UNIVERSITÀ DEGLI STUDI DI PADOVA
School of Agricultural Science and Veterinary Medicine

Second Cycle Degree (MSc) in Food and Health

Simultaneous quantification of HT-2, T-2, diacetoxyscirpenol (DAS) and neosolaniol (NEO) mycotoxins by LC-MS/MS in different cereal matrices

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ACADEMIC YEAR 2022-2023

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Abbreviations and acronyms

EC	EUROPEAN COMMISSION
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
WHO	World Health Organization
TDI	Tolerable daily intake
BCAs	Biological control agents
NEO	Neosolaniol
DAS	diacetoxyscirpenol
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MS	Mass spectrometry
LC-MS/MS	Liquid chromatography−tandem mass spectrometry
QqQ	Triple quadrupole
CID	Collision-induced dissociation
MRM	Multiple reaction monitoring
IS	Ion spray voltage
CUR	Curtain gas
CAD	Collision gas
GS	Ion source gas
RT	Retention time
DP	Decluttering potential
EP	Entrance potential
CE	Collision energy
CXP	Collision cell exit potential
S/N	Signal to noise ratio
LOD	Limit of detection
LOQ	Limit of quantification
R^2	Coeficient of determination
RSD_r%	Relative standard deviation for repeatability
RSD_R%	Relative standard deviation for reproducibility

Abstract

The contamination of foods and feeds with mycotoxins, secondary metabolites produced by some fungal species, is an important issue that has been studied for several years because of its potential negative effect for the health of people and animals. Cereal crops are one of the most susceptible products to contamination by several mycotoxins such as aflatoxins, fumonisins, ochratoxins, deoxynivalenol, zearalenone. However, there is a group of these secondary metabolites, known as emerging mycotoxins, that can potentially contaminate cereals and for which there are no legal limits yet because previous studies have shown a low prevalence and concentration of these mycotoxins. This group of mycotoxins includes HT-2, T-2, diacetoxyscirpenol (DAS), and neosolaniol (NEO), for which only recommendations have been set by the European Union. This study stablished a simultaneous protocol to quantify HT-2, T-2, DAS, and NEO mycotoxins in different cereal matrices using the LC-MS/MS technique and applying validation parameters such as specificity, repeatability, reproducibility, and recovery. The detection and quantification limits were optimized taking into account the recommendations established by the European Union for these mycotoxins. After standardization of the LC-MS/MS conditions, we obtained values of repeatability and reproducibility parameters lower than 25% for all the mycotoxins analysed. Analysing three different food matrices of cereals and various concentration levels of mycotoxins, we obtained recovery values between 78% and 98%. Besides, we obtained detection limit (LOD) values of 0.5 pg/μl, based on the concentrations established for instrumental LOD of 0.1 pg/μl and 0.25 pg/μl. Regarding the quantification limit (LOQ) values of the method, the obtained RSD_r% values confirmed that they were acceptable according to the European Union recommendations in the case of T-2 and HT-2 mycotoxins. For NEO and DAS mycotoxins, the values were compared with similar studies, obtaining similar results, so they can be considered for future studies. Finally, the method was put into practice with the analysis of naturally contaminated samples, and it was possible to identify and quantify each mycotoxin of interest. In conclusion, we were able to develop and validate a rapid and highly sensitivity method for the simultaneous quantification of T-2, HT-2, DAS, and NEO mycotoxins in different types of food and feed matrices such as cereals.

Riassunto

La contaminazione di alimenti e mangimi con micotossine, metaboliti secondari prodotti da alcune specie fungine, è un importante tema studiato da diversi anni per i suoi potenziali effetti negativi sulla salute delle persone e degli animali. Le colture cerealicole sono uno dei prodotti più suscettibili alla contaminazione da parte di numerose micotossine quali aflatossine, fumonisine, ocratossine, deossinivalenolo, e zearalenone. Tuttavia, esiste un gruppo di questi metaboliti secondari, noti come micotossine emergenti, che possono potenzialmente contaminare i cereali e per i quali non esistono ancora limiti legali perché studi precedenti hanno mostrato una bassa prevalenza e concentrazione di queste micotossine. Questo gruppo di micotossine comprende HT-2, T-2, diacetossiscirpenolo (DAS) e neosolaniolo (NEO), per i quali sono state stabilite solo raccomandazioni dall'Unione Europea. Questo studio ha stabilito un protocollo simultaneo per quantificare le micotossine HT-2, T-2, DAS e NEO in diverse matrici di cereali utilizzando la tecnica LC-MS/MS e applicando parametri di validazione quali specificità, ripetibilità, riproducibilità e recupero. I limiti di rilevazione e quantificazione sono stati ottimizzati tenendo conto delle raccomandazioni stabilite dall'Unione Europea per queste micotossine. Dopo la standardizzazione delle condizioni LC-MS/MS, abbiamo ottenuto valori dei parametri di ripetibilità e riproducibilità inferiori al 25% per tutte le micotossine analizzate. Analizzando tre diverse matrici alimentari di cereali e diversi livelli di concentrazione di micotossine, abbiamo ottenuto percentuali di recupero comprese tra il 78% e il 98%. Inoltre, abbiamo ottenuto valori del limite di rilevabilità (LOD) di 0,5 μg/Kg, sulla base delle concentrazioni stabilite per LOD strumentali di 0,1 μg/Kg e 0,25 μg/Kg. Per quanto riguarda il limite di quantificazione (LOQ) del metodo, i valori di RSD_r% hanno confermato che i valori erano accettabili secondo le raccomandazioni dell'Unione Europea nel caso delle micotossine T-2 e HT-2. Per le micotossine NEO e DAS, i valori ottenuti sono stati confrontati con studi simili, ottenendo risultati simili, in modo che possano essere presi in considerazione per ricerche futuri. Infine, il metodo è stato testato in pratica analizzando campioni naturalmente contaminati, ed è stato possibile identificare e quantificare ciascuna micotossina di interesse. In conclusione, siamo stati in grado di sviluppare e validare un metodo rapido e altamente sensibile per la quantificazione simultanea delle micotossine T-2, HT-2, DAS e NEO, in diversi tipi di matrici alimentari e mangimi a base di cereali.

CHAPTER 1- Introduction

1.1 Mycotoxins - historical review

Human beings, since its inception, have been exposed to a constant risk related to food consumption, so it has been necessary to take some measures in this regard to minimize the risks of food intake (Valle, 2010). One of many risks has been mycotoxins, which according to the definition established by the FAO, WHO, EFSA and other control entities share the criteria established by Pitt et al. (2012) where it is mentioned that "mycotoxins are metabolites produced by fungi that when swallowed, inhaled, or absorbed through the skin, reduce activity, cause illness or death in humans and animals (not excluding birds)" (Pitt, et al., 2012).

Much of the historical background related to mycotoxins and food can be traced back to the 7th and 8th centuries BCE in Rome where the Robigalia festival was established in honour of the God Robigus, to whom it was necessary to make a sacrifice so that he would not attack the fields of crops and instead protect the grain and the trees (Valle, 2010). This event was celebrated on the time of the year when crops were most likely to be attacked by pests such as rust or mildew (Valle, 2010). Over the years one of the most evident diseases caused by mycotoxins was ergotism, according to some records it is mentioned that in the Middle Ages ergotism was used as a medical purpose but if it was only used in small amounts, on the contrary, if used in large quantities, it caused serious damage or even death (Valle, 2010). Among the main symptoms that were displayed we can mention: abortions, hallucinations, necrosis, loss of limbs, sensations of itching or tingling through the skin, distortions of the face, paralysis, convulsions, dementia, among others (Valle, 2010).

But the general interest in mycotoxins increased in 1960, when a liver disease in farm animals was declared in Great Britain that affected the turkey population (Valle, 2010). It was called "Disease X of Turkeys" causing the death of more than one hundred thousand turkeys; where it was later discovered that this disease to be caused by aflatoxins produced by fungi of the genus *Aspergillus* (Valle, 2010). After several subsequent tests, these toxins were found to be hepatocarcinogenic in animals and humans, prompting research on mycotoxins (Valle, 2010). However, it was also evidenced that the methodologies used at that time, such as conventional microbiological methods, were not robust enough to confirm the presence of mycotoxins (Valle, 2010).

Within the framework of the European Union as of 2009, The EFSA presents the preparation of a final opinion where a series of 30 risk assessments is carried out to examine the presence of undesirable substances in animal feed (European Food Safety Authority, 2022). Years later (2017), the EFSA meets with its Italian partners to discuss the problem of climate change and mycotoxins, highlighting the relationship in which changes in temperature, humidity, rainfall, and carbon dioxide production influence in the behaviour of fungi and, consequently, in the production of mycotoxins (European Food Safety Authority, 2022). In the following year, the EFSA evaluates the presence of mycotoxins in food, especially in cereals, to determine their impact on human and animal health (European Food Safety Authority, 2022). In addition, an opinion is published where the establishment of a group indicative value based on health criteria for mycotoxins and their modified forms is considered (European Food Safety Authority, 2022). In 2020, a scientific opinion is presented confirming the presence of naturally produced mycotoxins by moulds that can be found in a variety of food products, including cereals, meat preserves, fresh and dried fruit, and cheese, therefore, this aspect must be considered of vital importance for public health (European Food Safety Authority, 2022).

Thus, mycotoxins are considered one of the most important natural contaminants in food, due to the high incidence in products of plant origin such as cereals and spices, as well as meat and dairy products, representing a public health problem due to the high toxicity of these substances on human and animal health (Trombete, Saldanha, Direito, & Fraga, 2013). Speaking specifically of cereals, it can be mentioned that they are one of the foods that are commonly consumed as an essential source of energy, minerals, fibber, and vitamins throughout the world (Khodaei, Javanmardi, & Mousavi, 2021). However, these products are susceptible to infection by various fungi in the agricultural phase, storage and even in food processing. Mycotoxin diversity and contamination in cereals depends on poor storage conditions, climate, temperature, insect damage and drought (Khodaei, Javanmardi, & Mousavi, 2021). Some of the physical and chemical properties of cereals, such as pH, chemical composition, and water activity, increase the occurrence and concentration of mycotoxins (Khodaei, Javanmardi, & Mousavi, 2021).

Currently, more than 300 mycotoxins are known, however, about 30 are considered more important due to their high toxicity and wide presence in food (Trombete, Saldanha, Direito, & Fraga, 2013). In cereals, the presence of fungi of the genera

Aspergillus, *Penicillium* and *Fusarium* are directly related to food contamination by mycotoxins, in addition to causing important crop diseases (Trombete, Saldanha, Direito, & Fraga, 2013). The main mycotoxins produced by different species of fungi and widely occurring in cereals are trichothecenes (mainly deoxynivalenol - DON, T-2, and HT-2 toxin), zearalenone (ZEA), ochratoxin A (OTA), fumonisins and aflatoxins from group B and G (Trombete, Saldanha, Direito, & Fraga, 2013).

Moreover, within the world of mycotoxins, there are those that have not received much interest over the years, but the evidence of their incidence is increasing rapidly; however, they have not yet been submitted under legislative parameters that allow the regulation of concentration limits in food (Zhao, et al., 2018). These mycotoxins are known as “emerging”, data on the toxicity and occurrence of emerging mycotoxins are limited, and further investigation of these compounds is needed for proper risk assessment (Vaclavikova, et al., 2013). However, some studies have been carried out that describe their possible implications for food safety, mentioning that this type of mycotoxins could generate great harmful effects in humans and animals if they are exposed to very low concentration levels, especially in human risk groups such as children and elderly (EUROPEAN COMMISSION, 2002). Within this group are the HT-2, T-2, NEO, and DAS mycotoxins that have been found with greater predominance in cereal food and feed (Panasiuk, Jedziniak, Piatkowska, & Bocian, 2018). The increase in this type of mycotoxins is due to several factors such as climate change, poor crop handling and mismanagement of storage spaces (Panasiuk, Jedziniak, Piatkowska, & Bocian, 2018).

1.2 Importance of the study

That is why when studying this type of emerging mycotoxins in cereal matrices an analytical challenge is involved, since cereals presenting compounds such as organic acids, sugars, chlorophyll, and others, can be difficult to eliminate by preparing extracts of sample (Panasiuk, Jedziniak, Piatkowska, & Bocian, 2018). Therefore, it is important to develop a suitable method that, in addition to being sensitive and specific, allows the detection of low levels of mycotoxins in various samples and reduces the number of sample preparation steps and analysis time. Moreover, due to the increase in the prevalence of emerging mycotoxins in cereals and feed, it is important that studies aiming at developing new methodologies for their identification increase and

serve as support for the generation of regulations by the respective control entities, contributing to better control and management of cereal crops, reducing economic losses and their eventual negative effect on the food security of people and animals

1.3 Objectives of the study

Due to their potential risk to human and animal health, this study has the general objective of establishing a simultaneous protocol to quantify HT-2, T-2, DAS, and NEO mycotoxins in different cereal matrices using the LC-MS/MS analytical technique. Specifically, the instrumental conditions were set up to determine the most appropriate separation condition, ionization parameters, and product ions to carry out the quantification and confirmation of each analyte. Then, the method validation was sought, evaluating parameters of linearity, specificity, repeatability, reproducibility, and recovery. In order to give evidence of method suitability for food/feed analysis, the parameters recommended by the European Union for the mycotoxins for which there are records were considered, and for those for which there are no records, reference parameters from previous investigations were considered.

CHAPTER 2- General aspects of the main mycotoxins

2.1 Aflatoxins

Aflatoxins are a type of mycotoxins, produced by fungus species of the genus *Aspergillus*, the generic term Aflatoxin can refer to four different types of mycotoxins, known as B1, B2, (produced by *Aspergillus flavus* and *Aspergillus parasiticus*), G1 and G2 (produced by *Aspergillus parasiticus*) (Morreres, 2016). After entry into the body, Aflatoxin B1 and B2 are metabolized in the liver to create a hydroxylated metabolite, Aflatoxin M1 (Morreres, 2016). Regarding their chemical structure, aflatoxins are coumarins linked to a bifuran unit plus a pentanone (AFB) or lactone (AFG) ring (Schuler & Gomez, 2018). The production of this type of mycotoxins is favoured by high humidity levels (>15%), and temperature ranges that vary between 20 to 30° C (Morreres, 2016).

Aflatoxins have great carcinogenic, teratogenic, and mutagenic activity, which makes them an important public health issue and hence the importance of their control and prevention (Martinez, 2013). These compounds have been shown to induce tumour formation, mainly in the liver, but also in other organs such as the lung, kidney, and colon (Morreres, 2016). In addition to these toxic effects, it is also important to highlight its teratogenic and indirect immunosuppressive potential (Morreres, 2016).

Whitin the food sector, cereals are among the crops most susceptible to contamination by aflatoxigenic fungi; indeed, it is estimated that 25 % of world cereal crops are contaminated by mycotoxins, this fact is especially relevant for rice, since it is the second most consumed cereal worldwide (Romero, Ramirez, Garcia, & Madrid, 2022). In addition, AFB1 are present in large quantities in other cereal products (Khaneghah, Es, Raeisi, & Fakhri, 2018), it is documented that AFB1 can infect cereal grains specifically maize and in some cases peanuts, where aflatoxin infections have been observed (Khaneghah, Es, Raeisi, & Fakhri, 2018). On the other hand, concern about wheat contamination by mycotoxins has intensified in recent years, as can be seen from the increase in the number of publications on the subject (Trombete, Saldanha, Direito, & Fraga, 2013).

The high risks of aflatoxins for animal and human health, added to their prevalence in cereals, have prompted the establishment of different legislations to limit the content of aflatoxins in cereals, among other susceptible foods, for animal and human consumption (Romero, Ramirez, Garcia, & Madrid, 2022). One of the most restrictive

legislations is Commission Regulation (EC) No. 2023/915, which has replaced Commission Regulation (EC) No. 2023/915. It mentions the maximum values for aflatoxins that range from lower values such as 4 µg/Kg for cereals processed for human consumption and higher values of 15 µg/Kg for Hazelnuts and Brazil nuts ((EU) Comission Regulation, 2023)

2.2 Fumonisins

Fumonisins are a group of mycotoxins produced by *F. moniliforme*, there are 6 types of Fumonisins: B1, B2, B3, B4, A1 and A2. However, the most frequent and important due to their toxicity are B1 and B2 (Martinez, 2013). Fumonisins (B1 and B2) are metabolites produced by the species of *Fusarium proliferatum* and *Fusarium verticillioides* that have a long-chain hydrocarbon unit (Zain, 2011), the free amino group of the B series seems to be responsible for the biological and toxicological activity of these compounds (Martinez, 2013). The minimum, optimum and maximum growth temperatures vary between 2.5 to 5.0; 22.5 to 27.5 and 32.0 to 37.0° C; respectively (Martinez, 2013).

Through several studies it has been determined that fumonisins (B1 and B2) are carcinogenic metabolites, but fumonisin B1 is the most toxic, shown to promote tumours in rats (Kaushik, 2013), cause equine leukoencephalomalacia (Kaushik, 2013) and porcine pulmonary oedema (Kaushik, 2013). Although FB1 has been identified with significant health threats in cattle and many other animals, the evidence for the same in humans is currently inconclusive (Lumsangkul, Chiang, Lo, Fan, & Ju, 2019). Some studies raised concerns that FB1 exposure could contribute to serious adverse health outcomes, such as cancers and birth defects (Lumsangkul, Chiang, Lo, Fan, & Ju, 2019).

Fumonisins were initially detected in cereals, some studies have determined that fumonisins are considered to have a very low bioavailability, however, it has been proposed that the bioavailability of fumonisins can increase considerably when heated during the processing of foods (Reddy, et al., 2010). Fumonisins have been isolated at high levels in cornmeal and corn grits, including in products from some supermarkets such as the one in Charleston, South Carolina, the city with the highest incidence of esophageal cancer among African Americans in the United States (Reddy, et al., 2010). Recently, several new sources of fumonisins have been reported in corn flake products, Portuguese cornbread, tea black, herbals, medicinal plants,

and the polenta that is usually consumed in northern Italy (Waskiewicz, Beszterda, & Golinski, 2012). Therefore, these mycotoxins not only represent a significant risk to human and animal health, but also affect food safety, reduce livestock production, and are closely associated with economic losses (Lumsangkul, Chiang, Lo, Fan, & Ju, 2019).

Within the European Union (EU), exist the Commission Regulation has established the regulatory limits for fumonisins in food, based on the sum of FB1 and FB2 this is the regulation (EU) 2023/915 of April 25, 2023; where it can be seen that the highest permissible level corresponds to unprocessed maize grains with a value of 4000 μg/Kg, while the lowest value recorded is 800 μg/Kg, corresponding to cereals intended for consumption at breakfast and snacks based on maize ((EU) Comission Regulation, 2023). For products intended for animal feed, the European Union establishes a Recommendation document (2006/576/EC), where considering a humidity value of 12%, the regulatory limit of fumonisins in products intended for animal production, based on in the sum of FB1 and FB2 is 60 mg/Kg for maize and maize products ((EU) Comission Recommendation, 2016).

2.3 Ochratoxins

Ochratoxins are mycotoxins derived from the growth of *Aspergillus ochraceus* and *Aspergillus ostianius*, as well as *Penicillium verrucosum*. Grain contamination is highly variable and is influenced by regional and climate conditions during and after harvest (Petzinger & Ziegler, 2000). These toxins can occur in grains stored at 15±19% humidity and temperatures between 8–37° C (Petzinger & Ziegler, 2000).

Ochratoxins under natural conditions are formed into ochratoxin A and ochratoxin B (Petzinger & Ziegler, 2000). Ochratoxin B is less toxic than ochratoxin A and does not inhibit protein biosynthesis (Petzinger & Ziegler, 2000). Ochratoxin A possesses a chlorine atom at C5 of the dihydro-methyl-iscoumarin ring system, which is lacking in ochratoxin B (Petzinger & Ziegler, 2000). Furthermore, the presence of the chlorine atom and a phenolic OH group increases the toxicity of ochratoxin A (Petzinger & Ziegler, 2000).

Ochratoxin A is carcinogenic, genotoxic, teratogenic, immunotoxic and nephrotoxic (Petzinger & Ziegler, 2000). This mycotoxin represents a great importance regarding human health, some of the effects are caused also influence at the molecular level (Petzinger & Ziegler, 2000). In addition, recent reports of genotoxicity studies in cells

from the human uterus show considerably higher sensitivity than expected from the results of studies in animal cells, so there is a direct influence of the exposure time and the amount of toxin dose to which one is exposed, where the values for humans are much higher than for animals, concluding as a result that humans are much more exposed to the toxin (Petzinger & Ziegler, 2000).

Within the food field, in addition to cereals such as barley, maize, rye and oats, ochratoxins have occasionally or frequently been found in other plant products such as beans, figs, olives, nuts and spices, which represent sources of chronic contamination that cannot be considered negligible (Petzinger & Ziegler, 2000). Ochratoxin A is also found in animal-derived food products such as pork and poultry meat, as a result of feeding the animals contaminated fodder (Duarte, Pena, & Lino , 2010). However, based on the most recent assessment of OTA intake by European consumers and previous investigations, cereals have been found to be the most important dietary source of this mycotoxin, contributing around 50% of intake (Duarte, Pena, & Lino , 2010).

In the case of cereals, regulation (EU) 2023/915 of April 25, 2023, of the European Union mentions that the maximum level for unprocessed cereal grains is 5 μg/Kg, meanwhile the lowest level It is for products derived from unprocessed cereal grains and cereals marketed to the final consumer with a value of 3 μg/Kg ((EU) Comission Regulation, 2023). In the case of products intended for animal feed, the recommendation guide of the European Union Commission (2006/576/EC) establishes that the maximum limit must be presented at a value of 0.25 mg/Kg considering a moisture percentage of 12% ((EU) Comission Recommendation, 2016).

2.4 Zearalenone (ZEA)

ZEA is one of the most prevalent and potent contaminants of grains and cereal products that pose a serious threat to food safety (Rai, Das, & Tripathi, 2019). Species of fungi of the Fusarium genera such as *F. graminearum (Gibberella zeae)*, *F. culmorum*, *F. crookwellense*, *F. semitectum* and *F. equiseti* are causing contamination of cereal crops worldwide (Rai, Das, & Tripathi, 2019). Despite having a lactone ring, ZEA is known to be heat stable up to 150° C and does not degrade during food and feed processing (Rai, Das, & Tripathi, 2019). There are 5 known metabolites of ZEA, which are known as a-zearalenol (a-ZEA), b-zearalenol (b-ZEA), a-zearalanol (a-ZAL), b-zearalanol (b-ZAL), and zearalenone (ZON) (Rai, Das, & Tripathi, 2019). The

temperature, precipitation and carbon dioxide concentrations are essential for the formation of this mycotoxin in crops, so that in temperature ranges from 15 to 25° C a high growth and production capacity is displayed, determined that ZEA contamination in crops is more common in areas with colder climates (Han, et al., 2022) with is associated with a humidity value between of 16-90% (Rai, Das, & Tripathi, 2019).

In toxicologist terms, one of the most impact of the mycotoxin ZEA has been to cause reproductive toxicity (Rai, Das, & Tripathi, 2019). The ZEA is known to be extensively absorbed orally in rabbits, rats, mice, and humans (Rai, Das, & Tripathi, 2019). The hormonal action of the ZEA is greater than of any other natural non-steroidal estrogen due to its structural similarity to 17b-estradiol, so ZEA and its metabolites can bind to estrogen receptors (ER) with a high affinity, thus exerting estrogenic effects (Rai, Das, & Tripathi, 2019). Data on the toxicity of ZEA in humans are very scarce, however, several previous studies mention that the ZEA mycotoxin could cause hyperestrogenic syndrome in humans (Rai, Das, & Tripathi, 2019). The ZEA mycotoxin is also known to exhibit potential for hepatotoxicity, hematotoxicity and immunotoxicity (Rai, Das, & Tripathi, 2019).

ZEA and their metabolites have been identified in many cereals, including maize, wheat, sorghum, barley, and (Rai, Das, & Tripathi, 2019). High incidences of ZEA have been reported in feed and food products from different countries of the European Union and Asia (Rai, Das, & Tripathi, 2019). However, only a few studies found the probable intake of ZEA to cross the tolerable daily intake (TDI) value provided by the EU (Rai, Das, & Tripathi, 2019). Several aspects such as temperature, vegetation time, contamination by fungal strains, impact the amount of ZEA accumulated in crops (Rai, Das, & Tripathi, 2019). Based on global contamination data over the last 20 years suggest that ZEA contamination in cereals and cereal-based foods also represents a significant risk in the economic sector (Rai, Das, & Tripathi, 2019). Due to these and several additional causes, novel strategies have recently been developed within cereal crops such as the use of biological control agents (BCAs) that suppress the growth and colonization of harmful pathogens, where it has been shown, for example, that *Epicoccum* and *Sordaria*, which are endophytic fungi, can control the growth of *Fusarium graminearum* and, therefore, the production of ZEA in foods such as corn (Mahato, et al., 2021).

Considering that the presence of the ZEA mycotoxin is widely distributed in cereals, the European Union (EU) has formulated specific regulations for ZEA in food. The EU

legislation in its regulation 2023/915 defines that the maximum permissible limits for ZEA must be 100 μg/Kg for unprocessed cereals except for rice, on the other hand, for unprocessed corn grains the established maximum limit is reported in 350 μg/Kg, this being the upper limit to be considered ((EU) Comission Regulation, 2023). For cereal-based products such as bread, pastries, cookies, except rice products, a value of 50 μg/Kg is established, this being the lower limit to be considered ((EU) Comission Regulation, 2023). For products for animal consumption, the recommendation (2006/576/EC) inform that the values established in cereal feed that contains corn is 3 mg/Kg, while for cereal products that do not contain corn, the value is 2 mg/Kg ((EU) Comission Recommendation, 2016).

CHAPTER 3- Trichothecenes

3.1 General Aspects

Trichothecene mycotoxins are the largest group of toxins produced particularly by molds belonging to the genus *Fusarium* (Flores, Lizarraga, De Cerain, & Gonzales, 2015). Several genera of taxonomically unrelated fungi such as *Chephalosporium, Myrothecium, Stachybotrys, Trichoderma, Trichothecium, and Verticimonosporium,* can also form trichothecenes (Krska, Baumgartner, & Josephs, 2001). However, *F. sporotrichoides, F. poae and F. equiseti* are known to produce type A trichothecenes, while *F. graminearum and F. culmorum* are the most important type B trichothecene-producing molds (Krska, Baumgartner, & Josephs, 2001).

Trichothecenes are polycyclic sesquiterpenoids that have a C9-C10 double bond, an epoxide between the C12 and C13 carbon atoms, and a variable number of hydroxyl and acetoxy groups in the molecule (Santini, Ferracane, Somma, Aragon, & Ritieni, 2009). They are classified into four types, namely A, B, C, D, considering the functional groups present in the molecule, only some of them have been found as natural contaminants of cereal products (Santini, Ferracane, Somma, Aragon, & Ritieni, 2009). Type A trichothecenes include HT-2 toxin (HT-2), T-2 toxin (T-2), diacetoxyscirpenol (DAS), and neosolaniol (NEO) and differ from type B trichothecenes by the absence of a carbonyl group at position C (8), these are: deoxynivalenol (DON, also known as vomitoxin), nivalenol (NIV) and its acetylated derivatives, respectively 3-acetyldeoxynivalenol (3AcDON) and fusarenon-X (FUS-X) (Santini, Ferracane, Somma, Aragon, & Ritieni, 2009). DON and NIV are the commonly found trichothecenes, followed only by T-2, HT-2, and DON-acetylated derivatives, according to previously investigated data (Santini, Ferracane, Somma, Aragon, & Ritieni, 2009).

Trichothecene mycotoxins are non-volatile compounds of low molecular weight (Wannemacher & Wiener, 1997). This group of mycotoxins is relatively insoluble in water but highly soluble in acetone, ethyl acetate, chloroform, dimethyl sulfoxide (DMSO), ethanol, methanol, and propylene glycol (Wannemacher & Wiener, 1997). When kept as crystalline powders or liquid solutions, trichothecene mycotoxin compounds are stable when exposed to air, light, or both (Wannemacher & Wiener, 1997). In addition, these mycotoxins are not autoclaved, but require heating at 500° C for 10 minutes or 250° C for 30 minutes for complete inactivation (Wannemacher &

Wiener, 1997). A 3% to 5% solution of sodium hypochlorite could also be an effective inactivating agent (Wannemacher & Wiener, 1997). In addition, the mycotoxin is produced at alkaline pH, with high temperatures ranging between 20-37° C, and the water activity is close to 0.98–0.995 aw (Ahad, Zhou, Lepp, & Pauls, 2017).

3.2 Toxicity

Trichothecene mycotoxins are toxic to humans and other organisms in general, they present an acute toxicity that varies according to the toxin and the animal species studied, so the ingestion of this toxin in very low doses can cause important effects on the health of living organisms (Wannemacher & Wiener, 1997). Once trichothecene mycotoxins enter the systemic circulation, regardless of the route of exposure, they rapidly affect tissues (Wannemacher & Wiener, 1997). The clinical symptoms and signs of trichothecene poisoning can vary depending on whether the exposure is acute or chronic (Wannemacher & Wiener, 1997). Acute oral, parenteral, dermal, or aerosol exposures to trichothecene mycotoxins cause gastric and intestinal lesions (Wannemacher & Wiener, 1997). Central nervous system toxicity causes anorexia and nausea; suppression of the function of the reproductive organs; and acute vascular effects leading to hypotension and shock. In addition, trichothecene mycotoxins have been found to inhibit protein, DNA and RNA synthesis, and to have immunosuppressive and cytotoxic effects (Lattanzio, Pascale, & Visconti, 2009). Specific symptoms, such as local skin necrosis and inflammation and corneal lesions have been observed in animal models (Wannemacher & Wiener, 1997). In cattle, clinical signs of trichothecene infection include food refusal and vomiting, growth retardation, reproductive disorders, blood disorders, dermatitis, oral lesions, and depressed immune response (Meneely, Ricci, Van Egmond, & Elliott, 2011). However, one species may be more sensitive to the effects of these mycotoxins than others, since, for example, ruminants are less affected due to their ability to metabolize trichothecenes into less toxic metabolites, also species of plants have mechanisms by which they can reduce the toxicity of trichothecene mycotoxins by incorporating the toxin into the plant matrix or by chemical modification (Meneely, Ricci, Van Egmond, & Elliott, 2011). However, they can also cause minor effects such as stunting, wilting, chlorosis, necrosis, and germination inhibition (Meneely, Ricci, Van Egmond, & Elliott, 2011).

3.3 Importance in foods

DON being the most frequent Fusarium toxin, has been documented in several studies, where its presence has been registered in cereals, specifically in corn, oats, barley, wheat, bran, and white flour (Lankova, et al., 2008). The durum wheat flour, which is used almost exclusively for pasta production, is susceptible to *Fusarium* infection and is often heavily contaminated with DON (Krska, Baumgartner, & Josephs, 2001). On other hand, the contamination by this mycotoxin in wheat is considered a constant concern in many countries due to the high occurrence and high rates found in both raw material and derivatives (Trombete, Saldanha, Direito, & Fraga, 2013). For the reduction and decontamination of mycotoxins in products intended for human consumption, different methodologies based on physical, chemical, and microbiological processes have been developed and adapted, such as the use of chemical agents, biotransformers, adsorbents, methods based on colour classification, radiation ionizing, ozonation and others (Trombete, Saldanha, Direito, & Fraga, 2013). However, despite the mentioned methods that are considered promising, the main procedure for the control of wheat contamination with mycotoxins is still the use of Good Agricultural Practices throughout the production chain, since, when the mycotoxins, these will not be easily eliminated during food processing (Trombete, Saldanha, Direito, & Fraga, 2013). Studies carried out in Croatia in a comprehensive survey focused on trichothecene mycotoxins in cereals, mentioned that with a total analysis of 465 samples for DON, T-2 and diacetoxyscirpenol (DAS), the target trichothecenes they were detected in 41, 17 and 27 % of the samples, respectively (Hajslova, et al., 2007). In addition, in recent studies, the frequency of trichothecenes has been shown to increase substantially (14-fold), as recorded in Western Canada between the years 1998 and 2004 (Marin, et al., 2021). The increased frequency may be partly due to pathogenic superiority of these mycotoxins in wheat, since trichothecene-producing strains have shown to be more aggressive and could have the capability to produce more spores, having a faster growth rate (Marin, et al., 2021).

3.4 Legislation

The current community legislation on maximum levels of mycotoxins in food products of the European Community in their regulation 2023/915 mentions values referent to

mycotoxin DON of 1250 µg/Kg for unprocessed cereal grains except for rice ((EU) Comission Regulation, 2023). On the other hand, for durum wheat grains, unprocessed oat grains and corn grains, a value of DON of 1750 µg/Kg is established ((EU) Comission Regulation, 2023). For cereals marketed to the final consumer as cereal flour, semolina, bran, and germ, except for rice and its derivative products, a value of 750 µg/Kg is reported, a value also applied to pasta with a water content of 12 %((EU) Comission Regulation, 2023). For products such as bread, pastries, biscuits, cereal snacks and breakfast cereals, the maximum value allowed is 500 µg/Kg ((EU) Comission Regulation, 2023). For products intended for animal feed, considering a moisture content of 12 %, is possible to mentioned that the values of DON for products that contain corn are established in a value of 8 mg/Kg, meanwhile for the products without content of corn inside the product is registered a value of 12 mg/Kg ((EU) Comission Recommendation, 2016).

For T-2 and HT-2 toxins, the recommendation established by the European Union Commission (2013/165/UE), where it is mentioned that for the sum of T-2 and HT2 in unprocessed foods such as barley and corn the value is 200 µg/Kg, for oats 1000 µg/Kg, wheat, rye and other cereals 100 µg/Kg ((EU) Comission Recommendation, 2013). Cereal grains for direct human consumption such as oats, also can present a value of 200 µg/Kg, for corn 100 µg/Kg and other cereals the values are established at 50 µg/Kg ((EU) Comission Recommendation, 2013). For products that have a cereal base and are intended for human consumption, there are establish values of 100 µg/Kg for cereal bran, 50 µg/Kg for other milled cereal products, 75 µg/Kg for breakfast cereals and 25 µg/Kg for bakery products, cereal snacks, and pasta ((EU) Comission Recommendation, 2013). For other cereal-based products such as compound feed, except cat feed, the maximum values are 250 µg/Kg ((EU) Comission Recommendation, 2013).

For diacetoxyscirpenol and neosolaniol mycotoxins, no regulatory values are currently registered regarding their presence or content in food (Knutsen, et al., 2018). However, in point 32 of regulation (EC) 1881/2006 already expired and now preceded by regulation in force (EU) 2023/915, it was mentioned that regarding the trichothecenes diacetoxyscirpenol and neosolaniol, there is limited information available and in the few studies carried out, low levels of these mycotoxins are shown, therefore maximum limits for these mycotoxins could not be defined ((EU) Comission Recommendation, 2006). In the current regulation (EU) 2023/915, these mycotoxins

are not mentioned, but according to a document issued by the EFSA as a scientific opinion, possible quantification limits can be proposed with the LC-MS/MS methodology considering a LOQ from 0.05 to 125 µg/Kg (Knutsen, et al., 2018).

CHAPTER 4- Analytical methods of detection

4.1 General review

The discovery of aflatoxins and their effects on the health of animals and humans arose just over 50 years ago, this fact marked a pivotal moment in the study of these metabolites (Shephard, 2016). The determination of mycotoxins has always been a challenge within the field of analytics because mycotoxins are found at low levels in food matrices, in addition are difficult to extracting them from complex materials and presenting a wide variety of structures and chemical properties (Shephard, 2016). Due to the complexity of the determination of mycotoxins, analytical methods have been directed to the study of mycotoxins individually, such as ZEA, or with other mycotoxins in a group, selecting those that present chemical and structural similarities, such as aflatoxins or fumonisins (Shephard, 2016). Analytical approaches for the determination of mycotoxins can be carried out through methods based on chromatographic techniques as well as methods based on immunochemistry (Anfossi, Giovannoli, & Baggiani, 2016).

Methods based on chromatographic techniques aim at the quantitative determination of mycotoxins and involve detection by liquid chromatography (LC) or gas chromatography (GC) coupled to ultraviolet (UV), fluorescence (FLD) or mass spectrometry (MS) (Anfossi, Giovannoli, & Baggiani, 2016). The use of a sophisticated instrument set-up, combined with extensive sample preparation allows for the determination of a wide range of mycotoxins with increased sensitivity (Anfossi, Giovannoli, & Baggiani, 2016). On the other hand, immunochemical-based methods such as the ELISA test are commercially available for all regulated mycotoxins and are the most widely used analytical tool to ensure food safety throughout the food chain (Anfossi, Giovannoli, & Baggiani, 2016). In addition, this type of technique is continuously under development, where various formats are presented with the aim of providing fast, portable, and easy-to-operate systems (Anfossi, Giovannoli, & Baggiani, 2016).

Typically, detecting all regulated mycotoxins for all food commodities requires a big quantity of protocols, so instruments capable to perform multi-analyte analysis in a specific manner, such as mass spectrometers capable to perform tandem mass spectrometry (MS/MS) experiments, have become one of the methodologies for responding these analytical challenges with better results (Anfossi, Giovannoli, &

Baggiani, 2016). The use of MS detectors also offers the possibility of obtaining information on the analytes structure and allowing the identification of new unknown mycotoxins (Anfossi, Giovannoli, & Baggiani, 2016). Therefore, the application of this analytical technique in multi-residue analyses, where the presence of mycotoxins in food and feed has been evaluated, allows confirming that the recovery of regulated mycotoxins can be achieved with a high frequency (Anfossi, Giovannoli, & Baggiani, 2016). For this reason, the advantages of mass spectrometry, including high sensitivity, selectivity, precision, and performance, make it the technique of choice for multi-residue analysis (Anfossi, Giovannoli, & Baggiani, 2016).

4.2 Liquid Chromatography

Liquid chromatography is a separation technique in which components in a sample are separated based on their different affinity between two phases, a stationary phase contained in a column and a liquid mobile phase that flows through the column (Martinez, 2013). Therefore, the chromatographic system consists of four components: a sample introduction device, a pump capable to operate at high pressure, a compartment for column allocation, and a detector (Ardrey, 2003). The chromatographic process occurs as a result of change of mobile phase compositions, that in consequence cause repeated absorption or desorption steps of analytes along the stationary phase (Martinez, 2013). The separation occurs as consequence of the different distribution coefficients between the components of a sample, so the choice of the column and the adequate mobile phase is very important (Martinez, 2013). There are several different chromatographic techniques, one of them is the so called “high-performance liquid chromatography” (HPLC), where a liquid mobile phase is pumped at high pressure (up to 400 bar (4×10^7 Pa)) to guarantee a constant flow rate and therefore a reproducible chromatography onto a column capable of withstanding the high pressures generated (Ardrey, 2003). Chromatographic separation is generated when the components of a mixture interact to different degrees with the mobile and/or stationary phases, therefore, they take different times to move from the sample introduction position to the position in which they are detected (Casanave, Araujo, & Lopez, 2012).

4.3 Liquid chromatography coupled to mass spectrometry (LC-MS)

The LC-MS is an analytical methodology where a mass spectrometry detector is coupled to a chromatographic separation system, constituting a tool that allows problems of identification (with a high level of confidence) and quantification of substances to be solved (Martinez, 2013). During the historical evolution of this methodology years, to main problems have been solved: *a)* removing the large amount liquid from the mobile phases before entering the high vacuum region of the MS; and *b)* transforming the molecules in solution into ions in the gas phase without thermal degradation (Martinez, 2013). The sensitivity of the LC-MS analysis will depend on the analyte being analysed and the interface used (Martinez, 2013) and for this reason several instrumental configuration and ion sources have been developed. For instance, LC-MS instruments equipped with Electrospray (ESI) ion source are suitable for the analysis of polar substances, with low to high molecular weights and enough thermostability (Martinez, 2013).

4.4 Mass spectrometer

Mass spectrometry (MS) is an analytical technique employed to qualitatively and quantitatively determines the structure and the molecular mass of analyte in relation to its mass and charge m/z (Ardrey, 2003). Different mass spectrometric analysers are available (quadrupoles, ion traps, Time of Flight, ion mobility) allowing this technique to identify chemical compounds of which there is no knowledge or allowing to quantify known compounds in a very specific and sensible manner. (Ardrey, 2003). A MS analysis can be schematized in the following steps: 1) the ionization, in which the neutral molecule of the analytes passes into a charged gaseous state by an ion source (Grimalt, 2009). 2) the separation of the molecular ions based on their m/z ratio (Grimalt, 2009). For this step to develop correctly, it is necessary to work with a high vacuum, allowing the free movement of ions in space, without the interference of other species with which they may collide (Grimalt, 2009). 3) the ions detection and creation of mass spectra (Grimalt, 2009). The main distinction between the various mass spectrometers is found in the type of analyser used (Martinez, 2013). The analyser is the part of the instrument that allows the separation, working generally at very low pressures, of the ions in the gas phase that have been formed in the ionization source, based on their mass/charge ratio (m/z) (Martinez, 2013). The most relevant analysers

that can be found today are the triple quadrupole (QqQ), time of flight (TOF), ion trap (IT, LIT), magnetic sector, ion mobility (IM) and Fourier transform cyclotron resonance (FT-ICRMS) (Martinez, 2013).

4.5 Triple quadrupole (QqQ)

When it is desired to use MS for the identification of chemical compounds based on their structure, more information is necessary than that obtained by the ions generated in the ionization process (Martinez, 2013). Therefore, this additional information can be obtained from the fragmentation of these ions, that is, from tandem mass spectrometry experiments (Martinez, 2013). Speaking specifically of the quadrupole mass filter instruments, performing MS/MS necessarily implies the addition of two extra quadrupoles to a linear quadrupole instrument (Martinez, 2013). Thus, fragmentation is produced by collision of the selected ion or precursor ion with an inert gas molecules, which are generally argon molecules (Martinez, 2013). This process is called collision-induced dissociation (CID) and occurs in two stages: in the first, the translational energy of the ion is converted into internal energy after colliding with the inert gas molecules; in the second, this internal energy is used to break the ion into several fragments that will be the product ions (Martinez, 2013). Thus, in the first quadrupole it is possible to isolate an ion of determined m/z that passes to the second quadrupole, used as a collision cell, where the fragmentation of the ions takes place when colliding against the inert gas molecules (Martinez, 2013). Fragment ions are then separated into the third quadrupole, before being detected. When working with a triple quadrupole instrument, different scanning modes can be used depending on the final objective of the analysis, thus, in MS mode, a scan of all ions called *full scan* can be performed or a specific ion can be selected to be monitored (Selected Ion Monitoring, SIM) (Martinez, 2013). When working in MS/MS mode, product ion scans, precursor ion scans, neutral losses, or multiple reaction monitoring (MRM) can be performed, increasing sensitivity and selectivity of the process (Shi, et al., 2017). The MRM mode is used for the most sensitive quantification of known compounds, it allows to obtain a very good signal/noise ratio since practically all background ions are filtered out (Vogeser & Parhofer, 2007).

CHAPTER 5- Materials and Methods

5.1 Materials

5.1.1 Chemicals and reagents

- Acetonitrile HPLC-LC grade
- Water HPLC-LC grade
- Formic Acid 0.1 %
- Ammonium formate 10 mM
- Ammonium acetate 7.5 M
- QuEChERS salt commercial sachet type DisQuE pn 186006813: 4 g Mg SO4, 1 g NaCl, 1,5 g Sodium citrate.
- QuEChERS salt commercial centrifugate tube (15ml) type DisQuE pn 186008080: 750 g Mg SO4, 150 g C18-Silica, 150 g Alumina-N, 250 mg PSA.
- Mycotoxin standards:
    - T-2 in acetonitrile (100 μg/ml)
    - HT-2 in acetonitrile (100 μg/ml)
    - Neosolaniol in acetonitrile (100 μg/ml)
    - Diacetoxyscirpenol in acetonitrile (100 μg/ml)

5.1.2 Laboratory tools

- HPLC. Standard configuration with column phase Kinetex 2.6μm XB-C18 LC Column 100X2.1mm.
- SCIEX triple-quadrupole 3500 mass spectrometer, connected to the HPLC system.
- Mill for crushing or pulverizing food samples.
- Balance with resolution 0,001 g
- Orbital Shaker (280 rpm)
- Plastic tubes of 50ml and 15 ml
- Glass vials of 1,5 ml
- Syringe and membrane filters PTFE with a porosity of 0,45 μm

5.1.3 Softwares

- Analyst v. 1.7

- Analyst SCIEX OS

5.2 Methodology

5.2.1 Calibration Curve

The calibration curve was prepared from a stock solution of mycotoxins, these solutions were stored at -4° C in the dark. A calibration mix with an equal amount of each mycotoxin standard was prepared in mobile phase A 75% and mobile phase B 25% as follow: prepare the following calibration solutions with a nominal value of 250 pg/μl, 100 pg/μl, 50 pg/μl, 15 pg/μl, 5 pg/μl, 1 pg/μl, 0.25 pg/μl, 0.1 pg/μl. To construct the calibration curves, the peak area of each toxin was plotted against its concentration. The calibration curves were calculated using the linear regression method using the Analyst v. 1.7 program and Analyst SCIEX OS software.

5.2.2 Extraction procedure

For the extraction of mycotoxins, seven types of cereal matrices were tested: wheat grain for direct human consumption, pasta, corn flakes, breakfast cereal and soft wheat ¨Gentile rosso¨. The last two samples like yellow corn flour for polenta was purchased at a supermarket in the area around the laboratory, and feeds for animal consumption was collected from a nearby farm. A protocol already validated by the host laboratory (3A Laboratori) was used with some modifications:

5 g± 0.1 o samples were weighted in tubes of 40 ml, then 10ml of water and 10 ml of formic acid/acetonitrile 10:90 was added. Samples were shacked for 60 minutes to 280 rpm in a horizontal position to avoid the formation of bubbles. Later, 2 g of QuEChERS salts were added, samples were shacked strong with the hand for 1 minute and centrifugated for 5 minutes to 2500 rpm. 5 ml of the organic supernatant were transferred into the new tubes of 15ml with QuEChERS salts and shacked strong with the hand for 1 minute. After then, the tubes were centrifugate for 5 minutes to 2500 rpm. The extract was finally filtered through PTFE 0,45 μm and transferred into a glass microtubes to be analyzed by LC-MS/MS.

Some samples were concentrated to 2.5 to increase the quantity of mycotoxins (NEO) and improve the quality of the peaks in the chromatographic process, so these samples were evaporated and resuspend in a 400μl of solvent (mobile phase A 75%

and mobile phase B 25%), mixed in the vortex, filtered again with PTFE 0,45 μm filters and the extracts were transferred to the glasses microtubes to be analyzed by LC-MS/MS.

5.2.3 Analysis of real samples and spiking experiments

Samples of conventional wheat grain, pasta and corn flakes were firstly analyzed to investigate the absence of the mycotoxins, then these were used as blank samples. To perform the method validation, these samples were enriched with an appropriate amount of standard mycotoxin before undergoing the extraction process. For other samples, like breakfast cereal and feed for animal consumption very few amounts of target mycotoxins were detected. However, this samples were not considered as blank samples, but were enriched with higher levels of mycotoxin standards. Once the method was validated, real samples of yellow corn flour for polenta and soft wheat "Gentile rosso", also were analyzed to quantify T-2, HT-2, NEO, and DAS. This samples were not enriched with mycotoxin standards because were used as a studied cases to verify the presence or absence of the mycotoxins studied.

5.2.4 Instrumental optimization and performance validation

5.2.4.1 LC parameters

The chromatographic analysis has been carried out using a gradient elution with an aqueous mobile phase (A) composed by ammonium formate 10mM and 0.1% formic acid and an organic mobile phase (B) composed by acetonitrile with 0.1% formic acid. The chosen gradient is reported in Table 5.1.

Table 5.1 Gradient

Total Time (min)	Flow Rate (μl/min)	A (%)	B (%)
0.00	450	90.0	10.0
1.00	450	90.0	10.0
7.10	450	2.0	98.0
8.50	450	2.0	98.0
8.60	450	90.0	10.0
13.00	450	90.0	10.0

5.2.4.2 MS parameters

These parameters were obtained using a SCIEX triple-quadrupole 3500 mass spectrometer. All analyses were performed with the following settings: Scan Type: Multiple Reaction Monitoring (MRM) ; positive polarity, Ion Spray Voltage (IS) of 4500 V, Curtain Gas (CUR) of 35, Collision Gas (CAD) of 8, probe temperature of 400° C, Ion Source Gas 1 (GS1) of 60, the Ion Source Gas 2 (GS2) of 65. Direct infusion analysis was performed at 5 µl min−1 adding by HPLC flow a solution of ammonium formate and 0.1 % formic acid at a flow rate of 450 µl min−1. Data acquisition and processing were performed using Analyst software v. 1.7 and Analyst SCIEX OS software.

5.2.4.3 Selection of the transition ions

The following parameters were optimized: Retention Time (RT), Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Collision Cell Exit Potential (CXP), to obtain the entire spectrum of MS/MS fragmentation. Of all resulting transitions, the two with the highest response were selected. The identification of quantifier and qualifier ions between the two transitions was considered taking into account the slopes of the curves, where the line with the highest slope was selected as the quantifier ion and the second line as the qualifier ion.

5.2.5 Method Validation

In order to guarantee the analytical quality of the results obtained during the study, the following validation parameters have been analyzed: specificity, repeatability, recovery, and detection and quantification limits.

5.2.5.1 Specificity

In order to verify that no interference coincides in the elution region of the analytes of interest, blank matrices samples were analyzed, that there are no overlaps in the peaks. Blank samples and process blank samples were injected to verify that no interference is generated during the process, considering a minimum signal/noise ratio (S/N) of 3 and a maximum signal/noise ratio (S/N) of 10.

5.2.5.2 Repeatability and reproducibility

The precision of the method was evaluated under repeatability (RSD_r%) and reproducibility (RSD_R%) conditions that are based on concentration studies. For repeatability, the mean and standard deviation of the concentrations indicated by the chromatographic system are considered, applying the Horwitz equation specified in the regulation (EU) No 519/2014 ((EU) Comission Regulation, 2014). Meanwhile for reproducibility, the coefficient of variation of the quantified analyte concentration in known concentrations is considered. These concentrations were obtained by carrying out at least 9 replicates with 3 different levels (T-2 and HT-2: 5 μg/Kg, 75 μg/Kg, 300 μg/Kg; NEO and DAS: 1 μg/Kg, 15 μg/Kg, 60 μg/Kg) using the following food matrices: corn flakes, breakfast cereal and pasta.

5.2.5.3 Recovery

The tests carried out to calculate the recovery were based on the fortification of the matrices (wheat grain, pasta, and feed for animal consumption) with the addition of the mycotoxin standards T-2, HT-2, NEO, and DAS considering different concentration levels (HT-2 and T-2: 15-250 μg/Kg and >250 μg/Kg; NEO and DAS: 1-50 μg/Kg and >50 μg/Kg). Were obtained 3 replicates from each sample submitted to the test, then the results obtained were calculated in percentage values.

5.2.5.4 Detection and Quantification limits

The limits of detection and quantification were based on the determination of the signal to noise (S/N). An analytical signal greater than 3 (S/N≥3) was considered for the instrumental LOD value for all mycotoxins, however the instrumental LOQ value was estimated as a peak having the S/N ≥10, these instrumental parameters were applied using the solutions of mycotoxins standards. The method LOD and LOQ were tasted using 9 replicates of the corn flakes matrix and was defined that the firsts points of the calibration curve were established as LOD. On the other hand, the LOQ values of the method for T-2 and HT-2 were determined considering the recommendation established by the European Union Commission (2013/165/UE) ((EU) Comission Recommendation, 2013), meanwhile for the mycotoxins NEO and DAS, the LOQ value of the method was obtained considering the scientific article published (Kim, Jung, Nam, Lee, & Yoo, 2022).

CHAPTER 6- Results

6.1 Calibration Curve

The analysis of the calibration curves for each of the mycotoxins studied produced the coefficient of determination (R^2) presented in Table 6.1, where all the resulting values are greater than or equal to 0.99. In addition, their respective intercepts and slopes could be determined from the calibration curves as shown in Annex 1, Annex 2, Annex 3, and Annex 4 under the tested conditions.

Table 6.1 R^2 values of the standard curves of HT-2, T-2, NEO, and DAS

	HT-2	T-2	NEO	DAS
R^2	0.99351	0.99526	0.99650	0.99682

6.2 Instrumental optimization and performance validation

The respective optimal conditions were established by a direct infusion of standards mycotoxins. Product ion scan analysis were performed to obtain the MS/MS spectra of mycotoxins and the two most intense product ions were selected (see Annex 9, Annex 10, Annex 11, Annex 12, Annex 13, Annex 14, Annex 15, Annex 16). Of the two transitions, the transition 1 corresponds to a quantifier ion and the transition 2 corresponds to a qualifier ion (see Annex 5, Annex 6, Annex 7, Annex 8). Table 6.2 shows the values of retention time, product ions, DP, EP, CE and the optimal CXP for each mycotoxin tested.

Table 6.2 MRM Transitions and Retention Times for each Mycotoxin (T2, HT2, DIA, NEO)

Analyte	Ion Type	RT (min)	Precursor ion	Product ions	DP	EP	CE	CXP
T2	Quantifier	6,7	484.3	305.2	60	10	19	12
	Qualifier		$[M+NH_4]^+$	215.2			27	11
HT2	Quantifier	6	442.2	263.1	60	10	19	10
	Qualifier		$[M+NH_4]^+$	215.2			19	10
NEO	Quantifier	4,2	400.1	169.1	60	10	38	6
	Qualifier		$[M+NH_4]^+$	185.1			27	6
DAS	Quantifier	5,7	384,1	307.2	60	10	16	12
	Qualifier		$[M+NH_4]^+$	247.1			19	12

6.3 Method validation

6.3.1 Specificity

According to the chromatographic conditions developed, in none of the chromatograms of the blank samples submitted to analysis were interferences detected in the area associated with the retention time of the mycotoxins HT-2, T2, NEO and DAS, considering a signal to noise (S/N) between 3 to 10.

6.3.2 Repeatability and reproducibility

The results obtained for the repeatability and reproducibility analysis are shown in Table 6.3, where it can be seen that the RSD_r% values ranged from 10% to 20%, considering a level range that goes from 1 μg/Kg to greater than or equal to 250 μg/Kg, while for the RSD_R% parameter the values oscillated between 15.6 % and 25%, taking

into account a level range that goes from 15 μg/Kg to greater than or equal to 250 μg/Kg. All these values correspond to different concentration levels of HT-2, T-2, NEO, and DAS mycotoxins using 3 different food matrices (corn flakes, breakfast cereal and pasta).

Table 6.3 Repeatability and reproducibility values for HT-2, T2, NEO and DAS mycotoxins

Analyte	Level range (μg/Kg)	RSD_r%	RSD_R%
HT-2	15-250	20	25
	>250	12.5	16.3
T-2	15-250	22	15.6
	>250	10	15.6
NEO	1-50	20	
	>50	15	16
DAS	1-50	10	
	>50	15	16

6.3.3 Recovery

The results corresponding to the recovery can be seen in Table 6.4, where for each matrix studied (wheat grain, pasta, feed for animal consumption) and mycotoxin concentration level considered ranging from 5 μg/Kg to 300 μg/Kg, recovery percentages ranging from 78% to 98% were obtained.

Table 6.4 Recovery percentages of HT-2, T-2, NEO, and DAS mycotoxins

Analyte	Matrix	Number of replicates	Level (μg/Kg)	Recovery%
HT-2	Wheat grain	3	50	78
	Pasta	3	300	94
	Feed for animal consumption	3	40	98
T-2	Wheat grain	3	50	89
	Pasta	3	300	68
	Feed for animal consumption	3	40	85
NEO	Wheat grain	3	20	89
	Pasta	3	200	95
	Feed for animal consumption	3	40	82
DAS	Wheat grain	3	5	86
	Pasta	3	50	88
	Feed for animal consumption	3	40	87

6.3.4 Detection and Quantification limits

The resulting limits of detection (LOD) are shown in Table 6.5, where data based on an instrumental LOD and a method LOD are reported, as well as the signal to noise values for each analyte. These LOD values vary between ranges that go from 0.1 pg/μl

to 0.5 pg/μl. On the other hand, Table 6.6 shows the data referring to the quantification values (LOQ) of the method (ranges from 1 μg/Kg to 5 μg/Kg), signal to noise (ranges from 22 to 97), and RSDr% at method LOQ (ranges from 8.5% to 20%).

Table 6.5. Instrumental Limit of Detection (LOD) for HT-2, T-2, NEO, and DAS mycotoxins

Analyte	Instrumental LOD (pg/μl)	Signal to Noise at instrumental LOD (S/N)	Method LOD (μg/Kg)
HT-2	0,25	4,3	0,5
T-2	0,1	10,7	0,5
NEO	0,25	13,4	0,5
DAS	0,1	8,1	0,5

Table 6.6 Limit of Quantification of the method for HT-2, T-2, NEO, and DAS mycotoxins using 9 replicates in a corn flakes food matrix

Analyte	Method LOQ (μg/Kg)	Signal to Noise at method LOQ (S/N)	RSDr% at method LOQ (μg/Kg)
HT-2	5	22	10%
T-2	5	97	15%
NEO	1	26	20%
DAS	1	63	8.5%

6.3.5 Samples studied as case study

The results obtained in Table 6.7 show the analysis of two samples (soft wheat "Gentile rosso" and yellow corn flour for polenta) naturally contaminated by mycotoxins

HT-2, T-2, NEO, and DAS, generating specific values of the concentrations calculated for each of these analytes. These values were detected from 0.3 μg/Kg to 18.8 μg/Kg.

Table 6.7 Calculated concentrations of HT-2, T-2, DAS, and NEO in two food matrices

Sample Name	Analyte	Calculated Concentration (μg/Kg)
Soft wheat “Gentile rosso”	HT-2	18,8
	T2	3
	NEO	0,3
	DAS	0,3
Yellow corn flour for polenta	HT-2	0,9
	T2	0,5
	NEO	0,3
	DAS	0,3

CHAPTER 7- Discussion

In the host lab where I performed my thesis (3A Laboratori), an extraction method and a chromatographic method (LC-MS/MS) were set up and employed with the purpose of detecting and quantifying other mycotoxins that have not been part of this study. Therefore, this study aimed to optimize this method for 4 emerging mycotoxins (HT-2, T-2, NEO, and DAS). For the extraction method, some modifications were added, such as the concentration of the samples that contained the NEO mycotoxin and its reactivation in a solvent that contained 75% mobile phase A and 25% mobile phase B. These types of modifications contributed especially to the chromatographic process where the peak shape, and consequently the S/N, was improved for NEO. Besides, they contributed to decrease the interference effect of the matrix on the analytes studied, and to increase the concentration of NEO mycotoxin in the matrices studied, as shows in the Annex 17 and 18, where it is possible to see the chromatographic difference between the sample with NEO concentrated or without concentration.

During the instrumental optimization of the LC-MS/MS parameters, the fragmentation pattern for each molecule under analysis was obtained by product ion analysis. Then, the selection of the two most abundant fragments and their relative collision energy (representing ion transitions) was obtained. MRM transitions (Annex 9, Annex 10, Annex 11, Annex 12, Annex 13, Annex 14, Annex 15, Annex 16) were chosen in order to contribute to the specificity of the chromatographic method, allowing the observation of adequate peaks to carry out the validation process, providing reliable results of quantification and identification of the analytes, and avoiding the risk of obtaining false positives and false negatives (Basantes, Estrada, & Flores, 2022). The lack of interferences in all the matrices (wheat grain for direct human consumption, pasta, corn flakes, breakfast cereal and soft wheat ¨Gentile rosso", yellow corn flour for polenta and feed for animal consumption) and the adequate symmetry of the chromatograms obtained suggest that the studied method was also selective for each mycotoxin compounds (HT-2, T-2, NEO, DAS), since the transition ions were stable and the signal intensity was adequate using the MRM mode, generating a good resolution of the monitored analytes (Medina, 2020). In addition, it was possible to appreciate that, in the injections of the blank matrix, no signals were generated that could interfere with the determination of the analytes studied.

The validation studies were carried out for the development of analytical methods to demonstrate that the method under study is capable of detecting an analyte reliably

and accurately, in a certain matrix (Nuñez, 2018). For this purpose, several parameters were evaluated: one of them was the linearity of the analytical procedure, that was determined by means of the calibration curve where, for all the HT-2, T-2, NEO and DAS mycotoxins, it was possible to visualize a linear behaviour in a concentration range from 0.1 pg/µl to 250 pg/µl by the regression analysis of the calibration line presenting linear correlation coefficients greater than 0.99 ($r \geq 0.99$). This gives a result in accordance with the SANTE/11813/2017 Guide, where the linear correlation coefficient must be greater than or equal to 0.98 to consider the method to be linear (EURL, 2020). Therefore, this result indicates that there is a linear correlation between the results of the areas obtained as a function of the total concentration of the mycotoxins studied (Limachi, 2021).

Regarding the precision parameters of the method, the repeatability and reproducibility values obtained for HT-2 and T-2 mycotoxins show to be acceptable values in accordance with the provisions of regulation in accordance with the provisions of regulation (EU) No 519/2014, where the criteria established for the repeatability parameter (RSDr%) that considers a concentration ranging from 15-250 µg/Kg must be lower than or equal to 30%, while for a concentration level greater than 250 µg/Kg the RSD_r% value must be lower than or equal to 25%. Meanwhile, to consider the reproducibility value (RSD_R%), the regulation mentions that for a concentration level between 15-250 µg/Kg the RSD_R% value must be $\leq$ 50% and for concentrations greater than 250 µg/Kg the RSD_R% value should be $\leq$ 40% ((EU) Comission Regulation, 2014). Thus, it can be mentioned that the study has the capacity to generate a series of repeated measurements of the same analyte carried out under foreseen conditions established by the method, where the use of the calibration curves in the established range will allow the interpolation of values relative to concentrations of HT-2 and T-2 mycotoxins in cereal samples.

Regarding the repeatability and reproducibility of the mycotoxins NEO and DAS, RSD_r% values lower than 20% and RSD_R% values lower than 16% were obtained. These values cannot be compared with a legal parameter due to the absence of regulations for these mycotoxins. However, similar studies applied to these mycotoxins have managed to reach similar values for RSD_r% and RSD_R%, giving results such as <15 % (Kim, Jung, Nam, Lee, & Yoo, 2022), <18% (Spanjer, Rensen, & Scholten, 2008) and <20 % (Sulyok, Berthiller, Krska, & Schumacher, 2006), so the

values obtained in this study share a similarity with other studies and therefore could serve as a potential information for the development of a regulation in the future.

In terms of recovery: for HT-2 and T-2 mycotoxins, were considered the levels 15-250 μg/Kg and >50 μg/Kg, the resulting value was obtained in a range of 68% to 98%, which is within the ranges recommended by regulation (EU) No 519/2014. This result indicates that the optimized method is highly efficient to recover the mycotoxin in food matrices, and to determine a reliable amount of HT-2 and T-2 mycotoxins contained in the samples analyzed ((EU) Comission Regulation, 2014). In addition, these values show that the recoveries do not depend on the type of matrix evaluated since no significant differences were observed among matrices. On the other hand, observing the recovery values obtained for the NEO and DAS mycotoxins, values ranging from 82% to 95% were obtained considering the two levels that were analyzed in this study (1-50 μg/Kg >50 μg/Kg). So, comparing these values obtained for the mycotoxins NEO and DAS with a similar study (Kim, Jung, Nam, Lee, & Yoo, 2022), a similar result was obtained (83-93%), thus suggesting that the analytical method applied is appropriate for the identification and quantification of this type of trichothecene mycotoxins in cereals.

Regarding the method LOQ values for HT-2 and T-2 mycotoxins, the determined values of 5 μg/Kg is an acceptable value because it is within the ranges allowed by current regulation (2013/165/UE) ((EU) Comission Recommendation, 2013). In the same way, the method LOQ value for the NEO and DAS mycotoxins (1 μg/Kg) shows to be reliable, being this value comparable with that obtained in the study by Kim et al. (2022), that is 1.3 μg/Kg LOQ NEO and 0.8 LOQ DAS μg/Kg, so it can be considered as a basis for further studies. Regarding the instrumental LOD values and method LOD values of all the mycotoxins studied, it can be mentioned that, by being able to measure values between 0.25 pg/μl, 0.5 pg/μ and 1 pg/μl with a signal to noise value ≥ 3, the equipment has a great sensitivity to detect these mycotoxins (Morreres, 2016). In particular, the LOD values used in this study are much lower than those obtained by Kim et al. (2022). However, there are studies that set detection and quantification limits that are even lower, such as the study (Knutsen, et al., 2018), so, increasing the detection and quantification sensitivity of the method can be improved (Morreres, 2016). It should also be considered that the RSDr% value obtained for the LOQ of the method is within the permissible range registered in the regulation (<30%) ((EU)

Comission Regulation, 2014), so the repeatability of the method can be confirmed under the same conditions.

For the samples that were submitted to the analysis as study cases, it was evidenced that in their white matrix there was the presence of HT-2, T-2, NEO, and DAS mycotoxins (Annex 19 and Annex 20). However, it should be noted that only the soft wheat “Gentile rosso” sample can be considered positive for the presence of the HT-2 mycotoxin since it presents a value of 18.8 μg/Kg, being a concentration higher than the maximum permissible limit reported in the recommendation 2013/165/UE ((EU) Comission Recommendation, 2013). Therefore, for the rest of the samples where the presence of mycotoxins was detected, it can be mentioned that values lower than the LOQ were identified, but they are adapted to the LOD values analyzed by this study. Therefore, the mycotoxin presence at a very low concentration could be distinguished from the background noise with a certain degree of confidence (Esteo, 2018). Thus, the instrument is sensitive enough to detect mycotoxins at very low concentrations (Morreres, 2016). In addition, these results help to generate a broader source of information regarding the detection of these mycotoxins in terms of sensitivity. Despite the fact that the concentrations of the mycotoxins NEO and DAS are much lower than those established for mycotoxins HT-2 and T-2, it was possible to validate the parameters with the use of the same methodology and equipment (Kim, Jung, Nam, Lee, & Yoo, 2022).

CHAPTER 8- Conclusions

A rapid and quantitative method for the simultaneous determination of HT-2, T-2, NEO, and DAS mycotoxins in seven types of food matrices (cereals and feed) has been developed, with brief sample preparation and extraction using QuEChERS.

Instrumental optimization was achieved by standardizing all ionization parameters, allowing the selection of the two precursor ions with the highest signals, showing the absence of interference in the area close to the retention time, so the analysis was considered specific.

For all the analytes, a good linearity was found, with correlation coefficients higher than 0.99 in all cases and in all matrices submitted to the study.

The repeatability and reproducibility parameters proved to be compatible with what is required by the recommendations established by the European Union and with the studies used to validate the method.

The recovery percentages obtained in the different cereal matrices showed to be within the limits required for HT-2 and T-2 mycotoxins, while for NEO and DAS mycotoxins the values are very similar to those reported by other studies.

This quantitative method has several advantages such as a simple pretreatment, high sensitivity due to low LOQs and LODs obtained, so it can be applied to the determination and quantification of mycotoxins in routine analysis of foods such as cereals and cereal-based products.

The use of a triple quadrupole mass spectrometer shows a high performance in identifying the 4 mycotoxins at low levels (μg/Kg) in cereal and feed matrices, so it can be used to promote the development of the LC-MS/MS methodology, generating improvements and innovations for the identification of mycotoxins at even lower levels.

CHAPTER 9- Quoted literature

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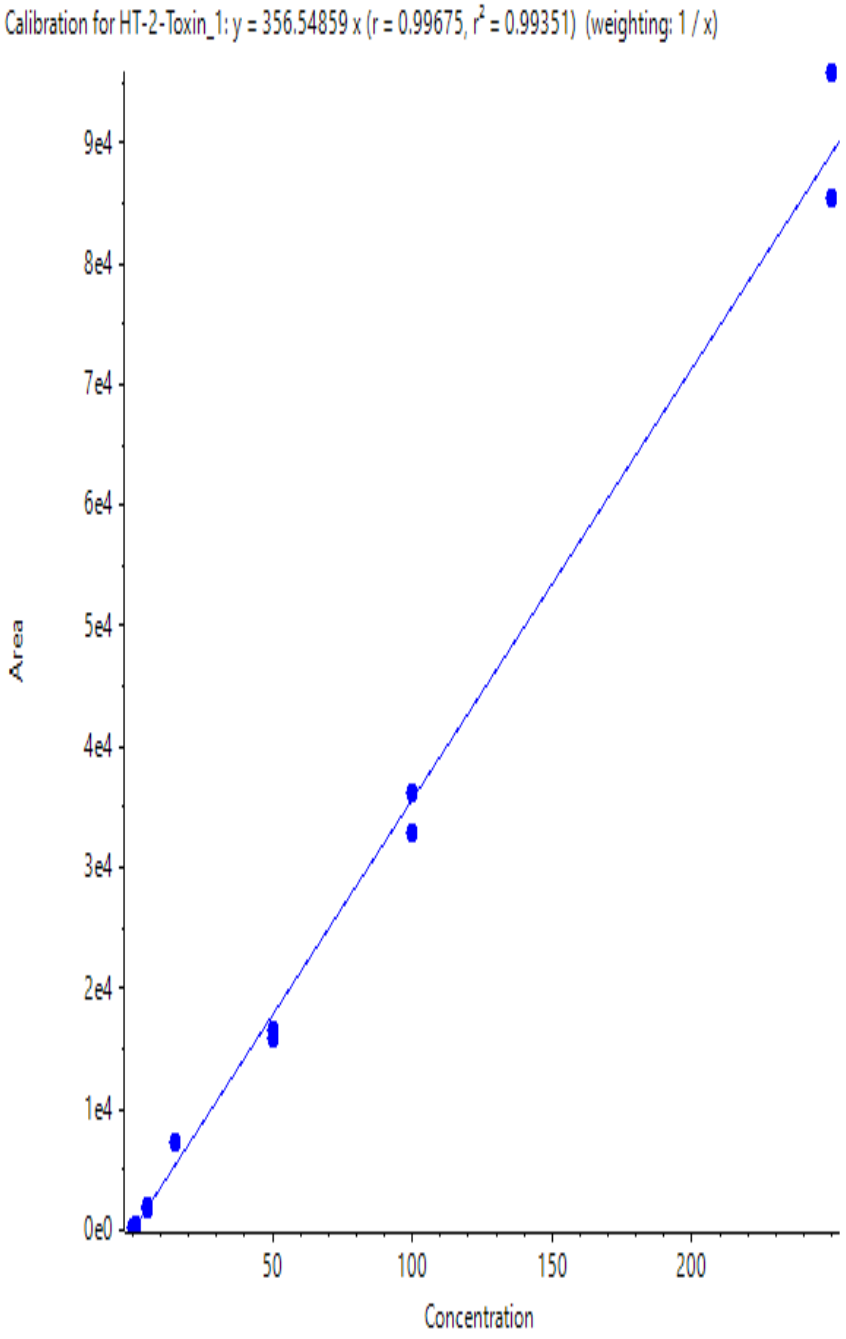
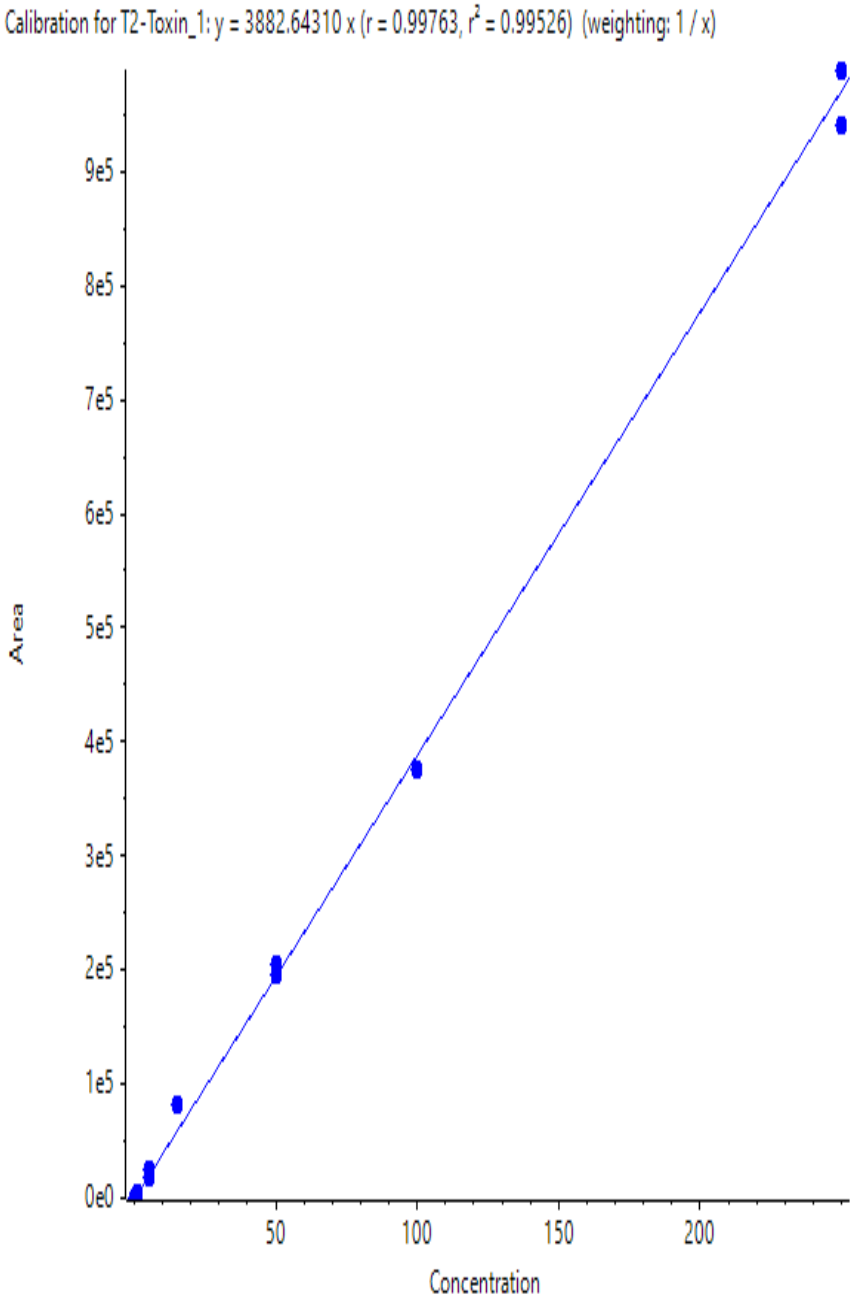
CHAPTER 10- Annexes

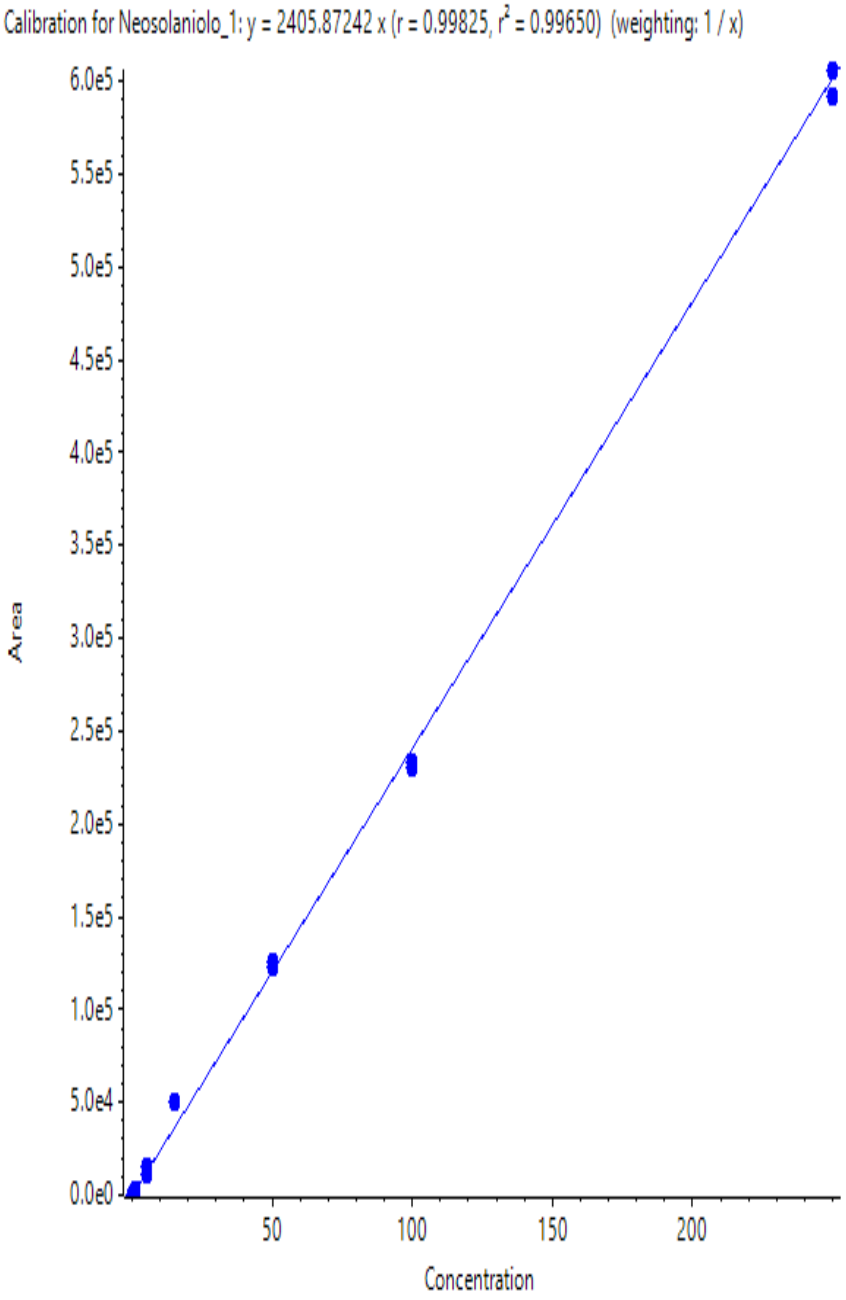
ANNEX 1- Calibration curve of mycotoxin HT-2



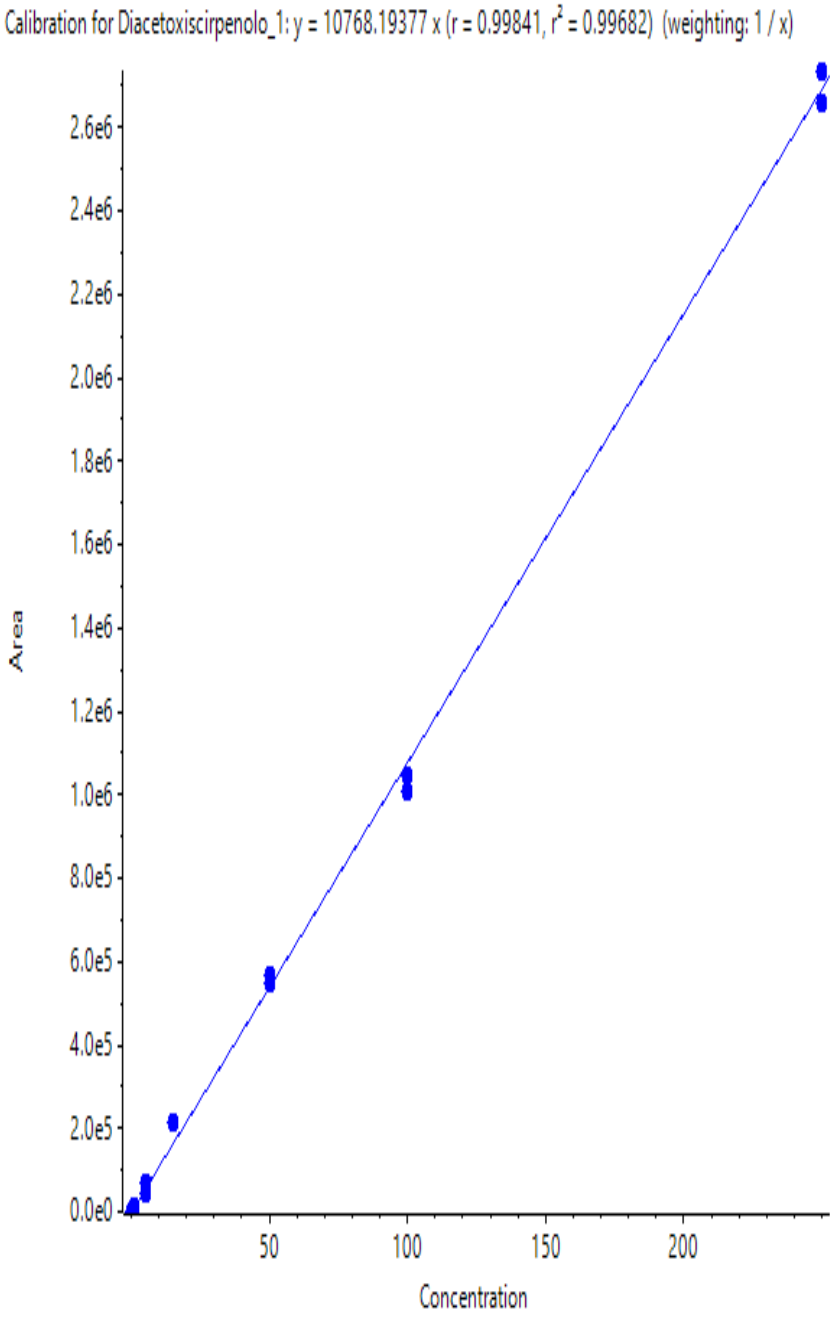
ANNEX 2- Calibration curve of mycotoxin T-2



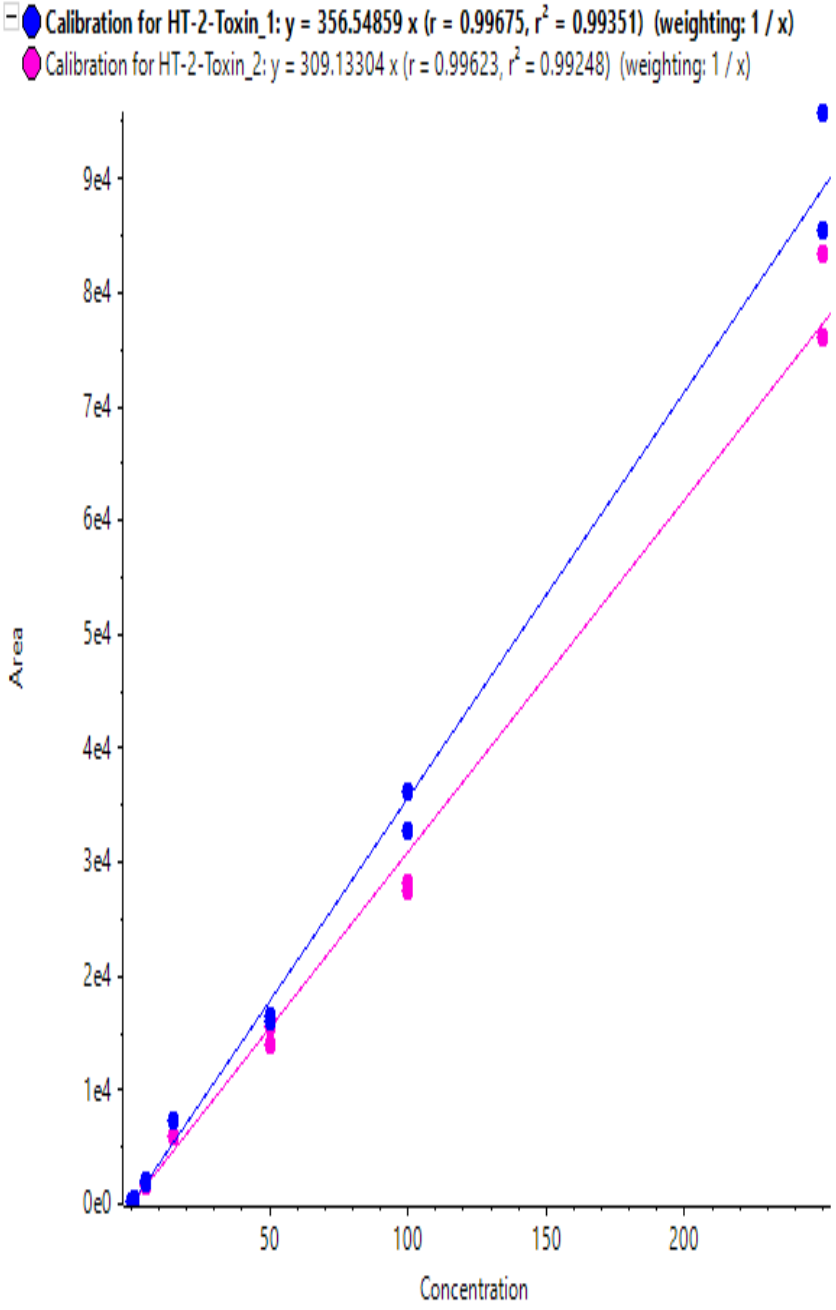
ANNEX 3- Calibration curve of mycotoxin NEO



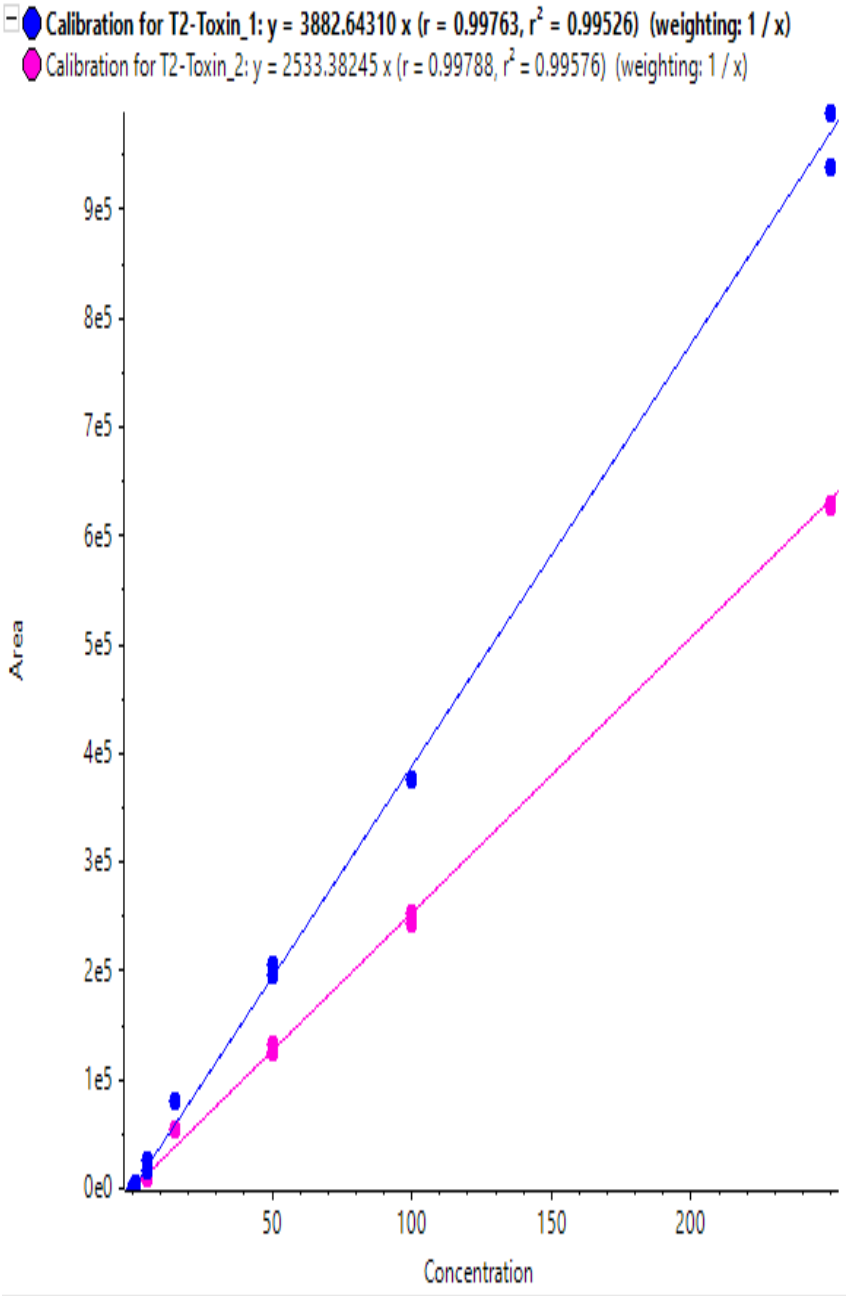
ANNEX 4- Calibration curve of mycotoxin DAS



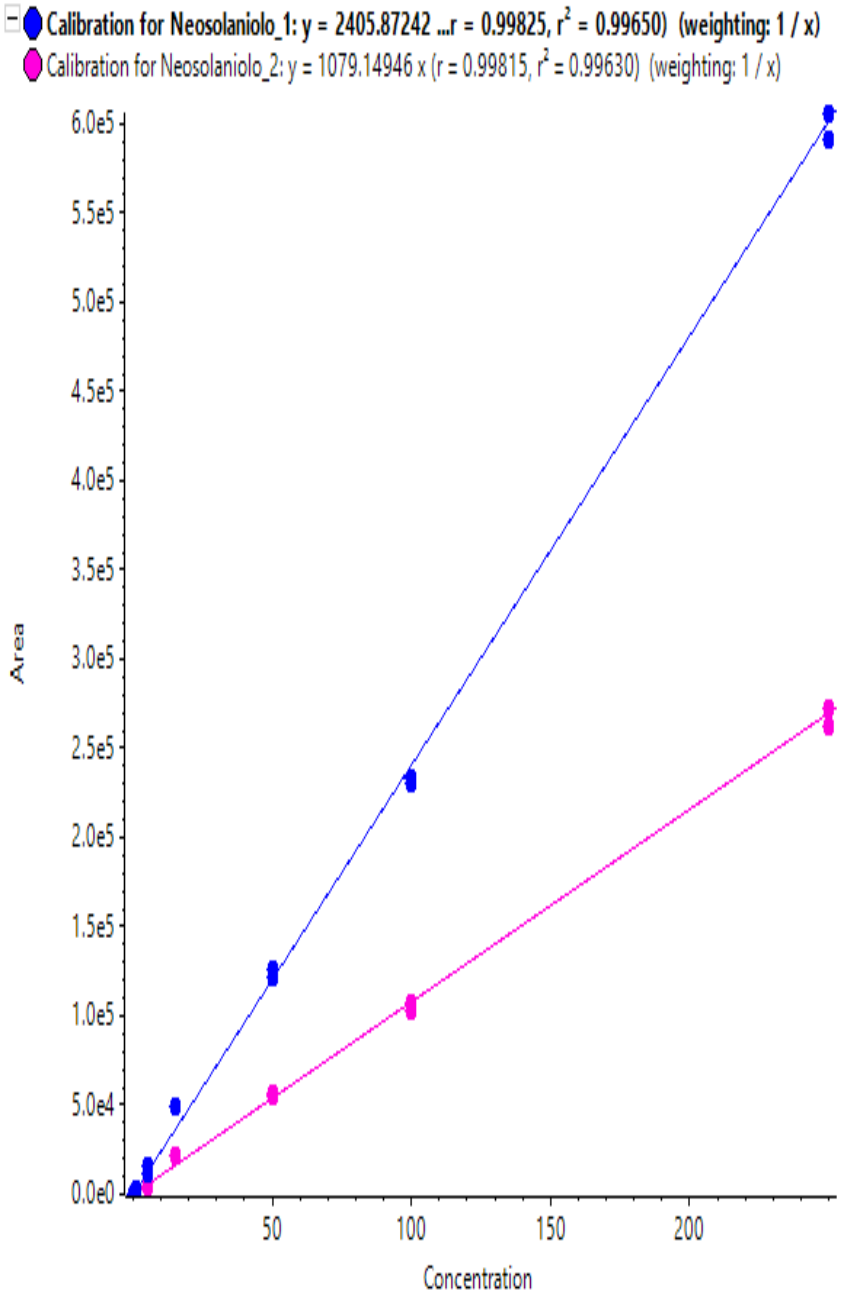
ANNEX 5- Calibration curve of mycotoxin HT-2 with the quantifier (HT-2-Toxin 1) and qualifier (HT-2-Toxin 2) ion transitions



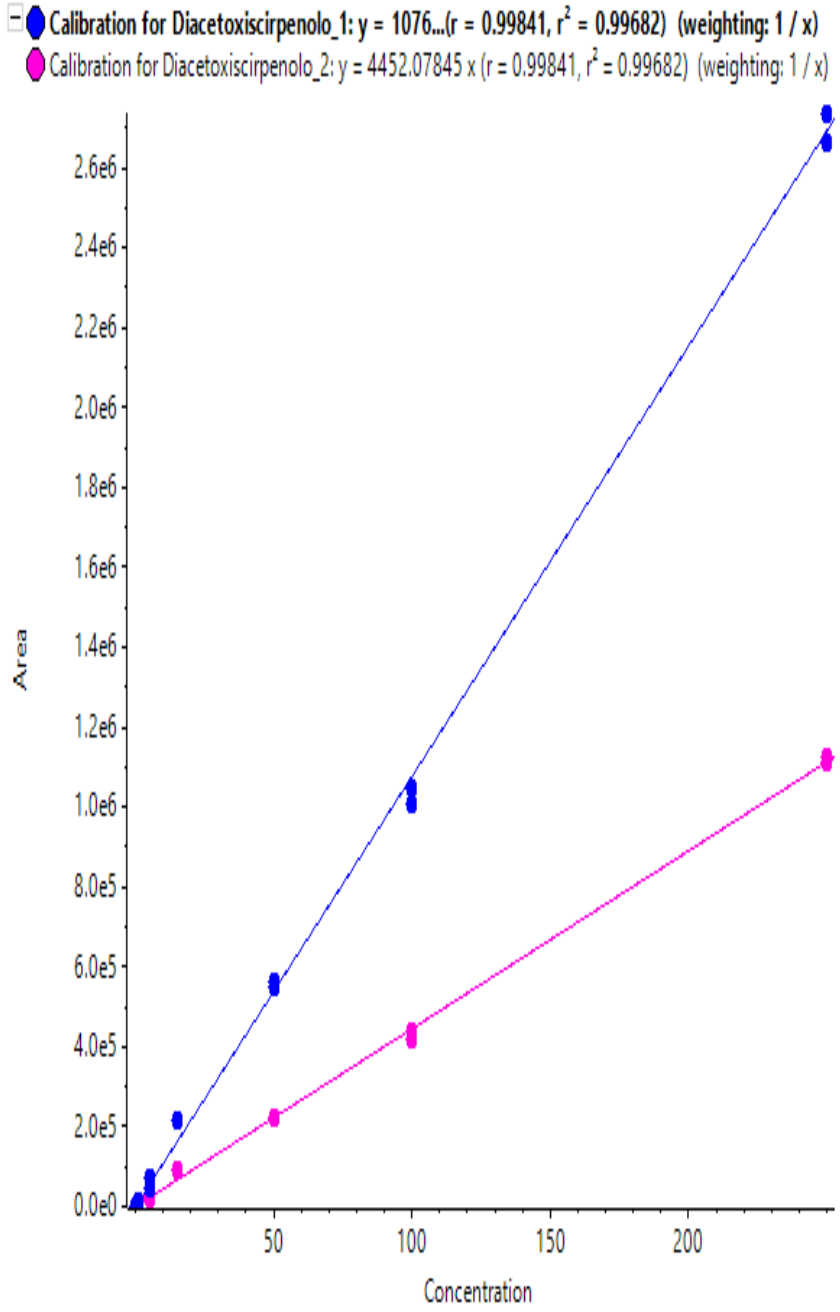
ANNEX 6- Calibration curve of mycotoxin T-2 with the quantifier (T2-Toxin 1) and qualifier (T2-Toxin 2) ion transitions



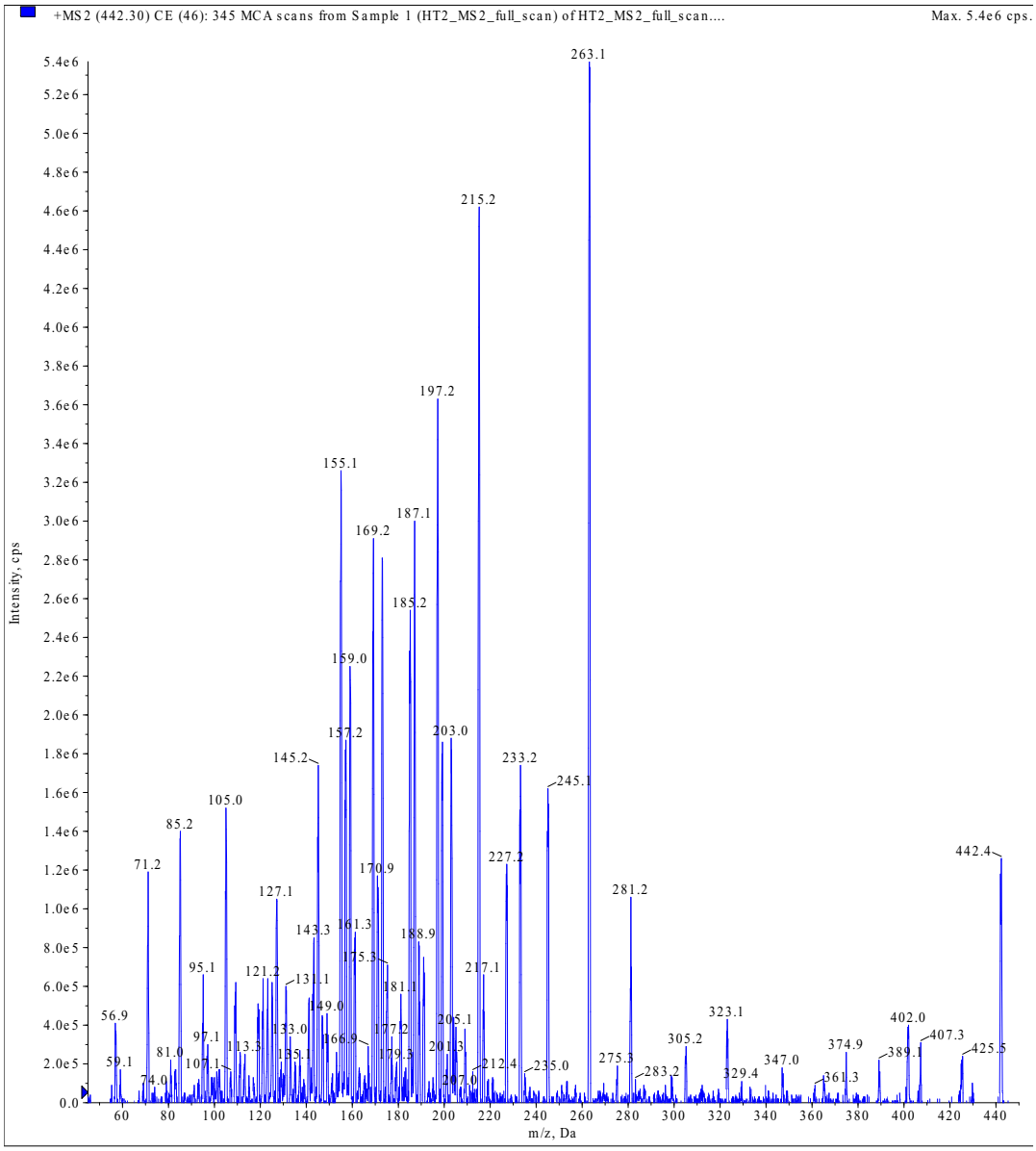
ANNEX 7- Calibration curve of mycotoxin NEO with the quantifier (Neosolaniolo_1) and qualifier (Neosolaniolo_2) ion transitions



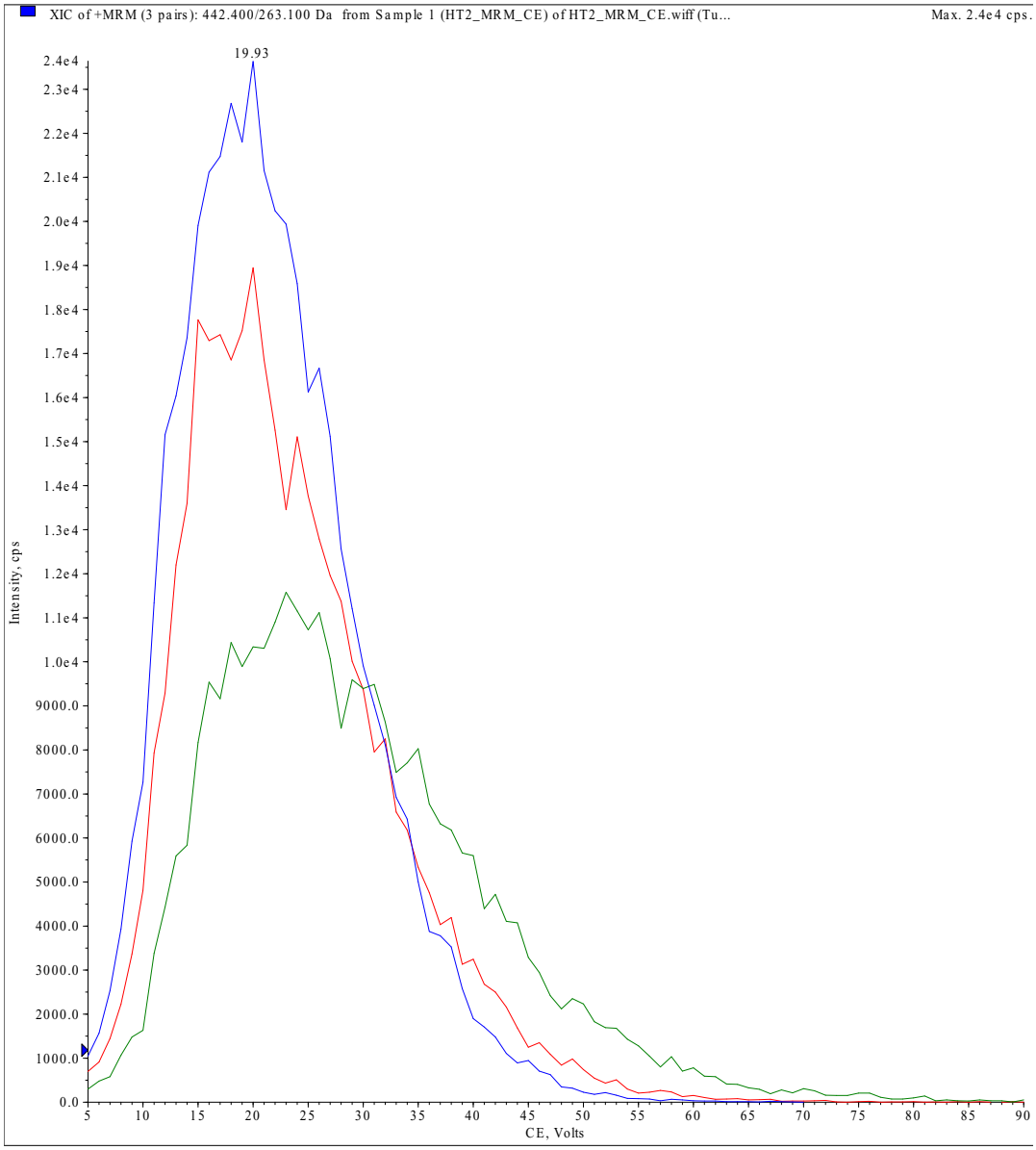
ANNEX 8- Calibration curve of mycotoxin DAS with the quantifier (Diacetoxiscirpenolo_1) and qualifier (Diacetoxiscirpenolo_2) ion transitions



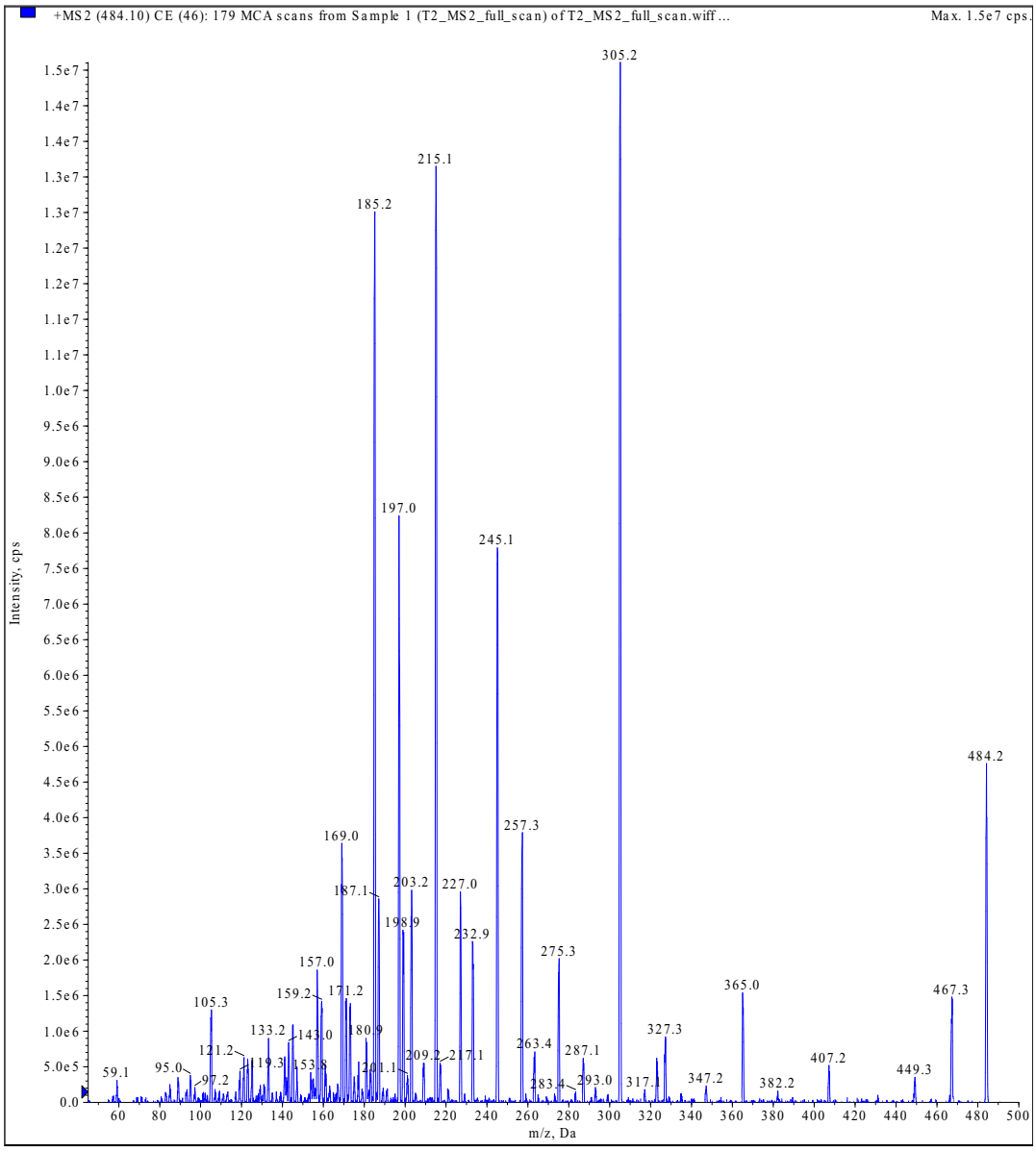
ANNEX 9- Product ion scan of mycotoxin HT-2



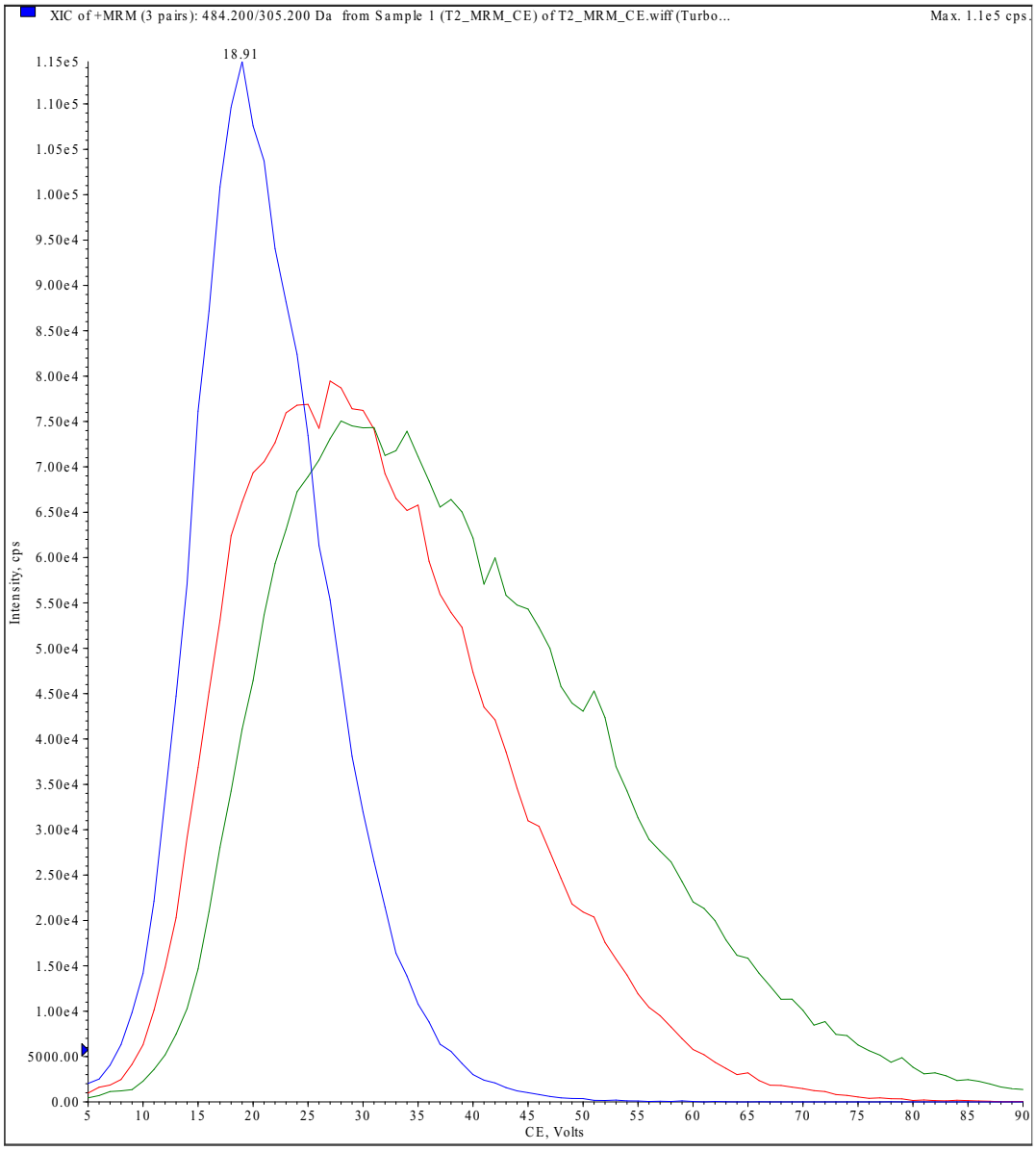
ANNEX 10- Intensity of the Energy collision of mycotoxin HT-2



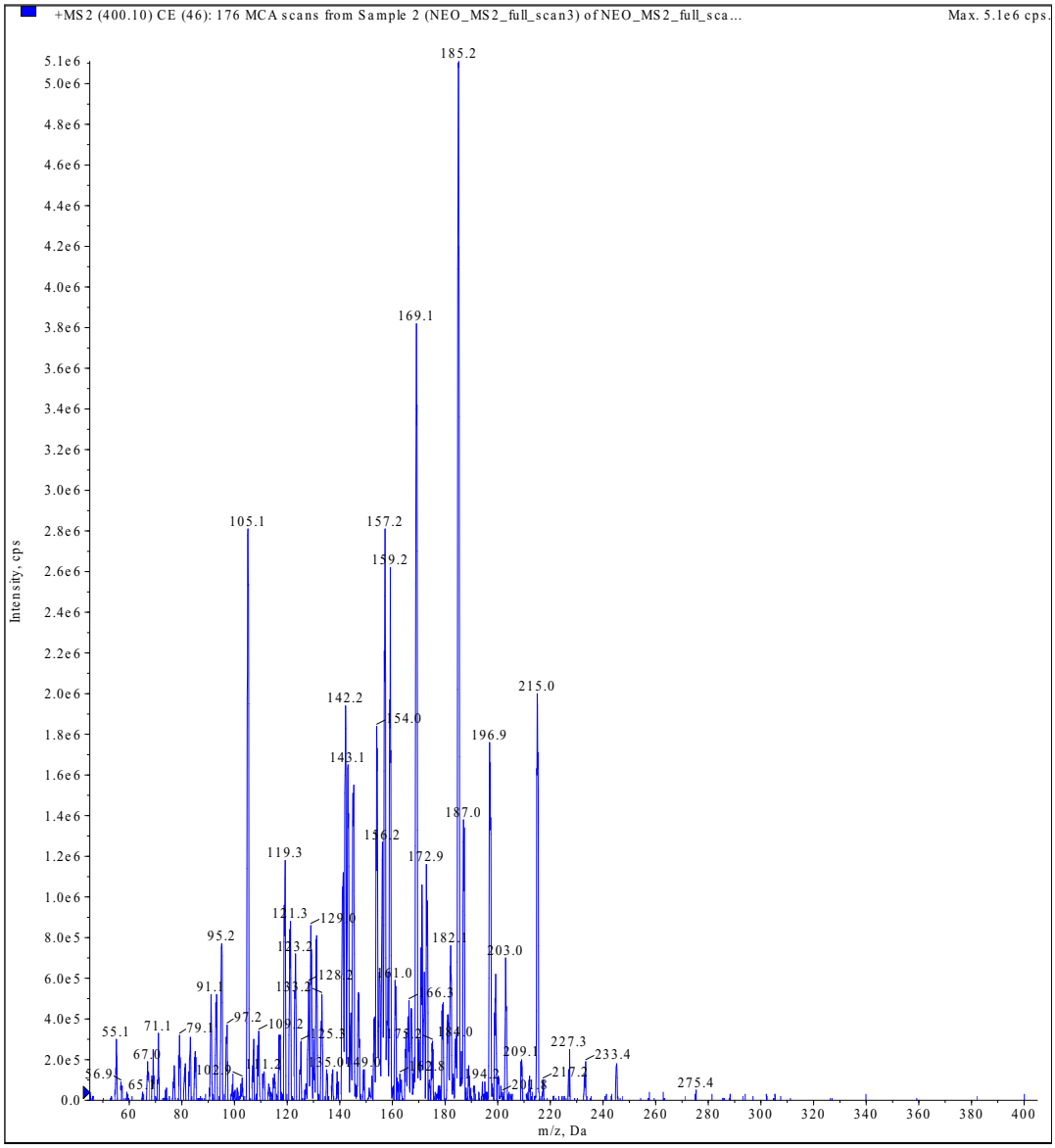
ANNEX 11- Product ion scan of mycotoxin T-2



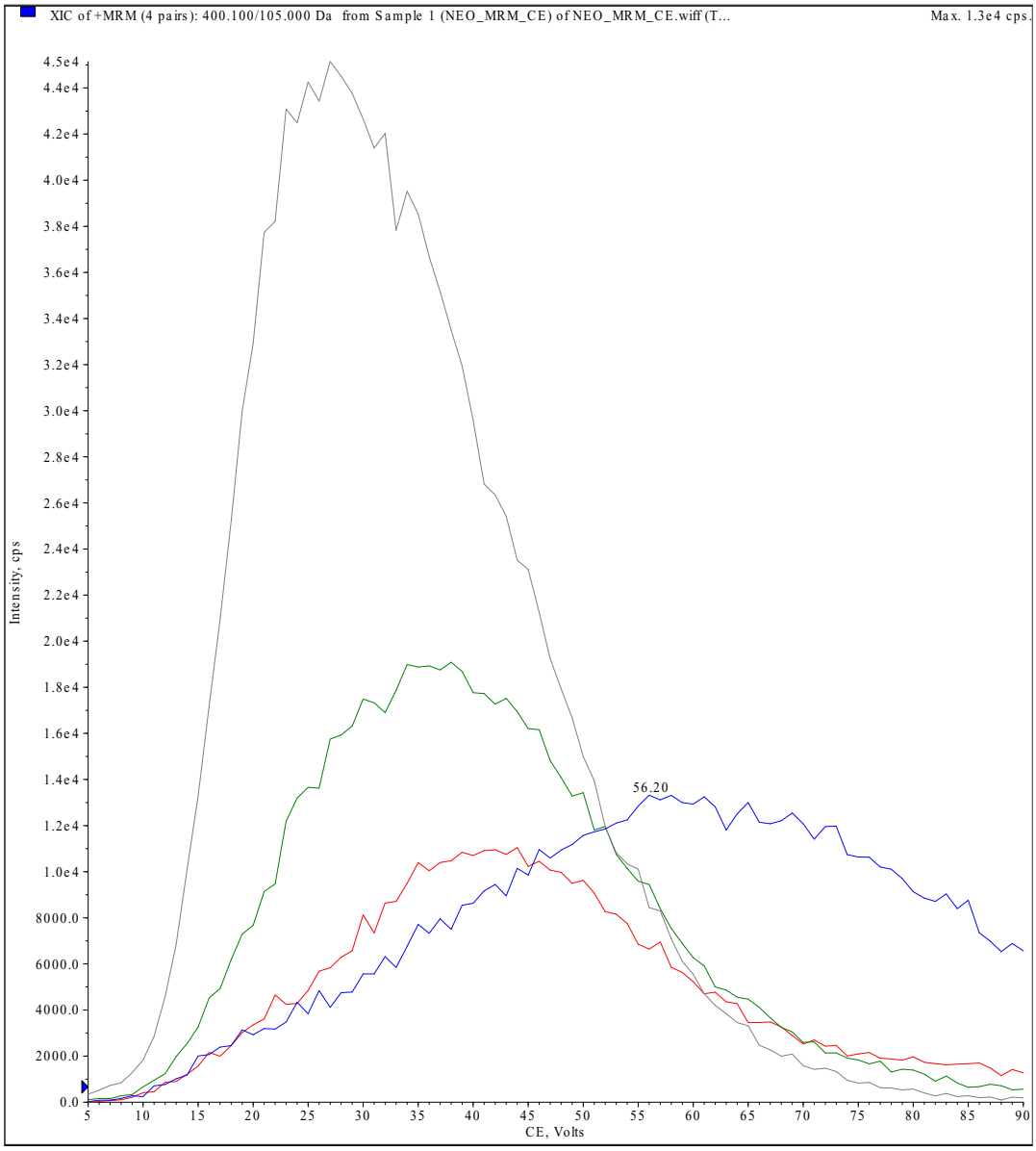
ANNEX 12- Intensity of the Energy collision of mycotoxin T-2



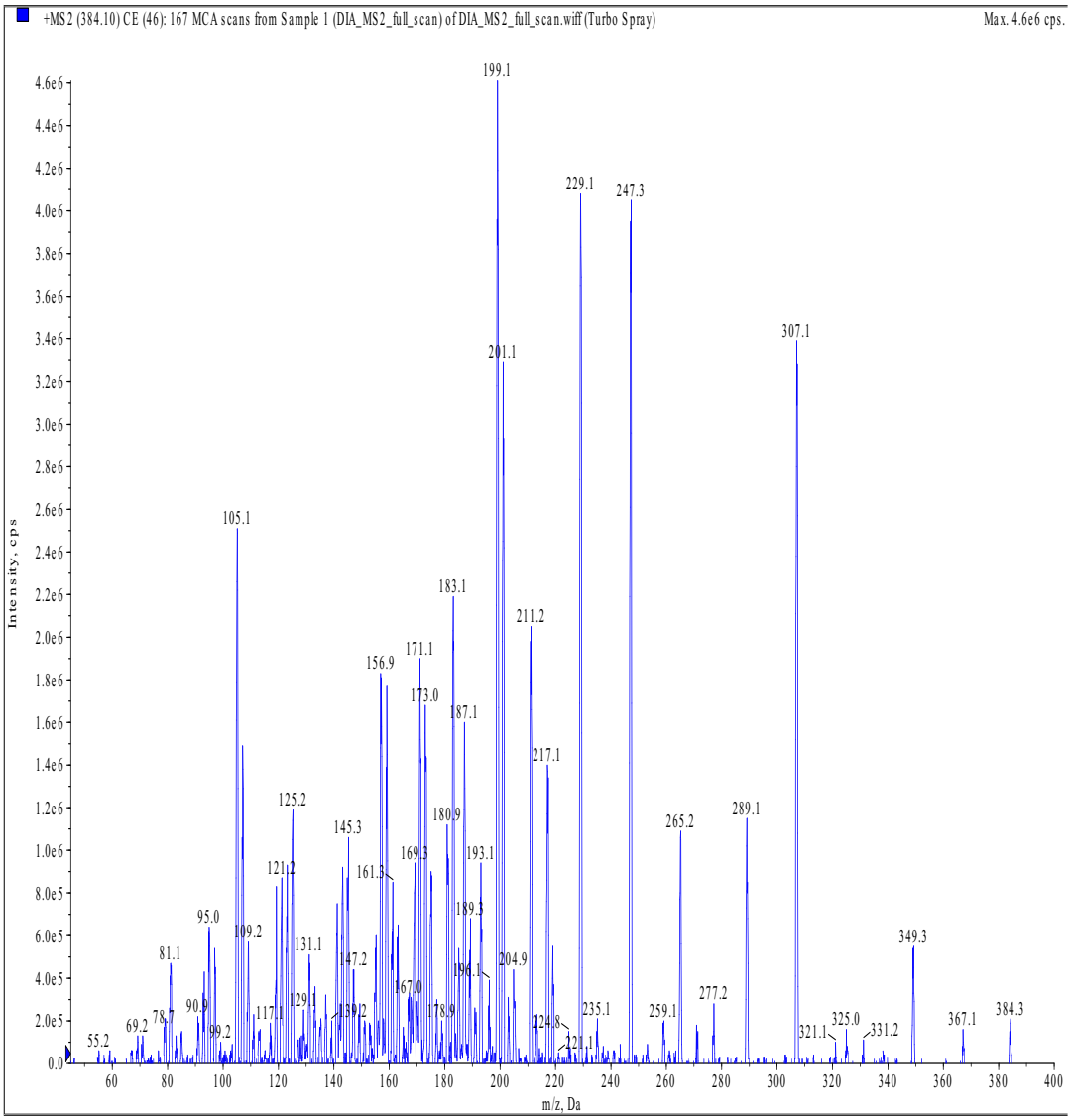
ANNEX 13- Product ion scan of mycotoxin NEO



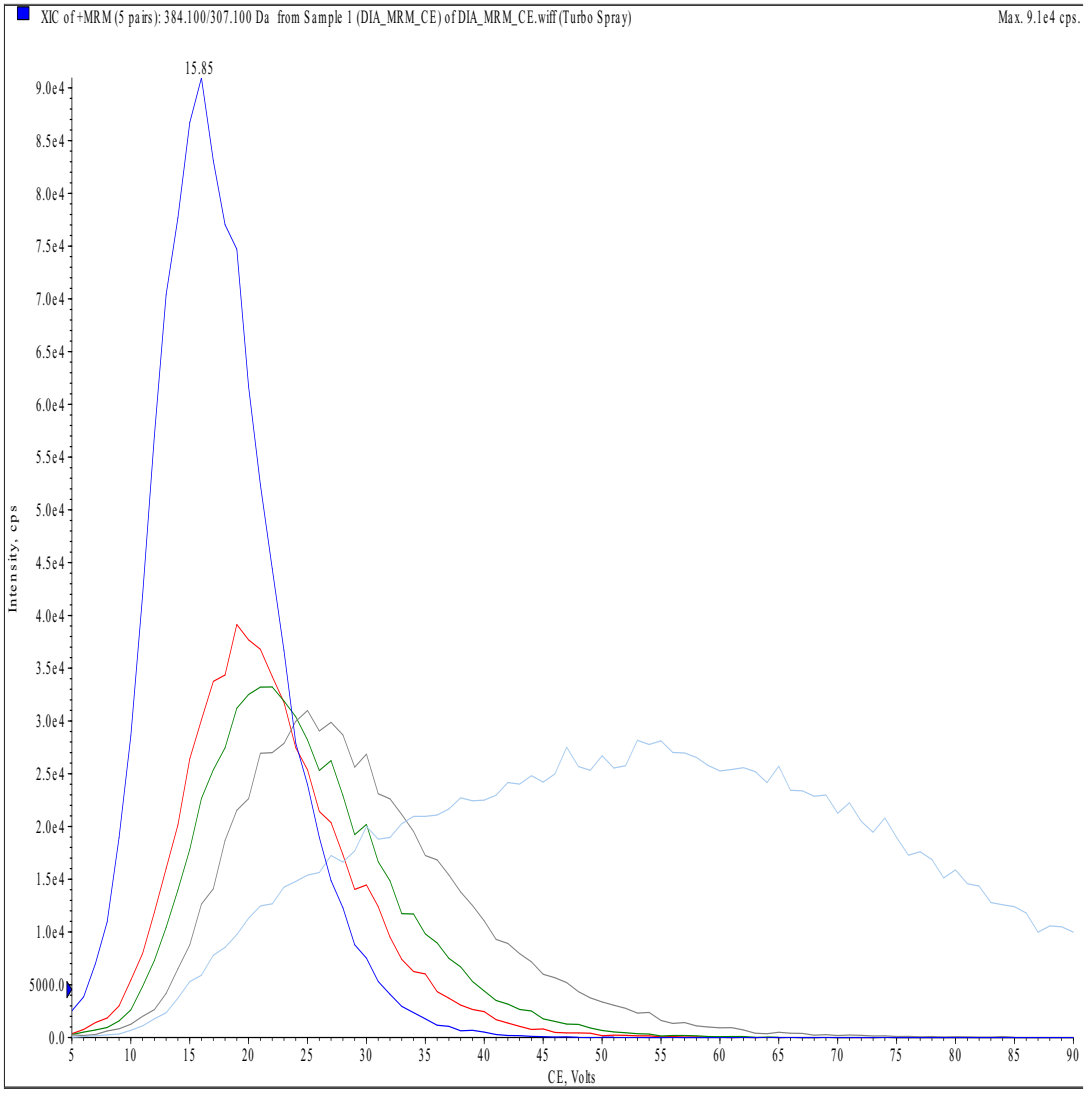
ANNEX 14- Intensity of the Energy collision of mycotoxin NEO



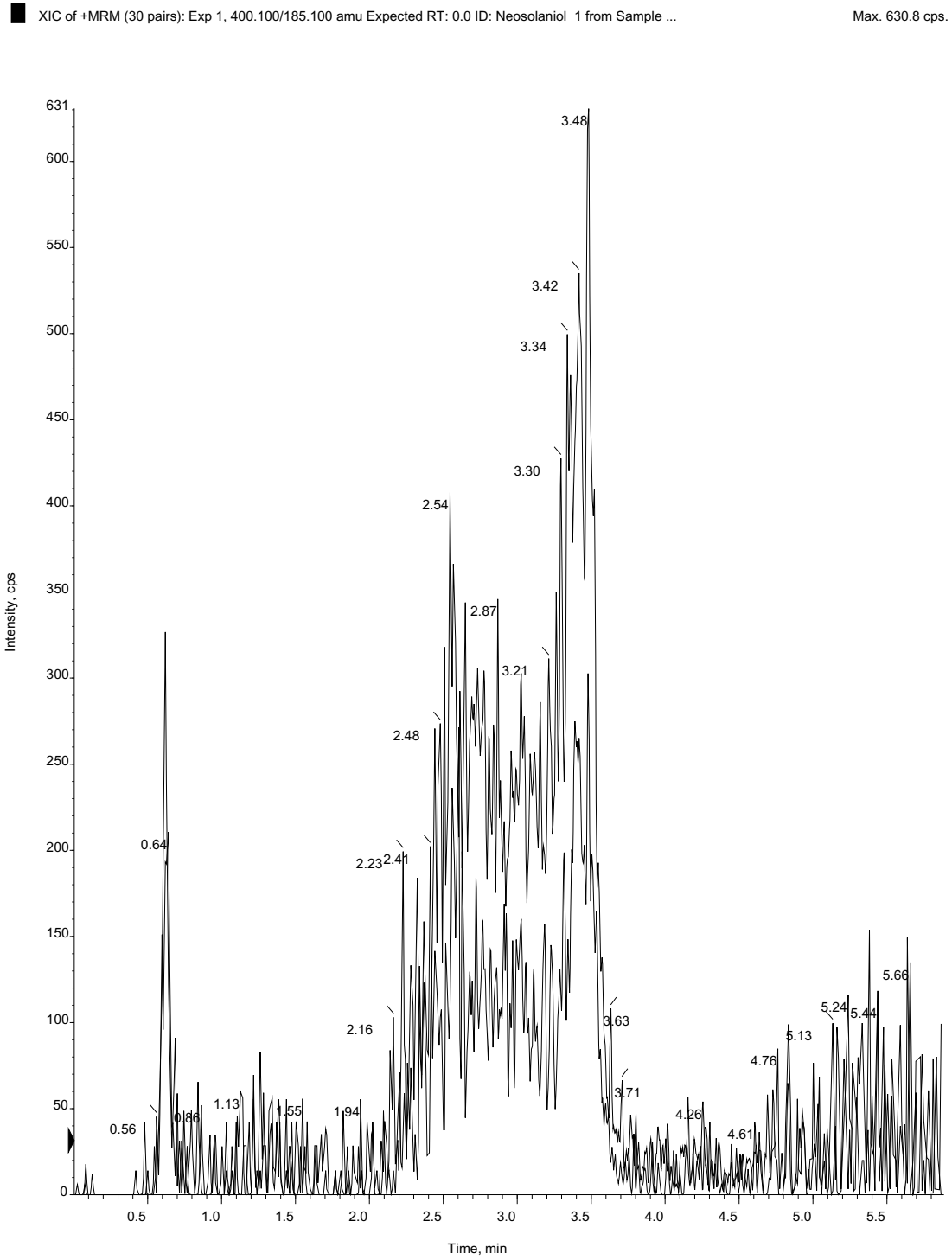
ANNEX 15- Product ion scan of mycotoxin DAS



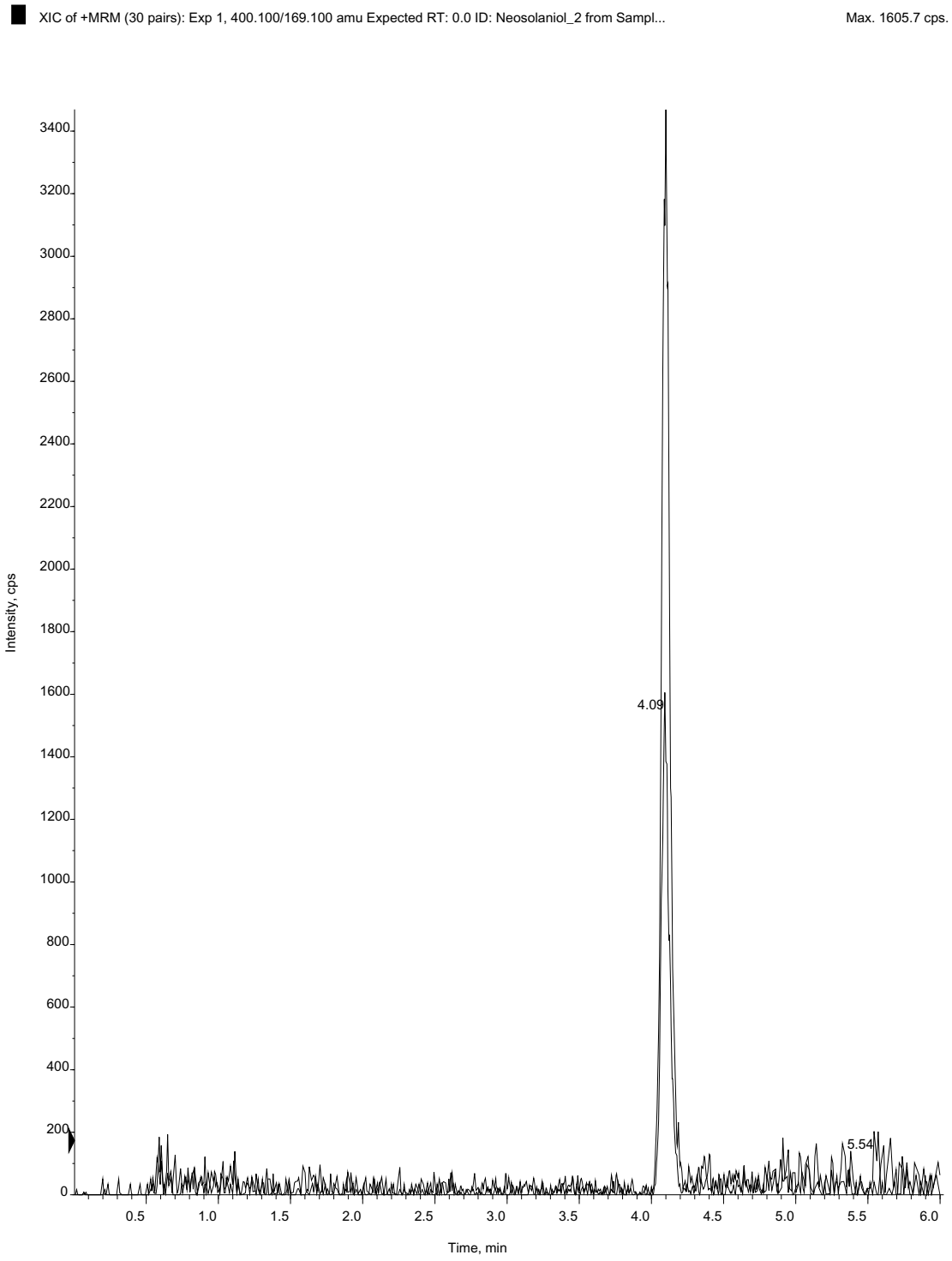
ANNEX 16- Intensity of the Energy collision of mycotoxin DAS



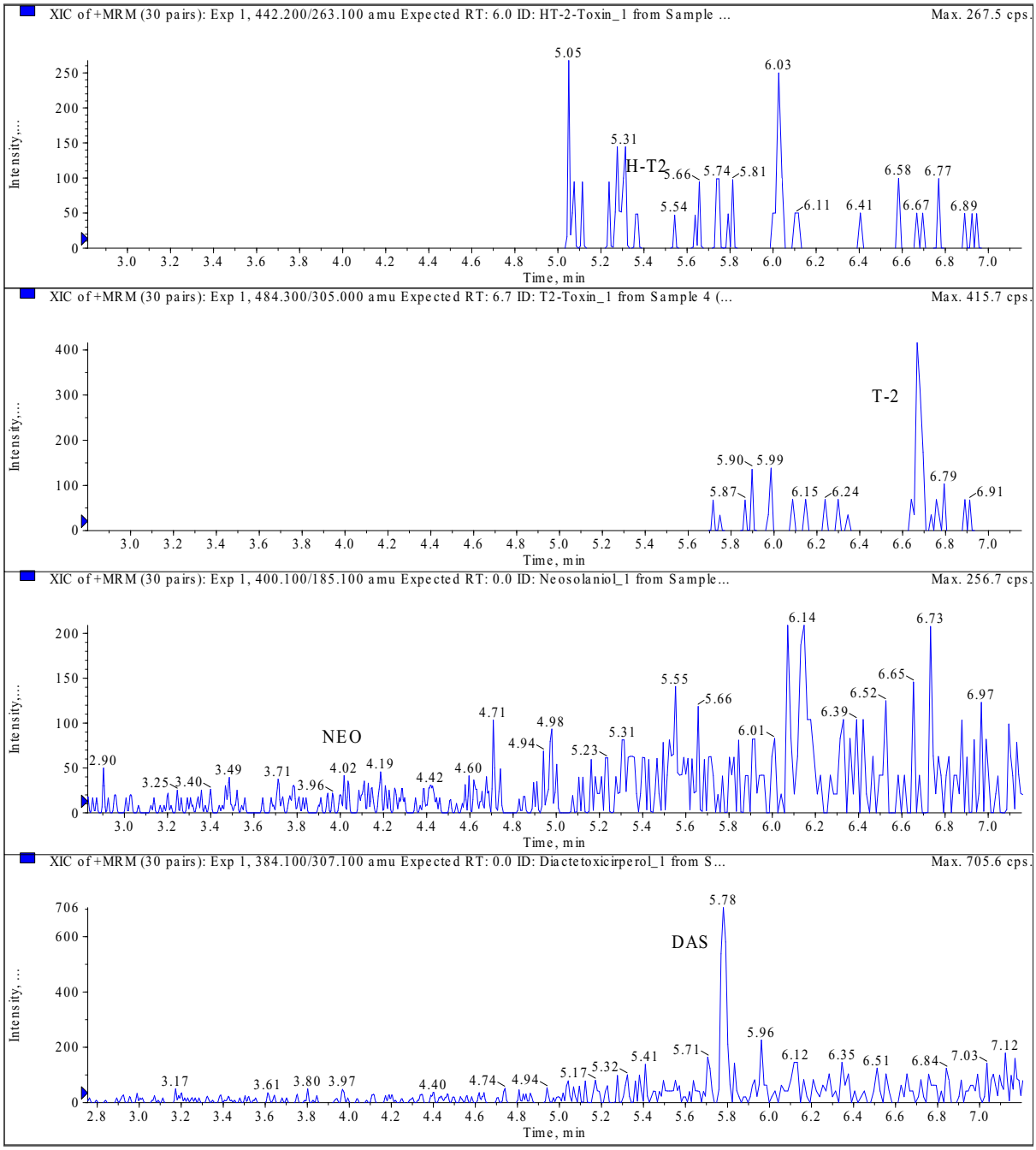
ANNEX 17- Chromatograph of mycotoxin NEO without concentration steep in a cereal breakfast matrix.



ANNEX 18- Chromatograph of mycotoxin NEO with a concentration steep in a cereal breakfast matrix.



ANNEX 19- Chromatograph of yellow corn flour for polenta food matrix with a presence of mycotoxins HT-2, T-2, NEO, and DAS.



ANNEX 20- Chromatograph of soft wheat "Gentile rosso" food matrix with a presence of mycotoxins HT-2, T-2, NEO, and DAS.