfoodmicro.2007.07.035

CAST (Ed.). (2003). Mycotoxins: Risks in plant, animal, and human systems. Council for Agricultural Science and Technology.

Chernushevich, I. V. (1999). Orthogonal injection provides a high- efficiency interface for transferring ionsfrom a continuous beam to a pulsed mode and makes it easier to obtain high resolution. 10.

Chernushevich, I. V., Ens, W., & Standing, K. G. (1999). Peer Reviewed: Orthogonal-Injection TOFMS for Analyzing Biomolecules. Analytical Chemistry, 71(13), 452A-461A. https://doi.org/10.1021/ac990524l

Chin, J. P., Megaw, J., Magill, C. L., Nowotarski, K., Williams, J. P., Bhaganna, P., Linton, M., Patterson, M. F., Underwood, G. J. C., Mswaka, A. Y., & Hallsworth, J. E. (2010). Solutes determine the temperature windows for microbial survival and growth. Proceedings of the National Academy of Sciences, 107(17), 7835–7840. https://doi.org/10.1073/pnas.1000557107

Cinar, A., & Onbaşı, E. (2019). Mycotoxins: The Hidden Danger in Foods. Mycotoxins and Food Safety. https://doi.org/10.5772/intechopen.89001

Creppy, E. E., Chiarappa, P., Baudrimont, I., Borracci, P., Moukha, S., & Carratù, M. R. (2004). Synergistic effects of fumonisin B1 and ochratoxin A: Are in vitro cytotoxicity data predictive of in vivo acute toxicity? Toxicology, 201(1), 115–123. https://doi.org/10.1016/j.tox.2004.04.008

De Baere, S., Goossens, J., Osselaere, A., Devreese, M., Vandenbroucke, V., De Backer, P., & Croubels, S. (2011). Quantitative determination of T-2 toxin, HT-2 toxin, deoxynivalenol and deepoxy-deoxynivalenol in animal body fluids using LC–MS/MS detection. Journal of Chromatography B, 879(24), 2403–2415. https://doi.org/10.1016/j.jchromb.2011.06.036

Deligöz, E., & Bilge, N. (2017). Threat Coming with Milk: Aflatoxin. Turkish Journal of Agriculture - Food Science and Technology, 5(8), 846–857. https://doi.org/10.24925/turjaf.v5i8.846-857.1111

Downard, K. (2004). Mass Spectrometry. https://doi.org/10.1039/9781847551306

Engelhardt, G., Zill, G., Wohner, B., & Wallnöfer, P. (1988). Transformation of the Fusarium mycotoxin zearalenone in maize cell suspension cultures. Naturwissenschaften, 75(6), 309–310.

European Commission. (2002a). 657/EC implementing Council Directive 96/23/EC concerning the performances of analytical methods and the interpretation of results. Official Journal of the European Communities, L221.

European Commission. (2002b). Commission Decision of 14 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C(2002) 3044) (Text with EEA relevance) (2002/657/EC), CELEX1. https://op.europa.eu:443/en/publication-detail/-/publication/f18ec4c7-8d1d-4593-94c6-6f85e9cfe014/language-en/format-PDF

European Commission. (2002c). Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Off. J. Eur. Comm, 140, 10–21.

European Commission. (2006). Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (including amendments as of 13 November 2012). Official Journal of the European Communities, 364(5), 5–24. https://eur-lex.europa.eu/eli/reg/2006/1881/oj

European Commission. (2013). Commission Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. Official Journal of the European Communities, 165.

Faktor, J., Dvorakova, M., Maryas, J., Procházková, I., & Bouchal, P. (2012). Identification and characterisation of pro-metastatic targets, pathways and molecular complexes using a toolbox of proteomic technologies. Klinická Onkologie : Casopis Ceské a Slovenské Onkologické Spolecnosti, 25 Suppl 2, 2S70-7.

Fang, J. (2017). Peptide Mapping with Higher Confidence The Application Capability of IMS QTof Mass Spectrometry with Four-dimension (4D) Peak Detection (RT, m/z, intensity, and CCS values). 9.

Fiebig, L., & Laux, R. (2016). A collision cross section and exact ion mass database of the formulation constituents polyethylene glycol 400 and polysorbate 80. International Journal for Ion Mobility Spectrometry, 19(2), 131–137. https://doi.org/10.1007/s12127-016-0195-2

García-Moraleja, A., Font, G., Mañes, J., & Ferrer, E. (2015). Simultaneous determination of mycotoxin in commercial coffee. Food Control, 57, 282–292. https://doi.org/10.1016/j.foodcont.2015.04.031

Gonçalves, R.A., Hofstetter, U., Schatzmayr, D., & Jenkins, T. (2018). Mycotoxins in Southeast Asian aquaculture: Plant-based meals and finished feeds. World Mycotoxin Journal, 11(2), 265–275. https://doi.org/10.3920/WMJ2017.2239

Gonçalves, R.A., Schatzmayr, D., Hofstetter, U., & Santos, G. A. (2017). Occurrence of mycotoxins in aquaculture: Preliminary overview of Asian and European plant ingredients and finished feeds. World Mycotoxin Journal, 10(2), 183–194. https://doi.org/10.3920/WMJ2016.2111

Gonçalves, Rui A., Naehrer, K., & Santos, G. A. (2016). Occurrence of mycotoxins in commercial aquafeeds in Asia and Europe: A real risk to aquaculture? Reviews in Aquaculture, 10(2), 263–280. https://doi.org/10.1111/raq.12159

Gustavo González, A., & Ángeles Herrador, M. (2007). A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. TrAC Trends in Analytical Chemistry, 26(3), 227–238. https://doi.org/10.1016/j.trac.2007.01.009

Hooft, J. M., Elmor, A. E. H. I., Encarnação, P., & Bureau, D. P. (2011). Rainbow trout (Oncorhynchus mykiss) is extremely sensitive to the feed-borne Fusarium mycotoxin deoxynivalenol (DON). Aquaculture, 311(1–4), 224–232. https://doi.org/10.1016/j.aquaculture.2010.11.049

ICH. (1995). Validation of Analytical Procedures: Text and Methodology. ICH Topic Q 2 (R1). CPMP/ICH/381/95. European Medicines Agency London.

IUPAC. (1997). Compendium of Chemical Terminology (2ed ed.). Blackwell Scientific Publications. https://doi.org/10.1351/goldbook.T06250

Jennifer. (2019, May 26). The Ultimate Guide To Mycotoxins and What Makes Mold Toxic | Mold Help For You. Https://Moldhelpforyou.Com/. https://moldhelpforyou.com/mycotoxins/

Jonker, M. A., & Egmond (Eds.). (2004). Worldwide regulations for mycotoxins in food and feed in 2003. Food and Agriculture Organization of the United Nations.

Kovalsky, P., Kos, G., Nährer, K., Schwab, C., Jenkins, T., Schatzmayr, G., Sulyok, M., & Krska, R. (2016). Co-Occurrence of Regulated, Masked and Emerging Mycotoxins and Secondary Metabolites in Finished Feed and Maize—An Extensive Survey. Toxins, 8(12), 363. https://doi.org/10.3390/toxins8120363

Kralj Cigić, I., & Prosen, H. (2009). An overview of conventional and emerging analytical methods for the determination of mycotoxins. International Journal of Molecular Sciences, 10(1), 62–115.

Krska, R., Schubert-Ullrich, P., Molinelli, A., Sulyok, M., MacDonald, S., & Crews, C. (2008). Mycotoxin analysis: An update. Food Additives & Contaminants: Part A, 25(2), 152–163. https://doi.org/10.1080/02652030701765723

Laganà, A. (2018). LC-MS/MS Method for Mycotoxin Analysis. MDPI.

Lim, C. W., Lai, K. Y., Yeo, J. F., Tai, S. H., & Chan, S. H. (2015). Quantitative assessment of moniliformin in cereals via alternative precipitation pathways, aided by LC-LIT-MS and LC-Q-TOF-MS. Food Chemistry, 174, 372–379. https://doi.org/10.1016/j.foodchem.2014.11.069

Lim, C., & Webster, C. D. (2001). Nutrition and Fish Health. 393.

Ludwig Huber. (1998). Validation and Qualification in Analytical Laboratories. Taylor & Francis.

Magan, N., Medina, A., & Aldred, D. (2011). Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. Plant Pathology, 60(1), 150–163. https://doi.org/10.1111/j.1365-3059.2010.02412.x

Manning, B. B. (2010). Mycotoxins in Aquaculture Feeds. SRAC Publication, 5002, 4.

Meca, G., Luciano, F. B., Zhou, T., Tsao, R., & Mañes, J. (2012). Chemical reduction of the mycotoxin beauvericin using allyl isothiocyanate. Food and Chemical Toxicology, 50(5), 1755–1762. https://doi.org/10.1016/j.fct.2012.02.070

Merel, S., Regueiro, J., Berntssen, M. H. G., Hannisdal, R., Ørnsrud, R., & Negreira, N. (2019). Identification of ethoxyquin and its transformation products in salmon after controlled dietary exposure via fish feed. Food Chemistry, 289, 259–268. https://doi.org/10.1016/j.foodchem.2019.03.054

Miller, J. D. (1998). GLOBAL SIGNIFICANCE OF MYCOTOXINS. 18.

Miraglia, M., Marvin, H. J. P., Kleter, G. A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., De Santis, B., Dekkers, S., Filippi, L., Hutjes, R. W. A., Noordam, M. Y., Pisante, M., Piva, G., Prandini, A., Toti, L., van den Born, G. J., & Vespermann, A. (2009). Climate change and food safety: An emerging issue with special focus on Europe. Food and Chemical Toxicology, 47(5), 1009–1021. https://doi.org/10.1016/j.fct.2009.02.005

Monbaliu, S., Van Poucke, C., Detavernier, C., Dumoulin, F., Van De Velde, M., Schoeters, E., Van Dyck, S., Averkieva, O., Van Peteghem, C., & De Saeger, S. (2010). Occurrence of Mycotoxins in Feed as Analyzed by a Multi-Mycotoxin LC-MS/MS Method. Journal of Agricultural and Food Chemistry, 58(1), 66–71. https://doi.org/10.1021/jf903859z

Morgavi, D. P., & Riley, R. T. (2007). An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with Fusarium toxins. Animal Feed Science and Technology, 137(3), 201–212. https://doi.org/10.1016/j.anifeedsci.2007.06.002

Nazari, F., Sulyok, M., Kobarfard, F., Yazdanpanah, H., & Krska, R. (2015). Evaluation of Emerging Fusarium mycotoxins beauvericin, Enniatins, Fusaproliferin and Moniliformin in Domestic Rice in Iran. Iranian Journal of Pharmaceutical Research : IJPR, 14(2), 505–512. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4403067/

Nič, M., Jirát, J., Košata, B., Jenkins, A., & McNaught, A. (Eds.). (2009). Internal standard in chromatography. In IUPAC Compendium of Chemical Terminology (2.1.0). IUPAC. https://doi.org/10.1351/goldbook.I03108

Paglia, G., Angel, P., Williams, J. P., Richardson, K., Olivos, H. J., Thompson, J. W., Menikarachchi, L., Lai, S., Walsh, C., Moseley, A., Plumb, R. S., Grant, D. F., Palsson, B. O., Langridge, J., Geromanos, S., & Astarita, G. (2015). Ion mobility-derived collision cross section as an additional measure for lipid fingerprinting and identification. Analytical Chemistry, 87(2), 1137–1144. https://doi.org/10.1021/ac503715v

Parenty, A. D. C., Button, W. G., & Ott, M. A. (2013). An Expert System To Predict the Forced Degradation of Organic Molecules. Molecular Pharmaceutics, 10(8), 2962–2974. https://doi.org/10.1021/mp400083h

Pereira, V., Fernandes, J., & Cunha, S. (2014). Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis. Trends in Food Science & Technology, 36(2), 96–136.

Peter, V. (2019). Encyclopedia of Food Chemistry. Elsevier.

Prichard, E., & Barwick, V. (2007). Quality Assurance in Analytical Chemistry. John Wiley & Sons.

Prosperini, A., Meca, G., Font, G., & Ruiz, M. (2012). Study of the cytotoxic activity of beauvericin and fusaproliferin and bioavailability in vitro on Caco-2 cells. Food and Chemical Toxicology, 50(7), 2356–2361.

Rao, T. N. (2018). Validation of Analytical Methods. Calibration and Validation of Analytical Methods - A Sampling of Current Approaches. https://doi.org/10.5772/intechopen.72087

Regueiro, J., Negreira, N., & Berntssen, M. H. G. (2016). Ion-Mobility-Derived Collision Cross Section as an Additional Identification Point for Multiresidue Screening of Pesticides in Fish Feed. Analytical Chemistry, 88(22), 11169–11177. https://doi.org/10.1021/acs.analchem.6b03381

Regueiro, J., Negreira, N., Hannisdal, R., & Berntssen, M. H. G. (2017). Targeted approach for qualitative screening of pesticides in salmon feed by liquid chromatography coupled to traveling-wave ion mobility/quadrupole time-of-flight mass spectrometry. Food Control, 78, 116–125. https://doi.org/10.1016/j.foodcont.2017.02.053

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses—An overview. International Journal of Food Microbiology, 119(1), 3–10. https://doi.org/10.1016/j.ijfoodmicro.2007.07.019

Rochat, B. (2018). Quantitative and Qualitative LC-High-Resolution MS: The Technological and Biological Reasons for a Shift of Paradigm. Recent Advances in Analytical Chemistry. https://doi.org/10.5772/intechopen.81285

Sahoo, P. K., & Mukherjee, S. C. (2001). Effect of dietary β-1,3 glucan on immune responses and disease resistance of healthy and aflatoxin B1-induced immunocompromised rohu (Labeo rohita Hamilton). Fish & Shellfish Immunology, 11(8), 683–695. https://doi.org/10.1006/fsim.2001.0345

Serrano, AB, Font, G., Mañes, J., & Ferrer, E. (2013). Emerging Fusarium mycotoxins in organic and conventional pasta collected in Spain. Food and Chemical Toxicology, 51, 259–266.

Serrano, Ana, Capriotti, A., Cavaliere, C., Piovesana, S., Samperi, R., Ventura, S., & Laganà, A. (2015). Development of a Rapid LC-MS/MS Method for the Determination of Emerging Fusarium mycotoxins Enniatins and Beauvericin in Human Biological Fluids. Toxins, 7(9), 3554–3571. https://doi.org/10.3390/toxins7093554

Snyder, L. R., Kirkland, J. J., & Dolan, J. W. (2011). Introduction to modern liquid chromatography. John Wiley & Sons.

Spanjer, M. C., Rensen, P. M., & Scholten, J. M. (2008). LC–MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. Food Additives & Contaminants: Part A, 25(4), 472–489. https://doi.org/10.1080/02652030701552964

Speijers, G. J. A., & Speijers, M. H. M. (2004). Combined toxic effects of mycotoxins. Toxicology Letters, 153(1), 91–98. https://doi.org/10.1016/j.toxlet.2004.04.046

Steen, H., Küster, B., & Mann, M. (2001). Quadrupole time-of-flight versus triple-quadrupole mass spectrometry for the determination of phosphopeptides by precursor ion scanning: QqTOF vs QqQ for phosphopeptide precursor ion scanning. Journal of Mass Spectrometry, 36(7), 782–790. https://doi.org/10.1002/jms.174

Stöckl, D., D'Hondt, H., & Thienpont, L. M. (2009). Method validation across the disciplines—Critical investigation of major validation criteria and associated experimental protocols. Journal of Chromatography B, 877(23), 2180–2190.

Tandem mass spectrometry. (2020). In Wikipedia. https://en.wikipedia.org/w/index.php?title=Tandem_mass_spectrometry&oldid=944084490

Tola, M., & Kebede, B. (2016). Occurrence, importance and control of mycotoxins: A review. Cogent Food & Agriculture, 2(1), 1191103. https://doi.org/10.1080/23311932.2016.1191103

Turner, N. W., Subrahmanyam, S., & Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. Analytica Chimica Acta, 632(2), 168–180. https://doi.org/10.1016/j.aca.2008.11.010

Vendl, O., Berthiller, F., Crews, C., & Krska, R. (2009). Simultaneous determination of deoxynivalenol, zearalenone, and their major masked metabolites in cereal-based food by LC–MS–MS. Analytical and Bioanalytical Chemistry, 395(5), 1347–1354. https://doi.org/10.1007/s00216-009-2873-y

Williams, J. P., & Hallsworth, J. E. (2009). Limits of life in hostile environments: No barriers to biosphere function? Environmental Microbiology, 11(12), 3292–3308. https://doi.org/10.1111/j.1462-2920.2009.02079.x

Xu, B., Jia, X., Gu, L., & Sung, C. (2006). Review on the qualitative and quantitative analysis of the mycotoxin citrinin. Food Control, 17(4), 271–285. https://doi.org/10.1016/j.foodcont.2004.10.012

Young, J. C., Zhu, H., & Zhou, T. (2006). Degradation of trichothecene mycotoxins by aqueous ozone. Food and Chemical Toxicology, 44(3), 417–424. https://doi.org/10.1016/j.fct.2005.08.015

Ytrestøyl, T., Aas, T. S., & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. Aquaculture, 448, 365–374. https://doi.org/10.1016/j.aquaculture.2015.06.023

Zöllner, P., & Mayer-Helm, B. (2006). Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography–atmospheric pressure ionisation mass spectrometry. Journal of Chromatography A, 1136(2), 123–169. https://doi.org/10.1016/j.chroma.2006.09.055