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EVALUATION METHODS OF MYCOTOXIGENIC CONTAMINANTS IN FEED RAW MATERIALS - A REVIEW

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Abstract

Mycotoxigenic contaminants in feed raw materials are a serious problem in animal production due to their negative impact on animal performance and health. The most hazardous genera of mycotoxigenic fungi that can contaminate feed raw materials are Aspergillus, Fusarium, and Penicillium. However, not all fungi of these genera are mycotoxin producers. Therefore, to differentiate contaminated from healthy feed it is important to apply fast detection methods. This review paper aims to present fast qualitative detection methods of mycotoxigenic contaminants. Studies are focused on molecular techniques such as PCR (polymerase chain reaction), as well as chromatographic method such as TLC (thin layer chromatography). Using the PCR method, there can be detected either the fungal species and genera, either the presence of certain genes or gene clusters encoding for mycotoxin synthesis. Mycotoxin detection through TLC is an affinity-based method, in which non-volatile compounds are detected based on their retention factor (Rf) depending on the stationary and mobile phase used. The mycotoxigenic contaminants reviewed in this paper are Aspergillus, Fusarium and Penicillium and their producers.

Key words*: feed, mycotoxins, PCR (polymerase chain reaction), TLC (thin layer chromatography).*

INTRODUCTION

Feed raw materials are exposed to various microbial contaminants, some responsible for toxic secondary metabolites called mycotoxins. Highly damaging are those fungi from *Aspergillus*, *Fusarium* and *Penicillium* genera. These pathogens are able to contaminate feed raw materials in a wide variety of their stages and conditions. Fungal infection can be installed either in the field, during harvest, while being stored in various settings, or by improper handling (Sîrbu et al., 2020; Twarużek et al., 2021).

Mycotoxin contamination levels are highly influenced by the mold type, ecologic factors and phytosanitary management practices. Once a substrate is contaminated, although the mycotoxins-producing molds are suppressed, the mycotoxins still persist, due to their stability over time and slow degradability (Cheli et al., 2013). Due to their toxicity, most hazardous mycotoxins have concentration limits that are governed by European Union regulations, which indicate their maximum levels in feeds and feeding stuffs (European Commission, 2016).

According to Food and Agricultural Organization (FAO) of the United Nations, mycotoxins are contaminating a quarter of world's crop production and compromising over one billion metric tons of food items and raw materials each year. Due to their damaging potential, in addition to food quality evaluation, timely assessment of these contaminants and identification of the most important toxigenic species are crucial for the improvement of management methods to ensure food and feed safety (Suanthie et al., 2009).

To maintain a high quality of the feed raw materials at any stage of their production (e.g., crop planting, culturing, harvesting, processing, storage, and transportation) requires periodic surveillance and monitoring of the mycotoxigenic contaminants, both fungi and toxic compounds. Molecular techniques based

on PCR are highly effective to detect mycotoxigenic fungi mostly by using species specific primers or by detecting the genes responsible for mycotoxins synthesis. As well, the chromatographic methods, such as TLC, are highly efficient in detecting various mycotoxins (Kim et al., 2011).

Considering the toxigenic potential of the samples, the PCR techniques are safer, and compared to the microbiologic methods, they are less time consuming. The PCR techniques not only that are quick, by they are also specific and sensitive if correct performed. Their versatility allows simultaneous amplification of species-specific genes, structural or regulatory genes involved in mycotoxin production pathways, by multiplex PCR. As well, by qPCR (quantitative PCR or Real Time PCR) the contamination level could be detected, while by RT-PCR (Reverse Transcription PCR) the expressed genes can be detected and even quantified through Quantitative Reverse Transcription PCR (RT-qPCR). All of these methods can be performed on various sample type including feed matrices (Rahman et al., 2020).

Chromatographic techniques have been used extensively to separate mycotoxins from other simultaneous produced non-toxic fungal metabolites (Betina, 1985). This is due to the fact that chromatographic methods, such as Liquid Chromatography (LC) and Gas Chromatography (GC), are able to isolate and reveal these compounds in their pure form. However, of all chromatographic methods used for studying mycotoxins, TLC is by far the most popular as it can detect toxins without complex or expensive equipment.

The present review aims to provide a comprehensive summary of the mycotoxigenic contaminants, relevant for public food and feed safety. Both fungi and related toxins are considered. A general description of mycotoxins detection through TLC is also presented, while PCR techniques are described for fungal identification and mycotoxin encoding genes detection.

Mycotoxigenic fungi in feed

The main toxigenic fungi found on feedstuffs belong to the *Aspergillus, Fusarium*, and *Penicillium* genera. Aside them there are also *Alternaria*, *Claviceps, Cephalosporium, Monascus, Myrothecium, Stachybotrys, Trichothecium, Verticimonosporium* etc. These fungi can be detected using traditional culture-based methods or by molecular techniques. To reduce the drawbacks of the culture-based methods, such as labor, time, costs, and for much more rapid and precise detection and identification, PCR methods are preferred. Therefore, most detection procedures are now DNA-based. A wide range of protocols allow the identification of a single species, multiple species belonging to the same genus or mixed populations of different genera (De Saeger et al., 2011). Moreover, molecular techniques based on PCR are able to provide valuable information on susceptible mycotoxin producing fungi (Stępień et al., 2012).

Molecular detection of fungi

There is a huge need for fungal contaminants detection in food and feed due to the safety concern they are raising, especially if they are mycotoxins producers. Classical microbiologic approaches for their detection, evaluation, identification and quantification have a number of drawbacks, as they are time-consuming, labour-intensive, dependent on the target, have poor result reliability, which can determine some difficulties in standardization (Pegels et al., 2012).

For early stage detection of fungal phytopathogens with high risk for human and animal health, scientists have increased the use of molecular techniques. Various PCR techniques can now provide relevant information regarding food and feed microbial contamination. Among these, beside the conventional PCR, there is the nested PCR, multiplex PCR, PCR restriction fragment length polymorphism (PCR-RFLP), amplified fragment length polymorphism PCR (AFLP-PCR), quantitative or real-time PCR (qPCR), droplet digital PCR (ddPCR), as well as some magnetic capture hybridization PCR (MCH-PCR), co-operational PCR (Deepa et al., 2021).

Food and feed microbiology are highly benefiting from the molecular technologies for partial and whole genome sequencing, which offer a wealth of data that are crucial in fungal identification and classification (Munaut et al., 2011).

PCR detection based on conserved DNA regions of taxonomic interest

The creation of species-specific PCR techniques based on conserved genes was mainly motivated by taxonomic and phylogenetic considerations. In the beginning, they were created to evaluate any probable sequence polymorphism between species belonging to the same genus and for further research on their evolutionary relationships. The level of conservation of some of the reported genes, however, was occasionally too high for complete resolution within a genus, so the conserved genes make the best templates for real-time PCR primers and probes when classifying because they enable the simultaneous detection, identification, and quantification of the target fungus species (Anantharajah et al., 2021).

The internal transcribed spacer region (ITS1-5.8S-ITS2) of the ribosomal RNA gene cluster (Figure 1) was selected as the official DNA barcoding area for fungi since it is the most frequently sequenced marker in fungi and has universally effective primers (Usyk et al., 2017).

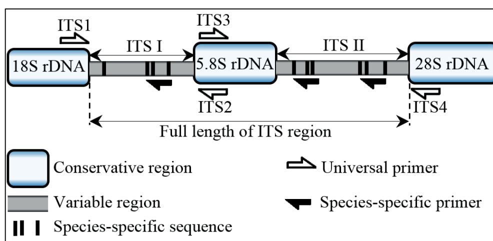

Figure 1. Primer sites within the ribosomal RNA genes of fungi (Horisawa et al., 2009; Srivastava et al., 2014)

The highly conserved 18S, 5.8S, and 28S subunits, on which universal primers are easily created, constitute the ribosomal genes. Two internal transcribed spacer regions (ITS1 and ITS2) divide them one from another, and repeated modules are connected by the intergenic spacer (IGS), these last three sequences were heavily used to distinguish different taxa because of their considerable diversity (Richard et al., 2008). In addition, compared to single gene copies, their multi-copy property is undeniably advantageous in the detection and measurement of extremely small quantities of DNA. Indeed, the sensitivity can be more crucial than a single gene copy for assays by more than 100 times (Edwards et al., 2002).

Moreover, based on the DNA sequence variety identified inside the internal transcribed spacer portions of the ribosomal DNA, several primers were created for *Aspergillus* species differentiation (Henry et al., 2000).

Several other coding regions are found interesting in fungal detection. They can help for taxonomic purposes, or molecular detection protocols of certain contaminants and pathogens (Table 1).

First of all, there is calmodulin, a calcium modulated protein present in all eukaryotic cells. Beside intracellular calcium signaling, it acts on several signaling metabolic pathways and gene expression regulation. Calmodulin encoding gene (*CaM*) is a good choice for taxonomic and phylogenetic studies due to its modest size and high degree of evolutionary conservation (Chin & Means, 2000).

Microtubules primary cytoskeletal elements present in all eukaryotic cells. The alfa and beta tubulin subunits play key roles in several vital cellular mechanisms. The α- and β-tubulin coding genes exhibit some evolutionary sequence variations between genera or species, which make them useful for molecular detection techniques (Munaut et al., 2011).

Translation elongation factors TEF1-α (or EF-Tu) and TEF2 (or EF-G) also exhibited remarkable evolutionary conservation, which makes their coding regions useful molecular differentiation of closely related species (Nouripour-sisakht et al., 2017).

The actin genes also facilitate deep-level phylogeny (Voigt & Wöstemeyer, 2000). Moreover, by analyzing various conserved regions within the genomes and combining the results, more reliable identification can be obtained.

For *Aspergillus* and *Penicillium* identification at species level, other target genes were also used. Phylogeny of *PB1* (RNA polymerase II largest subunit), *RPB2* (RNA polymerase II second largest subunit), *Tsr1* (putative ribosome biogenesis protein) and *Cct8* (putative chaperonin complex component TCP-1) genes were also employed (Hocking et al., 2006; Houbraken & Samson, 2011).

Table 1. Sequences of the most frequently used primers in fungal phylogeny studies

Locus	Gene/ region	Primer	Sequence (5’-3’)	T (°C)	Fragment size (bp)	Target fungi	Reference
Internal transcribed spacer (ITS)	*ITS1-5,8S-ITS2*	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	55	565 to 613	*Aspergillus*	Henry et al., 2000; Als-huhaib et al., 2018
					~595	*Fusarium*	Zarrin et al., 2016
					~570	*Penicillium*	Demirel et al., 2013
		ITS1F ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC		574-576	*Fusarium*	Manter & Vivanco, 2007
Small Subunit (SSU, 18S) of the rRNA	*18S rRNA*	NS1 NS4	GTAGTCATATGCTTGTCTC CTTCCGTCAATTCCTTTAAG	52	~1200	all fungi	White et al., 1990
Large Subunit (LSU, 28S) of the rRNA	*28S rRNA*	LROR LR6	ACCCGCTGAACTTAAGC CGCCAGTTCTGCTTACC	52	~1200	all fungi	Vilgalys et al., 1990; Rehner & Samuels, 1995
β-tubulin	*β-tub*	βtub-F βtub-R	TGACGGGTGATTGGGATCTC CGTCCGCTTCTTCCTTGTTT	68	198	*A. fumigatus*	Serrano et al., 2011; Zarrin et al., 2017
		Tub-F Tub-R	CTCGAGCGTATGAACGTCTAC AAACCCTGGAGGCAGTCGC	60	340	*Fusarium* *Aspergillus*	Atoui et al., 2012
	Bt1 (benA)	Bt1a Bt1b	TTCCCCCGTCTCCACTTCT TCATG GACGAGATCGTTCATGTTGAACTC	55	450	*Aspergillus*	Al-Aayedi et al., 2020
					~ 540	various fungi	Glass & Donaldson, 1995
	Bt2 (tub2/BenA)	Bt2a Bt2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	58	~ 550	various fungi	Glass & Donaldson, 1995
					340	*F.oxysporum*	
					415-580	*Aspergillus*	Ćurčić et al., 2000
					~ 580	*Penicillium*	Yin et al., 2017
Calmodulin	*CaM*	CMD5 CMD6	CCGAGTACAAGGAGGCCTTC CCGATAGAGGTCATAACGTGG	55	475-595	*Aspergillus*	Hong et al, 2017
		CMD5 CMD6	CCGAGTACAAGGARGCCTTC CCGATRGAGGTCATRACGTGG		~ 580	all fungi	Hong et al, 2005; Yin et al., 2017
		CF1 CF4	GCCGACTCTTTGACYGARGAR TTTYTGCATCATRAGYTGGAC	55	~ 750	*Penicillium*	Yin et al., 2017
Translation elongation factor 1-alpha	tef1	EF1-983F EF1-2218R	GCYCCYGGHCAYCGTGAYTTYAT ATGACACCRACRGCRACRGTYTG	66 >>56 touchdown (9 cycles) 56 (in the following 36 cycles)	~1000	all fungi	Rehner & Buckley, 2005
		EF1-1018F EF1-1620R	GAYTTCATCAAGAACATGAT GACGTTGAADCCRACRTTGTC		~600	all fungi	Stielow et al., 2015
		ef1 ef2	ATGGGTAAGGARGACAAGAC GGARGTACCAGTSATCATGTT	53	~700	*Fusarium*	Geiser et al., 2004; Maciá-Vicente et al., 2008.
RNA polymerase II subunit 1	RPB1	RPB1af RPB1cr	GARTGYCCDGGDCAYTTYGG CCNGCDATNTCRTTRTCCATRTA	52	~1200	various fungi	Stiller, & Hall, 1997; Matheny et al., 2002

Table 1. Sequences of the most frequently used primers in fungal phylogeny studies (continuation)

Locus	Gene/ region	Primer	Sequence (5'-3')	T (°C)	Fragment size (bp)	Target fungi	Reference
RNA polymerase II second largest subunit	RPB2-1	5F	GAYGAYMGWGATCAYTTYGG	61	~1000	*Penicillium*	Yin et al., 2017
		7CR	CCCATRGCTTGYTTRCCCAT				
RNA polymerase II second largest subunit	RPB2-2	5F eur	GAYGAYCGKGAYCAYTTCGG	61	~1000	*Penicillium*	Yin et al., 2017
		7CR eur	CCCATRGCYTGYTTRCCCAT				
Mini chromosome maintenance protein	MCM7	Mcm7-709for	ACIMGIGTITCVGAYGTHAARCC	56	~650	various fungi	Schmitt et al., 2009; Raja et al., 2011
		Mcm7-1348rev	GAYTTDGCIACICCIGGRTCWCCCAT				

T (°C) represents the primers' annealing temperature; R = A or G; Y = C or T; K = G or T; M = A or C; S = C or G; W = A or T; D = A, G or T; H = A, C or T; N = A, C, G or T; I=inosine.

The ITS is considered suitable for fungal barcoding, as sequences databases are converting a wide spectrum of species, with a confident number of strains within taxon. However, for some fungal genera with a wide species range (including *Aspergillus, Fusarium,* and *Penicillium*), the ITS region is not always precise enough for species identification. Therefore, the ITS sequencing should be coupled with the analysis of other conserved coding regions, such as actin, β-tubulin, calmodulin, translation elongation factors (Raja et al., 2017).

Other research studies focused on fungal identification combine the PCR amplification of ITS region with RFLP, using different restriction enzymes to cleave the ITS amplification product.

For phylogenetic analysis, mainly for highlighting intraspecific variation, anonymous DNA sequences can also be detected through PCR. Such target sequence of the genomic DNA may or may not contain functional conserved genes (Gherbawy & Voigt, 2010). AFLP and RAPD are the main two methods for detecting these so-called anonymous DNA sequences (Vos et al., 1995; Carter & Vetrie, 2004). Most RAPD or AFLP markers were transformed into PCR markers for effective detection and identification, as they can find species-specific DNA fragments.

Identifying toxigenic fungal contaminants

Some pathogenic fungi not only they can cause considerable yield losses, but due their mycotoxin synthesis they can also compromise the quality of the harvest. The main mycotoxin-producing genera are primarily *Aspergillus, Fusarium*, and *Penicillium* (Marasas et al., 2008). Most of the toxigenic fungi can produce more than a single mycotoxin, while a given mycotoxin can also be produced by fungi belonging to different genera (Perrone & Gallo 2017).

The mycotoxigenic *Aspergillus* species are able to produce aflatoxins (B1, B2, G1, G2), ochratoxin A, and, less commonly fumonisins, gliotoxin and patulin (Munaut et al., 2011).

Fusaria are mainly producing particularly for trichothecenes and fumonisins. There are four types of trichothecenes classified from type A to D. The type A include diacetoxyscirpenol (DAS), HT-2, T-2, and neosolaniol. Type B trichothecenes are deoxynivalenol (DON), nivalenol, (NIV), and their acetylated derivatives. The type C and D trichothecenes are a minor group of toxins which are produced by other fungi than *Fusarium* species (Munkvold, 2017).

The most important mycotoxins produced by *Penicillium* species, are ochratoxin A and patulin, while at a less extent is cyclopiazonic acid (Perrone & Susca, 2017).

However, the most hazardous mycotoxins are by far the aflatoxins and DON, with highest negative impact for trade, and for the animal and human health (Munkvold, 2017).

In order to identify and characterize the aflatoxigenic molds, some genes encoding for the enzymes involved in aflatoxin production have been cloned and sequenced. Through these studies molecular markers have been

found, and primer pairs for PCR and qPCR were developed for faster identification and quantification of the contamination level (Buslyk et al., 2022). Due to the hazardous, teratogenic, mutagenic, and carcinogenic effects of aflatoxins (Sicuia et al., 2014), multiplex PCR methods were created to amplify simultaneously different genes involved in mycotoxin synthesis (Table 2).

Table 2. Specific primers for detecting mycotoxigenic fungi

Mycotoxin	Coding gene	Primer	Primer's sequence (5'to 3')	Annealing temperature (°C)	Fragment size (bp)	Reference
Aflatoxin	*AflR*	AflR660 AflR1249	CGCGCTCCCAGTCCCCTTCATT CTTGTTCCCCGAGATGACCA	59	630	Sweeney & Dobson, 1998
	AflR	APA-450 APA-1482	TATCTCCCCCCGGGCATCTCCCGG CCGTCAGACAGGCACTGGACACGG	65	1032	Schnerr et al., 2002
	*Ord*1	Ord1501 Ord2226	TTAAGGCAGCGGAATACAAG GACGCCCAAAGCCGAACACAAA	58	719	Sweeney & Dobson, 1998
	*nor*1	nor1 nor2	ACCGCTACGCCGGCACTCTCGGCAC GTTGGCCGCCAGCTTCGACACTCCG	65	400	Geisen, 1996
	*ver*1	ver1 ver2	GCCGCAGGCCGCGGAGAAAGTGGT GGGGATATACTCCCGCGACAAGCC	65	537	Geisen, 1996
	*ver*1	VER-496 VER-1391	ATGTCGGATAATCACCGTTTAGATGGC CGAAAAGCGCCACCATCCACCCCAATG	65	895	Schnerr et al., 2002
	*omt*A	Omt1 Omt2	GTGGACGGACCTAGTCCGACATCAC GTCGGCGCCACGCACTGGGTTGGGG	65	797	Geisen, 1996
	*omt*A	OMT-208 OMT-1232	GGCCCGGTTCCTTGGCTCCTAAGC CGCCCCAGTGAGACCCTTCCTCG	65	1024	Schnerr et al., 2002
Fumonisin	Fum5	Fum5F Fum5R	GTCGAGTTGTTGACCACTGCG CGTATCGTCAGCATGATGTAGC	62	845	Bluhm et al., 2002
Patulin	*IDH*	IDH-1 IDH-2	CAATGTGTCGTACTGTGCCC ACCTTCAGTCGCTGTTCCTC	52	600	Paterson et al., 2010
15-acetyl-deoxynivalenol 15A-DON	*tri3*	Tri3F971 Tri3R1679	CATCATACTCGCTCTGCTG TTRTAGTTTGCATCATTRTAG	53	708	Quarta et al., 2006
3-acetyl-deoxynivalenol (3A-DON)	*tri3*	Tri3F1325 Tri3R1679	GCATTGGCTAACACATGA TTRTAGTTTGCATCATTRTAG	53	354	Quarta et al., 2006
DON	*tri5*	3551H 4056H	ACTTTCCCACCGAGTATTTC ATCCCTCAAAAACTGCCGCT	55	525	Quarta et al., 2005
various trichothecene	*tri5*	Tr5F Tr5R	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCG	60	544	Doohan et al., 1999
		Tox5-1 Tox5-2	GCTGCTCATCACTTTGCTCAG CTGATCTGGTCACGCTCATC	68	658	Niessen & Vogel, 1998
		HATri/F HATri/R	CAGATGGAGAACTGGATGGT GCACAAGTGCCACGTGAC	62	260	Edwards et al., 2001
	tri6	Tri6F Tri6R	CTCTTTGATCGTGTTGCGTC CTTGTGTATCCGCCTATAGTGATC	62	596	Bluhm et al., 2002
DON	*tri5-tri6*	N1-2 N2-2R	CTTGTTAAGCTAAGCGTTTT AACCCCTTTCCTATGTGTTA	55	200	Bakan et al., 2002
deoxynivalenol (DON)	*tri7*	GzTri7/f1 GzTri7/r1	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG (C/T) ACTGGTGC	60	~161	Lee et al., 2001
nivalenol	*tri7*	Tri7F340 Tri7R965	ATCGTGTACAAGGTTTACG TTCAAGTAACGTTCGACAAT	53	625	Quarta et al., 2006
NIV	*tri7*	GzTri/fl GzTri/rl	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG(C/T)ACTGGTGC	60	161	Lee et al., 2001

A problematic aspect regarding mycotoxin contamination is their chemical stability at high temperatures. Therefore, they are hardly destroyed by food and feed processing. But more important they are problematic due to their chronic effects. Some mycotoxins are now under regulation in several countries, while the risk related to emerging problems and/or new discovered mycotoxins requires urgent and wide investigations (Moretti et al., 2017).

Principles in mycotoxins extraction and analysis

To evaluate in desired feed stuff the mycotoxins spectrum and their level, several steps are required to be correctly performed. Sampling is the first step in collecting relevant results. Due to the uneven fungal and mycotoxin distribution within feed, proper sampling will influence the relevance of the results within the analyzed batch.

The collected samples within the same batch should be homogenized for proper accuracy, and sample division must be prepared according to the procedures to generate representative analytical samples. Grinding has an important impact on the extraction success, and it allows a better access of the solvents during the extraction of the desired compounds from the matrix (Köppen et al., 2010).

After sample preparation the desired compounds should be extracted from the matrix using appropriate solvents. Some protocols, especially designed to extract from large volume samples (kilogram) recommend to prepare the ground sample as a paste, by mixing with water, to improve the extraction when adding the solvents. Typically, organic solvents are used, sometimes diluted in acetic acid or water.

The most used solvents are chloroform, methanol (Al-Jaal et al., 2019), ethanol, benzene, acetonitrile, acetone, toluene, ethyl acetate, ethyl ether, dimethyl sulfoxide (Zhang et al., 2018), hexane, cyclohexane (Pereira et al., 2015), sometimes single or more commonly as mixtures (Agriopoulou et al., 2020).For higher sensitive detection methods, the crude extract should be cleaned-up before detection and quantitative determination (Köppen et al., 2010).

Mycotoxins can be analyzed using a variety of techniques, such as (i) spectrometry, (ii) TLC, (iii) enzyme linked immunosorbent assays (ELISA), (iv) high-performance liquid chromatography (HPLC) coupled with fluorescence detection (HPLC-FL), diode array detector (HPLC-DAD), ultraviolet and visible detector (HPLC UV-Vis), single mass spectrometry (HPLC-MS), tandem mass spectrometry (HPLC-MS/MS), thermo-spray MS (HPLC-TSP MS) (Valenta, 1998; Lawrence & Scott, 2000; Köppen et al., 2010).

Less recommended are gas chromatographic methods (GC) as mycotoxins are not volatile. Therefore, if GC is used, it must by coupled with electron capture (ECD), flame ionization (FI) or mass spectrometry (MS) (Turner et al., 2009). Moreover, by chemical derivatization of the samples, GC-MS could be performed for mycotoxin detection (Valenta, 1998).

The TLC method

The TLC is considered a popular analytical method due to its relative simplicity and affordability, high sensitivity, and fast separation time (Gololo et al., 2016). The procedure is carried out on a glass or aluminum plates, lightly covered with an adsorbent substance (e.g. silica gel or aluminum oxide), known as binding matrix. These thin coated plates are the stationary phase, while the mobile phase is the migration solution made of organic solvents. The samples should be loaded as spots on the bottom of the plate, which is then placed in to an enclosed tank loaded with the migration solution. During the TLC migration, the plates adsorb the mobile phase, through capillary action. The compounds from the migration solution are interacting with the analyte, making the substances form the samples to ascend the TLC plate at various rates (Figure 2). The compounds present in the sample are migrating at a different rate separating themselves one form each other while the migration front in ascending (De Saeger, 2011). Due to the migration variability of the extracted compounds, the mycotoxin can be detected based on their different retention factor. However, to identify the compounds, standard mycotoxins should be analyzed through the same protocol. An important factor in mycotoxin detection by TLC, is choosing the appropriate organic solvent for the samples subjected to analysis (Waksmundzka-Hajnos et al., 2008)

The Rf values are distinctive for each mycotoxin. However, they are influenced by the TLC characteristics. More precisely, the Rf value of a certain mycotoxin may differ with the extraction solvents used, type of the stationary phase, and mobile phase composition, respectively. Thus, the Rf values refer to the compounds' movement distance on the TLC plate. These values provide the critical information needed to detect the mycotoxins present in the samples, and represent the essence of TLC. The Rf is calculated as the ratio between the compounds' moving distance, and the solvent migration front. Based on this calculation algorithm the Rf values are unitless, and range from 0 to 1.

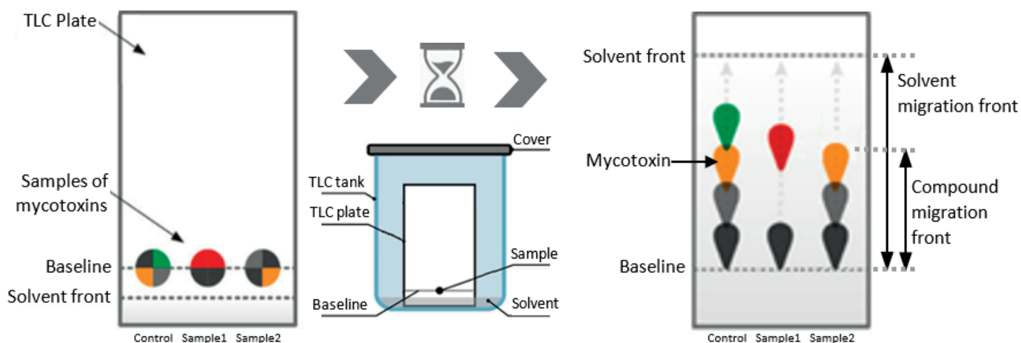

Figure 2. TLC performing steps in mycotoxin detection. The control containing standard mycotoxins and various samples are represented by the spots placed on the plate. Different colors are rebutted for each chemical component. Every component within a sample is separated from the others based on their ascent rates on the TLC plate during the solvent front migration. In the left side of the figure, the plate is represented after sample loading, while in the right side is represented after sample migration (https://www.mycotoxins.info/fileadmin/user_upload/MediaLibrary/Fig16_The-different-steps-in-the-TLC.jpg; https://theory.labster.com/tlc-procedure/)

In order to optimize a TLC protocol for mycotoxins detection it is recommended to use mycotoxin standards, which will provide the Rf values of the known compounds. Later on, the identification of the mycotoxins presence in the studied samples is more precise.

Well documented and reproducible TLC methods for *Aspergillus, Fusarium* and *Penicillium* mycotoxins are now available. The most important toxigenic compounds produced by these fungi can be separated and detected through TLC using the following solvent systems, toluene: ethyl acetate: 90% formic acid (6:6:1, v/v/v) or benzene: methanol: acetic acid (24:2:1, v/v/v) as described by Scott et al. (1970).

Aflatoxins, which are the most important *Aspergillus* mycotoxins, are well extracted in chloroform, at 4°C, overnight. An appropriate solvent mixture for TLC on silica gel plates is the 6:3:1 (v/v/v) mixture of chloroform: ethyl acetate/formic acid (Ciobotaru et al., 2014).

Similar to *Aspergillus*, *Fusarium* mycotoxin can also be extracted in chloroform (Vujanovic and Mansour, 2011). While for chromatographic elution on silica gel plates, an appropriate mobile phase is the mixture of toluene:ethyl acetate:formic acid (5:4:1, v/v/v) as described by (Ursan et al., 2018).

Mostly in mycotoxin detection, the last step in TLC evaluation of the extracted compounds, is the UV light exposure. Most compounds are better revealed under 254-365 nm UV light (Waksmundzka-Hajnos et al., 2008).

CONCLUSIONS

The unproper quality of animal feed due to mycotoxin exposure can cause acute or chronic illnesses, as well as occasionally fatalities. As a result, sensitive and precise analytical techniques and methods are required to acquire sufficient data on mycotoxin exposure levels, and to evaluate and determine the pertinent toxicological risk for both people and animals. Moreover, in order to support monitoring programs and the security of global trade, these analytical methods should also make it possible to measure such contaminants with high accuracy and precision at levels below the legal limits established by the European Union or other national or international legislation.

In order to meet performance criteria (accuracy and precision) and reliably approach the low detection limits required for risk assessment studies, a number of analytical techniques for the measurement of mycotoxins occurring in feeds have been developed and constantly refined.

The most effective methods for measuring the main known mycotoxins found in agricultural and feed commodities now is the classical methods for detecting based on chromatographic techniques such as HPLC and TLC. Furthermore, the most promising method for the simultaneous determination, evaluation and identification of many mycotoxins appears to be PCR technique.

Finally, a number of novel technologies, frequently combined with molecular methods, have been proposed for the quick analysis of mycotoxins in feed. However, more research is needed to validate these technologies and determine and evaluate whether they can be applied to real samples, particularly when mycotoxins are present at levels close to legal limits.

REFERENCES

Agriopoulou, S., Stamatelopoulou, E., & Varzakas, T. (2020). Advances in analysis and detection of major mycotoxins in foods. *Foods, 9*, 518. DOI: 10.3390/foods9040518

Al-Aayedi, A., Ali, S., Başbuğa, S., Atik, O., & Can, C. (2020). PCR-RFLP analyses of *Aspergillus* spp. isolated from pistachio in Turkey. *The Journal of Turkish Phytopathology, 49*(2), 25-33.

Al-Jaal, B., Salama, S., Al-Qasmi, N., & Jaganjac, M. (2019). Mycotoxin contamination of food and feed in the Gulf Cooperation Council countries and its detection. *Toxicon, 171*, 43–50.

Al-shuhaib, M. B. S., Albakri, A. H., Alwan, S. H., Almandil, N. B., Abdulazeez, S., & Borgio, J. F. (2018). Optimal pcr primers for rapid and accurate detection of Aspergillus flavus isolates. Microbial pathogenesis, 116, 351–355. DOI: 10.1016/j.micpath.2018.01.049

Anantharajah, A., Defour, J., Olive, N., Kabera, F., Croonen, L., Deldime, F., Vaerman, J., Barb, C., Bod, M., Scohy, A., Verroken, A., Rodriguez-Villalobos, H., & Kabamba-mukadi, B. (2021). How to choose the right real-time RT-PCR primer sets for the SARS-CoV-2 genome detection ? *Journal of Virological Methods, 295,* 114197. DOI: 10.1016/j.jviromet.2021.114197

Atoui, A., El Khoury, A., Kallassy, M., & Lebrihi A. (2012). Quantification of Fusarium graminearum and Fusarium culmorum by real-time PCR system and zearalenone assessment in maize. *International Journal of Food Microbiology, 154*, 59–65

Bakan, B., Giraud-Delville, C., Pinson, L., Richard-Molard, D., Fournier, E., & Brygoo, Y. (2002). Identification by PCR of Fusarium culmorum strains producing large and small amounts of deoxynivalenol. *Applied and Environmental Microbiology*, *68*(11), 5472–5479.

Betina, V. (1985). Thin-layer chromatography of mycotoxins. *Journal of Chromatography A*, *334*, 211–276.

Bluhm, B. H., Flaherty, J. E., Cousin, M. A., & Woloshuk, C. P. (2002). Multiplex polymerase chain reaction assay for the differential detection of trichothecene- and fumonisin-producing species of *Fusarium* in cornmeal. *Journal of Food Protection, 65*(12), 1955–1961. DOI: 10.4315/0362-028x-65.12.1955

Carter, N. & Vetrie, D. (2004). Applications of genomic microarrays to explore human chromosome structure and function. Human Molecular Genetics, 13(2): R297-302. DOI: 10.1093/hmg/ddh230.

Cheli, F., Campagnoli, A., & Dell'Orto, V. (2013). Fungal populations and mycotoxins in silages: From occurrence to analysis. *Animal Feed Science and Technology*, *183*(1–2), 1–16.

Chin D. & Means A.R. (2000). 'Calmodulin: a prototypical calcium sensor'. *Trends Cell Biol, 10*, 322–328.

Ciobotaru, O., Grosu, I.A., & Cornea C.P. (2014). The effect of feed contamination with mycotoxins on animals and ways for prevention and degradation of mycotoxins. *Scientific Papers. Animal Science and Biotechnologies, 46* (1), 94-98.

Ćurčić, N., Krulj, J., Bočarov Stančić, A., &Bodroža Solarov, M. (2021). PCR - based detection methods as a tool for identification of *Aspergillus* species. Poster presentation in *The International Bioscience Conference 25 – 26 November 2021, in Novi Sad, Serbia.* https://ibsc2021.pmf.uns.ac.rs/wp-content/uploads/2021/11/Curcic.pdf

De Saeger, S. (Ed.). (2011). Determining mycotoxins and mycotoxigenic fungi in food and feed. Elsevier.

Deepa, N., Adkar-Purushothama, C. R., & Sreenivasa, M. Y. (2021). Molecular technologies for the early detection of fungal phytopathogens associated with cereal crops. In *Microbes in Land Use Change Management* (pp. 137–154). Elsevier.

Demirel, R., Sariozlu, N.I., & İlhan, S. (2013). Polymerase Chain Reaction (PCR) identification of terverticillate *Penicillium* species isolated from agricultural soils in Eskişehir Province. *Brazilian Archives of Biology and Technology, 56* (6), 980-984. DOI: 10.1590/S1516-89132013005000004

Doohan, F. M., Weston, G., Rezanoor, H. N., Parry, D. W., & Nicholson, P. (1999). Development and use of a reverse transcription-PCR assay to study expression of Tri5 by Fusarium species in vitro and in planta. *Applied and Environmental Microbiology*, *65*(9), 3850–3854.

Edwards, S.G., Callaghan, J.O., & Dobson, A.D.W. (2002). PCR-based detection and quantification of mycotoxigenic fungi. *Mycological Research 106*(9), 1005–1025. DOI: 10.1017/S0953756202006354

Edwards, S.G., Pirgozliev, S.R., Hare, M.C., & Jenkinson, P. (2001). Quantification of trichothecene-producing Fusarium species in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. *Applied and Environmental Microbiology*, *67*(4), 1575–1580.

European Commission (2016). Commission Recommendation (EU) 2016/1319 of 29 July 2016 amending Recommendation 2006/576/EC as regards deoxynivalenol, zearalenone and ochratoxin A in pet food. *Off. J. Eur. Union*, *208*, 58–60.

Geisen, R. (1996). Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Systematic and Applied Microbiology*, *19*(3), 388–392.

Geiser, D.M., Jiménez-Gasco, M.D.M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A., & O'Donnell, K. (2004). FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. *European Journal of Plant Pathology, 110*(5-6), 473-479. DOI: 10.1023/B:EJPP.0000032386.75915.a0.

Gherbawy, Y., & Voigt, K. (2010). *Molecular identification of fungi*. Springer Berlin, Heidelberg, p 501. ISBN978-3-642-42576-9, DOI: 10.1007/978-3-642-05042-8

Glass, N. L., & Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology, 61*(4), 1323–1330.

Gololo, S.S., Mapfumari, N.S., Shai, L.J., & Mogale, M.A. (2016). Comparison of the TLC Rf values and UV-Visible spectral profiles of the leaf extracts of *Senna italica* collected from four districts in Limpopo province, South Africa. *Journal of Chemical and Pharmaceutical Research*, *8*(5), 144–148.

Henry, T., Iwen, P. C., & Hinrichs, S. H. (2000). Identification of Aspergillus species using internal transcribed spacer regions 1 and 2. *Journal of clinical microbiology, 38*(4), 1510–1515. https://doi.org/10.1128/JCM.38.4.1510-1515.2000

Hocking, A. D., Pitt, J. I., Samson, R. A., & Thrane, U. (2006). *Advances in food mycology*. Edited by A. D. Hocking, J. I. Pitt, R. A. Samson and U. Thrane, Published by: Springer Science/Business Media, Inc., New York. Trends in Food Science & Technology, 19, 116-117. DOI: 10.1016/j.tifs.2007.11.002.

Hong, S.B., Go, S.J., Shin, H.D., Frisvad, J.C., & Samson, R.A. (2005). Polyphasic taxonomy of Aspergillus fumigatus and related species. *Mycologia, 97*(6), 1316- 1329. DOI: 10.3852/mycologia.97.6.1316.

Hong, S. B., Go, S. J., Shin, H. D., Frisvad, J. C., & Samson, R. A. (2005). Polyphasic taxonomy of Aspergillus fumigatus and related species. *Mycologia*, *97*(6), 1316–1329. DOI: 10.3852/mycologia.97.6.1316

Horisawa, S., Sakuma, Y., & Doi S. (2009). Qualitative and quantitative PCR methods using species-specific primer for detection and identification of wood rot fungi. *Journal of Wood Science, 55*, 133-138. DOI 10.1007/s10086-008-1011-3

Houbraken, J., & Samson, R. A. (2011). Phylogeny of Penicillium and the segregation of Trichocomaceae into three families. *Studies in Mycology, 70*, 1–51. DOI: 10.3114/sim.2011.70.01

https://theory.labster.com/tlc-procedure/ - accessed in January 19, 2023

https://www.mycotoxins.info/mycotoxin-testing/analytical-methods/ - accessed in January 19, 2023

Kim, D. M., Chung, S. H., & Chun, H. S. (2011). Multiplex PCR assay for the detection of aflatoxigenic and non-aflatoxigenic fungi in meju, a Korean fermented soybean food starter. *Food Microbiology, 28*(7), 1402–1408.

Köppen, R., Koch, M., Siegel, D., Merkel, S., Maul, R., & Nehls, I. (2010). Determination of mycotoxins in foods: current state of analytical methods and limitations. *Applied Microbiology and Biotechnology, 86*(6), 1595–1612.

Lawrence, J.F., & Scott, P.M. (2000). Chapter 10. HPLC methods for the determination of mycotoxins and phycotoxins, In Techniques and Instrumentation in Analytical Chemistry, Editor(s): D BarcelÓ, Elsevier, Volume 21, Pages 413-456, DOI: 10.1016/S0167-9244(00)80016-5.

Lee, T., Oh, D.-W., Kim, H.-S., Lee, J., Kim, Y.-H., Yun, S.-H., & Lee, Y.-W. (2001). Identification of deoxynivalenol-and nivalenol-producing chemotypes of Gibberella zeae by using PCR. *Applied and Environmental Microbiology*, *67*(7), 2966–2972.

Maciá-Vicente, J. G., Jansson, H. B., Abdullah, S. K., Descals, E., Salinas, J., & Lopez-Llorca, L. V. (2008). Fungal root endophytes from natural vegetation in Mediterranean environments with special reference to Fusarium spp. *FEMS Microbiology Ecology, 64*(1), 90–105. DOI: 10.1111/j.1574-6941.2007.00443.x.

Manter D.K., Vivanco J.M. (2007). Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. Journal of *Microbiological Methods, 71*, 7 – 14

Marasas WFO, Gelderblom WCA, Vismer HF (2008) Mycotoxins: a global problem. In: Leslie JF, Bandyopadhayay R, Visconti A (eds) *Mycotoxins. CABI, Oxfordshire*, pp 29–40

Matheny, P.B., Liu, Y.J., Ammirati, J.F., Hall, B.D., 2002. Using RPB1 sequences to improve phylogenetic inference among mushrooms (Inocybe, Agaricales). *Am. J. Bot. 89*, 688–698.

Moretti A., Logrieco A., Antonia Susca (2017). Mycotoxins: An Underhand Food Problem. In: Antonio Moretti and Antonia Susca (eds.), *Mycotoxigenic Fungi: Methods and Protocols, Methods in Molecular Biology, 1542*, 3-12. DOI 10.1007/978-1-4939-6707-0_1.

Munaut, F., Van Hove, F., & Moretti, A. (2011). Molecular identification of mycotoxigenic fungi in food and feed. In *Determining mycotoxins and mycotoxigenic fungi in food and feed*. Woodhead Publishing Limited. DOI: 10.1533/9780857090973.4.298

Munkvold, G. (2017). Fusarium Species and Their Associated Mycotoxins. In: Antonio Moretti and Antonia Susca (eds.), Mycotoxigenic Fungi: Methods and Protocols, Methods in Molecular Biology, vol. 1542, pp. 51-106. DOI 10.1007/978-1-4939-6707-0_1.

Niessen, M. L., & Vogel, R. F. (1998). Group specific PCR-detection of potential trichothecene-producing Fusarium-species in pure cultures and cereal samples. *Systematic and Applied Microbiology*, *21*(4), 618–631.

Nouripour-Sisakht, S., Ahmadi, B., Makimura, K., Hoog, S., Umeda, Y., Alshahni, M. M., & Mirhendi, H. (2017). Characterization of the translation elongation factor 1-α gene in a wide range of pathogenic

Aspergillus species. *Journal of medical microbiology, 66*(4), 419–429. DOI: 10.1099/jmm.0.000450

Paterson, R. R. M., Archer, S., & Kozakiewicz, Z. (2010). A Gene Probe for the Patulin Metabolic Pathway with Potential for Use in Patulin and Novel Disease Control. *Biocontrol Science and Technology, 10*(4), 37–41. DOI: 10.1080/09583150050115098

Pegels, N., González, I., López-Calleja, I., Fernández, S., García, T., & Martín, R. (2012). Evaluation of a TaqMan real-time PCR assay for detection of chicken, turkey, duck, and goose material in highly processed industrial feed samples. *Poultry Science, 91*(7), 1709–1719.

Pereira, V., Fernandes, J.O., & Cunha, S.C. (2015). Comparative assessment of three cleanup procedures after QuEChERS extraction for determination of trichothecenes (type A and type B) in processed cereal-based baby foods by GC–MS. *Food Chem., 182*, 143–149

Perrone, G. & Gallo, A. (2017). Mycotoxins: An Underhand Food Problem. In: Antonio Moretti and Antonia Susca (eds.), *Mycotoxigenic Fungi: Methods and Protocols, Methods in Molecular Biology*, *1542*, 33-50. DOI 10.1007/978-1-4939-6707-0_1.

Perrone, G. & Susca, A. (2017). *Penicillium* species and their associated mycotoxins. In: Antonio Moretti and Antonia Susca (eds.), *Mycotoxigenic Fungi: Methods and Protocols, Methods in Molecular Biology*, *1542*, 107-119. DOI 10.1007/978-1-4939-6707-0_1.

Sîrbu, V.I., Popa, A., Israel-Roming F. (2020). Mycotoxins in feed: An overview on biological effects. AgroLife Scientific Journal, 9(2), 285-296.

Quarta, A., Mita, G., Haidukowski, M., Santino, A., Mulè, G., & Visconti, A. (2005) Further data on trichothecene chemotypes of European *Fusarium culmorum i Food Addict Contamin, 22*, 309–315.

Quarta, A., Mita, G., Haidukowski, M., Logrieco, A., Mulè, G., & Visconti A. (2006). Multiplex PCR assay for the identification of nivalenol, 3- and 15-acetyl-deoxynivalenol chemotypes in *Fusarium*. *FEMS Microbiology Letters, 259*(1), 7–13, https://doi.org/10.1111/j.1574-6968.2006.00235.x

Rahman, H. U., Yue, X., Yu, Q., Zhang, W., Zhang, Q., & Li, P. (2020). Current PCR-based methods for the detection of mycotoxigenic fungi in complex food and feed matrices. *World Mycotoxin Journal, 13*(2), 139–150.

Raja, H., Miller, A., Pearce C., & Oberlies N. (2017). Fungal identification using molecular tools: a primer for the natural products research community. *J Nat Prod, 80*(3), 756-770.

Raja, H., Schoch, C. L., Hustad, V., Shearer, C., & Miller, A. (2011). Testing the phylogenetic utility of MCM7 in the Ascomycota. *MycoKeys, 1*, 63-94.

Rehner, S. A., & Buckley, E. (2005). A Beauveria phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. *Mycologia, 97*(1), 84–98. DOI: 10.3852/mycologia.97.1.84

Rehner, S.A. & Samuels, G.J. (1995). Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany, 73*, S816–S823.

Richard, G., Kerrest, A., & Dujon, B. (2008). Comparative Genomics and Molecular Dynamics of DNA Repeats in Eukaryotes. *Microbiology and Molecular Biology Reviews, 72*(4), 686–727. DOI: 10.1128/MMBR.00011-08

Schmitt, I., Crespo, A., Divakar, P. K., Fankhauser, J. D., Herman-Sackett, E., Kalb, K., Nelsen, M. P., Nelson, N. A., Rivas-Plata, E., Shimp, A. D., Widhelm, T., & Lumbsch, H. T. (2009). *Persoonia Molecular Phylogeny and Evolution of Fungi, 23*, 35-40.

Schnerr, H., Vogel, R. F., & Niessen, L. (2002). Correlation between DNA of trichothecene-producing Fusarium species and deoxynivalenol concentrations in wheat-samples. *Letters in Applied Microbiology*, *35*(2), 121–125.

Scott P.M., Lawrence J.W., & van Walbeek W (1970). Dtection of Mycotoxins by Thin-Layer Chromatography: Application to Screening of Fungal Extracts. *Applied Microbiology, 20*(5), 839-842.

Serrano, R., Gusmão, L., Amorim, A., Araujo, R. (2011). Rapid identification of Aspergillus fumigatus within the section Fumigati. *BMC Microbiol, 11*, 82 DOI: 10.1186/1471-2180-11-82

Sicuia O. A., Israel-Roming F., Ciobotariu O., Matei A., Zamfir M., Ciucă M., & Cornea C. P. 2014, antifungal action of lactic acid bacteria isolated from plant materials against mycotoxigenic fungi. *Scientific Bulletin. Series F. Biotechnologies, XVIII*, 234-240.

Srivastava, M., Shahid, M., Singh, A., Kumar, V., Pandey, S, et al. (2014). To Develop Strain Specific Molecular Marker for Easy and Proper Identification of Fungal Species Based on Molecular Characters: A Review. *Journal of Molecular Biomarkers & Diagnosis, 5*, 172. DOI:10.4172/2155-9929.1000172

Stępień, Ł., Gromadzka, K. & Chełkowski, J. (2012). Polymorphism of mycotoxin biosynthetic genes among Fusarium equiseti isolates from Italy and Poland. *J Appl Genetics 53*, 227–236 DOI: 10.1007/s13353-012-0085-1

Stielow, J. B., Lévesque, C. A., Seifert, K. A., Meyer, W., Iriny, L., Smits, D., Renfurm, R., Verkley, G. J., Groenewald, M., Chaduli, D., Lomascolo, A., Welti, S., Lesage-Meessen, L., Favel, A., Al-Hatmi, A. M., Damm, U., Yilmaz, N., Houbraken, J., Lombard, L., Quaedvlieg, W., & Robert, V. (2015). One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia, 35*, 242–263. DOI: 10.3767/003158515X689135.

Stiller, J.W., & Hall, B.D. (1997). The origin of red algae: Implications for plastid evolution. *Proc. Natl. Acad. Sci. U.S.A., 94*, 4520-4525.

Suanthie, Y., Cousin, M. A., & Woloshuk, C. P. (2009). Multiplex real-time PCR for detection and quantification of mycotoxigenic *Aspergillus, Penicillium* and *Fusarium. Journal of Stored Products Research, 45*(2), 139–145.

Sweeney, M. J., & Dobson, A. D. W. (1998). Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *International Journal of Food Microbiology*, *43*(3), 141–158.

Twarużek, M., Skrzydlewski, P., Kosicki, R., & Grajewski, J. (2021). Mycotoxins survey in feed materials and feeding stuffs in years 2015–2020. *Toxicon, 202*, 27–39.

Ursan M., Boiu-Sicuia O.A., Voaides C., Stan V., Bubueanu C., & Cornea C.P., 2018. The potential of new streptomyces isolates as biocontrol agents against *Fusarium* spp. *"Agriculture for Life, Life for Agriculture" Conference Proceedings, 1*(1): 594-600. DOI: 10.2478/alife-2018-0093.

Usyk, M., Zolnik, C. P., Patel, H., Levi, M. H., & Burk, R. D. (2017). Novel ITS1 Fungal Primers for Characterization of the Mycobiome. mSphere, 2(6), e00488-17. DOI: 10.1128/mSphere.00488-17

Valenta, H. (1998). Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. *Journal of Chromatography A, 815*, 75–92.

Vilgalys, R., & Hester, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *Journal of bacteriology, 172*(8), 4238–4246. DOI: 10.1128/jb.172.8.4238-4246.1990.

Voigt, K., & Wöstemeyer, J. (2000). Reliable amplification of actin genes facilitates deep-level phylogeny. *Microbiological Research, 155*(3), 179–195. DOI: 10.1016/S0944-5013(00)80031-2

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., & Kuiper, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research, 23*(21), 4407–4414. DOI: 10.1093/nar/23.21.4407

Vujanovic, V. & Mansour, M. (2011). Chemotaxonomic diagnostics: Combining sucrose-water agar with TLC to discriminate Fusarium graminearum 3-acetyl-DON and 15-acetyl-DON chemotypes. *Mycotoxin Research, 27*, 295-301. DOI: 10.1007/s12550-011-0107-6.

Waksmundzka-Hajnos, M., Sherma, J., & Kowalska, T. (Eds.) (2008). Thin Layer Chromatography in Phytochemistry (1st ed.). CRC Press. DOI: 10.1201/9781420046786

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: *Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds., PCR Protocols. A Guide to Methods and Applications*, Academic Press, San Diego, 315-322. DOI: 10.1016/b978-0-12-372180-8.50042-1

Yin, G., Zhang, Y., Pennerman, K. K., Wu, G., Hua, S. S. T., Yu, J., Jurick, W. M., Guo, A., & Bennett, J. W. (2017). Characterization of Blue Mold Penicillium Species Isolated from Stored Fruits Using Multiple Highly Conserved Loci. *Journal of fungi (Basel, Switzerland), 3*(1), 12. DOI: 10.3390/jof3010012

Zarrin, M., Rashidnia, Z., Faramarzi, S., & Harooni, L. (2017). Rapid identification of *Aspergillus fumigatus* using Beta-tubulin and rodletA genes. *Open Access Macedonian Journal of Medical Sciences, 5*(7), 848–851. DOI: 10.3889/oamjms.2017.203

Zarrin, M., Ganj, F., & Faramarzi, S. (2016). Analysis of the rDNA internal transcribed spacer region of the *Fusarium* species by polymerase chain reaction-restriction fragment length polymorphism. *Biomedical Reports, 4*(4), 471–474. DOI: 10.3892/br.2016.615

Zhang, L.; Dou, X.-W.; Zhang, C.; Logrieco, A.F.; Yang, M.-H. (2018). A Review of Current Methods for Analysis of Mycotoxins in Herbal Medicines. *Toxins, 10*, 65. DOI: 10.3390/toxins10020065