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Verification Study of a Commercial ELISA Kit for Aflatoxin B_1 Detection in Corn

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Controlo de Qualidade

Trabalho realizado sob orientação do Professor Doutor José Fernandes e da Doutora Sara Cunha.

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DECLARATION

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ABSTRACT

Animal feed is a potential route for contaminants like mycotoxins to enter into the human food chain. Hence, a close monitoring is fundamental and should be performed with adequate analytical methods.

In this study, a commercial ELISA kit for aflatoxin B_1 detection in corn samples, RIDASCREEN® Aflatoxin B_1 30/15, was assessed by the evaluation of some performance parameters. Plus, specific requirements for the appliance of this kit as a screening method at the concentration of 20 µg/kg were also determined. Lastly, corn samples were analysed with the kit and with an LC-MS/MS method, and the results were compared.

Basically, the following results were achieved: limit of detection of 1.1 µg/kg and limit of quantification of 2.5 µg/kg; repeatability of 9.3 %; 18.0 % of intermediate precision; trueness of 101.8 %; relative expanded uncertainty of ± 0.46 µg/kg; cut-off value of 14 µg/kg and a very low rate of false suspect results, for screening. Finally, the analysis of real samples showed the applicability of the kit. Overall, RIDASCREEN® Aflatoxin B1 30/15 applied to corn revealed to be an effective tool for the quantification of this mycotoxin between 2.5 and 50 µg/kg or just for its screening at 20 µg/kg.

Keywords: verification study, ELISA, aflatoxin B_1, corn, LC-MS/MS.

Resumo

As rações usadas em alimentação animal constituem uma potencial porta de entrada na cadeia alimentar humana de contaminantes químicos, como é o caso das micotoxinas. Assim sendo, a sua monitorização adequada é fundamental, devendo ser realizada com recurso a métodos analíticos apropriados.

Neste trabalho, um kit comercial de ELISA para a deteção de aflatoxina B_1, RIDASCREEN® Aflatoxin B_1 30/15, aplicado a milho foi alvo de estudo, pela avaliação de alguns parâmetros de desempenho. Adicionalmente, alguns requisitos específicos para a aplicação do kit como a estratégia de rastreio para uma concentração alvo de 20 µg/kg foram também determinados. Por último, amostras de milho foram analisadas com o kit e com um método de LC-MS/MS e os resultados devidamente comparados.

Resumidamente, alcançaram-se os seguintes resultados: limite de deteção e de quantificação de 1.1 e 2.5 µg/kg; repetibilidade de 9.3 %; 18.0 % de precisão intermédia; justeza de 101.8 %; incerteza relativa expandida de ± 0.46 µg/kg; valor-limite de 14 µg/kg e uma baixa taxa de falsos suspeitos, como método de rastreio. Finalmente, a análise de amostras reais mostrou a aplicabilidade do kit. Globalmente, o kit RIDASCREEN® Aflatoxin B1 30/15 aplicado ao milho revelou-se uma ferramenta efetiva para a quantificação desta micotoxina entre 2.5 e 50 µg/kg ou apenas para o seu rastreio ao nível de 20 µg/kg.

Palavras-chave: estudo de verificação, ELISA, aflatoxina B_1, milho, LC-MS/MS.

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LIST OF ACRONYMS AND ABREVIATIONS

α	Significance level
15-AcDON	15-acetyldeoxynivalenol
3-AcDON	3-acetyldeoxynivalenol
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
AFs	Aflatoxins
AMIS	Agricultural Market Information System
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
ASE	Accelerated solvent extraction
CBS	Commodity Balance Sheet
CRM	Certified reference material
DAD	Diode array detection
DLLME	Dispersive liquid-liquid micro-extraction
DON	Deoxynivalenol
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ESI +	Positive ionization mode
ESI -	Negative ionization mode
EU	European Union
FAO	Food and Agriculture Organization
FB_1	Fumonisin B_1

FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
FBs	B-series fumonisins
FLD	Fluorescence detector
FMs	Fumonisins
FPIA	Fluorescence polarization immunoassay
FT-NIR	Fourier-transform near-infrared
GAP	Good agricultural practices
GC	Gas chromatography
GC-MS	Gas chromatography coupled with a mass spectrometry detector
GIPSA	The United States Department of Agriculture's Grain Inspection, Packers and Stockyards Administration
GMP	Good manufacturing practices
GPD HLB	Grade polypropylene depth hydrophilic-lipophilic balance
HACCP	Hazard Analysis and Critical Control Points
HESI	Heated electrospray ionization
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography coupled with a diode array detector
HPLC-FLD	High-performance liquid chromatography coupled with a fluorescence detector
HPLC-UV	High-performance liquid chromatography coupled with an ultraviolet detector
HRMS	High-resolution mass spectrometry
HT-2	HT-2 toxin
IACs	Immuno-affinity columns
IARC	International Agency for Research on Cancer
IL-DLLME	Ionic-liquid-based dispersive liquid-liquid micro-extraction
IS	Internal standard
IT	Ion trap

IR	Infrared spectroscopy
k	Coverage factor
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled with a mass spectrometry detector
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry detector
LFD	Lateral flow device
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave-assisted extraction
MeCN	Acetonitrile
MeOH	Methanol
mIAC	Multiple immuno-affinity column
MIPs	Molecularly imprinted polymers
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix solid-phase dispersion
n	Number of replicate observations
N	Number of certified reference materials considered
NCI	Negative chemical ionization
NIR	Near-infrared
NM	Not mentioned
OD	Optical density
OTA	Ochratoxin A
OTB	Ochratoxin B
OTs	Ochratoxins
PCI	Positive chemical ionization
PLE	Pressurized liquid extraction
PSA	Primary secondary amine

QqQ	Triple quadrupole
QTRAP	Hybrid quadrupole/linear ion trap detector
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
r	Repeatability limit
RMS_{bias}	Root mean square of the bias
RSD	Relative standard deviation
RSD_r	Relative standard deviation of repeatability
RSD_{Rw}	Relative standard deviation of intermediate precision
R_w	Intermediate precision limit
SAX	Strong anion exchangers
SCX	Strong cation exchangers
STD	Standard deviation
STD_r	Repeatability standard deviation
STD_{Rw}	Intermediate precision standard deviation
SERS	Surface-enhanced Raman spectroscopy
SFE	Supercritical fluid extraction
SIM	Single ion monitoring
SLE	Solid-liquid extraction
SPE	Solid-phase extraction
SPME	Solid-phase micro-extraction
SRM	Selective reaction monitoring
T-2	T-2 toxin
TMCS	Trimethylchlorosilane
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TMSIM	Trimethylsilylimidazole
TOF	Time-of-flight
TRCs	Trichothecenes
U	Expanded uncertainty

u_{bias}	Standard uncertainty associated with bias
u_c	Combined standard uncertainty
u_{CRM}	Standard uncertainty associated with the certified value of the certified reference material
UHPLC	Ultra-high-performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
u_{Rw}	Standard uncertainty associated with intermediate precision
USE	Ultrasonic extraction
UV	Ultraviolet
ZEN	Zearalenone
α-ZEL	α-Zearalenol
β-ZEL	β -Zearalenol

INTRODUCTION

1. Animal feeding

Feed is described by the European Commission as any substance or product, including additives, whether processed, semi-processed or unprocessed, intended to be used for oral feeding to animals (European Commission, 2011). It can be classified into four groups (Food Standards Agency, 2017a):

- **Forages** – silage made from grass or cereal crops;
- **Cereals and other home-grown crops** – feeds with a high energy and/or protein content;
- **Compound feeds** - manufactured mixtures of single feed materials, minerals and vitamins;
- **Products and by-products of the human food and brewing industries** – residues of vegetable processing, spent grains from brewing and malting and by-products of the baking, bread-making and confectionery industries.

Livestock diets typically include, in its majority, a combination of feeds, designed to meet not only animals nutritional needs with minimal costs, but also to provide everything they need for their health, welfare and production (Adams, 2006; Food Standards Agency, 2017a). However, it is possible to state cereals and cereal-based products as one of the most commonly used ingredients in animal feed, supplying most of the nutrients for livestock (Awika, 2011; GRACE Foundation, 2016; Pinotti, Ottoboni, Giromini, Dell'Orto, & Cheli, 2016; Wilkinson, 2011). In developed countries, up to 70 % of the cereal harvest is used in the daily diet of animals, whereas in developing countries this commodity is mainly used for human consumption (Oliveira, Zannini, & Arendt, 2014). Plant protein sources such as the by-products from the extraction of oil from oilseed crops are also predominantly present in animal feeding, complementing cereal grains usually poor in protein (Capper, Berger, Brashears, & Jensen, 2013; FAO, 2002; GRACE Foundation, 2016; Wilkinson, 2011).

Cereals for feeding industry globally include maize, wheat, barley, sorghum and oats grains (Awika, 2011; Capper et al., 2013). Essentially maize but also wheat are considered key global agricultural commodities in regard to farm animals diets (GRACE Foundation, 2016; Perry, 1984; Ray, Mueller, West, & Foley, 2013; Streit, Naehrer, Rodrigues, & Schatzmayr, 2013). In fact, the majority of world maize domestic utilization (approximately 55%) goes into animal feed (**Figure 1**), because maize and products derived thereof are widely used feed raw materials (Awika, 2011; Cowieson, 2005; Heuzé & Tran, 2016a; Kosicki, Błajet-Kosicka, Grajewski, & Twaruzek, 2016; Perry, 1984; Streit et al., 2013). Concerning the use of wheat in feedingstuffs, it can be observed that it is only around 20%

since wheat is used mainly for human consumption (**Figure 2**). Nevertheless, in the European Union (EU) almost half of the wheat utilization is in feed (FAO, 2016b; Heuzé, Tran, Renaudeau, Lessire, & Lebas, 2015). Therefore, wheat grains and several by-products are also seen as suppliers of various significant materials in livestock feeding (Heuzé & Tran, 2015; Heuzé et al., 2015; Perry, 1984).

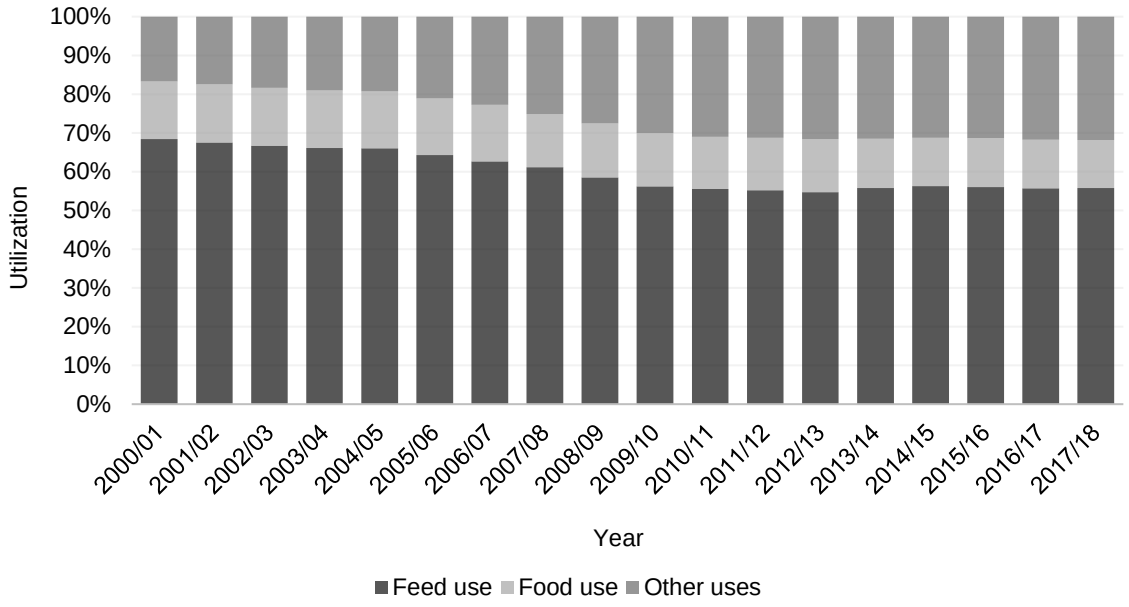

Figure 1 – World maize domestic utilization between 2000/01 and 2016/17 (AMIS, 2016b).

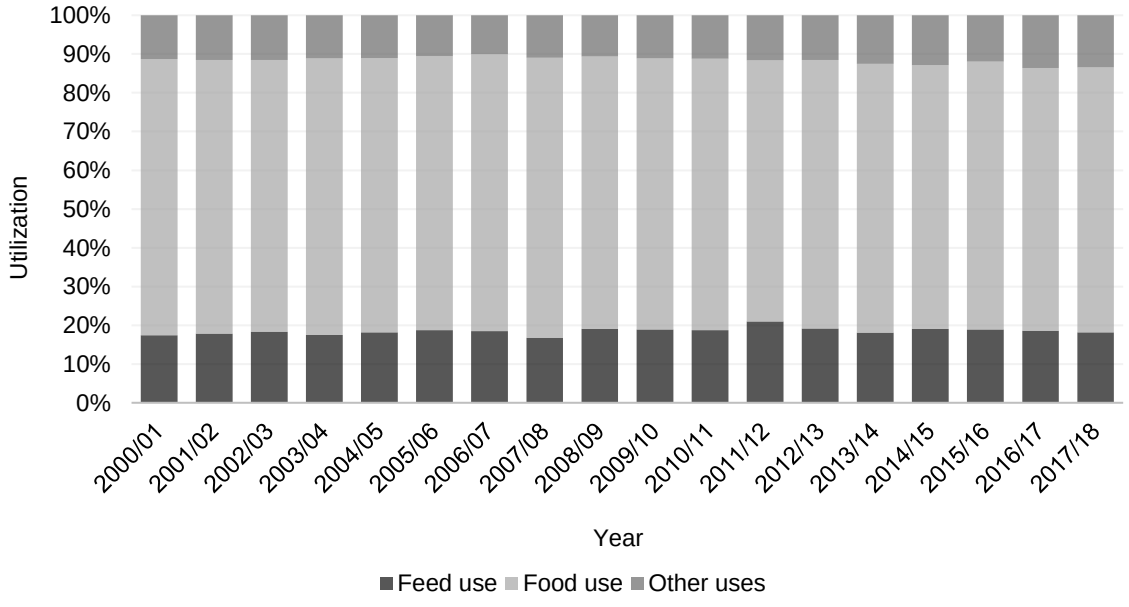

Figure 2 – World wheat domestic utilization between 2000/01 and 2016/17 (AMIS, 2016b).

Oilseed crops like soybeans, cottonseed, sunflower, sesame, and oil palm can be used as vegetable protein sources, in animal feed manufacture (Capper et al., 2013; FAO, 2002). However, soybean products remain universally accepted as the most important and

preferred feed commodities since they are a high-quality protein source in livestock diets (FAO, 2002; GRACE Foundation, 2016; Heuzé & Tran, 2016b; Martín-Pedrosa et al., 2016; Newkirk, 2010; Perry, 1984). Actually, soybean meal, which is the by-product of oil extraction from soybeans, represents two-thirds of the total world output of protein feedstuffs (Heuzé & Tran, 2016b). In regard to soybeans utilization in feed, it was not possible to find relevant information in the Food and Agriculture Organization (FAO) - Agricultural Market Information System (AMIS) Commodity Balance Sheet (CBS). However, through the literature, it is possible to understand that apart from the main use of soybean for oil production, it is widely used as a component of animal feed.

Global demand for agricultural crops has been increasing over the years, with an expected growth of 84 % between 2000 to 2050 (FAO, 2009; GRACE Foundation, 2016; Kruse, 2010; Ray et al., 2013; Tilman, Balzer, Hill, & Befort, 2011). In regard to the feed crops under discussion, we can observe **Figure 3**, based on data obtained from the FAO-AMIS CBS (AMIS, 2016a). It represents the world production of maize, wheat, and soybeans between 2000/01 (2002/03, in the case of soybeans) and 2017/18. Taking this data into account, it is possible to state that the production of these materials has been generally following a growing linear trend, throughout these years. Actually, between 1961 and 2008, (Ray et al., 2013) claimed that the average rates of yield change per year were 1.6 % for maize, 0.9 % for wheat and 1.3 % for soybean. Regarding the individual production of the three keys crops for livestock feed production here considered, data acquired from the FAOSTAT database for food and agriculture was gathered (FAO, 2016a). Maize, the leading crop in the world (Awika, 2011), is mostly produced in America and Asia (**Figure 4**), namely in the United States of America, Brazil and in China mainland. Asia and Europe, more precisely China mainland, India, Russian Federation, and France are the regions where wheat is produced in bigger amounts (**Figure 5**), although this cereal grain is the most widely grown (Heuzé & Tran, 2015). Soybeans are almost entirely produced in America (**Figure 6**), with a focus in the United States of America, Brazil, and Argentina.

The development mentioned before in world feed crop production is intended to meet the rapid growth and intensification of the livestock industry, propelled by the rising demand for livestock products (FAO, 2002, 2009; Food Standards Agency, 2017a). This is, in turn, driven essentially by increases in world population and urbanization as well as changes in lifestyles and food preferences (FAO, 2002, 2009; Ray et al., 2013; Tilman et al., 2011). Consequently, animal feed safety has become even more of a concern for both producers and governments since feed consumption is, eventually, a potential route for hazards to reach the human food chain (FAO, 2002, 2009; FAO & WHO, 2007; Krska, Richard, Schuhmacher, Slate, & Whitaker, 2012). Thus, in accordance with the Directive

2002/32/EC, the quality and safety of products intended for animal feed must be assessed prior to its feeding, ensuring they do not represent any danger to human health, animal health or the environment or do not adversely affect livestock production (FAO & WHO, 2007; The European Parliament and The Council of the European Union, 2015). Among the undesirable substances laid down in this Directive, mycotoxins presence have been increasingly targeted becoming one of the most important hazards in feed raw materials, also due to the verified growth in their formation (E. M. Binder, Tan, Chin, Handl, & Richard, 2007; Tima, Brückner, Mohácsi-Farkas, & Kiskó, 2016).

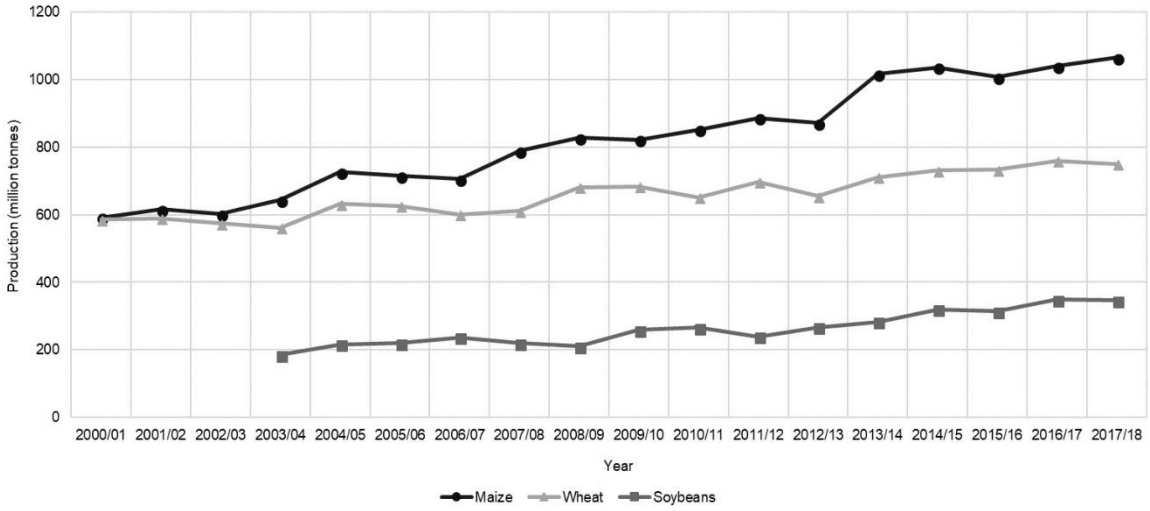

Figure 3 – World production of maize, wheat and soybeans between 2000/01 (2002/03, in the case of soybeans) and 2016/17 (AMIS, 2016a).

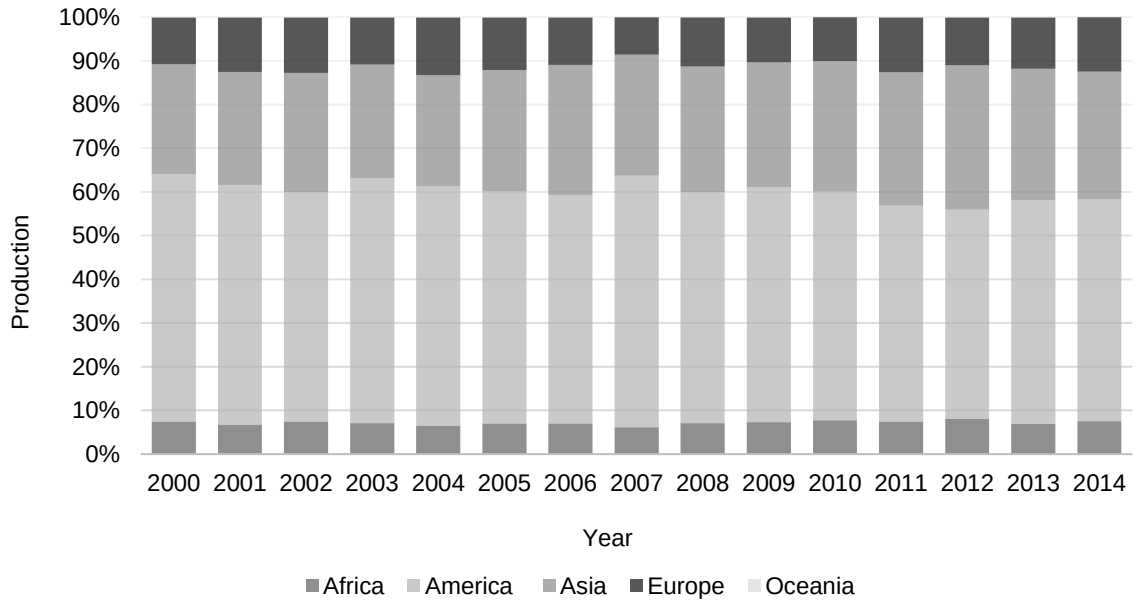

Figure 4 – Geographical maize production between 2000 and 2014 (FAO, 2016a).

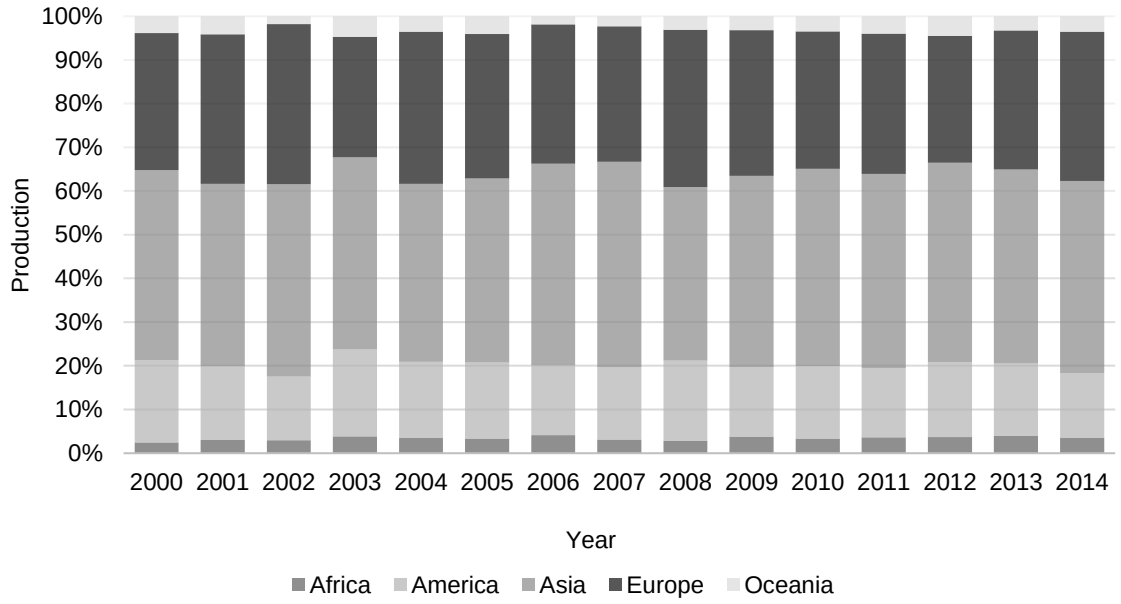

Figure 5 – Geographical wheat production between 2000 and 2014 (FAO, 2016a).

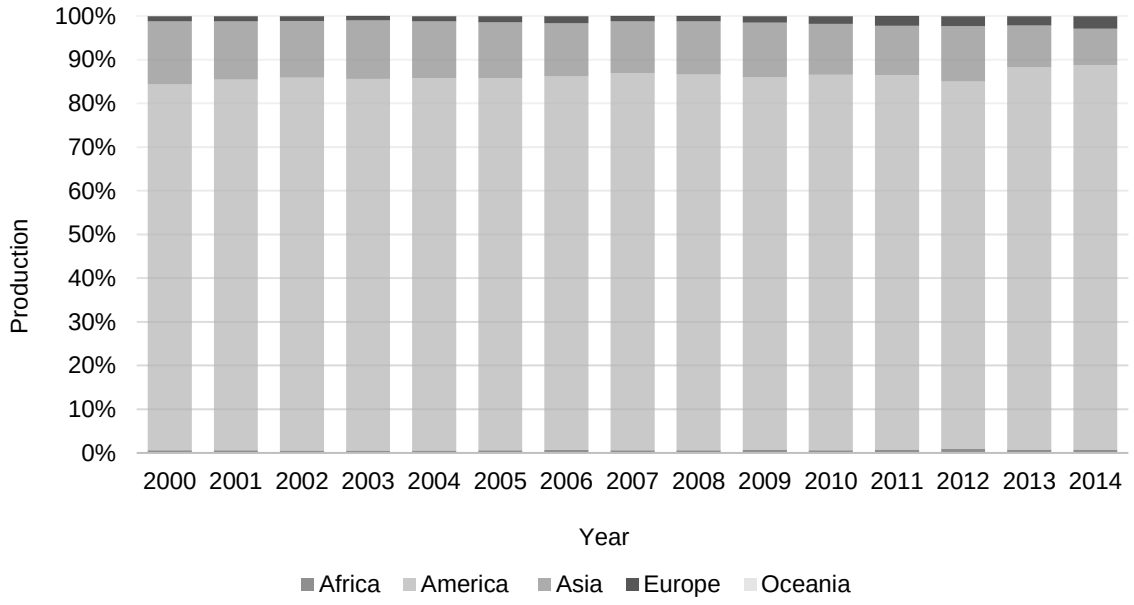

Figure 6 – Geographical soybeans production between 2000 and 2014 (FAO, 2016a).

From now on, important mycotoxins classes, their toxicity, and implications will be addressed. Further, EU legislation covering these metabolites will be under discussion, along with mycotoxins occurrence and methods for their determination. Lastly, a small contextualization about methods validation will be part of this introduction.

2. Prevalent mycotoxins classes and toxicity

Mycotoxins are a relatively large and chemically diverse group of toxic secondary metabolites of low molecular weight (Adams, 2006; CAST, 2003; Pereira, Fernandes, &

Cunha, 2014). They are typically produced by filamentous fungi especially those belonging to the genus *Aspergillus*, *Penicillium,* and *Fusarium*, although *Claviceps* and *Stachybotrys* are also important mycotoxins producers. Approximately 300 to 400 mycotoxins have been identified and reported so far (CAST, 2003; Dzuman, Zachariasova, Veprikova, Godula, & Hajslova, 2015; Pinotti et al., 2016). However, regarding their prevalence in feeds and its known effects on livestock health only a few groups of mycotoxins are considered of safety and economic concern. Namely, aflatoxins (AFs), fumonisins (FMs), ochratoxins (OTs), trichothecenes (TRCs) and zearalenone (ZEN) (FAO & WHO, 2007; Pinotti et al., 2016; Smith, Madec, Coton, & Hymery, 2016). Other mycotoxins, such as patulin, citrinin, and emerging mycotoxins are beyond the scope of this work. With these relevant classes in mind, a brief introduction about each one will be provided, along with the associated toxicological effects. It is important to emphasise that symptoms of mycotoxicoses result from mycotoxin exposure in sufficient quantities or over a long enough period of time, also depending on various other factors such as species, age, gender and nutrition (CAST, 2003; Groopman, Kensler, & Wu, 2013; Marroquín-Cardona, Johnson, Phillips, & Hayes, 2014; Pereira et al., 2014).

2.1. Aflatoxins

Aspergillus flavus and *A. parasiticus* are the main species of aflatoxin-producing fungi, although *A.* nomius and *A. pseudotamarri* are known to produce them, as well. AFs group encompasses several different toxins, however, only 4 types are the most abundant: aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) (**Figure 7**) (Bordin, Sawada, Rodrigues, da Fonseca, & Oliveira, 2014; CAST, 2003; Sirhan, Tan, & Wong, 2013). Their natural fluorescence under ultraviolet light (B for blue and G for green) and their relative chromatographic mobility give them a name. Metabolic products derived from AFs, as aflatoxin M_1 (AFM_1) and M_2 (AFM_2), are also referred to as important contaminants of this class (CAST, 2003; Piotrowska, Śliżewska, & Biernasiak, 2013; Rocha, Freire, Maia, Guedes, & Rondina, 2014).

AFs represent the group of fungal toxins of greatest concern in terms of human toxicity since their toxic effects can advert from their entry in the human food chain in two ways. Directly, after human exposure by consumption of contaminated crops or finished processed food products, since AFs are very stable and may resist food processing operations. Indirectly, from tissues, eggs, milk, and dairy products of animals fed with aflatoxin-contaminated feeds, through excretion of the hydroxylated derivative of AFB_1, AFM_1. Actually, AFB_1 is the most commonly occurring aflatoxin and most potent hepatocarcinogen, being classified by the International Agency for Research on Cancer

(IARC) as a human carcinogen (group 1) and AFM_1 as possibly carcinogenic to humans (group 2B) (Dimitrieska-Stojković et al., 2016; Groopman et al., 2013; IARC, 2016; Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Smith et al., 2016; Streit et al., 2012). Concerning livestock health, AFs are also a major problem causing acute death to chronic disease. Clinical signs of animal intoxication include gastrointestinal dysfunction, anaemia, jaundice, haemorrhage and an overall decrease in productive parameters, such as reduction in weight gain, lower feed efficiency, decreased egg or milk production, inferior carcass quality and increased susceptibility to environmental and microbial stressors. Ultimately, prolonged exposure to low dietary levels of AFs can result in extensive functional and structural liver lesions, including cancer. It is important to note that nursing animals are, as well, exposed to the AFB_1 toxic metabolite secreted in milk (CAST, 2003; Kovalsky et al., 2016; Marin et al., 2013; Streit et al., 2012).

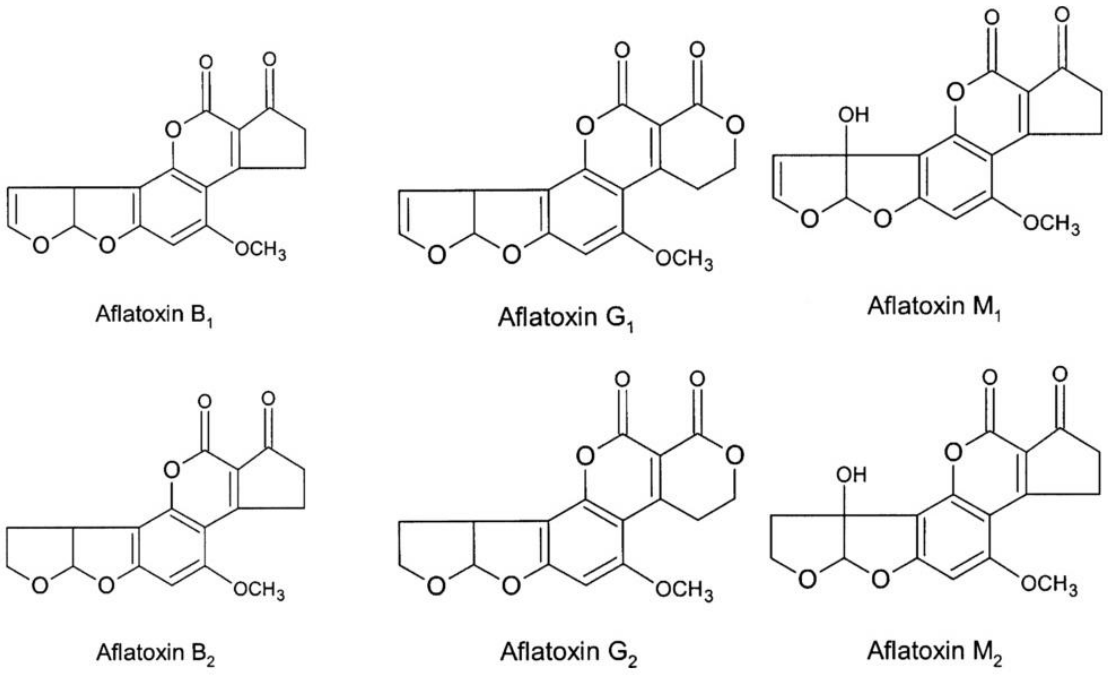

Figure 7 – Chemical structure of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2.

2.2. Fumonisins

FMs are commonly classified as *Fusarium* toxins since they can be produced by several species of this genus, with *F. verticillioides* (previously classified as *F. moniliforme*) and *F. proliferatum* as the central producing species. However, *A. niger* was recently found to produce FMs, too (Pitt, Taniwaki, & Cole, 2013; Rocha et al., 2014; Streit et al., 2012). Within the 16 fumonisin analogues known to date, the B-series FMs (FBs), which compromise fumonisin B_1, B_2, B_3 and B_4, are the most important ones (Anukul, Maneeboon, Roopkham, Chuaysrinule, & Mahakarnchanakul, 2014; Rocha et al., 2014; Streit et al., 2012).

Fumonisin B_1 (FB_1) (**Figure 8**) is reported as the predominant and most toxic member of FMs family and has been recognised as a possible human carcinogen (group 2B) (IARC, 2016; Marroquín-Cardona et al., 2014; Streit et al., 2012). Fumonisin B_2 (FB_2) (**Figure 8**) is also toxicologically significant. Apparently, the carcinogenic character of FBs is not related to direct DNA damage by FMs, being then associated with the disruption of sphingolipid biosynthesis due to structural similarities of these toxins with the backbone precursors of sphingolipids (Groopman et al., 2013; Marin et al., 2013; Rocha et al., 2014). In animals, ingestion of feed contaminated with FBs can cause significant disease in horses, swine, and rabbits that are more considerably sensible than cattle and poultry (CAST, 2003; Marin et al., 2013; Murugesan et al., 2015). Leukoencephalomalacia syndrome appears mainly in horses triggering primary symptoms like lethargy, blindness, and decreased feed intake and, ultimately, convulsions and death. In pigs, FB_1 is associated with pulmonary oedema whose clinical signs typically include reduced feed consumption, dyspnoea, weakness, cyanosis, and death (Groopman et al., 2013; Marin et al., 2013; Rocha et al., 2014). Besides, these mycotoxins have also shown hepatotoxicity (CAST, 2003; Groopman et al., 2013).

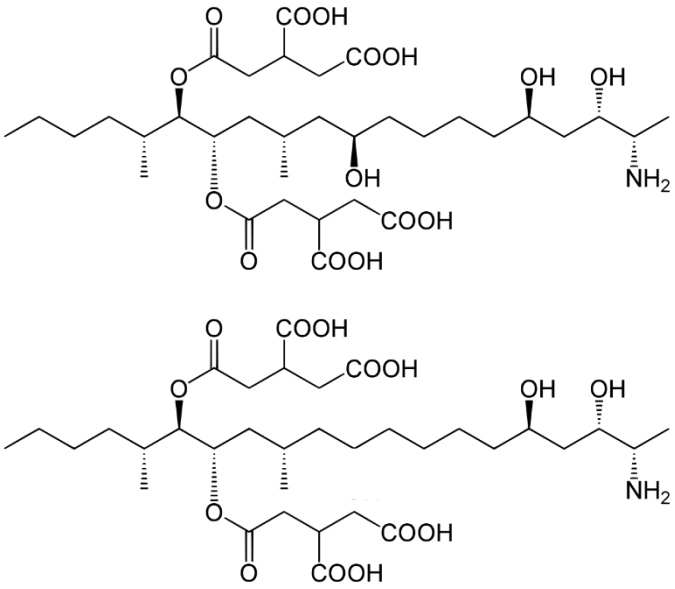

Figure 8 – Chemical structure of FB_1 and FB_2.

2.3. Ochratoxins

Production of OTs (ochratoxin A (OTA) and ochratoxin B (OTB)) occurs essentially by fungi belonging to the genus *Aspergillus* and *Penicillium*, namely by species as *A. ochraceus, A. carbonarius, P. verrucosum* and *P. nordicum* (CAST, 2003; Milani, 2013; Piotrowska et al., 2013; Rocha et al., 2014). Major producers of this toxin depend on the weather, with *P. verrucosum* being the prevalent fungi in temperate regions and *A.*

ochraceus, in warmer regions (Streit et al., 2012). In this group of structurally related mycotoxins, the main one is OTA (CAST, 2003; Marroquín-Cardona et al., 2014).

OTA (**Figure 9**) is linked with potent nephrotoxic effects in animals as a consequence of exposure to naturally occurring levels in feed since the major target organ are the kidneys (CAST, 2003; Groopman et al., 2013; Marin et al., 2013; Marroquín-Cardona et al., 2014). In fact, OTA has been associated with endemic nephropathy in swine (Marroquín-Cardona et al., 2014; Rocha et al., 2014). High dietary doses of this toxin may cause liver damage and necrosis of intestinal and lymphoid tissue (CAST, 2003; Groopman et al., 2013). Regarding humans toxicity, OTA has been implicated in a fatal kidney disease typical in the Balkan countries (Balkan endemic nephropathy) and has been classified as possibly carcinogenic (group 2B) (CAST, 2003; IARC, 2016; Marin et al., 2013; Marroquín-Cardona et al., 2014). Additionally, there has been a public health concern respecting the transfer of OTA to animal-derived food (Streit et al., 2012).

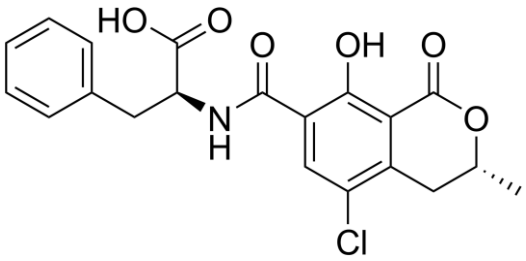

Figure 9 – Chemical structure of OTA.

2.4. Trichothecenes

TRCs are produced to a great extent by *Fusarium* species, although not exclusively, since some *Cephalosporium*, *Myrothecium*, *Stachybotrys,* and *Trichoderma* species also produce these mycotoxins. This is a large class of fungal metabolites with more than 150 structurally related compounds, which are chemically divided into 4 types (A to D) (CAST, 2003; Kovalsky et al., 2016; Marin et al., 2013). TRCs from the type A and B are the most important. Type A-TRCs mainly includes HT-2 and T-2 toxins (HT-2 and T-2), while type B-TRCs are frequently represented by deoxynivalenol (DON), its derivatives 3-acetyldeoxynivalenol (3-AcDON) and 15-acetyldeoxynivalenol (15-AcDON) and nivalenol (Juan, Ritieni, & Mañes, 2013; Rodríguez-Carrasco, Ruiz, Font, & Berrada, 2013).

HT-2 and T-2 (**Figure 10**), produced by *F. sporotrichioides*, *F. langsethiae*, *F. acuminatum*, and *F. poae*, although not being very prevalent, are the most toxic members of type A-TRCs (Groopman et al., 2013; Kovalsky et al., 2016; Marin et al., 2013; Streit et al., 2012). They were found to inhibit protein and DNA synthesis and weaken cellular immune response, in animals (Groopman et al., 2013; Streit et al., 2012). Symptoms

include decreased feed intake and weight gain, bloody diarrhoea, haemorrhaging, oral lesions, low egg, and milk production, abortion and death, in some cases (Groopman et al., 2013; Kovalsky et al., 2016; Marin et al., 2013; Streit et al., 2012).

DON (**Figure 11**), produced primarily by *F. graminearum* and *F. culmorum*, is one of the least acutely toxic TRCs but, as it is highly incident, is considered being very relevant in animal husbandry (CAST, 2003; Groopman et al., 2013; Ran et al., 2013; Streit et al., 2012). Exposure to DON affects more severely monogastric animals, especially swine, and may cause feed refusal, vomiting, and anorexia, apart from symptoms described previously for HT-2 and T-2 (CAST, 2003; Kovalsky et al., 2016; Marin et al., 2013). Overall, ingestion of low to moderate levels of this mycotoxin by animals leads to increased susceptibility to pathogens and to a poor performance (CAST, 2003; Marin et al., 2013). DON was categorized by IARC as not classifiable as to its carcinogenicity to humans (group 3) (IARC, 2016).

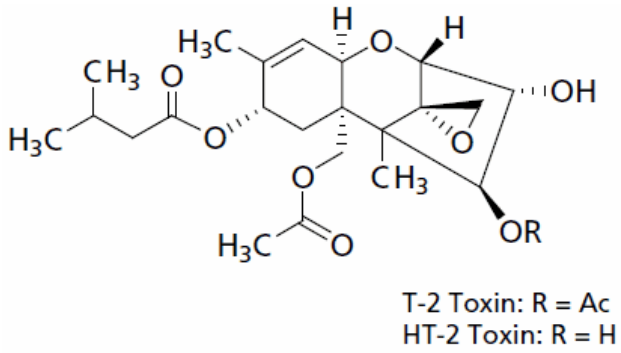

Figure 10 – Chemical structure of HT-2 and T-2.

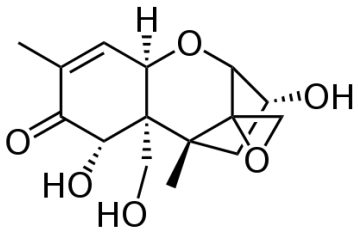

Figure 11 – Chemical structure of DON.

2.5. Zearalenone

ZEN (**Figure 12**) is a *Fusarium* mycotoxin produced particularly by *F. graminearum* but also by *F. culmorum*, *F. cerealis*, *F. equiseti*, among others (Marin et al., 2013; Rocha et al., 2014). This mycotoxin has α-Zearalenol (α-ZEL) and β –Zearalenol (β-ZEL) as its derivatives (González Peyera et al., 2014). ZEN is classified commonly as a non-steroidal estrogen, once it has structural similarities with the female sex hormone, estradiol. This chemical characteristic gives it the capability to bind to estrogen receptors, causing adverse effects associated with reproductive disorders and hyperestrogenism, in humans and

breeding animals (CAST, 2003; Piotrowska et al., 2013; Rocha et al., 2014; Streit et al., 2012). According to IARC, ZEN belongs to group 3, which means it is not classifiable regarding its carcinogenicity to humans (IARC, 2016).

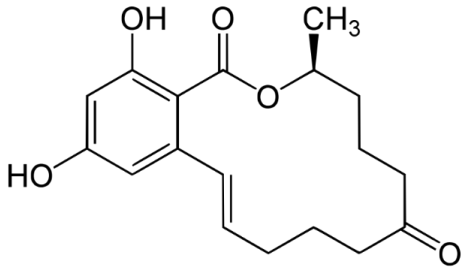

Figure 12 – Chemical structure of ZEN.

3. Mycotoxins economic and commercial implications

Adverse health effects caused by animal consumption of mycotoxin-contaminated feed, which include occult conditions (for example, growth retardation, impaired immunity, and decreased disease resistance), chronic to acute disease and even death, affect animal performance to a great extent (CAST, 2003; Marroquín-Cardona et al., 2014; Pinotti et al., 2016). Therefore, a threat to feed supply chain security like mycotoxins becomes a global concern and a significant constraint to animal production systems (Grenier & Applegate, 2013; Pinotti et al., 2016). These metabolites cause perturbations in the feed industry due to the decrease in the quality of commodities and also because of the rejection and disposal of highly contaminated crops (CAST, 2003; Marroquín-Cardona et al., 2014; Pinotti et al., 2016). Generally, large costs on the economy of these industries arise from mycotoxin contamination. Apart from de aforementioned problems, economic losses might be associated with increased costs with health care, finding alternative feed sources, prevention strategies, investment in testing methods and with regulatory costs (CAST, 2003; Oliveira et al., 2014; Pinotti et al., 2016; Smith et al., 2016). Additionally, mycotoxins presence may impact on international commodity trade, propelled by increasing globalization (Bordin et al., 2014; CAST, 2003).

4. Legislation covering mycotoxin contamination

In an attempt to avoid the adverse effects and implications above discussed, several worldwide institutions and organisations have restricted allowed levels of certain mycotoxins in animal feeds, since truly mycotoxin-free feedingstuffs are impossible to guarantee. Naturally, the limits and the mycotoxins targeted by legislation vary from country to country since different scientific, economic and political factors influence this decision-making process (CAST, 2003; Kovalsky et al., 2016; Krska et al., 2012; Smith et al., 2016).

Particularly, in the EU, legislation (regulation or recommendation) established so far covers AFs, FBs, OTA, some type A, and B TRCs and ZEN in different feeding matrices. Directive 2002/32/EC specifies maximum content for AFB_1 in products intended for animal feed (The European Parliament and The Council of the European Union, 2015). Guidance values for DON, FBs, OTA, and ZEN contamination were set in Commission Recommendation 2006/576/EC (The Commission of the European Communities, 2006a). Finally, for HT-2 and T-2, indicative levels in cereals and cereal products were issued in Commission Recommendation 2013/165/EC (The European Commission, 2013a). **Table 1** summarizes the values given in these legislations concerning mycotoxin contamination of feed for farm animals. It can be seen that cereals are the most contemplated matrix and that products intended for feeding pigs have the lowest limits since these animals are very susceptible to mycotoxins. Besides, these values are among the most stringent in the world, which may hinder the export of feed commodities from some developing countries to their European trading partners (CAST, 2003; Schatzmayr & Streit, 2013; Smith et al., 2016).

Table 1 – EU legislation (maximum content, guidance values, and indicative levels) on mycotoxins in products intended for livestock feed (The Commission of the European Communities, 2006a; The European Commission, 2013a; The European Parliament and The Council of the European Union, 2015).

Mycotoxins	Products intended for livestock feed	Levels [a] (mg/kg)	Legislation
AFB_1	Feed materials	0.02	Directive 2002/32/EC
	Complementary and complete feed with the exception of:	0.01	
	− compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals	0.005	
	− compound feed for cattle (except dairy cattle and calves), sheep (except dairy sheep and lambs), goats (except dairy goats and kids), pigs (except piglets) and poultry (except young animals)	0.02	
DON	Feed materials		Commission Recommendation 2006/576/EC
	− Cereals and cereal products with the exception of maize by-products	8	
	− Maize by-products	12	
	Complementary and complete feedingstuffs with the exception of:	5	
	− complementary and complete feedingstuffs for pigs	0.9	
	− complementary and complete feedingstuffs for calves (< 4 months), lambs and kids	2	
$FB_1 + FB_2$	Feed materials		Commission Recommendation 2006/576/EC
	− Maize and maize products	60	
	Complementary and complete feedingstuffs for:		
	− pigs, horses (*Equidae*), rabbits	5	
	− poultry, calves (< 4 months), lambs and kids	20	

[a] – Feed/ feedingstuff moisture content of 12 %.

(continued on next page)

Table 1 *(continued)*

Mycotoxins	Products intended for livestock feed	Levels [a] (mg/kg)	Legislation
OTA	Feed materials − Cereals and cereal products Complementary and complete feedingstuffs for: − pigs − poultry	 0.25 0.05 0.1	Commission Recommendation 2006/576/EC
T-2 + HT-2	Unprocessed cereals − Barley (including malting barley) and maize − Oats (with husk) − Wheat, rye and other cereals Cereal products for feed and compound feed − Oat milling products (husks) − Other cereal products − Compound feed	 0.2 1 0.1 2 0.5 0.25	Commission Recommendation 2013/165/EC
ZEN	Feed materials − Cereals and cereal products with the exception of maize by-products − Maize by-products Complementary and complete feedingstuffs for: − piglets and gilts (young sows) − sows and fattening pigs − calves, dairy cattle, sheep (including lamb) and goats (including kids)	 2 3 0.1 0.25 0.5	Commission Recommendation 2006/576/EC
[a] – Feed/ feedingstuff moisture content of 12 %.			

5. Mycotoxins occurrence

Mycotoxin contamination is widespread among numerous raw agricultural commodities, such as cereals but they can also be found in nuts, fruits, spices, and processed foods like coffee, wine, and beer since these metabolites are highly chemically stable (CAST, 2003; Marin et al., 2013; Oliveira et al., 2014; Schatzmayr & Streit, 2013; Smith et al., 2016). Animal products namely milk, meat, and eggs represent another possible source of mycotoxins as discussed earlier (Pinotti et al., 2016; Smith et al., 2016). With regards to feed raw materials and animal feed, these contaminants have been described as ubiquitous, because they may arise under very different conditions but also due to trade flows that end up distributing mycotoxins outside their naturally occurring areas (Kovalsky et al., 2016; Pinotti et al., 2016; Schatzmayr & Streit, 2013; Smith et al., 2016).

The incidence of mycotoxins is influenced by several factors, with their production possibly starting in the field throughout the crop growing cycle and increasing during harvesting, drying, processing, and storage steps, depending strongly on various environmental conditions. These comprehend not only climatic factors, such as temperature and moisture content which are the main aspects modulating fungal growth and mycotoxins production, but also pH, bioavailability of micronutrients and insect damage, for example (CAST, 2003; FAO, 2007; Marroquín-Cardona et al., 2014; Piotrowska et al., 2013; Rodríguez-Carrasco et al., 2013; Smith et al., 2016). Others factors like geographic location, agricultural practices, harvest year and the length and conditions of storage affect the extent of contamination of a particular commodity (Alkadri et al., 2014; CAST, 2003; Pereira et al., 2014; Smith et al., 2016). However, the substrate susceptibility to fungal invasion plays a major role in mycotoxin production (Guerre, 2016). Despite their unavoidable and unpredictable nature, *Aspergillus* and *Penicillium* are commonly classified as storage fungi, while *Fusarium* is often regarded as field fungi (Bryden, 2012; CAST, 2003; Kovalsky et al., 2016). Moreover, *Aspergillus* usually are widespread in regions with tropical and subtropical climates, growing with low water activity (CAST, 2003; Piotrowska et al., 2013), *Penicillium* occurs typically in cool temperate zones (Groopman et al., 2013; Pitt et al., 2013) and *Fusarium* species are native to areas with temperate climates, growing with relatively high water activity (Marin et al., 2013; Marroquín-Cardona et al., 2014). However, such distribution patterns should not be seen as a rule, since favourable environmental conditions for fungal proliferation are expected to appear in unusual places and circumstances, due to the global warming scenario (Grenier & Applegate, 2013; Marroquín-Cardona et al., 2014; Smith et al., 2016).

In the feed manufacturing process, safety complications includes aspects like the practice of mixing different batches of distinct raw ingredients, which creates a new matrix with an entirely new risk profile and the fact that the majority of mycotoxins are stable compounds that are not destroyed during storage, milling or high-temperature feed manufacturing process (Abdou, Othman, El-Bordeny, Ibrahim, & Abouzeid, 2016). For these reasons, the knowledge of the occurrence and distribution of mycotoxins in animal feeds is of extreme importance, giving the opportunity to determine the direct risk posed to animals.

Overall, it seems that the common association between certain raw materials and a specific mycotoxin contamination profile has led researchers to favour the determination of these same contaminants. However, in addition to the fact that mycotoxins formation is a complex and multifactor phenomenon, worldwide contamination and distribution patterns of fungi and their secondary metabolites are predicted to be significantly affected by climate change scenarios, as a result of the appearance of favourable environmental conditions for fungal proliferation in uncommon places (Grenier & Applegate, 2013; Marroquín-Cardona et al., 2014; Smith et al., 2016).

Mycotoxins may appear in concentrations that exceed the strict limits established by EU and, once formed, are stable during harvesting and storage. This draws attention to the need for applying prevention and control strategies such as Hazard Analysis and Critical Control Points (HACCP) approaches, good agricultural and manufacturing practices (GAP and GMP) and quality control from the field up to the final product (Greco, Franchi, Golba, Pardo, & Pose, 2014; Stoev, 2015). However, contaminated feed might be redirected to less vulnerable animal species or, ultimately, detoxification methods can be used, involving the addition of feed additives “that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action” (Aiko & Mehta, 2015; E. M. Binder et al., 2007; The Commission of the European Communities, 2009). These substances have to be authorised under the feed additive Regulation 1831/2003 amended by Regulation 386/2009 (Food Standards Agency, 2017b). This way hygienic and nutritional quality of feed is guaranteed, ensuring safety and productivity of the farm animals (E. M. Binder et al., 2007; Greco et al., 2014).

5.1. Co-occurrence

Natural contamination of raw ingredients and feeds with an array of mycotoxins is a frequent scenario around the world, which can be explained by the ability of moulds to simultaneously produce different kinds of mycotoxins and because commodities may be concurrently infected with numerous fungal species or in rapid succession. Besides,

compound feed is made up of a mixture of several raw ingredients, making it particularly vulnerable to multiple mycotoxins contamination (Pinotti et al., 2016; Smith et al., 2016). Usually, combinations of two mycotoxins, such as DON with ZEN and AFB_1 with FBs, are reported more frequently (CAST, 2003; Smith et al., 2016).

Multiple mycotoxin contaminations pose great concern since it is completely clear that adverse effects on animal health and performance can be additive and/or synergistic, which means that the overall toxicity is not only the sum but the multiple of the mycotoxins' individual toxicities (Pinotti et al., 2016; Zachariasova, Dzuman, Veprikova, Hajkova, & Jiru, 2014). This means that the study of just one of these impurities provides insufficient information about the risk associated with a respective feedstuff and that attention toward mycotoxin co-occurrence should be increased (Gutleb et al., 2015; Streit et al., 2012). Nevertheless, legislation over the world and in Europe only considers mycotoxin mono-exposure data and does not address relevant mycotoxin combinations (**Table 1**), which can be considered a loophole that should be taken into account in the future.

6. Mycotoxins testing methods

Evaluation of mycotoxin contamination on feed materials and feed is a direct requirement of the adoption of legislation limits for these impurities, providing information to producers, manufacturers, traders, and researchers (Abdou et al., 2016; Cheli, Battaglia, Gallo, & Dell'Orto, 2014; Kovalsky et al., 2016; Xie, Chen, & Ying, 2016). Moreover, the analysis is fundamental in the potential risks assessment for livestock and for global trade of commodities, in the diagnosis of mycotoxicosis and in monitoring mycotoxin mitigation strategies (Bryden, 2012; Keller et al., 2016). The determination of this contaminant is quite complex since they represent a structurally diverse chemical compound group that frequently appears in low concentrations and in a vast range of matrices, and sometimes, in various combinations (Alkadri et al., 2014; Bryden, 2012; Pereira et al., 2014). Nevertheless, sufficiently reliable, accurate, sensitive and selective methods are available for the qualitative and quantitative analysis of these secondary metabolites. Generally, three steps are involved in testing for mycotoxins: sampling, sample preparation and analytical procedure (Pereira et al., 2014; Xie et al., 2016).

6.1. Sampling

Obtaining sufficiently representative samples of a batch, i.e., sampling, is crucial in the entire process of mycotoxins determination. In fact, this step accounts for the greatest source of error since the analytes under discussion often appear unevenly distributed and in trace levels (Wagner, 2015; Xie et al., 2016). Thus, sampling plans for different

commodities were established by several agencies. In EU, Regulation No 691/2013 amending Regulation No 152/2009 describes methods of sampling, in feed materials, for the official control of AFs and other mycotoxins. Briefly, representative laboratory samples are prepared from the sampling points by i) selecting one or more characteristic lots; ii) repeatedly collecting incremental samples at numerous single positions in the lot; iii) forming an aggregate sample by combining the incremental samples by mixing; iv) preparing the final samples by representative dividing (The European Commission, 2013b; Turner et al., 2015).

6.2. Sample preparation

The sample preparation steps, grinding and sub-sample, accomplish the conversion of the aggregate sample into a representative sub-sample, from which is prepared the laboratory sample. Ideally, a subsampling mill is used, performing these two processes simultaneously. However, a conventional grinder can also be used, where the aggregate sample is crushed, and then a sub-sample is taken. Sample preparation error can be diminished by increasing the fineness of grind and using larger subsamples (IARC Scientific Publications, 2012; Turner et al., 2015; Xie et al., 2016). From the Annex II of the Regulation No 401/2006, it is possible to withdraw the criteria for sample preparation, although it is for the official control of the levels of mycotoxins in foodstuffs (The Commission of the European Communities, 2006b).

6.3. Analytical procedure

Analytical procedure, for the majority of the methods, includes a step of sample pre-treatment where mycotoxins are solvent-extracted from the laboratory sample and the extract is purified from the unwanted co-extracted matrix components, followed by an optional sample concentration step, before the final separation and detection steps (Pereira et al., 2014; Turner et al., 2015; Xie et al., 2016).

In the next sections, is provided an overview of the different sample pre-treatment techniques and separation and detection methods, namely screening, and chromatographic procedures, reported on mycotoxin analysis in maize, wheat, soybeans, their by-products, and animal feed, published in the last years. Additionally, enzyme-linked immunosorbent assay, gas and liquid chromatography methods, applied in this field of analysis, in 2016, were revised and the number of reports published in this year and in 2013 was compared, in order to assess the evolution of the application of these methods.

6.3.1. Sample pre-treatment

Sample pre-treatment makes it possible to obtain an enriched extract of the compounds of interest, as clean as possible, reducing matrix effects. As there is a great diversity of these techniques, a careful choice as to be made depending on the type of matrix, the physicochemical properties of the target analyte(s) and the final detection method (Pereira et al., 2014; Turner et al., 2015; Xie et al., 2016).

Extraction with solvents

Classical solid-liquid extraction

In solid-liquid extraction (SLE), a solvent or a mixture of solvents is intended to extract the analyte quantitatively from the solid sample, with as little additional compounds as possible (Xie et al., 2016). To obtain an accurate determination in the final step, some parameters of this method need to be wisely controlled, namely the solvent type and its volume, the ratio between the sample and the extraction solvent, the temperature and the time of extraction (Pereira et al., 2014). Regarding the choice of the solvent, it depends on the chemical properties of the matrix and on the analyte to be extracted (Pereira et al., 2014; Xie et al., 2016). As the majority of the mycotoxins are soluble in polar and slightly polar solvents and insoluble in apolar solvents, mixtures of organic solvents, like acetone, acetonitrile (MeCN), chloroform, dichloromethane, ethyl acetate or methanol (MeOH) are often used. Small amounts of diluted acids (formic acid, acetic acid, and citric acid) or water is usually added to improve the extraction efficiency, since an acid solution can break interactions between the toxins and other sample constituents like proteins or sugars, and water increases penetration of the solvent into the material (Pereira et al., 2014). Following the addition of the extraction solvent, shaking is used to favour the procedure, and then centrifugation or filtration is normally carried out, before concentration and/or clean-up steps (Pereira et al., 2014; Xie et al., 2016). Occasionally, non-polar solvents as hexane and cyclohexane are used before or after the extraction to remove lipophilic components. SLE, the oldest sample pre-treatment method using solvents, was the most widely used technique in the articles here reviewed despite being a tedious and labour-intensive process (Pereira et al., 2014). Since the selection of a suitable extraction solvent is a challenging process during the optimization of a method, it is common to test different extraction mixtures in order to understand which one is capable of yielding the highest recovery rates (Sifou et al., 2016). For example, (Sifou et al., 2016) tried MeCN/ water/ formic acid (89:10:1 v/v), MeOH/ water/ formic acid (89:10:1 v/v), water/ MeCN (84:16 v/v), MeCN/ water/ acetic acid (79:20:1 v/v), MeOH (100 %) and MeCN (100 %) to extract OTA in poultry feed samples, concluding that MeOH (100 %) provides the most efficient extraction.

Instrumental solvent extraction

Other solid-liquid extraction techniques, that use a different type of energy source to favour the process, have been used for the determination of mycotoxins in raw feed ingredients, like microwave-assisted extraction (MAE) and ultrasonic extraction (USE). Although these instrumental solvent extraction techniques consume less solvent and frequently provide better extraction efficiencies (in terms of extraction yield an/or recovery) comparing to classical SLE, their use did not became widespread maybe because optimization and routine use are difficult, adding the required initial investment in special equipment (Pereira et al., 2014).

Microwave-assisted extraction

MAE is a relatively quick process that through highly localized temperature and pressure causes selective migration of target compounds from the material to the surroundings, using microwave energy (S. Chen & Zhang, 2013; Pereira et al., 2014). According to (Pereira et al., 2014), this extraction technique is only suitable for thermal resistant analytes, which in the case of mycotoxins is not a problem as this is one of their properties. A pre-treatment technique using MAE followed by solid-phase extraction (SPE) was successfully developed by (S. Chen & Zhang, 2013) to determine AFs in grains and grain products with LC coupled to a fluorescence detector (FLD). To perform MAE, 12 mL of MeCN was added to 3 g of sample. This mixture was then heated at 80 ºC for 15 minutes and 350 psi.

Ultrasonic extraction

USE uses acoustic cavitation to cause molecular movement of solvent and sample, aggressively improving the transfer of the analytes from the matrix into the solvent with improved efficiency. This technique is carried out with an ultrasonic bath and the duration of the ultrasound application depends on the matrix (Xie et al., 2016). Generally, USE enables the reduction of the extraction time, consumes low solvent, is economical and offers a high level of automation compared to traditional extraction methods (Cun Li, Wu, Yang, & Huang-Fu, 2012; Xie et al., 2016). In mycotoxin contamination analysis in raw feed ingredients and feed, during 2016, (Fan et al., 2016) and (Yan Wang et al., 2016) applied USE. (Fan et al., 2016) ultrasonicated the sample together with MeCN 50 % for 40 minutes at 40 ºC, in order to quantify DON and its derivatives in the feed with an ultra-high-performance liquid chromatography (UHPLC) coupled to MS/MS method. (Yan Wang et al., 2016) used an ultrasonic water bath for 20 minutes at room temperature to extract with MeCN/ water (84:16 v/v, containing 1 % acetic acid), AFs, FBs, DON and ZEN from corn, further analysed by ultra-performance liquid chromatography (UPLC) coupled to MS/MS.

Clean-up methods

Solid-phase extraction

SPE is a technique commonly applied to solid matrices as purification and/or concentration step, after the extraction of mycotoxins (Pereira et al., 2014). Basically, the sample is passed through a cartridge or a disk filled with a solid sorbent where the analytes are adsorbed, then the undesirable compounds are washed and, at last, the analytes are eluted with solvents of different polarities (Pereira et al., 2014; Xie et al., 2016). There is a wide variety of solid phases commercially available and the choice depends on the matrix, the analytes, the interferences and on the solvent used to extract the analytes. Typically, reverse-phase materials (e.g., C18), strong cation or anion exchangers (SCX, SAX) or polymeric materials with combined properties are used in conventional SPE procedures (Pereira et al., 2014; Turner et al., 2015; Xie et al., 2016). This method has the great advantage of enabling the extraction, pre-concentration, and purification in one step without increasing the matrix content in the final extract (Xie et al., 2016). In addition, SPE is safe, efficient and reproducible. However, as it is not possible to use a single cartridge for all mycotoxins, it is a relatively expensive method and its performance can be affected by several conditions like pH, type of solvent and ionic strength of the sample (Pereira et al., 2014). For the analysis of FB_1 in soya bean meal and feed and T-2 in corn, (Abdou et al., 2016) developed a high performance liquid chromatography (HPLC) coupled to FLD (HPLC-FLD) in which the clean-up was performed with a Sep-Pak C18 column eluted with 15 mL of MeOH/ water (60:40 v/v). In an LC coupled to tandem MS (MS/MS) method (LC-MS/MS, this C18 reverse-phase SPE column was only used by (Chilaka, De Boevre, Atanda, & De Saeger, 2016) to determine FBs, DON and 15-AcDON, ZEN and its metabolites and HT-2 in maize. Relatively to SAX columns, they were merely employed to purify FBs and further detect them with HPLC-FLD, in soya bean seeds and processed soya bean powder (Egbuta, Mwanza, Phoku, Chilaka, & Dutton, 2016) and in maize (Guo et al., 2016; Kamala et al., 2016). Plus, for example, Grade Polypropylene Depth Hydrophilic-Lipophilic Balance (GPD HLB) SPE column was applied in UHPLC-MS/MS, by (Fan et al., 2016), to determine DON and its derivatives in feed after the extraction with MeCN 50 %.

Enhanced solid-phase extraction

Recently, new sorbent materials in the area of mycotoxin analysis have been developed and commercialized which are more selective for target molecules and provide higher recoveries (Pereira et al., 2014).

Immuno-affinity columns

Immuno-affinity columns (IACs) are a particular case of SPE, based on the principle of antigen-antibody interactions (Turner et al., 2015; Xie et al., 2016). So, the columns are formed by an activated solid phase support which has immobilised antibodies for a given mycotoxin or mycotoxin class (Pereira et al., 2014; Xie et al., 2016). When the extract passes through the column, the target mycotoxin acts as the antigen and binds selectively to the specific antibodies. A rising step removes the impurities and then a miscible solvent or the antibody denaturation elutes the mycotoxins (Pereira et al., 2014). IACs allow a highly selective purification, resulting in cleaner extracts with minimal interfering matrix components and low LOQ (Eva M. Binder, 2007; Xie et al., 2016). Although this is an automated sample clean-up method, it is time and solvent consuming, requires a high level of expertise and the use of expensive disposable cartridges (Xie et al., 2016). Moreover, in the presence of low concentrations of organic solvents, the denaturation of the antibodies is verified, which means that the extract must be an aqueous solution containing little or no organic solvent. Besides, there is the possibility of occurring non-specific interactions due to cross-reactivity with other mycotoxins (Pereira et al., 2014). Commercially available columns are mostly designed for only one type of toxin but nowadays, multiple IACs (mIACs) for the simultaneous determination of different types of mycotoxins are also accessible. However, this multiple format needs changes in development and format before being applied to a large number of mycotoxins (Pereira et al., 2014; Xie et al., 2016). IACs were broadly applied to clean extracts in HPLC methods for analysis of mycotoxins in raw feed ingredients and feed, in 2016. Differently, in multi-mycotoxins LC-MS/MS surveys, mIACs were just used by (Hu et al., 2016) in feed, and (Z. Zhang, Hu, Zhang, & Li, 2016) in corn and wheat.

Multifunctional columns

Multifunctional columns (MFCs) allow the performance of a one-step purification process where compounds, like proteins, fats, pigments, etc., that may interfere with the analytical method are retained in the solid phase, letting the analytes of interest to pass through the column, at the same time (Eva M. Binder, 2007; Pereira et al., 2014; Xie et al., 2016). MycoSep®/MultiSep® columns, suitable for mycotoxins, are filled by adsorbents such as charcoal, celite, ion-exchange resins, polymers, and other materials, packed into a plastic tube between two filter discs (Pereira et al., 2014). Commercially available there are many types of MFCs, whose choice depends on the matrix and also on the analytes (Pereira et al., 2014). Overall this is a simple and quick process because it does not require the washing and elution steps (Pereira et al., 2014; Xie et al., 2016). Plus, MFCs eliminate the problems of irreversible adsorption or premature elution of analytes from the sorbent material (Xie et

al., 2016). On the other hand, these are single-usage columns, which do not allow any sample concentration and the purification is not always effective, depending on the matrix (Pereira et al., 2014). In raw feed ingredients and feed analysis for mycotoxin contamination during 2016, MycoSep® 226 and 227 and MultiSep® 211 were the MFCs most used. For example, (Wu et al., 2016) applied MycoSep® 226 to clean extracts for the subsequent determination of AFB_1 in corn and by-products, wheat and by-products, soybean meal and diverse feeds with HPLC-FLD. MycoSep® 227 was used by (Hietaniemi et al., 2016) for TRCs analysis in wheat with a GC-MS method. Finally, (Kosicki et al., 2016) reported the employment of MultiSep® 211 column to purify maize and feed extracts to further quantify FBs with LC-MS/MS. Additionally, MycoSep® 224 and MycoSep® 225 columns were used for the determination of ZEN and DON, respectively, in wheat with HPLC coupled to diode array detection (DAD) (HPLC-DAD), by (Calori-Domingues et al., 2016).

Molecularly imprinted polymers

Molecularly imprinted polymers (MIPs) represent a purification method based on the chemical creation of simulated binding sites using a template molecule for the analytes of interest, in a cross-linked polymer matrix. The target molecule is retained as a result of the shape recognition (Pereira et al., 2014; Turner et al., 2015; Xie et al., 2016). This technique has some potential given its high selectivity and great stability to heating and pH shifts, being considered a cheaper alternative for clean-up (Pereira et al., 2014; Xie et al., 2016). However, can occur inconsistent molecular recognition, polymer swelling in unfavourable solvents, slow binding kinetics and potential sample contamination by template draining (Pereira et al., 2014). Furthermore, MIPs have remained as a research tool with little adoption by commercial companies. Their development and optimization require considerable time, which includes finding the best template molecule for imprinting and testing the resultant material in relevant applications (Shephard, 2016). (Q. Wang, Chen, et al., 2016) developed a solid-state electrochemiluminescence sensor that combined with MIP technique allowed ultrasensitive determination of OTA. This sensor was successfully applied to OTA determination in real corn samples, obtaining recoveries ranging from 88.0 % and 107.9 %.

Combined extractive/clean-up extraction

QuEChERS

The QuEChERS method, which means Quick, Easy, Cheap, Effective, Rugged, and Safe, even though it was not initially developed for the analysis of mycotoxins, it has been successfully applied with this objective (Dzuman, Zachariasova, et al., 2014; Turner et al., 2015). It involves a micro-scale extraction using MeCN, followed by a salting-out step of the

analytes into the MeCN phase and then a purification based on a quick dispersive SPE. Basically, in the extraction step, $MgSO_4$ and NaCl are used to reduce sample water, while in the purification step simple sorbent materials like primary secondary amine (PSA), C18 and alumina are used to retain the matrix and co-extracted compounds (Dzuman, Zachariasova, et al., 2014; Pereira et al., 2014; Turner et al., 2015). With the aim of ensuring an efficient extraction of mycotoxins, the original method may suffer some modifications as for example, changes in the salts used, in their quantity or in the amount of C18, or addition of formic acid to the extraction solvent, water or MeOH. Plus, in dried matrices, a swelling step with water is recommended to make samples more accessible to the extraction solvent (Pereira et al., 2014). QuEChERS is a versatile and easy to handle method that needs small amounts of organic solvents and allows the introduction of several modifications throughout the dispersive SPE step, to extend the range of mycotoxins to be analysed. However, this technique cannot be easily automated and usually achieves an enrichment factor very poor, leading sometimes to the need of an additional enrichment step (Pereira et al., 2014). Concerning the determination of mycotoxins by HPLC methods, in 2016, in raw feed ingredients and feed, just (Xu et al., 2016) applied a modified QuEChERS procedure to extract DON and its derivatives from wheat. The extraction was performed with water, MeCN and salts ($MgSO_4$ and NaCl), followed by the use of *n*-hexane to remove fat. Oasis® MAX SPE cartridge was used to clean-up the extract before the injection in the UHPLC-DAD system. This method allowed to obtain good recoveries, between 80.0 % and 102.2 %. In LC methods, QuEChERS technique was practiced more frequently and here are some examples. (Bryła et al., 2016) prepared wheat samples for multi-mycotoxins determination with UHPLC combined with high-resolution MS (HRMS), applying a modified QuEChERS procedure. Extraction solvent consisted of a mixture of water and 10 % formic acid in MeCN, to which $MgSO_4$, NaCl, sodium citrate dihydrate and sodium citrate dibasic sesquihydrate were added. Then, to eliminate the lipid faction, hexane was used. Finally, $MgSO_4$, C18 silica gel, neutral alumina, and PSA were added to perform clean-up. With (León, Pastor, & Yusà, 2016), which aimed multi-mycotoxins analysis in feed, a QuEChERS-based approach performed in one step was chosen. So, water along with MeCN containing 1 % acetic acid and $MgSO_4$, NaCl, sodium citrate and disodium citrate sesquihydrate were used. The extract was then analysed by a UHPLC-HRMS system. (Yan Wang et al., 2016) optimized QuEChERS to extract and purify AFs, FBs, DON and ZEN from corn before the analysis by UPLC-MS/MS. The authors concluded that the best choice was doing USE with MeCN/ water (84:16 v/v, containing 1 % acetic acid) without the addition of salt and purification with the cleaning agent C18.

Matrix solid-phase dispersion

Matrix solid-phase dispersion (MSPD) consists on mixing a small amount of sample with an abrasive solid support material that has been derivatized to produce a bound organic phase on its surface (SPE sorbent), using a mortar and a pestle. The resulting mixture is packed in a glass column or a cartridge and then the adsorbed residues are selectively eluted with a series of organic solvents (Pereira et al., 2014; Xie et al., 2016). This method uses small amounts of sorbent and solvents, reducing the cost and time of analysis. However, this method is difficult to automate and often an additional purification step is required, which might become time-consuming when a great number of samples need to be analysed (Pereira et al., 2014). Many factors influence the efficiency and selectivity of MSPD like the suitability of the sorbent, sample: sorbent ratio, solvents, and relative concentrations and properties of the analytes (Pereira et al., 2014; Xie et al., 2016). Sorbent choice depends on the polarity of the analyte and on the possible co-extracted components of the matrix (Pereira et al., 2014). Reverse-phase materials such as C18 and C8 are dispersion sorbents commonly applied, due to their lipophilic character that enables good disruption, dispersion, and retention of lipophilic species (Pereira et al., 2014). However, normal-phase, non-bonded sorbents like florisil, amino, phenyl, and silica have also been proposed as a dispersant in some MSPD applications (Xie et al., 2016; Ye, Lai, & Liu, 2013). According to (Ye et al., 2013), this technique is extensively applied to solid and semisolid samples for the extraction of drugs, pesticides, pollutants, among others. However, in mycotoxins quantification, MSPD is an unconventional alternative to classical SPE. In the field of analysis here reviewed, (Ye et al., 2013) developed a new simple and efficient MSPD procedure coupled to HPLC-DAD for the determination of FB_1 and FB_2 in corn. Various conditions were optimized, namely the type, volume and pH of the eluting solvent, the dispersion sorbent and the ratio of dispersing material to the matrix. They concluded that 10 mL of MeOH with 10 mM formic acid is the eluting solvent that provides better recoveries, with a C18 sorbent in a 2:1 ratio of sample:sorbent.

Special extraction techniques

These techniques do not need any additional purification step because the extraction procedure results in a clean and enriched extract, ready to be analysed (Pereira et al., 2014). In the matrices under review, from 2013 to 2016, dispersive liquid-liquid microextraction (DLLME) was the only special extraction technique employed.

Dispersive liquid-liquid micro-extraction

DLLME is a novel miniaturized extraction technique in which there is a rapid injection of a mixture of extraction solvent (organic) and dispersive solvent (water-organic miscible) into

an aqueous solution that contains the analytes. This leads to the formation of a cloudy solution and in consequence of the very large surface area formed between the two phases, the analytes are enriched rapidly and efficiently, in the extraction solvent. After centrifugation, they can be separated in the sedimented phase (Pereira et al., 2014; Xie et al., 2016). Although DLLME is more appropriate for aqueous samples, it is possible to apply this method to solid samples after an adequate pre-treatment (Pereira et al., 2014). Extraction efficiency is affected by many factors, like the type and volume of extraction solvent and of dispersive solvent, extraction time and effect of salt addition. Overall, DLLME is simple, rapid, efficient, inexpensive and environmentally safe since a very little volume of extraction solvent is needed (Pereira et al., 2014; Xie et al., 2016). Their limitations are related to the difficulty in automating, the necessity of using an aqueous solution containing the analytes (which in the case of solid matrices involves a previous extraction step) and with the low capacity to extract/concentrate polar analytes (Pereira et al., 2014). Additionally, the majority of the extraction solvents used in DLLME require the use of GC, due to incompatibility with HPLC systems (Pereira et al., 2014). A novel, rapid and efficient two-step micro-extraction technique, based on the combination of ionic-liquid-based DLLME (IL-DLLME) with magnetic SPE, was developed by (J. Zhao, Zhu, Jiao, Ning, & Yang, 2016), for the pre-concentration and separation of AFs in animal feedstuffs before HPLC-FLD. The ionic liquid extractant, 1-octyl-3-methylimidazolium hexafluorophosphate, was used in DLLME to extract AFs in the sample extracting solution medium. Then, hydrophobic pelargonic acid modified Fe_3O_4 magnetic nanoparticles were used as an efficient adsorbent to retrieve the AFs-containing ionic liquid from the DLLME step. Therefore, the target of the magnetic SPE was the ionic liquid instead of the mycotoxins. The authors compared the proposed method with other HPLC-FLD in which the clean-up was done with IAC and found no significant differences between data obtained by the two methods at the 95 % confidence level.

6.3.2. Separation and detection

A broad range of techniques can be used for this purpose and are generally divided into two categories: screening methods and chromatographic methods coupled to different detectors (Pereira et al., 2014; Xie et al., 2016). Currently, EU regulations do not require specific methods for the determination of mycotoxin levels, but any method of analysis should be characterised, whenever possible, by the criteria defined in Annex III of the Regulation (EC) No 882/2004 (The European Parliament and The Council of the European Union, 2004). Additionally, and although it is for the official control of the levels of mycotoxins in foodstuffs, Regulation No 401/2006 amended by Regulation No 519/2014 lays down, in the Annex II, the specific requirements that the method shall comply in relation

to individual mycotoxins (The Commission of the European Communities, 2006b; The European Commission, 2014).

Screening methods

Usually, screening assays are developed in the form of kits being extremely relevant tools for monitoring mycotoxin in feed ingredients and feed either by analysts for whom time is a constraint for decision making or by those for whom other methods may not be available due to cost or situation (Pereira et al., 2014; Shephard, 2016). These methods for single or whole mycotoxin classes compromise both qualitative tests that show the presence or absence of the target impurity and tests that yield semi-quantitative or quantitative results (Pereira et al., 2014; Venkataramana, Chandranayaka, Prakash, & Niranjana, 2014). Immunoassay-based methods, biosensors, and non-invasive techniques are among screening methods.

Immunoassay-based methods

Methods based on immunoassays are settled in the recognition of specific antibodies with mycotoxins that act like antigens (Pereira et al., 2014; Venkataramana et al., 2014). Detection is typically facilitated by the presence of a marker. This compound can be radioactive, chromogenic or fluorescent and reacts with an enzyme, generally horseradish peroxidase (HRP). Immunoassays without the marker are based on the natural fluorescence of some mycotoxins, or in measures of conductivity (Pereira et al., 2014). These tests are preferably employed for the first level screening and survey studies on mycotoxin contamination due to their simplicity, cheapness, sensitivity, and selectivity, although cross-reactivity with structural analogues can occur (Anfossi, Giovannoli, & Baggiani, 2016; Pereira et al., 2014). Plus, they do not require sophisticated equipment or skilled personnel (Venkataramana et al., 2014). However, the signal obtained from these techniques can be influenced by co-extractives and non-specific interactions or matrix effects (Shephard, 2016). Additionally, in the new scenario of mycotoxin investigation, immunoassay-based methods may have a potential limitation related to the overall selectivity for only one mycotoxin or a small group of compounds, making difficult the simultaneous determination of different compounds and the detection of unknown toxins and conjugated mycotoxins (Anfossi et al., 2016; Pereira et al., 2014). Nevertheless, these methods are in continuous development in various formats, aiming to provide rapid, portable and easy to operate systems (Anfossi et al., 2016). Enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay (LFIA) and fluorescence polarization immunoassay (FPIA) are included in this category of screening methods (Pereira et al., 2014; Shephard, 2016).

Enzyme-linked immunosorbent assay

ELISA methods represent a commonly used immunoassay method to rapidly monitor mycotoxins, being routinely used by agro-food laboratories (Dzuman, Zachariasova, et al., 2014; Pereira et al., 2014; Xie et al., 2016). For all regulated mycotoxins are commercially available ELISA microtiter plate kits that have well-defined applicability, analytical range, and validation criteria (Anfossi et al., 2016; Venkataramana et al., 2014; Xie et al., 2016). There are several ELISA formats commonly accessible, however in this field of analysis the predominant form is the competitive one. This is a strategy normally used when the antigen is small and has only one antibody binding site (epitope), which is the case of mycotoxins (R-Biopharm AG, 2016a; Thermo Fisher Scientific, n.d.; Xie et al., 2016). The competitive format is characterised by the fact that the signal intensity is inversely correlated with the concentration of antigen in the sample (Bio-Rad Laboratories, 2017; Robinson & Pellenz, 2015). Within this format type, it is possible to distinguish the classical competitive ELISA and the competitive inhibition ELISA (Robinson & Pellenz, 2015). Classical competitive format consists in the immobilization of the antigen standard on the surface of the plate. Then, there is an incubation of the antibodies directed against the target mycotoxin with the sample. The antigens in the sample will compete with the immobilized ones for binding to these antibodies. After the washing step, the antibodies bounded to the analyte are rinsed away (Robinson & Pellenz, 2015). In this case, detection can be performed directly or indirectly, which mainly determines the sensitivity of an ELISA. Direct detection uses a enzyme-labelled primary antibody that reacts with the antigen, while a enzyme-labelled secondary antibody with affinity for the primary antibody is used in indirect detection (Thermo Fisher Scientific, n.d.). In the competitive inhibition format, the competition occurs between unlabelled antigens from samples and enzyme-labelled antigens (enzyme conjugate) for binding to an antibody directed against the target mycotoxin. In this format, the plate can be coated with capture antibodies with affinity for the analyte or for a primary antibody (Robinson & Pellenz, 2015; Thermo Fisher Scientific, n.d.). Common to both types of competitive assays is the addition of an adequate substrate that is allowed to incubate so that the enzyme conjugated with antibody or antigen (classical or inhibition format, respectively) can act and produce changes in a given parameter (Bio-Rad Laboratories, 2017; R-Biopharm AG, 2016a; Thermo Fisher Scientific, n.d.). A large variety of substrates are available, and the choice depends upon the required assay sensitivity and the instrumentation available for signal-detection, although a mixture of hydrogen peroxide and a chromogen are usually applied (R-Biopharm AG, 2016a; Thermo Fisher Scientific, n.d.). Indeed, the simplest detection is a visual colour change which provides qualitative and semi-quantitative results (Pereira et al., 2014). The last step of all assays is the addition of a stop solution causing the reaction between the enzyme and the substrate to stop. The

results are usually determined in a plate reader. The signal intensity weakens as the sample antigen concentration increases, since a larger quantity of analyte results in: fewer enzyme-labelled antibodies bound to the antigen adsorbed to the plate (classical format); or less enzyme-labelled antigens bound to the antibody on the plate (Bio-Rad Laboratories, 2017; R-Biopharm AG, 2016a; Robinson & Pellenz, 2015). Advantages of ELISA include, in addition to the specificity of antibody-antigen binding, a relatively low limit of detection (LOD), high sample throughput with low sample volume and minimal clean-up procedures, and ease of application (Venkataramana et al., 2014; Xie et al., 2016). However, this method is not so reliable in the case of complex matrices, is quite time-consuming and the kits are for single use and are not suitable for field-testing (Pereira et al., 2014; Turner et al., 2015; Venkataramana et al., 2014; Xie et al., 2016). Besides, the possibility of false positive and false negative results requires additional confirmatory analysis (Venkataramana et al., 2014; Xie et al., 2016). From **Table A.1** (see **Appendix**), where ELISA methods from articles published in 2016 in mycotoxin field are reviewed, it is possible to conclude that all analytes were quantified with competitive ELISA after SLE mainly with an aqueous solution of MeOH or with water. Additionally, absorbance was the detection method most used, followed by optical density (OD), while FLD was only used by (Liang, Huang, Yu, Zhou, & Xiong, 2016) to detect OTA in corn. Regarding mycotoxins studied with ELISA, the more targeted were AFs and DON.

Lateral flow immunoassay

LFIA or membrane-based test strips are commercially available in the form of kits providing mainly visual qualitative results that indicate the presence or absence of a specific mycotoxin below a predetermined fixed level (AHDB Beef & Lamb, 2016; Pereira et al., 2014). More recently, semi-quantitative detection is possible using a portable photometric strip reader (Shephard, 2016). In LFIA, the sample flows along the strip by capillary migration and two lines are formed, the test line, whose intensity is inversely correlated to the mycotoxin concentration and the control line that allows the assay validation (Pereira et al., 2014; Venkataramana et al., 2014). This is an inexpensive screening tool that enables rapid, one-step and *in situ* analysis (Pereira et al., 2014; Venkataramana et al., 2014; Xie et al., 2016). Nonetheless, LFIA show often false positive results due to matrix interferences and reproducibility and sensitivity problems (Pereira et al., 2014; Venkataramana et al., 2014). In the field of analysis here reviewed, various authors utilized this methodology during the year of 2016 and some examples are presented. (Y. Chen et al., 2016) developed and optimized a multiplex LFIA for the simultaneous on-site determination of AFB_1, ZEN, and OTA in corn. This device provided both qualitative and quantitative results. For visual detection, LOD was 10 µg/kg for AFB_1, 50 µg/kg for ZEN and 15 µg/kg for OTA and for

results judged with a strip reader LOD were 0.10 µg/kg, 0.42 µg/kg and 0.19 µg/kg, respectively. LFIA was also used by (Carvalho et al., 2016) to evaluate mycotoxin presence in corn silages. FM, DON, AF, OTA, ZEN, and T-2/HT-2 were quantified with Reveal Q+ kits from Neogen Corporation. LOD are 0.3 - 6.0 mg/kg to FM and DON, 2.0 - 150.0 µg/kg to AF, 2.0 - 20.0 µg/kg to OTA, 25.0 - 1200.0 µg/kg to ZEN and 50 - 600 µg/kg to T-2/HT-2.

Fluorescence polarization immunoassay

FPIA indirectly measures the rate of rotation of a fluorophore (tracer) in solution based on the competition between the free mycotoxin on the sample and the mycotoxin labelled with the tracer towards a specific antibody. When tracers bound to the antibodies their rotation is restricted and, consequently, fluorescence polarization value increases. So, if a sample has a high concentration in the target mycotoxin it competes with the tracer for the interaction with the antibody resulting in free tracers with a faster motion, i.e., a low fluorescence polarization signal. Basically, this value is inversely proportional to the amount of free mycotoxin in the sample. FPIA is reliable, rapid, easy to perform and relatively suitable for automation but their solution-based nature turns it less easy to use in field scenarios (Pereira et al., 2014; Porricelli et al., 2016; Venkataramana et al., 2014). Concerning mycotoxin analysis in raw feed ingredients and feed, (Chenglong Li et al., 2016) developed a homologous and high-throughput multi-wavelength FPIA for the multiplexed detection of DON, T-2 and FB1 in maize flour with a LOD of 242.0 µg/kg, 17.8 µg/kg, and 331.5 µg/kg, respectively.

Biosensors and biosensor-based methods

Biosensors or immuno-sensors are analytical devices composed by one antibody as recognition element that reacts in a sensitivity and selectivity way towards the target mycotoxin and by a transducing element, which is responsible to convert the change of the physical variable produced by the reaction into a measurable signal (Pereira et al., 2014; Venkataramana et al., 2014). In fact, antibodies are the most widely used recognition element in sensors but there is an extensive range of other of these components (Lin & Guo, 2016; Turner et al., 2015). Alternatives to this classical element include, among others, enzymes, peptides, aptamers, and MIPs (Turner et al., 2015). Similarly, various transducing elements are available comprising techniques commonly applied with an optical or electrochemical nature along with piezoelectric and magnetic systems (Lin & Guo, 2016). Optical detectors can be based on surface plasmon resonance, fluorescence, optical waveguide light mode spectroscopy or total internal reflection ellipsometry. Electrochemical detectors are based on potentiometry with a carbon working electrode, differential pulse

voltammetry, conductometry, etc (Pereira et al., 2014). These methods are very promising since they provide results in a faster way, have a low price, high-throughput, greater sensitivity and are portable (Pereira et al., 2014; Turner et al., 2015; Venkataramana et al., 2014). However, they rely on specialist analytical equipment and their low selectivity and reproducibility make it necessary to confirm the results (Pereira et al., 2014; Turner et al., 2015). Plus, their applicability to routine analysis need to be further investigated (Pereira et al., 2014). Several authors developed biosensors and biosensors-based methods for mycotoxin analysis in raw feed ingredients and feed, during 2016. For example, electrochemical immunosensors were designed by (Ma et al., 2016; X. Zhang et al., 2016) to determine AFB_1 in maize and by (Lu, Seenivasan, Wang, Yu, & Gunasekaran, 2016) for FB_1 and DON determination in the same matrix. (Plotan et al., 2016) applied innovatively biochip array technology to multi-mycotoxin semi-quantitative screening in a large variety of feed ingredients, obtaining an overall average recovery of 104 %. An optical aptasensor was developed based on hybridization chain reaction amplification strategy and fluorescent perylene probe/ DNA composites, by (B. Wang, Wu, et al., 2016), for ultrasensitive detection of OTA. The application to corn samples demonstrated the feasibility and potential of the proposed enzyme-free amplification method, in the practical applications of agricultural products. (B. Wang, Chen, et al., 2016) developed a novel and ultrasensitive aptamer-based biosensor for the detection of AFB_1, in corn. For this, fluorescent nitrogen-doped carbon dots were synthesized and assembled on aptamer modified gold nanoparticles.

Non-invasive methods

Some non-invasive methods have been developed to measure mycotoxin contamination allowing simple, rapid and *in situ* analysis. Thus, it enables to promptly make decisions and avoid possible loss of an entire lot. However, due to high matrix dependence and lack of appropriate calibration materials, the application of this methods is still limited. The non-destructive approach includes infrared spectroscopy (IR) techniques and Raman spectroscopy (Pereira et al., 2014; Xie et al., 2016).

Infrared spectroscopy

Promising IR techniques include near-infrared (NIR) spectroscopy in combination or not with Fourier-transform (FT-NIR). Basically, NIR spectroscopy is based on the measurement of the absorption or reflectance of a given incident NIR radiation in the sample. The exposition to radiation in this region of the spectrum causes a change in the energy of chemical bonds involving hydrogen (for example, C-H, N-H, O-H, and S-H). However, the bands observed in NIR spectral region are very difficult to assign to specific compounds because of the complexity of the samples and also due to spectra overlapping and

interference from other functional chemical groups. This implies the application of modern chemometrics methods in the calibration development process. The detection of the NIR radiation absorbed by the sample is conducted by transmittance, reflectance, interaction and/or transflectance measurement (Pereira et al., 2014; Xie et al., 2016). This promising technique requires minimal or no sample pre-treatment and is environmentally friendly so that it does not require reagents nor produces chemical waste (Girolamo, Cervellieri, Visconti, & Pascale, 2014; Xie et al., 2016). Plus, NIR is highly accurate, needs little expert training and has the ability to analyse both large and small quantities of feeds, which avoids errors associated with inconsistent sampling (Coufal-Majewski et al., 2016). Beyond the difficulties in interpretation of spectral data posed by this technique, other drawbacks are related with the fact that NIR is only useful at high contamination levels as well as the system is heavily dependent on the establishment of an accurate calibration procedure (Coufal-Majewski et al., 2016; K. Lee, Herrman, Nansen, & Yun, 2013; Pereira et al., 2014). A non-destructive detection of DON by ultraviolet-visible-near infrared diffuse reflection spectroscopy in unprocessed, solid maize kernels was investigated by (Smeesters, Meulebroeck, Raeymaekers, & Thienpont, 2016). They proposed a two-stage measurement methodology, enabling to efficiently monitor the local DON-contamination on a large number of maize kernels. Plus, (Kos et al., 2016) presented a novel chemometric classification for FTIR spectra of mycotoxin-contaminated maize at regulatory limits. They investigated the classification ability of a decision tree at 1750 µg/kg for DON in maize, which corresponds to the regulatory limit set by the EU for unprocessed maize, in food.

Raman spectroscopy

The principle behind Raman spectroscopy relies on the irradiation of a substance with monochromatic light to further detect the loss of energy in the form of scattered light. Thus, information about the vibrational transition energy of the molecules is provided by this technique. Symmetrical vibrations of the covalent bonds in non-polar groups (e.g., C=C) enhance the sensitivity of Raman spectroscopy (K. Lee et al., 2013; K. M. Lee, Herrman, & Yun, 2014; Mignani et al., 2016). This method provides a unique expression of the molecular structure and so, it is considered to be as a molecular fingerprint, providing more useful qualitative and quantitative information on chemical functional groups of mycotoxin compounds and its derivatives than the conventional spectroscopic techniques (K. M. Lee et al., 2014; Mignani et al., 2016). Despite this advantages, Raman spectroscopy has received remarkably little attention for detection of mycotoxins in grains and oilseed (K. M. Lee et al., 2014). In 2016, (K.-M. Lee & Herrman, 2016) investigated the potential and feasibility of a surface-enhanced Raman spectroscopy (SERS) method as an alternative accelerated technique to screen ground maize contaminated with FMs. Chemometric

models developed based on SERS spectra showed an acceptable predictive performance and ability for qualitative and quantitative analysis.

Chromatographic methods

Chromatographic separation coupled with a suitable detection system is a widely used strategy to analyse quantitatively mycotoxin contamination, to confirm unambiguously positive findings and also to serve as a reference method to validate other tests. These are methods highly selective, accurate and reproducible that need expensive instrumentation and expertise in the field of chromatography. In analysis of feed, LC is the most common method, though GC and thin layer chromatography (TLC) are still considered (Anfossi et al., 2016; Venkataramana et al., 2014; Xie et al., 2016).

Thin layer chromatography

Contrary to what happens in developed countries, TLC is a method that is still commonly used in countries under development, especially if coupled to an ultraviolet (UV) or fluorescence scanner (Shephard, 2016; Xie et al., 2016). TLC allows qualitative, quantitative and semi-quantitative determination of naturally fluorescent mycotoxins. The qualitative confirmation can be done through the retention factor value and the fluorescence colour, after comparison with an external standard. In semi-quantification, the sample is compared with authentic standards using the visual estimation of fluorescence of the separated spots under long wavelength UV light. So, with this approach, results in precision and reliability depend directly on skilled and experienced persons. Quantification is mainly achieved by measuring fluorescence intensity or absorbance when separated spots on the TLC plate are exposed to UV light. TLC can be applied both in one- and two-dimensional format. This method provides the possibility of rapidly analyse several samples in a short period of time, has a low cost per sample analysed and it is easy to estimate contamination levels (Xie et al., 2016). However, low sensitivity and reproducibility along with the need of large quantities of solvent, intensive laboratory procedures and difficulties in automation have led TLC to be commonly replaced by other chromatographic techniques (Turner et al., 2015; Xie et al., 2016). In 2016, (Betancourt & Denise, 2016) applied this method to screen AFs contamination in corn hybrids. TLC plates were exposed to UV light at a short wavelength (250 nm) and visual comparison to standards allowed the identification of positive samples. (Mona, Mona, & Nagwa, 2016) performed AFB_1 detection in cattle feed with TLC, where standard and test samples were inspected under long wave UV lamp (360 nm).

Gas chromatography

In GC, volatile compounds are separated in capillary columns with a variety of general or specific detectors. GC coupled with an MS detector (GC-MS) simultaneously allows the identification and quantification of compounds and, for these reasons, is preferably used in mycotoxin analysis (Pereira et al., 2014; Sigma-Aldrich, 2011). This methodology is applicable almost exclusively to *Fusarium* toxins (Anfossi et al., 2016). Actually, in 2016, (Bernhoft, Christensen, & Sandvik, 2016; Buśko et al., 2016; Hietaniemi et al., 2016), after extraction with MeCN and clean-up with MFCs, used this technique to detect TRCs mainly in wheat, (**Appendix**, **Table A.2**). GC-MS can be done by electron impact (EI) or chemical ionization either on positive (PCI) or negative mode (NCI) (Pereira et al., 2014). Advantages of GC are related to resolution and sensitivity but this method has several limitations (Pereira et al., 2014; Turner et al., 2015). They include the risk of contamination and column blockage, the degradation problems that sometimes result from the use of hot injection devices and the necessity of a derivatization step prior to analysis (Pereira et al., 2014; Turner et al., 2015; Venkataramana et al., 2014). This procedure aims to counteract the low volatility and the high polarity of many mycotoxins, allowing, therefore, their analysis. Silylation and acylation reactions are the most common approaches, converting mycotoxins in more volatile, less polar and thermally more stable derivatives. In silylation, the introduction of a silyl group by a silyl reagent is valuable for MS applications because it produces either more interesting diagnostic fragments or characteristic ions used for single ion monitoring (SIM). Alternatively, acylation is preferable when acylated compounds are more stable than silylated ones (Pereira et al., 2014; Sigma-Aldrich, 2011). In 2016, silylation was the derivatization method majorly applied, when detecting mycotoxins with GC-MS (**Appendix**, **Table A.2**).

Liquid chromatography

Liquid chromatographic methods are the mainstay separation of mycotoxin analysis. Several variations of LC are available offering good sensitivity, high dynamic range, and versatility. On the other hand, these methods suffer from portability, cost, and issues related to the sample type like the matrix effect, the choice of calibration and sample preparation (Turner et al., 2015; Xie et al., 2016).

HPLC is a well-established and prevalent method for identification and quantification of mycotoxins (Venkataramana et al., 2014). To date, both the normal-phase and reverse-phase columns have been used for this purpose. However, the great majority of separations are performed on reverse-phase columns because their manipulation is easier and watery mobile phases are less toxic. This HPLC procedure relies mostly on C18 columns and mobile phases composed of water, MeOH and MeCN mixtures in the proper ratios

(Shephard, 2016; Xie et al., 2016). HPLC has high separation power, is easy to use and suitable for automation (Xie et al., 2016). Traditionally, this chromatographic method is equipped with spectrometric detectors like UV (HPLC-UV) and fluorescence that depend on sample extract purification before analysis. These cheaper and mature techniques are usually optimized for a single analyte or a chemical group of analytes (Pereira et al., 2014; Shephard, 2016; Xie et al., 2016). From **Table A.3** (see **Appendix**), it is possible to see that HPLC-UV was left behind once only (Liu et al., 2016; Rao, Girisham, & Reddy, 2016; L. Wang, Shao, et al., 2016; Wu et al., 2016) applied this technique to quantify DON, ZEN and OTA in raw feed ingredients and feed. On the contrary, FLD was abundantly used in 2016, after SLE mostly with MeOH and clean-up by IACs, to analyse mainly AFs but also FBs, T-2, ZEN and OTA in those matrices. Commonly, pre- or post-column derivatization methods are used to improve mycotoxins fluorescence properties and consequently increase sensitivity. In the pre-column approach, trifluoroacetic acid is majorly applied, converting AFs in their corresponding hemiacetals derivatives which have stronger fluorescence. However, since this is a toxic and corrosive chemical and the derivatives formed have relative instabilities, this is not the preferred method. Additionally, post-column derivatization offers the advantage of being automated (Xie et al., 2016). Therefore, this strategy was more applied in 2016 to detect mycotoxins (**Appendix**, **Table A.3**). Different methods can be used such as bromination by an electrochemical cell (Kobra Cell), the addition of bromide or pyridinium hydrobromide perbromide and the formation of an iodine derivative. Although these post-column derivatization approaches produce molecules more fluorescent than their precursors, the use of bromine or iodine requires extra pumps and chemical reactors on the HPLC system and a long time to prepare the mobile phase. The use of post-column photochemical reactors is a novel derivatization methodology where the outlet of the HPLC is simply connected to ultraviolet permeable polytetrafluoroethylene tubing and wrapped over a high-intensity UV lamp. Stable and highly fluorescent derivatives are yielded from the reaction of mycotoxins with hydroxyl radicals from water, generated from UV light irradiation. This alternative technique is simple, the response is linear, has reproducibility and does not require chemical reagents, additional pumps or electrochemical cells being more economical than the conventional post-column derivatization (Xie et al., 2016). In 2016, (M. Lee et al., 2016) applied photochemical derivatization to enhance AFs, OTA and ZEN fluorescence in feed, (Ok, Jung, Lee, Peak, & Chun, 2016) used it to increase this property in AFs present in corn and (Wu et al., 2016) applied it to detect AFB1 in feed and raw feed ingredients (**Appendix**, **Table A.3**).

Recently, HPLC-DAD techniques arise but they are incapable of dealing with a large number of analytes in complicated samples (Xie et al., 2016). In 2016, (Calori-Domingues

et al., 2016) used this technique to quantify DON and ZEN in wheat, (Kim et al., 2016) determined DON and 3-AcDON in corn and feed, (Savi, Piacentini, Tibola, et al., 2016; Trombete et al., 2016) applied the method to detect DON in wheat and their by-products and DON and derivatives were quantified by UHPLC-DAD, by (Xu et al., 2016) (**Appendix**, **Table A.3**).

UHPLC/UPLC methods have been newly introduced. Columns filled with uniform particles of small size and instruments with high-pressure fluidic modules are used. This rising technique allows decreasing the run times and the solvent consumption resulting in more efficient chromatographic separations with higher sensitivity and resolution (Pereira et al., 2014; Shephard, 2016; Xie et al., 2016). In 2016, UHPLC/UPLC was exploited by (Bryła et al., 2016; Cogan et al., 2016; Degraeve et al., 2016; Dzuman et al., 2016; Fan et al., 2016; Jedziniak, Pietruszka, & Burek, 2016; Jong et al., 2016; León et al., 2016; Tibola, Fernandes, & Guarienti, 2016; Y. Wang, Dong, et al., 2016; Xu et al., 2016) to detect mycotoxins in feed and raw ingredients for feed (**Appendix**, **Table A.3** and **Table A.4**).

LC can be coupled to MS (LC-MS) or to MS/MS, which occurs via atmospheric pressure ionization (API) techniques such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI). This has resulted in a very versatile analytical tool whose applications include not only single mycotoxin analysis but, most importantly, true multi-mycotoxin determination (Pereira et al., 2014; Shephard, 2016; Xie et al., 2016). This is the current trend in this field since commodities can be contaminated with more than one mycotoxin, as discussed earlier.

Relatively to API methods, ESI is mostly well suited for the analysis of polar compounds, APPI is highly effective for the analysis of medium- and low-polar substances and APCI is often more sensitive when the majority of polar functional groups are of moderate polarity (Boyd, Basic, & Bethem, 2008; Pereira et al., 2014). In **Table A.4** (see **Appendix**), where are reviewed LC-MS methods, applied in 2016, in mycotoxins analysis in feed and raw feed ingredients, it can be seen that the vast majority used ESI interface in multi-mycotoxins applications. However, APCI and APPI methods have usually better performances in terms of chemical noise and signal suppression then ESI, despite being less used (Boyd et al., 2008). APCI is normally applied only to mycotoxins of the TRCs group, although its feasibility has also been examined in a few multi-mycotoxin methods (Pereira et al., 2014). Actually, (Hofgaard et al., 2016) employed this interface to quantify not only TRCs but also ZEN and FBs in wheat. Nowadays, most of the instruments offer combined interfaces (ESI/APCI) which have a compromised sensitivity between both modes but offer the main advantage of enabling the detection of polar and non-polar analytes in a single run (Pereira

et al., 2014). Normally, as a consequence of API, can be produced with protonated or deprotonated molecules (Pereira et al., 2014). With respect to ESI and mycotoxins, the protonated precursor ions are mainly formed, but additional information can be found in (Beltrán, Ibáñez, Sancho, & Hernández, 2009; Sulyok, Krska, & Schuhmacher, 2007; R.-G. Wang et al., 2015; Z. Zhao et al., 2015). In LC-MS/MS, the ionization process may have some problems and the analytical signal is unpredictably affected by the matrix effects. So, the use of isotope-labelled internal standards (IS), that are not naturally occurring in the samples and have identical chemical properties to the analytes, will compensate for both losses during the sample pre-treatment steps and for ion suppression or enhancement effects in the ion source. Despite being the best approach, these standards are only available for a limited number of mycotoxins and are very expensive (Åberg, Solyakov, & Bondesson, 2013; Pereira et al., 2014).

LC system can be combined with a single quadrupole, an ion trap (IT), a triple quadrupole (QqQ) or with a hybrid quadrupole/linear ion trap detector (QTRAP) (Pereira et al., 2014; Shephard, 2009). LC-MS/MS is enabled by QqQ and QTRAP (Boyd et al., 2008). As can be seen in **Table A.4** (see **Appendix**), QqQ instruments surpassed by far the remaining analysers, maybe due to improved signal to noise ratios from the additional selectivity of the second MS step (Boyd et al., 2008). In this field of analysis, IT was only used by (Kovalsky et al., 2016) to detect multi-mycotoxins in finished feed, maize and maize silage while (Dzuman et al., 2016; Hofgaard et al., 2016; Mngqawa et al., 2016) used QTRAP.

HRMS can be performed using time-of-flight (TOF) and Orbitrap analysers, that have a high mass accuracy (1 - 2 mg/kg), high resolving power (up to 200,000) and high dynamic range (about 5000) (León et al., 2016; Pereira et al., 2014). These instruments even operating in full scan mode are able to provide high sensibility, which makes easier the identification of analytes even when present at very low levels. Additionally, they have rapid spectral acquisition speed, allowing to record a virtually unlimited number of compounds (Pereira et al., 2014). Nevertheless, HRMS was not the preferred strategy in the quantitative analysis of mycotoxins in feed and raw feed ingredients during the year of 2016 (**Appendix**, **Table A.4**). Between the authors that chose these detectors, TOF was more frequently applied than Orbitrap, despite the known advantage of this last detector to screen unknown compounds in full scan mode, in parallel to the quantification of known analytes (Herebian, Zühlke, Lamshöft, & Spiteller, 2009).

Relying on the strengths of the exceptional sensitivity and separation capabilities of the modern LC-MS equipment, “Dilute and Shoot” (DaS) methods have been developed (Turner et al., 2015). They rely on sample dilution followed by a direct injection avoiding the

clean-up stage, which limits the potential loss of analytes. Besides, this is a rapid method that covers a wide range of polarities and so allows a wide range of mycotoxins and other secondary metabolites to be determined. On the negative side, DaS has the risk of having excessive and unpredictable interference from matrix which is a limitation as it can potentially overwhelm the sensitivity of the instrument (León et al., 2016; Shephard et al., 2013; Turner et al., 2015; Xie et al., 2016). For example, (Abia et al., 2013) applied the DaS technique to determine multiple mycotoxins occurrence in poultry feeds from Cameron.

MS detectors offer different advantages like exquisite sensitivity, robustness, information on chemical structure and specificity based on the mass-to-charge ratio (m/z). Additionally, a mass spectrum provides an ideal confirmatory technique. However, these methodologies require the expertise of professionals and time and labour-intensive sample pre-treatment steps (Shephard, 2016; Venkataramana et al., 2014; Xie et al., 2016).

Concerning ELISA and chromatographic methods applied in 2016 to determine mycotoxin contamination in feed and in the main feed raw ingredients, it can be seen in **Figure A.1** (see **Appendix**), that although chromatographic techniques coupled to classical detectors (UV and FLD) were expected to fall into disuse to be replaced by newer and sophisticated MS detectors, this scenario was not confirmed. In fact, LC-MS methods along with HPLC were substantially used in mycotoxins analysis in feed and raw feed ingredients here reviewed, during the year of 2016. Right after, came the application of ELISA methods.

7. Validation and verification of testing methods[1]

An objective evidence that a method fulfils the requirements for its intended application, meaning that it is fit for purpose, is established by validation (European Commission, 2015; National Association of Testing Authorities, 2013). This process is an essential component of measures and allows to assess the quality and the reliability of analytical results (Kirilov, Đokić, & Popov, 2013; Thompson, Ellison, & Wood, 2002). Different validation protocols have been established worldwide, for example by standardisation bodies and recognised technical organisations, since different methods have distinct requirements. Therefore, relevant guidelines to a sector should be consulted and followed, when applicable (Eurachem, 2014; National Association of Testing Authorities, 2013). Usually, typical validation parameters include working range, sensitivity, linearity (when applicable), selectivity, LOD and LOQ, precision (repeatability, intermediate precision and

[1] All the terms and definitions here applied, relating to this topic, generally followed the International Vocabulary of Metrology (Joint Committee For Guides In Metrology, 2012).

reproducibility), trueness (bias and recovery), robustness and measurement uncertainty (Eurachem, 2014; Shabir, n.d.; Thompson et al., 2002). Moreover, participation in a collaborative study may be required for official method recognition (Eurachem, 2014). Essentially, it should be ensured that validation studies are representative, in a way that the concentration ranges and matrix classes within the scope of the method are, as far as possible, conveniently covered (Thompson et al., 2002).

However, it may not always be practical or necessary to perform a full validation protocol, also because this process is quite a time consuming and expensive (Andreasson et al., 2015; National Association of Testing Authorities, 2013; Thompson et al., 2002). Therefore, the status of the method under consideration and the requirements for its proposed application will determine the extent to which a laboratory has to undertake validation (National Association of Testing Authorities, 2013; Thompson et al., 2002). A good example is the case of standard methods and commercial test kits, where basic validation work has already been carried out. Thus, the end-user laboratory only needs to verify that their analysts using their equipment in their laboratory environment have the ability to apply the method, obtaining the stated performance characteristics (Eurachem, 2014; National Association of Testing Authorities, 2013). Generally, verification studies of quantitative methods should cover precision and trueness, even because these are specific parameters for each facility that performs a method (AOAC INTERNATIONAL, 2007; Eurachem, 2014; National Association of Testing Authorities, 2013). Additionally, in trace analysis, laboratories should confirm if the achievable LOD and LOQ are fit for purpose (National Association of Testing Authorities, 2013). With respect to immutable performance characteristics, such as linearity, it does not need to be included in method verification (AOAC INTERNATIONAL, 2007). Plus, when the method is to be used within its scope and applied to samples identical to those for which it is validated, usually, there is no demand to verify its selectivity and robustness (AOAC INTERNATIONAL, 2007; Eurachem, 2014).

In the field of mycotoxins determination in feed, there is no specific directive or guidance document for the validation or verification of testing methods. However, in Regulation (EC) No 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules (The European Parliament and The Council of the European Union, 2004) it is defined that “whenever possible methods of analysis should be characterised by the following criteria: accuracy; applicability (matrix and concentration range); limit of detection; limit of determination; precision; repeatability; reproducibility; recovery; selectivity; sensitivity; linearity; measurement uncertainty and other criteria that may be selected as required”. Additionally, and although for the official control of the levels of mycotoxins in foodstuffs, Commission Regulation (EU) No 519/2014

(The European Commission, 2014) establishes the specific requirements with which the screening and confirmatory methods of analysis have to comply with for regulatory purposes assessment. Specifically, concerning screening methods, like ELISA, they can just be applied for a screening purpose, testing compliance with regulatory limits. In this way, the analysed samples are just classified either into “negative” or “suspect” in comparison with a screening target concentration, i.e., the concentration of interest for the detection of the analyte in a sample (The European Commission, 2014; von Holst & Stroka, 2014). Therefore, (The European Commission, 2014) foresees the determination of the cut-off level and the rate of false negative and false suspect results, to demonstrate the fitness-for-purpose of the screening method. The cut-off value, i.e., the threshold obtained with the screening method above which a sample is classified as suspect, with a false negative rate of 5 %, ends up reflecting the precision of the test (Lattanzio, Ciasca, Powers, & Holst, 2016; The European Commission, 2014). Plus, a low rate of false negative results is an indication of sufficient sensitivity and an adequate selectivity avoids false positive results. These parameters address, respectively, the safety of the screening method and the economic benefit of their application, because false positive results require the re-analysis with confirmatory methods, to check if the suspect samples are, effectively, non-compliant (Lattanzio et al., 2016; von Holst & Stroka, 2014).

8. Aims

The present study aimed at evaluating the performance of the commercially available ELISA kit for AFB_1 detection, RIDASCREEN® Aflatoxin B_1 30/15, in corn samples intended for livestock feed, since the quality of these methods usually varies. Therefore, an internal verification protocol was developed and applied to complement the information provided by the manufacturer and ensuring data quality in the end-user laboratory. The method was comprehensively evaluated according to LOD, LOQ, precision, trueness, and measurement uncertainty. Additionally, cut-off level and rate of false suspect results were estimated to demonstrate the applicability of this ELISA test to screen the presence of AFB_1 at the maximum permitted level of 20 µg/kg in feed materials intended for livestock feed, in EU. Finally, several real corn samples were subjected to analysis by RIDASCREEN® Aflatoxin B_1 30/15 kit and comparison of the results with those obtained with an LC-MS/MS method was made. The multiple mycotoxin contamination in the corn samples was likewise evaluated.

MATERIALS AND METHODS

1. Reagents and chemicals

Concerning ELISA experiments:

Analytical grade MeOH (CARLO ERBA Reagents) and qualitative filter paper 1300/80 (FILTER-LAB®) were supplied by Moreira da Costa & Santos (Porto, Portugal). Deionized water, AnalaR NORMAPUR® ISO 3696:1995, grade 3, was provided by VWR (Radnor, PA, USA).

Two lots of the commercially available kit RIDASCREEN® Aflatoxin B1 30/15 (Art. No. R1211), produced in R-Biopharm AG (Darmstadt, Germany) and purchased from Ambifood® (Porto, Portugal) were used in this study.

All the glassware exposed to mycotoxins was soaked in a bleach solution overnight before being washed.

Regarding LC-MS/MS method:

Mycotoxins standards namely, DON (1 mg, purity 95 %), FB_1 (1 mg, purity 98 %), T-2 (1 mg, purity 95 %), OTA (1 mg, purity 97 %), ZEN (1 mg, purity 98 %), and OTA-d_5 (0.5 mg, purity 95 %) were purchased from Toronto Research Chemicals (Toronto North York, ON, Canada). AFB_1 (1 mg, purity > 98 %), AFB_2 (1 mg, purity > 98 %), AFG_1 (1 mg, purity > 98 %), AFG_2 (1 mg, purity > 98 %), HT-2 (1 mg, purity > 98 %), 3-AcDON (1 mg, > 98 %) and 15-AcDON (2 mL, 100 µg/mL solution) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

MeCN, MeOH, acetic and formic acids were of HPLC grade and acquired at Merck (Darmstadt, Germany), as well as ammonium acetate (P.A.). Anhydrous magnesium sulphate ($MgSO_4$) was purchased from Sigma-Aldrich and sodium chloride (NaCl) from VWR, both treated at 500 ºC over 5h before use. C18 column (100 mg) was acquired at Agilent (Agilent technologies, USA). Ultrapure water (18.2 mΩ/cm) was purified by a Milli-Q gradient system from Millipore (Milford, MA, USA).

Stock solutions of 10 g/L of each standard were prepared and used for further dilutions. A working solution of IS, d_5-OTA, at 2 mg/L was prepared in MeCN. All standard solutions were stored at – 18 ºC when not in use.

2. Samples and sampling

For this study, 3 Certified Reference Materials (CRMs) were purchased from Ambifood® (Porto, Portugal). They are naturally AFs-contaminated corn samples from TRILOGY® (Washington, USA) with the following references, A-C-2223, A-C-2215 and A-C-292. **Table 2** presents relevant information about these materials, namely certified concentration, standard deviation (STD) and expanded uncertainty (U) using a coverage factor (k) of 2. CRMs were kept in the freezer pending analysis.

Plus, real corn samples were provided under CALSEG scope of activity between September of 2017 and March of 2018. Therefore, when sampling was at CALSEG responsibility, an accredited sampling (NP EN ISO/IEC 17025:2005) was assured, following the requirements of Commission Regulation (EU) No 691/2013 (The European Commission, 2013b). Most of the samples had to be milled and so, a Perten 3100 laboratory mill equipped with a 1.5 mm sieve (Huddinge, Sweden) was used to finely ground them. Prepared samples were vacuum-sealed in plastic bags and stored under refrigeration (at least below 8 °C) until the time of analysis.

Table 2 – Certified values of the 3 naturally AFs-contaminated corn samples from TRILOGY®.

CRM	Reference	Mycotoxins concentration (µg/kg)					STD (µg/kg)	U (k = 2)	
		AFB_1	AFB_2	AFG_1	AFG_2	AFs		µg/kg	%
1	A-C-2223	4.7	0.5	ND	ND	5.2	0.8	1.6	31.7
2	A-C-2215	19.1	1.9	ND	ND	21.0	2.9	6.2	29.4
3	A-C-292	47.1	2.7	1.7	ND	51.6	5.5	12.3	23.9
ND – Not detected.									

3. Testing methods

3.1. ELISA

ELISA kit used, RIDASCREEN® Aflatoxin B1 30/15, is intended for the quantitative analysis of AFB_1 in cereals and feed and work on the competitive inhibition format (**Figure 13**). More detailed kit specifications, focusing on corn, are summarized in **Table 3**.

Analysis carried out for this study were performed fully in line with R-Biopharm kit instructions (R-Biopharm AG, 2016b). Briefly, representative samples were well ground and mixed before pre-treatment steps, where 25 mL of MeOH 70 % was added to 5 g of grounded sample, and shaken vigorously by hand for 3 min. The resultant homogenate was then filtered through a filter paper and, afterwards, 1 mL of the filtrate was diluted with 1 mL of deionized water. To implement the test, 50 µL of standard solutions (provided with the kit) and diluted sample extract were added to the correspondent microwells. Then, 50 µL of

enzyme conjugate and antibody solution were added to each well. The plate was manually shaken and after an incubation of 30 min at room temperature, the wells were washed 3 times with 250 µL of PBS tween buffer. Then, 100 µL of substrate/ chromogen was added to each well and the plate was gently shaken and allowed to incubate for 15 min at room temperature. Finally, 100 µL of stop solution was added and the absorbance at 450 nm was measured using Stat Fax® 4700 ELISA microwell strip reader (Awareness® Technology, Inc., Palm City, USA). Data obtained was evaluated with an R-Biopharm software, RIDASOFT® Win.NET (Art. No. R9996). Calibration curves, generated through standard solutions, were fitted with cubic spline function and then AFB_1 concentration, in corn samples, was read, in µg/kg (**Figure 14**).

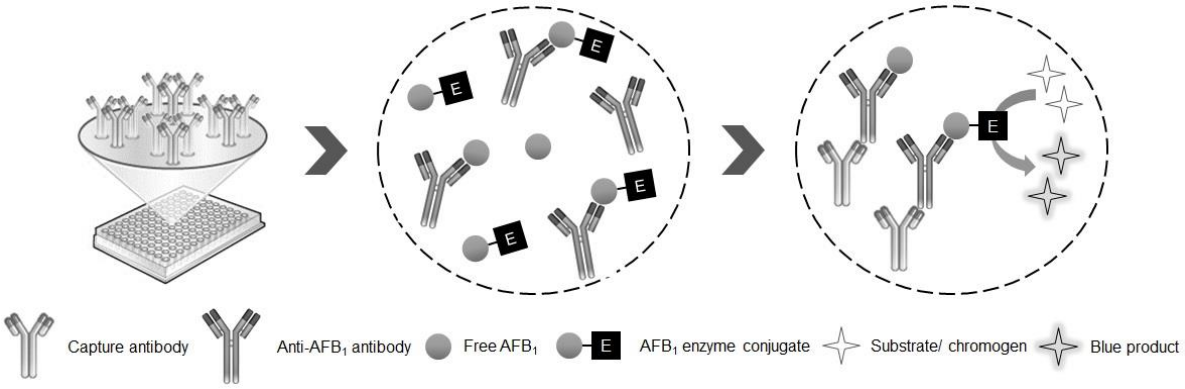

Figure 13 – Simplified schematic diagram of the principle of the competitive inhibition ELISA method of the RIDASCREEN® Aflatoxin B1 30/15 kit.

Table 3 – Declared characteristics and parameters of RIDASCREEN® Aflatoxin B1 30/15 ELISA kit.

Measuring range (µg/kg) or (µg/L)	**LOD; LOQ[1] (µg/kg) or (µg/L)**	**Selectivity[2] (%)**	**Average recovery[3] (%)**	**Average repeatability variation[3] (%)**	**Average reproducibility variation[3] (%)**
1.00 – 50.00	1; NM	100 (AFB_1) 13 (AFB_2) 29 (AFG_1) 3.2 (AFG_2) 1.5 (AFM_1)	87	< 10	< 10
[1] – for corn; [2] – in buffer system; [3] – for naturally contaminated corn CRMs. NM – Not mentioned.					

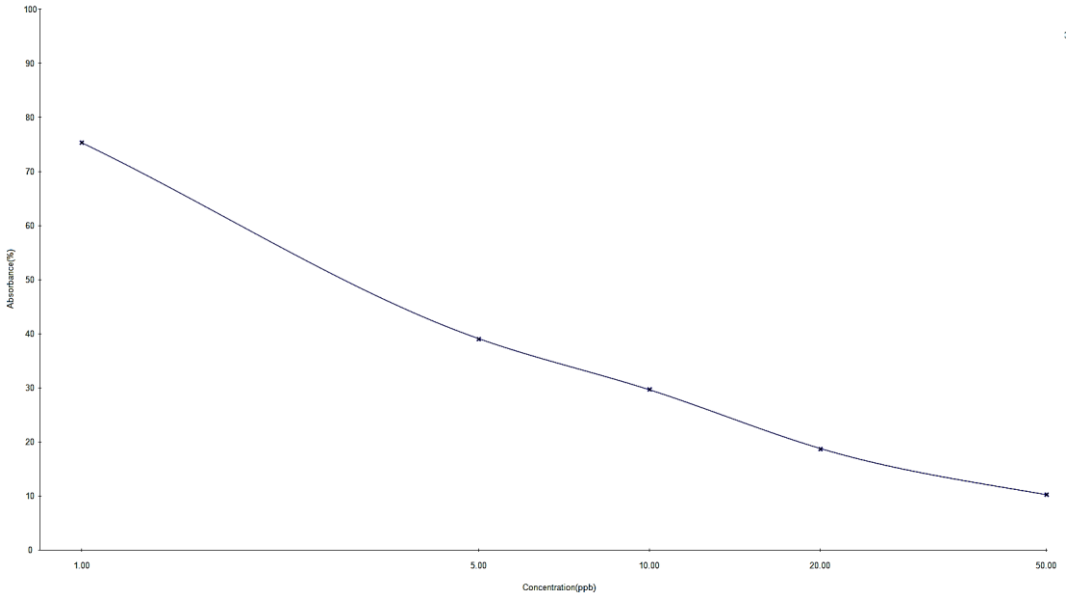

Figure 14 - Example of a calibration curve obtained with RIDASCREEN® Aflatoxin B1 30/15 kit, fitted with cubic spline function.

3.2. LC-MS/MS

3.2.1. Extraction and clean-up

Corn samples were subjected to a procedure for mycotoxins extraction based on a QuEChERS methodology with some modifications, previously developed by (Cunha, Sá, & Fernandes, 2018). Basically, to 1 g of ground and homogenized sample, it was added 20 µg/kg of OTA-d_5 (IS) and left open in a fume hood for approximately 15 min. After, 5 mL of water and 5 mL of MeCN acidified with 1 % formic acid (v/v) were added and the tube was shaken mechanically for 1 h. This step was followed by the addition of 2.0 g of $MgSO_4$ and 0.5 g of NaCl and then the mixture was shaken in a mechanical shaker for about 10 min and centrifuged at 3500 rpm for 3 min. Finally, 1 mL of the upper layer was evaporated to dryness under a gentle stream of nitrogen. Before the injection, the dry extract was reconstituted in 250 µL of mobile phase B (MeOH/ water/ acetic acid, 97:2:1 (v/v) and 5mM of ammonium acetate).

3.2.2. Instrumental and analytical conditions

The separation and quantification of the target mycotoxins were performed using an HPLC system Waters 2695 (Waters, Milford, MA, USA) coupled to a Micromass Quattro micro API™ triple quadrupole detector (Waters, Manchester, UK), equipped with the MassLynx 4.1 software for data processing. The HPLC conditions were used according to (Cunha et al., 2018). The chromatographic separation was achieved using a Kinetex® Phenomenex® C18 column (2.6 µm, 150 mm x 4.60 mm (i.d.)) with a pre-column from

Phenomenex (Torrance, CA, USA). The column was kept at 35 ºC and the autosampler maintained at ambient temperature (± 25 ºC), and the injection volume was 20 µL. The mobile phase consisted of a ternary mixture of mobile phase A (water/ MeOH/ acetic acid, 94:5:1 (v/v) and 5 mM ammonium acetate) and mobile phase B (MeOH/ water/ acetic acid, 97:2:1 (v/v) and 5 mM ammonium acetate), at a flow rate of 0.300 mL/min. The solvent gradient program was as follows: (1) 0 - 7.0 min, 95 % A and 5 % B; (2) 7.0 - 11.0 min, 35 % A and 65 % B; (3) 11.0 - 13.0 min, 25 % A and 75 % B; (4) 13.0 - 15.0 min, 0 % A and 100 % B; (5) 15.0 - 24.0 min, 95 % A and 5 % B; and (6) 24.0 - 27.0 min, 95 % A and 5 % B.

MS/MS acquisition was operated in positive-ion mode (ESI +) with multiple reaction monitoring (MRM). The optimized MS parameters were as follows: capillary voltage, 3.00 kV; source temperature, 150 ºC; desolvation temperature, 350 ºC; desolvation gas and cone gas flow, 350 and 50 L/h, respectively. High purity nitrogen (≥ 99.999 %, Gasin, Portugal) and argon (≥ 99.995 %, Gasin, Portugal) were used as a cone and collision gas, respectively. Dwell times of 0.1 s/scan were selected. For each analyte, two transitions were selected for identification and the corresponding cone voltage and collision energy were optimized for maximum intensity. The optimized MS/MS parameters for target mycotoxins are listed in **Table 4**.

3.2.3. Quality control

Linearity was determined in matrix-matched calibration curves in the range of 1 to 100 µg/kg or µg/L, using 4 to 7 calibration points. LOD and LOQ were determined by successive analyses of chromatographic extracts of sample solutions spiked with decreasing amounts of the analytes until reaching a signal-to-noise ratio of 3:1 and 10:1, respectively. Precision was assessed under repeatability conditions and expressed in terms of an average RSD_r, for each mycotoxin. At 3 concentration levels, 2 independent replicates of spiked samples were analysed on the same day by the same analyst under the same chromatographic conditions. Plus, method's trueness was evaluated with the recoveries obtained through the spiked sample used to construct the calibration curves.

Table 4 – Optimized parameters for mycotoxins analysis by LC-MS/MS.

Mycotoxin/ metabolite	Retention time (min)	Parent ion (m/z)	Product ions (m/z)	Cone energy (V)	Collision energy (V)
15-AcDON	8.77	339.1 $[M+H]^+$	137.1* 321.2	22	13
3-AcDON	8.77	339.2 $[M+H]^+$	203.2 231.2*	21 23	13
AFG_2	9.03	330.8 $[M+H]^+$	245.3 313.1*	35	30 24
AFG_1	9.37	329.0 $[M+H]^+$	243.0* 311.2	35	30
DON	9.60	297.0 $[M+H]^+$	203.3* 249.0	22 20	13 11
AFB_2	9.89	315.0 $[M+H]^+$	259.2* 287.3	40	33 35
AFB_1	10.32	313.0 $[M+H]^+$	241.2* 285.2	45	30
FB_1	16.30	722.5 $[M+H]^+$	334.2* 352.4	46 44	40 36
HT-2	16.31	442.1 $[M+H]^+$	215.3 263.2*	18	15
T-2	16.79	484.0 $[M+H]^+$	214.9* 245.2 305.2	21 23	18 15
OTA	17.16	404.0 $[M+H]^+$	239.1* 358.1	30 28	20 16
ZEN	17.19	319.2 $[M+H]^+$	187.0* 283.3	20	18 16
OTA-d_5	17.50	409.0 $[M+H]^+$	239.4* 257.1	32	22

* – Quantification ion.

4. Experimental design of ELISA verification study

The analytical quality of RIDASCREEN® Aflatoxin B1 30/15 kit, on corn samples, was assessed through the determination of some performance parameters: LOD and LOQ; precision expressed as repeatability and intermediate precision; trueness and measurement uncertainty. In the conducted verification study, it was provided, as far as possible, a realistic range and number of effects operating during the normal use of the method and, besides, a significant number of samples and replicates were used, whenever possible, trying always to cover the entire measuring range. The followed protocol is described in detail in the subsequent sections and was generally designed bearing in mind the criteria established by the Regulation (EC) No 882/2004 (The European Parliament and The Council of the European Union, 2004), and also some guidance documents in this field, namely (Eurachem, 2014; National Association of Testing Authorities, 2013). Regarding

measurement uncertainty, it was adopted the approach recommended in Nordtest Technical Report 537 (Magnusson, Näykki, Hovind, & Krysell, 2012).

Moreover, it was evaluated the performance of the RIDASCREEN® Aflatoxin B1 30/15 kit as a binary screening tool to asses if the presence of AFB_1 in corn exceeded the EU regulatory limit of 20 µg/kg, considering the requirements set in Commission Regulation (EU) No 519/2014 (The European Commission, 2014). Thus, the cut-off level and the rate of false positive results were calculated according to (The European Commission, 2014; von Holst & Stroka, 2014), as explained further.

4.1. LOD and LOQ

Experimental estimation of LOD and LOQ in corn was accomplished by analysing 20 test samples with a naturally low concentration of AFB_1. Then, by extrapolation of the calibration curve, the concentration values were obtained. Furthermore, the average concentration of the "blank samples" and the respective estimate of STD were found and an approximate value for the LOD and the LOQ was calculated according to **Equation 1** and **Equation 2**, respectively.

$$LOD = Average\ concentration + 3 \times STD \quad \textbf{(Eq. 1)}$$

$$LOQ = Average\ concentration + 10 \times STD \quad \textbf{(Eq. 2)}$$

4.2. Precision

Assay precision, under conditions of repeatability and intermediate precision, was evaluated through the performance of sufficient independent replicate measurements on 3 different corn CRMs, to cover the low, the medium and the high ranges of the quantification interval. Precision values were expressed numerically by measures of imprecision, like the absolute and the relative STD (RSD). Additionally, precision limits for repeatability and for intermediate precision were calculated, enabling to decide if there is a significant difference between results from the duplicate analysis, at a specified level of confidence.

4.2.1. Repeatability

Results used to estimate repeatability STD (STD_r) were obtained from the performance of 6 independent replicates in a single run and day, by the same analyst, on each material. The average, the STD and the RSD were calculated for each level of analyte concentration and, then, the overall average values of STD and RSD were determined as the repeatability. Plus, a repeatability limit (r) with a significance level (α) of 0.05 was calculated to each one of the 3 levels of concentration under study, according to **Equation 3**. The degrees of

freedom used were equal to the number of replicate observations (n) subtracted by 1 and the *t*-value was found using a two tails Student's *t*-distribution table.

$$r = \sqrt{2} \times t^{b}_{(n-1,\alpha)} \times STD_r \quad \textbf{(Eq. 3)}$$

4.2.2. Intermediate precision

Regarding the estimation of intermediate precision STD (STD_{Rw}), the same analytical steps were repeated for the 3 ranges of concentration by another trained analyst, within a time interval of 2 months. Furthermore, to cover long-term variations, a different batch of the kit was used whenever possible. Intermediate precision was then determined as the global average values of STD and RSD. Plus, for the 3 levels of concentration under study, an intermediate precision limit (R_w) with 11 degrees of freedom and at the 95 % confidence level (α of 0.05) was calculated according to **Equation 4**.

$$R_w = \sqrt{2} \times t^{b}_{(n-1,\alpha)} \times STD_{R_w} \quad \textbf{(Eq. 4)}$$

4.3. Trueness

Assessment of the trueness of data produced with ELISA kit was in practice expressed quantitatively as bias and as recovery. Therefore, the results from 6 independent replicate analyses of the several CRMs, under repeatability conditions, were used to estimate bias throughout the measuring range. Basically, to each material, the difference between the average of the obtained values (estimated concentration) and the reference value, was determined as bias and the relative form of it was also calculated, following **Equation 5**. Besides, recovery values were determined according to **Equation 6**. Additionally, a Student's two-tailed *t*-test was carried out to determine if the results obtained were significantly different from the certified value of AFB_1 concentration. **Equation 7** was used to calculate the *t*-value and a Student's *t*-distribution table provided the critical *t*-value with 5 degrees of freedom at the 95 % confidence level.

$$\%\, Bias = \frac{Bias}{Reference\ concentration} \times 100 \quad \textbf{(Eq. 5)}$$

$$\%\, Recovery = \frac{Estimated\ concentration}{Reference\ concentration} \times 100 \quad \textbf{(Eq. 6)}$$

$$t - value = \frac{Bias}{STD_r/\sqrt{n}} \times 100 \quad \textbf{(Eq. 7)}$$

4.4. Measurement uncertainty

Calculation of a reasonable estimate of the total uncertainty for a measurement result obtained with the method under study in corn samples was accomplished considering data from precision and trueness experiments. Therefore, standard uncertainty associated with intermediate precision (u_{Rw}) and with bias (u_{bias}) were combined according to **Equation 8**, to estimate the combined standard uncertainty (u_c). Regarding u_{Rw}, the value used was the average of the relative STD_{Rw} found for each level of concentration tested. In turn, the u_{bias} was calculated joining two components of the analysis performed on 3 CRMs (N) (**Equation 9**), the root mean square of the estimated relative bias (RMS_{bias}) (**Equation 10**) and the average of the relative standard uncertainties of the certified values (u_{CRM}). Finally, U was estimated, to a level of confidence of approximately 95 %, using a k of 2 (**Equation 11**). All the calculations were performed with relative values in µg/kg.

$$u_c = \sqrt{u_{Rw}^2 + u_{bias}^2} \quad \textbf{(Eq. 8)}$$

$$u_{bias} = \sqrt{RMS_{bias}^2 + u_{CRM}^2} \quad \textbf{(Eq. 9)}$$

$$RMS_{bias} = \sqrt{\frac{\sum_{i=1}^{N}(bias_i)^2}{N}} \quad \textbf{(Eq. 10)}$$

$$U = 2 \times u_c \quad \textbf{(Eq. 11)}$$

4.5. Cut-off level and rate of false suspect results

To determine the cut-off level, with a false negative rate of 5 % (confidence level of 95 %), results were taken from the replicate analysis under conditions of intermediate precision of the CRMs at the screening target concentration, 20 µg/kg. **Equation 12** was applied, with 11 degrees of freedom. Based on the cut-off value, the rate of false suspect results was estimated for 2 levels of negative samples. The values used derived from the analysis of 20 blank samples and from the intermediate precision experiments on the CRM with a low level of the analyte, corresponding to 25 % of the screening target concentration. Firstly, a *t*-value is calculated, separately for each level, as explained in **Equation 13** and then, based on the degrees of freedom of the experiments, the probability of the false suspect results for a one-tailed distribution was obtained using the spreadsheet function “TDIST”.

$$Cut-off = Average\ concentration_{CRM_2} - t^{u}_{(n-1,\alpha)} \times STD_{R_w} \quad \textbf{(Eq. 12)}$$

$$t-value = \frac{Cut-off-Average\ concentration_{negative}}{STD_{negative}} \quad \textbf{(Eq. 13)}$$

RESULTS AND DISCUSSION

1. LOD and LOQ

A total of 20 blank corn samples were subjected to extraction and analysis with the ELISA kit RIDASCREEN® Aflatoxin B1 30/15, to estimate the LOD and the LOQ of the method in this matrix. The main results obtained are shown in **Table 5** and indicate that acceptable limits were achieved. More specifically, LOD was found to be approximately 1.1 µg/kg, which is in accordance with what is declared by the manufacturer. Respecting the LOQ, a value of approximately 2.5 µg/kg can be defined for corn, according to the results, which is a limit perfectly suitable to the purpose of detecting AFB_1 in corn intended for livestock feed.

Table 5 – Summarized results for LOD and LOQ estimation for corn.

Sample	AFB_1 concentration[1] (µg/kg)	Average AFB_1 concentration (µg/kg)	STD (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
1	0.644	0.429	0.211	1.1	2.5
2	0.494				
3	0.528				
4	0.442				
5	0.585				
6	0.526				
7	0.419				
8	0.389				
9	0.562				
10	0				
11	0.349				
12	0.551				
13	0.249				
14	0				
15	0.571				
16	0.615				
17	0.530				
18	0				
19	0.453				
20	0.677				

[1] – Results are the average of independent duplicate analysis; results are out of the measuring range, obtained by extrapolation.

2. Precision

Parameters for precision achieved with the commercial kit under study were assessed at 3 concentration levels using CRMs. Therefore, it is important to note that this could lead to an underestimation of the variation that would be obtained for real test materials because these materials are frequently better homogenized (Thompson et al., 2002).

2.1. Repeatability

The results achieved for repeatability assays are presented in **Table 6**. It is possible to see that as the analyte concentration increased, STD_r was higher with values between 0.64 and 2.66 µg/kg. In relation to repeatability RSD (RSD_r), it ranged from 5.3 to 13.7 %, and the overall average was 9.3 % which is consistent with the manufacturer's repeatability claim of "< 10 %" also in corn CRMs. Regarding r, values of 2.3, 6.1 and 9.7 µg/kg were defined with a probability of 95 % for the low, the medium and the high level of concentration, respectively. Overall, this data suggests that with this ELISA kit and in our laboratory environment it is achievable a good precision under conditions of repeatability.

Table 6 – Repeatability data at 3 levels of AFB_1 concentration with CRMs.

Level	Replicate	AFB_1 concentration (µg/kg)	Average AFB_1 concentration (µg/kg)	STD_r (µg/kg)	Average STD_r (µg/kg)	RSD_r (%)	Average RSD_r (%)	r (µg/kg)
Low	1	4.40	4.67	0.64	1.66	13.7	9.3	2.3
	2	5.95						
	3	4.58						
	4	4.21						
	5	4.43						
	6	4.42						
Medium	1	18.55	18.86	1.67		8.9		6.1
	2	17.55						
	3	19.16						
	4	17.55						
	5	22.04						
	6	18.32						
High	1	48.87	50.52	2.66		5.3		9.7
	2	51.46*						
	3	47.30						
	4	53.12*						
	5	53.79*						
	6	48.56						

* – Results out of the measuring range, obtained by extrapolation.

2.2. Intermediate precision

Analysis of CRMs under conditions of intermediate precision yielded the results resumed in **Table 7**. As the AFB_1 concentration increased, STD_{Rw} was higher and the values obtained were in the range between 1.06 and 6.08 µg/kg. About the RSD of the intermediate precision (RSD_{Rw}), the overall average value found was of 18.0 % which is greater than the value reported by the manufacturer of "< 10 %" also in corn CRMs. Probably, this can be explained by the fact that our evaluation of intermediate precision covered more variation factors than the ones assessed by the kit producer that tested 2 CRM with low AFB_1

concentration, in 3 different days and with 3 batches of the kit (R-Biopharm AG, 2017). Furthermore, it was defined the R_w of 3.3, 12.8 and 18.9 µg/kg for the 3 levels of tested concentrations, meaning that in 95 % of the cases the difference between 2 values obtained by this method under conditions of intermediate precision will be lower or equal to these values.

Table 7 – Intermediate precision data at 3 levels of AFB_1 concentration with CRMs.

Level	n	Average AFB_1 concentration (µg/kg)	STD_{Rw} (µg/kg)	Average STD_{Rw} (µg/kg)	RSD_{Rw} (%)	Average RSD_{Rw} (%)	R_w (µg/kg)
Low	12	4.97	1.06	3.75	21.4	18.0	3.3
Medium	12	21.01	4.11		19.6		12.8
High	12	46.26	6.08		13.1		18.9

Concerning intermediate precision, The United States Department of Agriculture's Grain Inspection, Packers and Stockyards Administration (GIPSA) established some reference values in the document that states the requirements for the design criteria and performance specifications for quantitative AFs test kits. The maximum acceptable value of RSD_{Rw} is fixed, for concentrations of AFs below 100 µg/kg, following the **Equation 14** (GIPSA, 2016). For the AFB_1 concentrations tested under our study, reference values of 25, 20 and 18 % were determined to the low, the medium and the high levels of concentration, respectively. From **Table 7** it is possible to conclude that our results perfectly comply with these specifications set by GIPSA. Plus, it is also required by GIPSA that at least 95 % of the results for each concentration level must be within an acceptable range defined as the reference concentration minus or plus twice the maximum STD_{Rw} (derived from the maximum RSD_{Rw} (**Equation 14**). In **Figure 15** is a graphical presentation of the total results obtained for AFB_1 concentration in the tested CRMs and are defined the acceptable inferior and superior boundaries for the measured values, according to (GIPSA, 2016). It is possible to see that our data is in accordance with this last GIPSA requirement, with just 1 value from the measuring results of the low and medium level out of the superior limits. Taking all this information into consideration, we conclude that satisfactory intermediate precision parameters are attainable with RIDASCREEN® Aflatoxin B1 30/15 kit.

$$Maximum\ RSD_{Rw}(\%) = 31.572 \times Reference\ concentration^{-0.149} \qquad \textbf{(Eq. 14)}$$

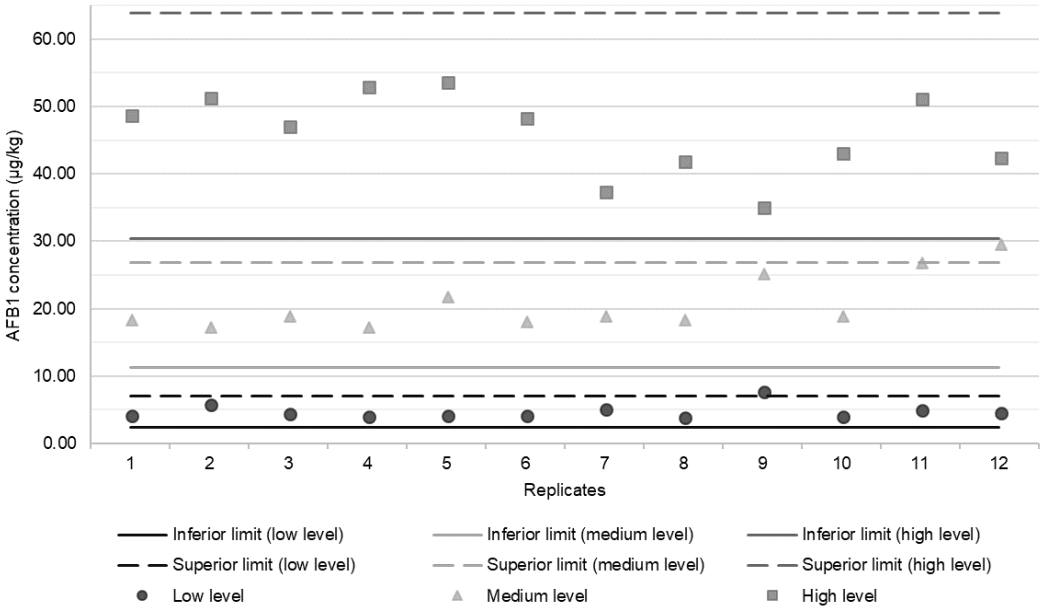

Figure 15 – Concentration values from AFB_1 measurements under conditions of intermediate precision on CRMs covering the low, the medium and the high ranges of the quantification interval (markers); acceptable concentration ranges for the tested levels of AFB_1 concentration defined according to (GIPSA, 2016) (lines).

3. Trueness

Trueness was also evaluated within the measuring range of this ELISA kit using CRMs that covered the low, the medium and the high level of AFB_1 concentration. In **Table 8** are shown the main results obtained and it can be concluded that this ELISA method is somewhat affected by matrix effects, meaning that due to cross-reactivities of structurally related compounds present in the sample, the target analyte concentration might be suppressed or enhanced (Dzuman, Vaclavikova, et al., 2014; National Association of Testing Authorities, 2013; Zheng, Humphrey, King, & Richard, 2005). In fact, underestimated results were found for the materials with a low and medium concentration of AFB_1, while overestimation was observed on the third CRM. However, estimated bias for each level of concentration cannot be considered relevant bearing in mind the stated STD of the acquired CRMs (**Table 2**). Concerning recovery, the overall average was found to be 101.8 % of the theoretical value (**Table 8**), which slightly contrasts with the 87 % that is reported by the manufacturer. Plus, in **Figure 16**, are expressed graphically the minimum and maximum recovery values obtained with all the CRMs applied in this study. It is possible to see that results on the material with the smallest concentration of AFB_1 gave both the lower and the higher values of recovery (89.6 and 126.6 %, respectively). The presence of some interferents in the sample extract is also reflected in the recoveries achieved in this study. Nevertheless, these are values perfectly acceptable. Usually, acceptance range for

this parameter is within 70 and 120 %, even though recovery values depend on the analytical procedure, the matrix and the analyte concentration (European Commission, 2015; Kirilov et al., 2013; National Association of Testing Authorities, 2013). With regard to the Student's *t*-test applied to test the null hypothesis that the estimated average concentration is equal to the reference value, for the 3 CRMs, in **Table 8** are expressed the calculated *t*-values. From a two tails Student's *t*-distribution table a critical *t*-value for a significance level of 0.05 and 5 degrees of freedom was found to be 2.571. Therefore, it is possible to conclude that for the CRM of low and medium concentration, the null hypothesis is not rejected, meaning that the estimated concentration is not significantly different from the reference value. For the third CRM applied, the calculated *t*-value exceeds the critical one and so, there is a statistical difference between the results obtained and the awaited ones, at the confidence level of 95 %. In practice, the performance obtained with this material was not so satisfactory which can be attributable to the fact that AFB_1 concentration is very close to the superior limit of this kit quantification range and some results had to be extrapolated. Probably, it would have been preferable to dilute the sample extracts in order to guarantee that the mycotoxin content was within the measurement interval. To conclude, it can be considered that the ELISA kit under study attains high trueness at the whole work range.

Table 8 – Trueness data at 3 levels of AFB_1 concentration with CRMs.

CRM	AFB_1 reference concentration (µg/kg)	n	Average AFB_1 concentration (µg/kg)	Average bias		Average recovery (%)		*t*-value
				(µg/kg)	(%)			
1	4.7	6	4.67	- 0.04	- 0.74	99.3	101.8	0.134
2	19.1		18.86	- 0.24	- 1.2	98.8		0.349
3	47.1		50.52	+ 3.42	+ 7.3	107.3		3.151

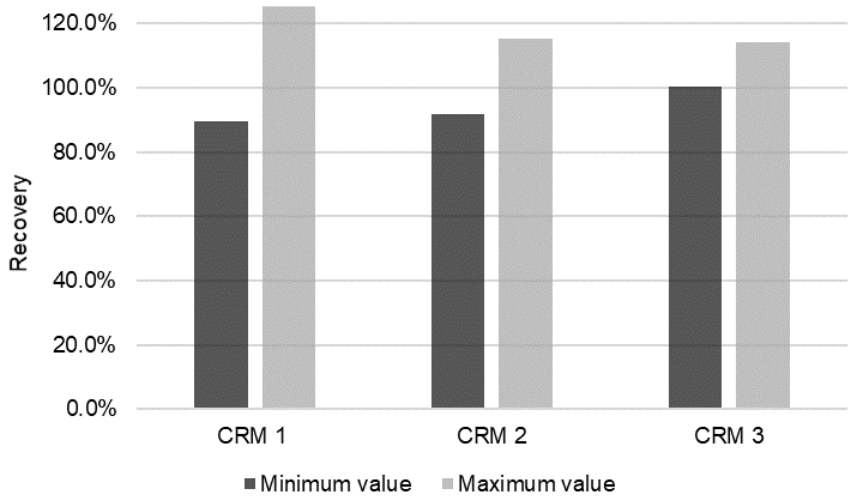

Figure 16 – Minimum and maximum recovery values obtained from repeated and independent analysis of CRMs, under conditions of repeatability.

4. Measurement uncertainty

In the estimation of measurement uncertainty associated with AFB_1 quantification in corn samples with RIDASCREEN® Aflatoxin B1 30/15 kit, were included the contributors that normally are considered the most significant, namely the overall precision and the bias (Eurachem & CITAC, 2012). Thus, results from intermediate precision and trueness experiments were used in calculations of measurement uncertainty and the main outcomes are resumed in **Table 9**. It should be noted that all the contributors to uncertainty were worked in terms of relative values, possibly eliminating the effect of concentration. A single value for U, over the whole measuring range, was estimated to be ± 0.46 µg/kg (relative). This provides an interval within which the concentration of AFB_1 determined with this method in corn is believed to lie, with an approximate level of confidence of 95 %. Uncertainty in the analytical result is especially important when assessing compliance with regulatory limits (Eurachem & CITAC, 2012).

Table 9 – Summary results for AFB_1 measurement uncertainty calculations.

Precision (µg/kg)	Bias (µg/kg)			Measurement uncertainty (µg/kg)	
Relative u_{Rw}	Relative RMS_{bias}	Relative u_{CRM}	Relative u_{bias}	Relative u_c	Relative U
0.180	0.0427	0.140	0.147	0.23	0.46

5. Cut-off level and rate of false suspect results

Considering the obtained average concentration of replicate analysis and the corresponding intermediate precision data, it was calculated a cut-off value of 14 µg/kg for the screening of AFB_1 presence at the regulatory limit of 20 µg/kg. With this established level, it is assured that the rate of false negative results is below 5 %, which is a mandatory performance criterion when applying screening methods for official control purposes (Lattanzio, 2016; von Holst & Stroka, 2014). Additionally, the statistical estimation of the rate of false suspect results showed very low values, below 0.1 % for blank samples and also for the CRM with a level of contamination rounding 5 µg/kg. Actually, looking at the results from these measurements, none was above the defined cut-off which means that this test classifies samples which do not contain AFB_1 or contain it at 25 % of the target concentration, correctly as negatives. However, it would have been more meaningful to determine the rate of false positive results on concentrations closer to the legal limit, for example at 50 or 75 %. Certainly, the estimated rates at these levels would reflect a scenario in which more samples result in suspect findings that require re-analysis with a confirmatory method. Regarding the fitness for the purpose of this test, it is not possible to completely conclude about it because this aspect has to address a cost evaluation of the benefits of

applying a screening method, considering that any suspect sample is always subjected to analysis by another method (von Holst & Stroka, 2014). This economic assessment was explored by (Lattanzio, Holst, & Visconti, 2013). Even so, the determination of these specific performance characteristics, according to Commission Regulation (EU) No 519/2014 (The European Commission, 2014), leads to the conclusion that this method is suitable for screening AFB_1 presence in corn at the regulatory maximum limit of 20 µg/kg.

6. Analysis of real corn samples

A total of 40 corn samples were analysed in duplicate either by ELISA and LC-MS/MS for comparison purposes regarding the AFB_1 presence, testing also the applicability of the kit in a real scenario and the occurrence of this mycotoxin in this feed material. Additionally, the applied LC-MS/MS method is able to detect other 11 mycotoxins and metabolites, proving a more complete idea about the entire mycotoxin contamination profile of the samples under study.

Regarding the analysis of the samples with RIDASCREEN® Aflatoxin B1 30/15 kit, obtained results are resumed in **Table 10**. From the 40 corn samples, the majority (37) had levels of AFB_1 below 1.1 µg/kg (LOD), of which 8 were presumably negatives, meaning that it was not detected this mycotoxin. Plus, the analysis also revealed that just 2 samples had AFB_1 content in levels that are detected by the method but are not quantifiable with acceptable precision and trueness, i.e., lower than 2.5 µg/kg (LOQ). Finally, it was only found in 1 corn sample an average concentration of 2.56 µg/kg. In this evaluation of contamination of real corn samples with AFB_1, the levels observed remained far below the maximum permitted level of 20 µg/kg in feed materials intended for livestock feed, in EU. Besides, if this kit was only used with a screening purpose with the previously calculated cut-off value of 14 µg/kg, all the samples would be classified as “negative”, without needing further confirmation.

Table 10 – Incidence of AFB_1 in real corn samples analysed with ELISA kit (n = 2).

Total samples	< LOD		< LOQ		≥ LOQ		
	Number	%	Number	%	Number	%	Concentration ± U (µg/kg)
40	37	92.5	2	5.0	1	2.5	2.56 ± 1.18

Concerning the LC-MS/MS method, matrix-matched calibration curves were constructed for 12 mycotoxins and metabolites by plotting the ratio response obtained from analyte/ response obtained from IS, against the concentration. The least-square method was employed to calculate regression parameters (**Table 11**). Satisfactory determination coefficients (R^2) were obtained with values higher than 0.9819, which confirms that analytical responses were linear over the tested range. **Table 11** also shows, for each

mycotoxin under study, the achieved: LOD, LOQ, average RSD_r, and average recovery. LOQ values ranged from 1.0 to 5.0 µg/kg, which is perfectly sensitive comparing to the levels established in EU legislation for the presence of these analytes in products intended for livestock feed (**Table 1**). Evaluation of precision under conditions of repeatability showed acceptable average RSD_r, with values ranging from 3.6 to 24.2 %. Finally, average recovery values obtained were between 84.2 and 141.2 %, revealing that this method has acceptable trueness. **Figure 17** presents a chromatogram of the analysis of a standard solution. Co-eluting peaks were not observed at the retention times of the targeted mycotoxins and, thus, method's selectivity can be considered adequate.

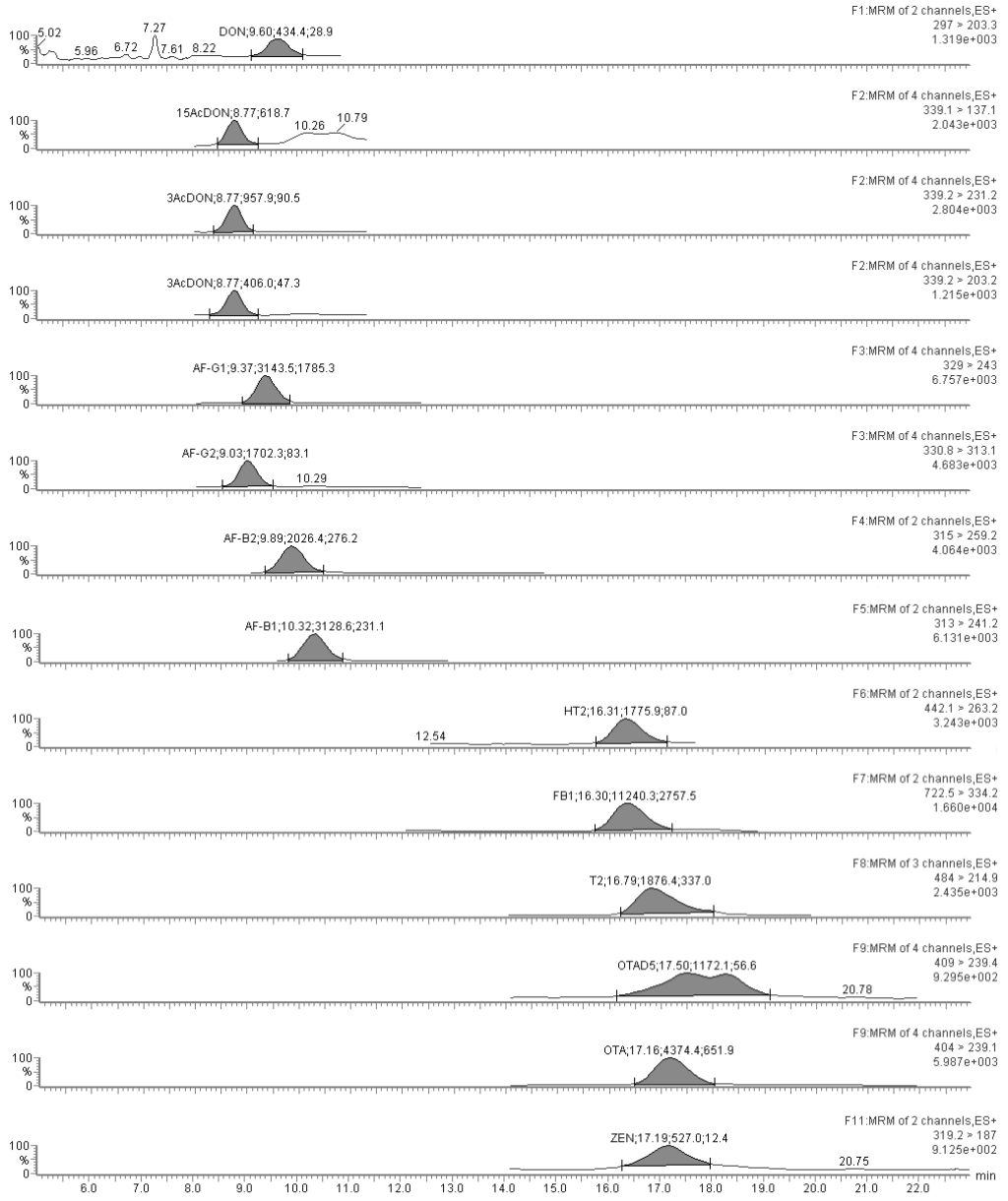

Figure 17 – LC-MS/MS chromatogram of the 12 targeted mycotoxins and metabolites in the standard solution of 100 µg/kg.

Table 11 – LC-MS/MS performance parameters for the analysis of 12 mycotoxins and metabolites in corn.

Mycotoxin/ metabolite	Linear range (µg/kg)	Slope	Intercept	R^2	LOD (µg/kg)	LOQ (µg/kg)	Average RSD_r (%)	Average recovery (%)
AFB_1	1.0 – 100	2.800	- 11.74	0.9950	0.3	1.0	6.8	141.2
AFB_2	2.5 – 100	1.960	- 14.64	0.9860	0.8	2.5	11.6	120.1
AFG_1	2.5 – 100	2.940	- 23.76	0.9819	1.3	4.2	8.2	123.1
AFG_2	1.0 – 100	1.525	- 8.196	0.9883	1.3	4.3	3.6	100.8
DON	2.5 – 100	0.3673	0.6798	0.9974	0.8	2.5	24.2	84.2
3-AcDON	1.0 – 100	0.8691	- 5.643	0.9874	0.3	1.0	10.7	84.2
15-AcDON	1.0 – 100	0.5558	- 2.491	0.9959	1.4	4.7	10.0	118.9
FB_1	1.0 – 100	9.507	31.67	0.9882	0.3	1.0	11.3	87.1
HT-2	5.0 – 100	1.668	- 15.52	0.9883	1.5	5.0	13.3	98.6
T-2	1.0 – 100	1.667	- 7.310	0.9907	0.3	1.0	7.2	135.1
OTA	1.0 – 100	3.893	- 16.14	0.9947	0.3	1.0	5.3	101.3
ZEN	1.0 – 100	0.4515	0.8048	0.9954	0.3	1.0	12.5	98.6

Mycotoxins were further quantified in real corn samples, using the matrix-matched calibration curves constructed and employing a stable isotopic labelled IS, aiming to overcome eventual drawbacks caused by matrix effects. **Table 12** shows the results found for these analytes and, basically, none of the samples was contaminated with mycotoxins in concentrations that could be quantitatively determined. However, with exception of AFG_1 and HT-2, the remaining mycotoxins were individually present in more than 50 % of the samples. FB_1 and OTA, mycotoxins produced by field and storage fungi, correspondingly, were the more incident, being detected in 39 and 37 of the 40 analysed samples, respectively. Respecting AFB_1 contamination in the analysed samples, it was shown a good agreement between the results found with the ELISA kit and with the LC-MS/MS method. Only 3 samples were classified with ELISA as having higher levels than the presumed, although concentrations found were never greater than 2.6 µg/kg. These results end up increasing the confidence in the ELISA kit under study.

Additionally, this method also provided information about the co-occurrence of the targeted mycotoxins, even at trace levels, leading to the conclusion that this was a ubiquitous scenario among all the samples. In fact, most of the samples were co-contaminated with compounds from the 5 mycotoxins classes under study (**Figure 18**). More specifically, several combinations of 2 or 3 mycotoxins were present at rates higher than 70.0 % (**Table 13**). For example, FB_1 co-occurred with OTA at a rate of 90.0 % and FB_1, OTA and ZEN were detected altogether in 80.0 % of the samples. On the other hand, the commonly reported co-occurrence of AFB_1 with FB_1 appeared in 52.5 % of the samples. Although the detected mycotoxins were not in worrying levels, these findings highlight the need to focus on multi-mycotoxins methods.

Table 12 – Incidence of 12 mycotoxins and metabolites in real corn samples analysed with LC-MS/MS (n = 2).

Mycotoxin/ metabolite	Total samples	< LOD		< LOQ	
		Number	%	Number	%
AFB_1	40	19	47.5	21	52.5
AFB_2		19	47.5	21	52.5
AFG_1		23	57.5	17	42.5
AFG_2		14	35.0	26	65.0
DON		9	22.5	31	77.5
3-AcDON		15	37.5	25	62.5
15-AcDON		15	37.5	25	62.5
FB_1		1	2.5	39	97.5
HT-2		32	80.0	8	20.0
T-2		9	22.5	31	77.5
OTA		3	7.5	37	92.5
ZEN		5	12.5	35	87.5

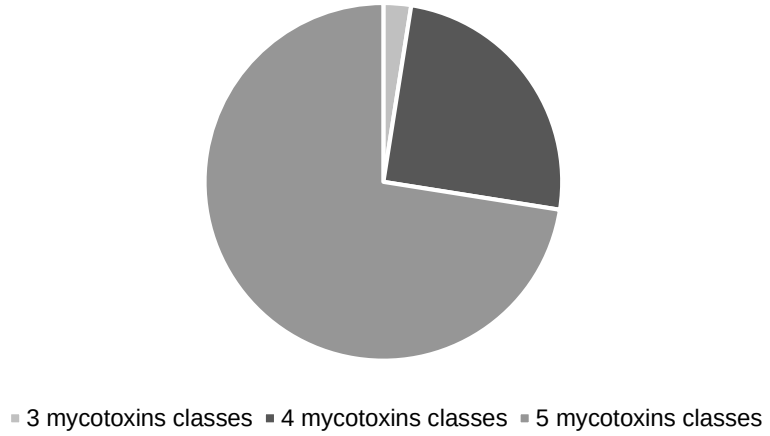

Figure 18 – Rate of samples (%) contaminated at trace levels with co-occurrent mycotoxins classes.

Table 13 – Combinations of co-occurring mycotoxins present in more than 70.0 % of the samples.

Co-occurring mycotoxins combinations	Rate of samples (%)
DON + FB_1	77.5
DON + OTA	72.5
FB_1 + OTA	90.0
FB_1 + T-2	75.0
FB_1 + ZEN	85.0
OTA + T-2	75.0
OTA + ZEN	82.5
DON + FB_1 + OTA	72.5
FB_1 + OTA + T-2	72.5
FB_1 + OTA + ZEN	80.0

CONCLUSIONS

In the present work, a commercial ELISA kit for AFB_1 detection, RIDASCREEN® Aflatoxin B1 30/15, was selected as a case study in corn samples. An internal verification protocol was therefore designed, evaluating the kit regarding the most important performance parameters. LOD, LOQ, precision under conditions of repeatability and of intermediate precision, trueness and measurement uncertainty were determined. Furthermore, other specific requirements (cut-off value and rate of false suspect results) were assessed for the kit application as a truly screening method with the purpose of testing compliance with the maximum permitted level, in EU, of 20 µg/kg in feed materials. Briefly, this study allowed to obtain the following outcomes:

- LOD and LOQ of 1.1 and 2.5 µg/kg, respectively, were defined for corn, which is perfectly acceptable for the intended purpose of the kit.
- Good repeatability data in CRM, with 9.3 % as the overall average of RSD_r, being consistent with the value declared by the manufacturer.
- Satisfactory intermediate precision results in CRM were achieved, with an average of RSD_{Rw} of 18.0 %; plus, our data perfectly complies with all the GIPSA requirements regarding this parameter.
- Trueness data was somehow affected by matrix components, however, estimated bias was not relevant enough to apply any corrections; globally, high trueness was attained since an overall average recovery value of 101.8 % was found.
- Measurement uncertainty, more precisely relative U, was estimated to be ± 0.46 µg/kg over the whole measuring range, calculated using a k of 2.
- The cut-off value for screening the presence of AFB_1 at 20 µg/kg was calculated to be 14 µg/kg with this method, assuring a rate of false negative results below 5 %; additionally, the rate of false suspect results was estimated to be very low for blank samples and samples contaminated at a level of 5 µg/kg, which reveals adequate selectivity.

Moreover, this study also contemplated the analysis of 40 real corn samples by the ELISA kit and by an LC-MS/MS method. Thus, the test kit applicability in a real scenario was proved and the samples contamination with other mycotoxins was also assessed. Regarding ELISA analysis, only 1 sample was contaminated with a quantifiable AFB_1 content of 2.56 ± 1.18 µg/kg. Clearly, all the levels found were far below the maximum permitted level established in the EU. Concerning LC-MS/MS experiments, they revealed that corn samples were not contaminated with any of the 12 analysed mycotoxins in quantifiable levels. Plus, the results obtained with the ELISA kit about AFB_1 were generally confirmed by LC-MS/MS. Finally, the application of this method also informed that

mycotoxins co-occurrence was ubiquitous among all the samples, even though these analytes were present at trace levels, below the LOQ.

Overall, RIDASCREEN® Aflatoxin B1 30/15 applied to corn achieved satisfactory performance characteristics in our laboratory environment, enabling, therefore, the routine use of this commercial kit with reliability. The kit showed effectiveness in quantifying AFB_1 between 2.5 and 50 µg/kg and proved also to be a suitable tool for screening the presence of this analyte at the limit of 20 µg/kg. As a final remark, it is important to mention that the application of this ELISA kit, or any other, in the routine analysis should be accompanied with the employment of an adequate internal and external quality control program. Furthermore, although approaches with immunoassay-based commercial kits are very valuable, they generally just target one mycotoxin which delivers insufficient information about the risk associated with a feedstuff. As a result, the development of simultaneously quick and multi-mycotoxins methods is a clear need in this field of analysis.

APPENDIX – ELISA and chromatographic methods applied in mycotoxins analysis in feed during 2016

Table A.1 – Overview of ELISA methods in mycotoxins analysis (2016).

Matrix	Mycotoxin/ metabolite	Sample pre-treatment	ELISA			Reference
		Extraction	Format	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Corn hybrids	FMs	MeOH	Direct competitive	OD	NM	(Betancourt & Denise, 2016)
Ground maize and gluten meal	FMs	MeOH 70%	Direct competitive	OD	Test kit G: 200; 250 Test kit K: 200; 500	(Coronel, Vicente, Resnik, Alzamora, & Pacin, 2016)
Mixed ruminant feed	AFs AFB_1 OTA FMs	MeOH 70 % MeCN 80 % NM MeOH 90 %	Direct competitive	OD	1.0; NM NM 0.4; NM 100; NM	(Ekici, Yildirim, & Yarsan, 2016)
Maize; wheat bran and dairy feeds	AFB_1	MeCN 80 %	Direct competitive	OD	NM	(Gizachew, Szonyi, Tegegne, Hanson, & Grace, 2016)
Corn silage; crushed yellow corn; wheat bran; wheat flour; soybean meal and full ration pellet for dairy cow	AFB_1	MeOH 70%	Competitive	Absorbance	1; NM	(Hashemi, 2016)
Maize silage	AFs ZEN DON T-2/ HT-2 OTA	MeOH 70% Distilled water NM Sodium bicarbonate buffer	Competitive	Absorbance	2.0; NM 10.0; NM 100.0; NM 10.0; NM 5; NM	(Jovaišienė et al., 2016)
Corn	OTA	0.02 M phosphate buffer (pH 6.5) with 60 % MeOH	Competitive	Fluorescence	0.0022; NM	(Liang et al., 2016)

NM – Not mentioned.

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Table A.1 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment	ELISA			Reference
		Extraction	Format	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Dairy concentrate feed	AFB_1 DON	MeOH 70 % Distilled water	Competitive	Absorbance	1.75; 3.61 18.5; 21.68	(Makau, Matofari, Muliro, & Bebe, 2016)
Maize and maize-based products	AFs FMs	Ethanol 65 %	NM	NM	2; NM 300; NM	(Nyangi et al., 2016)
DDGS	AFs	MeOH 80 %	Direct competitive	Absorbance	NM	(Oplatowska-Stachowiak et al., 2016)
Wheat silage	DON AFs	NM	Competitive	Absorbance	200; NM 2; NM	(Palacio, Bettucci, & Pan, 2016)
Wheat flour	DON	Distilled water	Competitive	Absorbance	< 200; 200	(Peruzzo & Pioli, 2016)
Soybean flour	ZEN	MeOH 70 %			17 - 41; 50	
Wheat	DON	Water	Direct competitive	Absorbance	233; NM	(Sanders et al., 2016)
Wheat dust					458; NM	
Durum wheat	DON	Distilled water	Competitive	Absorbance	18.5; NM	(Scala et al., 2016)
Dairy feed	AFB_1	NM	Competitive	OD	1; NM	(Senerwa et al., 2016)
Maize	AFB_1	MeCN 80 %	Competitive	OD	1; NM	(Sirma et al., 2016)
Wheat grains	DON	Distilled water	Competitive	Absorbance	< 200; 200	(Šliková, Gavurníková, Hašana, Mináriková, & Gregová, 2016)
Wheat	DON	Distilled or deionized water	Direct competitive	OD	100; 500	(Supronienė, Sakalauskas, Mankevičienė, Barčauskaitė, & Jonavičienė, 2016)
Maize	DON	NM	Indirect competitive	Absorbance	51; 143	(Tima, Brückner, et al., 2016)
Wheat	DON				54; 222	
Maize and wheat	ZEN T-2				17 - 41; 50 10 - 20; 50	

NM – Not mentioned.

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Table A.1 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment	ELISA			Reference
		Extraction	Format	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Swine feed	DON ZEN T-2	NM	Competitive	Absorbance	13; 200 17; 50 12; 50	(Tima, Rácz, Guld, Mohácsi-Farkas, & Kiskó, 2016)
Feed and raw materials	AFB_1	1 g of NaCl and MeOH 70 %	Competitive	Absorbance	1; NM	(Vita, Clausi, Franchino, & De Pace, 2016)
Wheat	DON T-2	Distilled water MeOH 70 %	Direct competitive	Absorbance	18.5; NM 3.5; NM	(Wagacha, Njeru, Okumu, Muthomi, & Mutegi, 2016)
Corn	FB_1	NM	Indirect competitive	Absorbance	1.15;	(X. C. Wang, Bao, et al., 2016)
Wheat silage	FMs ZEN	MeOH 80 % MeOH 60 %	Competitive	Absorbance	NM 12.5; NM	(Yazdi et al., 2016)
NM – Not mentioned.						

Table A.2 – Overview of GC-MS methods in mycotoxins analysis (2016).

Matrix	Mycotoxin/ metabolite	Sample pre-treatment			GC-MS			Reference
		Extraction	Clean-up	Derivatization	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Wheat; complete feed for pigs; complete farm-mixed wet feed for pigs	DON T-2 HT-2 T-2 + HT-2	NM	NM	NM	NM	NM	NM; 10	(Bernhoft et al., 2016)
Durum wheat	DON 3-AcDON 15-AcDON	MeCN 82 %	Charcoal/ Alumina/ Celite column	TMSIM-TMCS (100:1 v/v)	NM	SIM	0.01; NM	(Buśko et al., 2016)
Spring and winter wheat	DON 3-AcDON 15-AcDON T-2 HT-2	MeCN 84 %	MycoSep® 227 column	TMS ether derivatives	NM	NM	NM; 25	(Hietaniemi et al., 2016)

NM – Not mentioned; TMSIM – trimethylsilylimidazole; TMCS – trimethylchlorosilane; TMS – trimethylsilyl.

Table A.3 – Overview of HPLC methods coupled to classical detectors and DAD in mycotoxins analysis (2016).

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		HPLC			Reference
		Extraction	Clean-up	Derivatization	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Corn	T-2	MeOH 95 %	Sep-Pak C18 column	NA	Fluorescence	NM	(Abdou et al., 2016)
Soya bean meal and broiler finisher feed	FB_1						
Soya bean meal	AFB_2	MeOH 80 %	AflaTest® IAC				
Broiler starter feed	AFB_1 AFB_2						
Dairy cattle CFM	AFB_1 AFB_2						
Layer poultry feed	AFB_1 AFB_2						
Wheat	AFB_1 AFB_2 AFG_1 AFG_2 AFs	MeOH 80 %	Easi-Extract® AF IAC	Post-column derivatization	Fluorescence	0.031; 0.093 0.022; 0.066 0.032; 0.096 0.028; 0.084 0.091; 0.273	(Asghar, Ahmed, Iqbal, Zahir, & Nauman, 2016)
Maize	AFB_1 AFB_2 AFG_1 AFG_2 AFs	NM	IAC	Post-column derivatization	Fluorescence	NM; 0.1	(Bernhoft et al., 2016)
Complete feed for pigs	ZEN OTA			NA		NM; 3.0 NM; 0.1	

NA – Not applicable; NM – not mentioned.

(continued on the next page)

Table A.3 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		HPLC			Reference
		Extraction	Clean-up	Derivatization	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Corn silage	AFB_1 AFB_2 AFG_1 AFG_2	MeOH 80 %	C18 SPE column	Electrochemical post-column derivatization	Fluorescence	0.12; 0.4 0.015; 0.05 0.05; 0.16 0.03; 0.1	(Bahrami, Shahbazi, & Nikousefat, 2016)
Wheat	ZEN DON	MeCN 84 %	MycoSep® 224 column MycoSep® 225 column	NA	Photodiode array	10; 10 50; 100	(Calori-Domingues et al., 2016)
Corn	AFs AFB_1 AFB_2 AFG_1 AFG_2	MeOH 80 %	AflaTest® IAC	Pre-column derivatization with trifluoroacetic acid/ acetic acid/ water (2:1:7)	Fluorescence	NM	(Di Domenico et al., 2016)
Maize panel and complementary dairy cow feed	AFB_1	NM	AflaPrep® IAC SPE	Electrochemical post-column derivatization with potassium bromide	Fluorescence	0.005; 0.014	(Dimitrieska-Stojković et al., 2016)
Soya bean seeds and processed soya bean powder	AFs OTA	MeCN 90 %/ 4 % potassium chloride	IAC	NA	Fluorescence	NM	(Egbuta et al., 2016)
	FB_1	SPE isolute SAX columns					
Corn silage and concentrate cow feed	AFB_1	MeOH 80 %	IAC	Electrochemical post-column derivatization	Fluorescence	0.08; 0.3	(Ehsani, Barani, & Nasiri, 2016)
Maize kernel	FB_1 FB_2	Ultrapure water and MeCN	SAX column	Post-column derivatization with *o*-phthaldialdehyde	Fluorescence	4; 13 3; 10	(Guo et al., 2016)

NA – Not applicable; NM – not mentioned.

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Table A.3 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		HPLC			Reference
		Extraction	Clean-up	Derivatization	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Spring and winter wheat	ZEN	MeCN 84 %	MycoSep® 226 column	NA	Fluorescence	NM; 20	(Hietaniemi et al., 2016)
Crushed corn; crushed wheat; soybean meal; poultry feed	AFB_1 AFB_2 AFG_1 AFG_2	MeCN 90 % with 5 g of NaCl	AflaTest® IAC	NA	Fluorescence	0.03; 0.09 0.16; 0.48 0.17; 0.51	(Iqbal et al., 2016)
	ZEN	MeCN 90 %	ZearalaTest® IAC			0.05; 0.15	
Maize	AFB_1 AFB_2 AFG_1 AFG_2	MeOH 60 %	AflaStar® IAC	Electrochemical post-column derivatization with potassium bromide and nitric acid	Fluorescence	0.53; NM 0.15; NM 0.24; NM 0.01; NM	(Kamala et al., 2016)
	FB_1 FB_2	MeOH 75 %	SAX cartridge	Pre-column derivatization		53; NM 47; NM	
Maize; maize silage and complete feed samples for swine, poultry, and cattle	OTA	MeCN 60 %	OCHRAPREP® IAC	NA	Fluorescence	0.13; 0.40	(Kosicki et al., 2016)
	AFB_1 AFB_2 AFG_1 AFG_2	MeOH 80 %	AflaTest® IAC	Post-column derivatization		0.05; 0.15 0.02; 0.06 0.25; 0.75 0.08; 0.24	
Dairy cattle feed	AFB_1	1 g NaCl	AflaTest® IAC	NA	Fluorescence	NM	(Keller et al., 2016)
Corn; cattle feed and pig feed	DON 3-AcDON	MeCN 25 %	IAC	NA	Photodiode array	3.3; 11.0 8.3; 27.6	(Kim et al., 2016)

NA – Not applicable; NM – not mentioned.

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Table A.3 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		HPLC			Reference
		Extraction	Clean-up	Derivatization	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Chicken feeds	AFB_1 AFB_2 AFG_1 AFG_2 OTA ZEN	MeOH 80 % with 2.5 g NaCl	AOZ WB IAC	Post-column photochemical derivatization	Fluorescence	0.07; 0.26 0.02; 0.08 0.13; 0.32 0.02; 0.07 0.10; 0.65 1.30; 8.00	(M. Lee et al., 2016)
Maize; wheat; pig, chicken and duck complete feed	AFB_1	MeOH 80 %	CF AFLA IAC	NA	Fluorescence	0.5; 1.5	(Liu et al., 2016)
Maize; wheat and complete feed	ZEN DON	MeCN 84 % MeOH 60 %	ZearaStar IAC CF DON IAC		UV	10; 24 100; 260	
Maize	FB_1	NM	MultiSep® 211 SPE column	NA	Fluorescence	NM	(Magembe, Mwatawala, Mamiro, & Chingonikaya, 2016)
Wheat	AFB_1 AFB_2 AFG_1 AFG_2 AFs	MeOH 80 %	AflaClean® IAC	NA	Fluorescence	NM	(Namjoo et al., 2016)

NA – Not applicable; NM – not mentioned.

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Table A.3 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		HPLC			Reference
		Extraction	Clean-up	Derivatization	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Yellow corn	AFB_1	MeOH 70 % with 1 % NaCl	AflaTest® WB IAC	Pre-column derivatization with trifluoroacetic acid	Fluorescence	0.08; 0.23	(Ok et al., 2016)
	AFB_2					0.05; 0.15	
	AFG_1					0.12; 0.36	
	AFG_2						
	AFB_1			Post-column photochemical derivatization (PHRED cell)		0.02; 0.05	
	AFB_2					0.01; 0.02	
	AFG_1					0.03; 0.10	
	AFG_2					0.01; 0.03	
	AFB_1			Electrochemical post-column bromination derivatization (Kobra cell)		0.03; 0.08	
	AFB_2					0.01; 0.02	
	AFG_1					0.03; 0.10	
	AFG_2					0.02; 0.06	
Dehulled yellow corn	AFB_1			Pre-column derivatization with trifluoroacetic acid		0.08; 0.25	
	AFB_2					0.03; 0.11	
	AFG_1					0.13; 0.39	
	AFG_2					0.09; 0.27	
	AFB_1			Post-column photochemical derivatization (PHRED cell)		0.02; 0.06	
	AFB_2					0.01; 0.02	
	AFG_1					0.02; 0.05	
	AFG_2					0.01; 0.02	
	AFB_1			Electrochemical post-column bromination derivatization (Kobra cell)		0.04; 0.11	
	AFB_2					0.02; 0.05	
	AFG_1					0.05; 0.14	
	AFG_2					0.01; 0.04	

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Table A.3 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		HPLC			Reference
		Extraction	Clean-up	Derivatization	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Starter, broiler and layer feed	OTA	MeOH 60 %	IAC	NA	UV	NM	(Rao et al., 2016)
Mixed dairy cow feeds	AFB_1 AFB_2 AFG_1 AFG_2	MeOH 80 % with 5 g NaCl	AflaTest® IAC	NA	Fluorescence	0.054; 0.181 0.046; 0.153 0.059; 0.197 0.050; 0.168	(Sahin, Celik, Kotay, & Kabak, 2016)
Corn grain; corn grits; corn meal; corn flour	FB_1 FB_2	MeOH 80 %	SPE cartridge	NA	Fluorescence	2.5; 12.5 6; 31.3	(Savi, Piacentini, Marchi, & Scussel, 2016)
Milled wheat; finished flour; bran	DON	Water	DON-Test IAC	NA	Diode array	22; 77	(Savi, Piacentini, Tibola, et al., 2016)
Poultry feeds	OTA	MeOH 100 %	–	NA	Fluorescence	0.05; 0.15	(Sifou et al., 2016)
Wheat grains and whole-wheat flour	DON	Water	IAC	NA	Photodiode array	9.4; 31.3	(Trombete et al., 2016)
Feedstuffs	ZEN α-ZEL β-ZEL	0.05 M acetate buffer pH 4.7 + chlroform + base-acid treatment	Easi-Extract® ZEN IAC	NA	Fluorescence	1; NM 1; NM 4; NM	(Ueberschär, Brezina, & Dänicke, 2016)
Wheat; wheat shorts; wheat bran	DON	MeCN 84 %	MycoSep® 227 column	NA	UV	NM	(L. Wang, Shao, et al., 2016)

NA – Not applicable; NM – not mentioned.

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Table A.3 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		HPLC			Reference
		Extraction	Clean-up	Derivatization	Detection method	LOD; LOQ (µg/kg) or (µg/L)	
Corn; domestic and imported DDGS; corn germ meal; wheat; bran; wheat shorts and red dog; soybean meal; pig complete feed (powder and pellet); duck and cattle complete feed	AFB_1	MeOH 80 %	MycoSep® 226 column	Post-column photochemical derivatization	Fluorescence	0.5; 1.5	(Wu et al., 2016)
	ZEN	MeOH 60 %	CF AFLA IAC	NA		1.5; 4	
	DON				UV	0.02; 0.06	
Wheat flour	DON 3-AcDON 15-AcDON	Modified QuEChERS procedure		NA	UHPLC - Photodiode array	28.4; 94.7 48.0; 160.0 33.3; 111.1	(Xu et al., 2016)
Animal feedstuffs	AFB_1 AFB_2 AFG_1 AFG_2	IL-DLLME coupled to magnetic SPE	NA	NA	Fluorescence	0.632; NM 0.087; NM 0.422; NM 0.146; NM	(J. Zhao et al., 2016)

NA – Not applicable; NM – not mentioned.

Table A.4 – Overview of LC-MS methods in mycotoxins analysis (2016).

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Corn	AFB_1 ZEN OTA	MeCN/ water/ formic acid (79:20:1)	NA	ESI (±) TOF	NM	0.00001; 0.00005 0.005; 0.02 0.001; 0.003	(Amelin & Timofeev, 2016)
Animal feed	DON ZEN					0.005; 0.02	
Winter wheat	ZEN α-ZEL β-ZEL HT-2 T-2 DON 3-/15-AcDON AFB_1 AFB_2 AFG_1 AFG_2 OTA OTB FB_1 FB_2 FB_3	Modified QuEChERS procedure		UHPLC ESI (±) TOF	NM	NM; 2 NM; 1 NM; 25 NM; 20 NM; 4 NM; 50 NM; 25	(Bryła et al., 2016)
Maize	FB_1 FB_2 $FB_1 + FB_2$	NM	NM	NM	NM	NM; 10	(Bernhoft et al., 2016)

NA – Not applicable; NM – not mentioned.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Layer feed	T-2 HT-2 T-2 triol T-2 tetraol	MeCN 84 %	MycoSep® 227 column	ESI (+) QqQ	MRM	0.9; 2.9 7.1; 23.8 1.0; 3.4 7.5; 25	(Bernhardt, Valenta, Kersten, Humpf, & Dänicke, 2016)
Maize	FB_1 FB_2 $FB_1 + FB_2$	0.4 M phosphate buffer	NA	ESI (+) QqQ	NM	10; 30	(Bertuzzi, Mulazzi, Rastelli, & Pietri, 2016)
Maize silage; total mixed ration for dairy	DON ZEN FB_1 FB_2 T-2 HT-2 AFB_1 AFB_2 AFG_1 AFG_2 OTA	NM	NM	UPLC	NM	10.0; NM 1.0; NM 0.2; NM 	(Cogan et al., 2016)

NA – Not applicable; NM – not mentioned; SRM – selective reaction monitoring.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Maize	FB_1 FB_2 FB_3 DON 15-AcDON ZEN α-ZEL β-ZEL HT-2	MeCN/ water/ acetic acid (79:20:1)	C18 SPE column	ESI (+) QqQ	SRM	8.2; 16.4 11.5; 23 14; 28 7; 14 5; 10 3.25; 6.5 4.6; 9.2 5; 10 6.5; 13	(Chilaka et al., 2016)
Maize silage	ZEN α-ZEL β-ZEL FB_1 FB_2 DON 3-/15-AcDON HT-2 T-2 OTA AFB_1 AFG_1	MeCN 84 % with 1 % of acetic acid	NA	HESI (±) QqQ	SRM	3.4; 11.2 17.3; 57.7 10.4; 34.6 1.7; 5.8 3.9; 12.9 34.2; 113.9 1.6; 5.2 4.9; 16.2 0.29; 0.96 0.29; 0.97 0.05; 0.17	(Dagnac, Latorre, Fernández Lorenzo, & Llompart, 2016)
Wheat grain	DON 3-AcDON 15-AcDON	MeCN 84 %	MycoSep® 226 and multifunctional column	ESI QqQ	MRM	10; 30	(Dong et al., 2016)

HESI – Heated electrospray ionization; NA – not applicable; NM – not mentioned; SRM – selective reaction monitoring.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Maize	FB_1 + FB_2 DON ZEN HT-2 T-2	QuEChERS-based approach		UHPLC TOF	NM	NM	(Degraeve et al., 2016)
Maize	DON 15-AcDON 3-AcDON AFB_1 AFB_2 AFG_1 AFG_2 T-2 FB_1 FB_2 FB_3 HT-2 OTA ZEN α-ZEL β-ZEL	QuEChERS-like approach		UHPLC ESI (±) QTRAP	MRM	NM; 50 NM; 25 NM; 10 NM; 0.5 NM; 0.5 NM; 0.5 NM; 1 NM; 2.5 NM; 25 NM; 25 NM; 25 NM; 10 NM; 1 NM; 0.5 NM; 2.5	(Dzuman et al., 2016)
NM – Not mentioned.							

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
DDGS	DON	QuEChERS-like approach		UHPLC ESI (±) QTRAP	MRM	NM; 100	(Dzuman et al., 2016)
	15-AcDON					NM; 50	
	3-AcDON					NM; 25	
	AFB_1					NM; 1	
	AFB_2						
	AFG_1						
	AFG_2						
	T-2					NM; 2.5	
	FB_1						
	FB_2					NM; 25	
	FB_3						
	HT-2						
	OTA					NM; 1	
	ZEN					NM; 0.5	
	α-ZEL					NM; 2.5	
	β-ZEL						

NM – Not mentioned.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Formula feed	DON	MeCN 50 %	GPD HLB SPE cartridge	UHPLC ESI (±) QqQ	MRM	0.08; 0.10	(Fan et al., 2016)
	3-AcDON					2.09; 4.17	
	15-AcDON					0.57; 1.21	
Concentrated feed	DON					0.23; 0.52	
	3-AcDON					2.31; 4.85	
	15-AcDON					0.98; 1.86	
Premixed feed	DON					0.12; 0.24	
	3-AcDON					1.32; 2.98	
	15-AcDON					0.74; 1.86	
Corn silage	DON	MeCN with 1 % of acetic acid and deionized water with sodium acetate trihydrate	NA	ESI (+) QqQ	SRM	NM; NM	(Gallo et al., 2016)
Spring wheat	HT-2	MeCN 84 %	Oasis® HLB SPE column	APCI (+) QTRAP	MRM	50; 100	(Hofgaard et al., 2016)
	T-2					15; 30	
	DON					45; 90	
	3-AcDON						
	ZEN					30; 60	
	FB_1					40; 80	
	FB_2						

NA – Not applicable; NM – not mentioned; SRM – selective reaction monitoring.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Pig, cattle, chicken and rabbit feed	AFB_1	MeCN/ water/ acetic acid (80:18:2)	mIAC	ESI (±) QqQ	SRM	0.02; 0.06	(Hu et al., 2016)
	AFB_2						
	AFG_1					0.04; 0.12	
	AFG_2					0.03; 0.09	
	OTA					0.12; 0.36	
	ZEN					0.25; 0.75	
	T-2					0.12; 0.36	
Durum wheat	AFB_1	MeCN/ water/ formic acid (79:20:1)	NA	ESI (+) QqQ	MRM	2; 3.5	(Juan, Covarelli, Beccari, Colasante, & Mañes, 2016)
	AFB_2						
	AFG_1						
	AFG_2						
	OTA						
	HT-2					30; 70	
	T-2					7; 15	
	DON					30; 70	
	3-AcDON					30; 45	
	15-AcDON					15; 30	
	ZEN					30; 70	
	FB_1					35; 70	
	FB_2					70; 80	
	FB_3						

NA – Not applicable; SRM – selective reaction monitoring.

(continued on the next page)

Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Animal feed	DON 3-AcDON FB_1 FB_2 T-2 HT-2 OTA ZEN	QuEChERS		UPLC ESI (±) QqQ	MRM	50; 100 10; 50 6.0; 25 10; 25 1.0; 5.0 5.0; 10	(Jedziniak et al., 2016)
	AFB_1 AFB_2 AFG_1 AFG_2	MeCN/ water/ acetic acid (79:20:1)	AflaTest® IAC			0.50; 1.0	
Soya-based feed	All	NM	NM	UHPLC ESI (±) TOF UHPLC ESI (±) Orbitrap	NM	NM; 16	(Jong et al., 2016)
Feeds		QuEChERS		UHPLC ESI (±) HRMS/MS		NM; 0.5 - 50	
		MeCN/ water/ acetic acid	NA	MS/MS		2.5 - 500	

NA – Not applicable; NM – not mentioned.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Broiler feeds	AFB_1	MeCN/ water/ acetic acid (79:20:1)	NA	ESI (±) QqQ	MRM	0.39; 1.0	(Kongkapan, Poapolathep, Isariyodom, & Kumagai, 2016)
	AFB_2					0.48; 1.0	
	AFG_1					0.20; 1.0	
	AFG_2					0.78; 2.0	
	T-2					0.39; 1.0	
	OTA						
	ZEN					0.78; 2.0	
	DON						
Maize; maize silage and complete feed samples for swine, poultry, and cattle	DON	MeCN 80 %	Bond Elut® Mycotoxin column	API	NM	1.0; 3.0	(Kosicki et al., 2016)
	T-2					0.2; 0.6	
	HT-2					0.7; 2.0	
	ZEN					0.07; 0.20	
	FB_1		MultiSep® 211 SPE column			1.6; 5.0	
	FB_2						
	FB_3						

NA – Not applicable; NM – not mentioned.

(continued on the next page)

Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Finished feed for poultry, swine and ruminant, maize and maize silage	AFB_1 AFB_2 AFG_1 AFG_2 ZEN DON 3-AcDON 15-AcDON T-2 T-2 Tetraol T-2 Triol HT-2 FB_1 FB_2 FB_3 FB_4 FB_6 OTA	MeCN/ water/ acetic acid (79:20:1)	NA	ESI IT	NM	NM	(Kovalsky et al., 2016)

NA – Not applicable; NM – not mentioned.

(continued on the next page)

Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Poultry, swine, cattle, horse and lamb feed	DON AFG_2 AFG_1 AFB_2 AFB_1 T-2 ZEN OTA FB_1 FB_2	QuEChERS-based approach		UHPLC HESI (±) Orbitrap	Full scan	NM; 450 NM; 2.5 NM; 500 NM; 10 NM; 25 NM; 2500	(León et al., 2016)
Maize	ZEN α- ZEN β- ZEN	MeCN 75 %	Magnetic SPE with magnetic nanoparticles	API (+) UV-Vis DAD coupled with a MS detector	SIM	0.8; 2.5 1.0; 3.3 0.6; 1.9	(Moreno, Zougagh, & Ríos, 2016)

HESI – Heated electrospray ionization; NM – not mentioned; SIM – single ion monitoring.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Maize	DON 15-AcDON AFB_1 AFB_2 AFG_1 AFG_2 FB_1 FB_2 FB_3 OTA HT-2 T-2 α-ZEL β-ZEL ZEN	MeCN/ water/ formic acid (79:20:1)	NA	QTRAP	NM	NM; 100 NM; 50 NM; 1.0 NM; 2.0 NM; 10 NM; 2.5 NM; 25 NM; 10 NM; 5	(Mngqawa et al., 2016)
Wheat	ZEN DON	MeCN 80 %	SampliQ Amino SPE cartridge	ESI	NM	NM	(Qiu, Dong, Yu, Xu, & Shi, 2016)
Maize kernels	AFB_1	MeOH 70 %	NA	ESI (+) QqQ	MRM	0.344; 1.042	(Reid, Sparks, Williams, & Brown, 2016)
Whole wheat and white wheat flour	DON 3-AcDON 15-AcDON HT-2 T-2 ZEN	MeCN 84 %	NA	ESI (+) QqQ	NM	20; 40 150; 300 50; 100 75; 150 20; 40	(Stanciu, Juan, Miere, Loghin, & Mañes, 2016)

NA – Not applicable; NM – not mentioned.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Corn and wheat	DON	QuEChERS		ESI (+) QqQ	MRM	5.0; 15.0	(Sun et al., 2016)
	3-AcDON					2.0; 5.0	
	15-AcDON						
	FMs					15.0; 25.0	
	OTA					0.1; 0.4	
	ZEN					1.0; 4.0	
	HT-2					0.1; 0.4	
	T-2						
	AFB_1					0.03; 0.1	
	AFB_2						
	AFG_1					0.1; 0.3	
	AFG_2						
Wheat	DON	Solvent 1: MeCN/ water/ formic acid (80:19.9:0.1); solvent 2: MeCN/ water/ formic acid (20:79.9:0.1)	NA	UHPLC ESI (±) QqQ	MRM	NM; 200	(Tibola et al., 2016)
	3-AcDON					NM; 100	
	15-AcDON						
	ZEN					NM; 20	
Maize	FB_1	Solvent 1: MeCN 80 %; solvent 2: MeOH 80 %	FumoniTest® IAC	HESI (+) QqQ	NM	NM	(Vega, 2016)
	FB_2						

HESI – Heated electrospray ionization; NA – not applicable; NM – not mentioned.

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Table A.4 *(continued)*

Matrix	Mycotoxin/ metabolite	Sample pre-treatment		LC-MS			Reference
		Extraction	Clean-up	Ionization/ ion selection	Scan mode	LOD; LOQ (µg/kg) or (µg/L)	
Corn	AFB_1 AFB_2 AFG_1 AFG_2 FB_1 FB_2 FB_3 DON ZEN	QuEChERS		UPLC ESI (+) TOF	Full scan	0.05; 0.1 5; 15 50; 200 12; 25	(Y. Wang, Dong, et al., 2016)
Corn and wheat	AFB_1 AFB_2 AFG_1 AFG_2 OTA ZEN T-2	MeCN/ water/ acetic acid (80:19:1)	mIAC	ESI (±) QqQ	SRM	0.1; 0.3 0.04; 0.12 0.1; 0.3 0.04; 0.12 0.2; 0.6 0.1; 0.3 0.4; 0.12	(Z. Zhang et al., 2016)

SRM – Selective reaction monitoring.

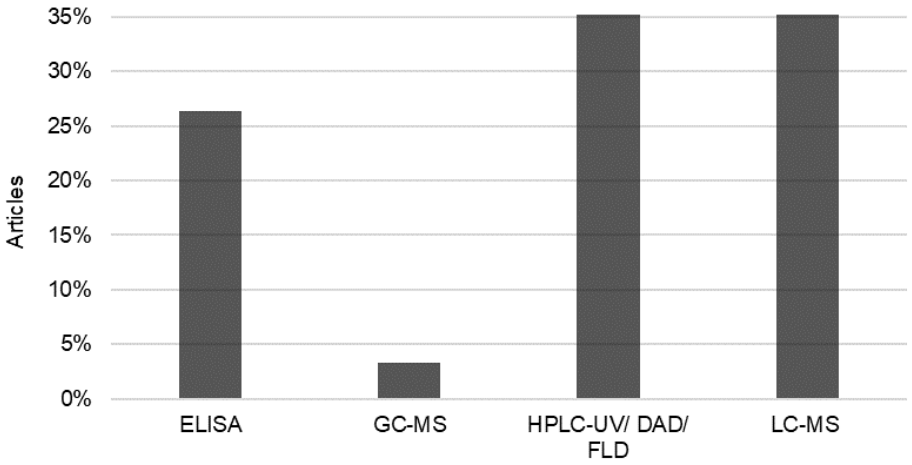

Figure A.1 – Survey on ELISA and chromatographic methods used in mycotoxins analysis (2016). *Sources:* PubMed, Google Scholar and Web of Knowledge.

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