UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS GRADUAÇÃO EM MEDICINA VETERINÁRIA

Natália Hoffmann Rossi

DETERMINAÇÃO SIMULTÂNEA DE MICOTOXINAS EM CEREAIS

Santa Maria, RS
2019

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de Doutor em Medicina Veterinária.

Orientador: Prof. Dr. Carlos Augusto Mallmann

Santa Maria, RS

2019

FICHA CATALOGRÁFICA

Ficha catalográfica elaborada através do Programa de Geração Automática da Biblioteca Central da UFSM, com os dados fornecidos pela autora.

Rossi, Natália Hoffmann
Determinação simultânea de micotoxinas em cereais / Natália Hoffmann Rossi.- 2019.
47 p.; 30 cm

Orientador: Carlos Augusto Mallmann
Coorientadores: Eduardo Furtado Flores, Paulo Dilkin
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Medicina Veterinária, RS, 2019

1. micotoxinas 2. lc-ms/ms 3. trigo 4. aveia 5. milho roxo I. Mallmann, Carlos Augusto II. Furtado Flores, Eduardo III. Dilkin, Paulo IV. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós- Graduação em Medicina Veterinária

A Comissão Examinadora, abaixo assinada,
aprova a Tese de Doutorado

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Elaborada por
Natália Hoffmann Rossi

Como requisito parcial para obtenção do grau de
Doutor em Ciência Rural

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Carlos Augusto Mallmann, Dr. (UFSM)
(Presidente/Orientador)

Mauricio Schneider Oliveira, Dr. (PRAXIS SCIENCE)

Matheus Augusto Gonçalves Nunes, Dr. (CORSAN)

Valderi Luiz Dressler, Dr. (UFSM)

Aline Lima Hermes Muller, Dr. (UFSM)

Santa Maria, 28 de fevereiro de 2019.

AGRADECIMENTOS

Ao professor Carlos Augusto Mallmann pela oportunidade e confiança para realização deste trabalho, a amizade e aprendizado.

Ao Dr. Mauricio Schneider Oliveira, por todas as colaborações no trabalho, ensinamentos, paciência, amizade, apoio e confiança.

Aos membros da banca Dr. Aline Lima Hermes Muller, Dr. Matheus Augusto Gonçalves Nunes, Dr. Valderi Luiz Dressler, as suas importantes contribuições.

Aos amigos do Instituto de Soluções Analíticas e Microbiológicas, especialmente Leandro Giacomini, Liziane Wovst, Patricia Lopes, Larissa Figliero, Solange Gerlach e Diego Sturza a amizade, coleguismo, compreensão, conselhos, momentos de descontração e auxílio durante cada etapa da realização deste projeto.

A todo o pessoal do LAMIC, especialmente ao Dr. Carlos Almeida, pelas discussões no desenvolvimento de minha pesquisa. E a Luciane Gressler pelas dicas e colaborações.

Ao Programa de Pós-Graduação em Medicina Veterinária a oportunidade de fazer parte de seu corpo discente. A secretária Maria Moro da Rosa, sempre muito solícita, disposta a ajudar e resolver qualquer contratempo.

A Deus por todas as fases da minha vida e cada pessoa em meu caminho. Aos meus amigos, que estão sempre presentes na minha vida, e torcem por mim, onde quer que estejam. A minha família, principalmente aos meus pais que sempre me incentivaram a estudar e demonstraram que esforço e trabalho são essenciais na vida, sendo os maiores exemplos da minha vida. Obrigada por tudo que sempre fizeram e fazem por mim durante toda a minha vida!

Muito obrigado.

RESUMO

DETERMINAÇÃO SIMULTÂNEA DE MICOTOXINAS EM CEREAIS

AUTOR: Natália Hoffmann Rossi

ORIENTADOR: Carlos Augusto Mallmann

Micotoxinas são metabólitos secundários produzidos por fungos filamentosos que podem estar presentes em inúmeros alimentos consumidos mundialmente. Quando alimentos contaminados são ingeridos por humanos ou animais podem ser considerados tóxicos. Diante disso, o objetivo do estudo foi aplicar um método quantitativo para as micotoxinas com e sem legislação vigente presentes em amostras de trigo e aveia em 2016 e 2017 e milho roxo em 2017. Foram analisadas 465 amostras, sendo 91 de aveia e 374 de trigo provenientes dos estados de Santa Catarina e Rio Grande do Sul e 63 amostras de milho roxo provenientes do Peru. Determinou-se a frequência de ocorrência e a concentração das micotoxinas alternariol (AOH), alternariolmetileter (AME), tentoxina, neosolaniol, nivalenol (NIV), vortmanina, 3-acetil deoxinivalenol, 15-acetil deoxinivalenol, deoxinivalenol (DON), zearalenona (ZEA), aflatoxina B1 (AB_1), aflatoxina B2, aflatoxina G1, aflatoxina G2, fumonisina B1, fumonisina B2, fumonisina B3, ocratoxina A, ocratoxina α, toxina T2, toxina HT2, diacetoxiscirpenol, fusarenon-x, ácido ciclopiazônico, gliotoxina, agroclavina e citroviridina. Foram encontrados níveis médios de micotoxinas dentro dos padrões da legislação brasileira, porém alguns metabólitos detectados não apresentam especificações para os cereais estudados. As principais micotoxinas encontradas em aveia foram AME e AOH, com frequência de ocorrência de 100 e 98%, respectivamente. As mesmas micotoxinas foram as principais encontradas em trigo,, seguidas de NIV, DON, ZEA e AB_1, dependendo do ano de coleta das amostras. No milho roxo não foram encontrados micotoxinas presentes na legislação brasileira. Todas as micotoxinas presentes na legislação apresentaram níveis abaixo do limite de quantificação. As principais micotoxinas encontradas em milho roxo foram AME e AOH, com frequência de ocorrência de 14,3 e 7,9%, respectivamente. Para as amostras de trigo e aveia conclui-se que em ambos os anos e cereais foram detectadas micotoxinas não incluídas na legislação, e alerta-se para a contaminação por micotoxinas que estão regulamentadas, em alguns casos acima dos níveis permitidos. A metodologia utilizada para determinação das micotoxinas foi cromatografia liquida de alta eficiência acoplada a espectrometria de massas. Esta é a primeira pesquisa de micotoxinas emergentes em aveia, trigo e milho roxo.

Palavras-chave: Micotoxinas. Cromatografia líquida. Espectrometria de massas. Aveia. Trigo. Milho roxo.

ABSTRACT

SIMULTANEOUS ANALYSIS OF MYCOTOXINS IN CEREALS

AUTHOR: Natália Hoffmann Rossi

ADVISER: Carlos Augusto Mallmann

Mycotoxins are secondary metabolites produced by filamentous fungi present in oat and wheat and several foodstuffs consumed worldwide. When contaminated food items are ingested, they may be toxic to humans and animals. Thus, this study aimed to apply a qualitative screening method and quantify the mycotoxins with and without current legislation present in oat and wheat samples in 2016 and 2017 and in purple maize in 2017. Four hundred and sixty-five samples of oat (91) and wheat (374) from the states of Rio Grande do Sul and Santa Catarina states and 63 samples of Peuvian purple maize from Peru were analyzed. The frequency of occurrence and concentration of the following mycotoxins were determined: alternariolmetileter (AME), alternariol (AOH), tentoxin, neosolaniol, nivalenol (NIV), wortmannin, deoxynivalenol (DON), 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, zearalenone (ZEA), aflatoxin B_1 (AFB_1), aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, fumonisin B_1, fumonisin B_2, fumonisin B_3, ochratoxin A, ochratoxin α, T-2 toxin, HT-2 toxin, fusarenon x, cyclopiazonic acid, gliotoxin, agroclavin and citreoviridin. Mean levels of mycotoxins within the standards of the Brazilian legislation were found, but some of the detected metabolites do not present specifications for the studied cereals. The main mycotoxins detected in oat were AME and AOH, with a frequency of occurrence of 100 and 98%, respectively. These were also the main mycotoxins found in wheat, followed by NIV, DON, ZEA and AFB_1, depending on the year of sample collection. For purple maize no mycotoxins were found in the Brazilian legislation. All mycotoxins present in the legislation had levels below the limit of quantification. The main mycotoxins found in purple maize were also AME and AOH, with frequency of occurrence of 14.3 and 7.9%, respectively It may be concluded that mycotoxins without guidance levels were detected in both cereals and years. Moreover, a warning must be made regarding the contamination at high concentrations by mycotoxins which are regulated, in some cases exceeding the permitted levels. For wheat and oatmeal samples, it may be concluded that in both years and cereals mycotoxins not included in the legislation were detected, and a warning must be made for the contamination by mycotoxins that are regulated, in some cases above the permitted levels. This is the first survey to analyze emerging mycotoxins in oats, wheat and purple corn.

Key words: Mycotoxin. Liquid chromatography. Mass spectrometry. Oats. Wheat. Purple corn.

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MANUSCRITO 1.

MANUSCRITO 2.

LISTA DE ABREVIATURAS E SIGLAS

3-DON - 3-acetil desoxinivalenol
15-DON - 15-acetil desoxinivalenol
AB_1 - Aflatoxina B1
AB_2 - Aflatoxina B2
ABNT - Associação Brasileira de Normas Técnicas
AG_1 - Aflatoxina G1
AG_2 - Aflatoxina G2
AGC – Agroclavina
AM_1 – Aflatoxina M1
AME - Alternariolmetileter
ANVISA - Agência Nacional de Vigilância Sanitária
AOH - Alternariol
CITREO - Citreoviridina
CPA - Ácido Ciclopiazônico
DAS - Diacetoxiscirpenol
DON - Deoxinivalenol
EC - European Commission
ESI - Electrospray Ionization
FAO - Food and Agriculture Organization
FB_1 - Fumonisina B1
FB_2 - Fumonisina B2
FB_3 - Fumonisina B3
FUSA-x - Fusarenona
GLI - Gliotoxina
HT2 - Toxina HT2
INMETRO - Instituto Nacional de Metrologia, Qualidade e Tecnologia
kg - Quilogramas
LC - Cromatografia Líquida
LC-MS - Liquid Chromatography Mass Spectrometry
LC-MS/MS - Liquid Chromatography Tandem Mass Spectrometry
LMT - Limite Máximo Tolerável
LQ - Limite de Quantificação
MAPA - Ministério da Agricultura, Pecuária e Abastecimento
MRM - Monitoramento de Múltiplas Reações
MS - Massa
NEO - Neosolaniol
NIV - Nivalenol
OMS - World Health Organization
OTA - Ocratoxina A
OTA α - Ocratoxina α
T-2 - Toxina T-2
TEN - Tentoxina
UFSM - Universidade Federal de Santa Maria
µg - Microgramas
v/v - Volume por volume
ZEA - Zearalenona
WORT –Vortmanina

SUMÁRIO

1 INTRODUÇÃO GERAL

Micotoxinas são metabólitos secundários produzidos por fungos filamentosos que podem desenvolver-se em vários gêneros alimentícios. Além disso, alimentos contaminados por micotoxinas podem causar uma resposta tóxica (micotoxicoses) quando ingeridos por animais ou humanos. Porém, nem todo crescimento fúngico resulta em formação de toxinas, e existência de fungos tampouco implica na presença de micotoxinas (BINDER et al., 2007; FINK-GREMMELS, MALEKINEJAD, 2007; VOSS, SMITH & HASCHEK, 2007). *Fusarium*, *Aspergillus* e *Penicillium* são os gêneros de maior ocorrência que podem contaminar, crescer e produzir essas toxinas em alimentos e ração animal, antes e durante a colheita ou no armazenamento (LUO, LIU e LI, 2018). *Fusarium* é um dos gêneros complexos, com espécies adaptadas a uma grande variedade de habitats. *Aspergillus* é um dos três gêneros fúngicos mais importantes e diversos que estão distribuídos universalmente (TANIWAKI, M.A.; PITT, I. J.; MAGAN N., 2018).

Outro gênero importante é o *Alternaria*, que são fungos patogênicos comuns de diversas culturas e que também infectam produtos processados (EFSA, 2011). *Alternaria alternata*, por exemplo, produz um grande número de micotoxinas, inclundoalternariol, alternariol monometil éter, tentoxina e ácido tenuazônico. Essas toxinas mostraram-se carcinogênicas para ratos e mutagênicas em testes *in vitro* (MAGAM & OLSEN, 2004).

Assim, para proteger consumidores e animais, os níveis de micotoxinas devem ser monitorados. Nesse sentido, o setor de produção de cereais tem empregado as boas práticas agrícolas para evitar a produção de toxinas (KABAK, DOBSON & VAR, 2006).

Apesar de a literatura fornecer uma ampla gama de resultados, tratando-se de efeitos de cada micotoxina individualmente, a contaminação por mais de uma toxina é mais provável do que de apenas uma (ALI, *et al.*, 1998; ESKOLA, PARIKKA & RIZZO, 2001; GONZALEZ *et al.*, 1999; PRONK, SCHOTHORST & EGMOND, 2002). Além disso, diversos lotes são misturados na fabricação de rações, alterando a contaminação individual de cada lote, com um novo perfil de risco (PENG, W.-X.; MARCHAL J.L.M.; VAN DER POEL A.F.B., 2019). Sendo assim, algumas doenças observadas, e/ou sinais clínicos dos animais muitas vezes não são respostas aos efeitos individuais de cada micotoxina, podendo existir efeitos aditivos, sinérgicos ou antagônicos (SPEIJERS & SPEIJERS, 2004).

A maioria dos estudos no Brasil envolvendo micotoxinas tem abordado apenas um limitado número delas por vez. Considerando que mais de uma toxina podem ocorrer concomitantemente, e que limites máximos toleráveis (LMT) foram estabelecidos pela

Agência Nacional de Vigilância Sanitária (ANVISA), é importante realizar o monitoramento de um maior número de micotoxinas para identificar quais metabólitos fúngicos estão coocorrendo nas amostras (HUSSEIN & BRASEL, 2001).

Toxinas emergentes não são comuns em análises de rotina. Por não serem tradicionalmente determinadas, não estão previstas em nenhuma legislação e não se conhece sua ocorrência e/ou prevalência. Essas micotoxinas vêm sendo avaliadas em estudos mais recentes. Tratando-se de Brasil, existem poucos estudos sobre tais toxinas. Cabe ressaltar que seus efeitos ainda não são conhecidos, umas vez que as mesmas não são tão pesquisadas (SPEIJERS, G. J. A.; SPEIJERS, M. H. M., 2004).

Devido ao fato de que uma única espécie de fungo presente em uma amostra pode produzir mais de uma micotoxina, é importante que os métodos de determinação sejam capazes de detectar e quantificar o maior número possível de micotoxinas (HUSSEIN & BRASEL, 2001).

Em humanos, micotoxinas podem induzir a efeitos agudos ou crônicos, tais como os teratogênicos, carcinogênicos, imunossupressivos, estrogênicos, anabolizantes, mutagênicos, hemorrágicos e nutricionais (KUMAR, V. *et al*., 2008). Já em animais podem causar diminuição da ingestão alimentar, piora da conversão alimentar, diminuição do ganho de peso, aumento na incidência de doenças e redução na capacidade reprodutiva (BINDER, 2007). Do ponto de vista econômico, as micotoxinas podem afetar o agronegócio, interferindo em relações comerciais e podendo reduzir a produção animal e agrícola (LEUNG, DÍAZ-LLANO & SMITH, 2006).

No Brasil, a ANVISA estabelece LMT para micotoxinas em alimentos através da RDC nº 7 de 2011 (ANVISA, 2011). Essa resolução possui quatro anexos que listam e classificam os alimentos e estabelecem os LMT de aflatoxinas (AB_1 + AB_2 + AG_1 + AG_2 e AM_1), ocratoxina A (OTA), desoxinivalenol (DON), fumonisinas (FB_1 + FB_2), patulina (PAT) e zearalenona (ZEA) admissíveis em alimentos prontos para oferta ao consumidor e em matérias-primas, para serem implementados até 2017. Até essa data o Brasil possuía regulamentação apenas para aflatoxinas em amendoins para alimentação humana. Os LMT estabelecidos foram baseados em resultados obtidos a partir de critérios estabelecidos pelo *Codex Alimentarius*, uma coletânea de orientações e recomendações sobre a segurança de alimentos reconhecida pela Organização Mundial da Saúde (OMS).

Por apresentarem toxicidade em baixas concentrações, é de extrema importância que as técnicas empregadas na detecção e quantificação das micotoxinas sejam sensíveis e confiáveis. Nesse aspecto, o resultado analítico torna-se um ponto crítico na tomada de

decisões, pois é a partir dele que se determinará se um dado alimento é próprio ou impróprio para o consumo.

Trigo (*Triticum aestivum*) é um dos grãos básicos mais importantes do mundo, sendo seu significado econômico e social do trigo derivado de sua ampla produção e capacidade superior de gerar diversos alimentos, como pães, massas, bolos e biscoitos. Tratando-se de Brasil, a produção concentra-se no Sul e Centro-sul do país, tendo como principais produtores o Paraná, Rio Grande do Sul e São Paulo. A região Sul é responsável por 90% da produção nacional (CONAB, 2018). No entanto, essa produção corresponde a 50% da necessidade do país, sendo necessária a importação do mesmo (TIBOLA et al., 2015).

A aveia (*Avena sativa*) é uma cultura semeada principalmente na região Sul do Brasil, responsável por 96% da produção total de aveia do país, com produção média de 2,1 t ha^{-1} (CONAB, 2018). A aveia é uma valiosa matéria-prima utilizada em particular para a produção de flocos, farelo e grumos. É uma fonte de fibra alimentar solúvel na forma de β-glucana, um componente-chave responsável pelos benefícios à saúde (CLEMENS & KLINKEN, 2014). Eles são fonte de muitos componentes valiosos da dieta com importância nutricional e biológica, isto é, proteínas, gorduras, fibras, carboidratos, minerais e vitaminas (PETERSON, 2001).

Zea mays L. variedade roxa é uma planta nativa da América. Foi um dos principais alimentos das muitas tribos indígenas na era pré-colombiana e lhe são atribuídas várias propriedades medicinais com potencial para uso industrial e exportação. No Peru, seu consumo é popular e massivo na forma de chicha morada e mazamorra morada. "Chicha Morada" é uma bebida típica, que é feita com água em que o milho roxo foi cozido. Os componentes químicos presentes no milho roxo são essências, ácido salicílico, gorduras, resinas, saponinas, sais de potássio e sódio, enxofre e fósforo, mas sobretudo compostos fenólicos (ARROYO et al., 2007).

O objetivo do presente trabalho foi avaliar as possíveis contaminações por micotoxinas que possam estar presentes em amostras de aveia, trigo e milho roxo, e desenvolver um método em que seja possível a quantificação simultânea das micotoxinas avaliadas. Quantificar a ocorrência e a concentração de algumas micotoxinas regulamentadas e emergentes presentes em amostras desses cereais. A metodologia utilizada para determinação das micotoxinas foi cromatografia liquida de alta eficiência acoplada a espectrometria de massas. Este é o primeiro estudo dessas toxinas em aveia e trigo no Brasil. Com base nos resultados pode-se traçar estratégias que modifiquem o atual cenário e aumentem a qualidade do produto final.

O presente trabalho será apresentado em formato de dois manuscritos publicáveis.

2 MANUSCRITO I - OCCURRENCE OF MYCOTOXINS IN OAT AND WHEAT IN THE SOUTH REGION OF BRAZIL

AUTHOR: Natália Hoffmann Rossi
ADVISER: Carlos Augusto Mallmann

Abstract: Mycotoxins are secondary metabolites produced by filamentous fungi present in oat and wheat and several foodstuffs consumed worldwide. When contaminated food items are ingested, they may be toxic to humans and animals. Thus, this study aimed to apply a qualitative screening method and quantify the mycotoxins with and without current legislation present in oat and wheat samples in 2016 and 2017. Four hundred and sixty-five samples of oat (91) and wheat (374) from Rio Grande do Sul and Santa Catarina states were analyzed. Frequency of occurrence and concentration of the following mycotoxins were determined: alternariolmetileter (AME), alternariol (AOH), tentoxin, neosolaniol, nivalenol (NIV), wortmannin, deoxynivalenol (DON), 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, zearalenone (ZEA), aflatoxin B_1 (AFB_1), aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, fumonisin B_1, fumonisin B_2, fumonisin B_3, ochratoxin A, ochratoxin α, T-2 toxin, HT-2 toxin, fusarenon x, cyclopiazonic acid, gliotoxin, agroclavin and citreoviridin. Mean levels of mycotoxins within the standards of the Brazilian legislation were found, but some of the detected metabolites do not present specifications for the studied cereals. The main mycotoxins detected in oat were AME and AOH, with a frequency of occurrence of 100 and 98%, respectively. These were also the main mycotoxins found in wheat, followed by NIV, DON, ZEA and AFB_1, depending on the year of sample collection. It may be concluded that mycotoxins without guidance levels were detected in both cereals and years. Moreover, a warning must be made regarding the contamination at high concentrations by mycotoxins which are regulated, in some cases exceeding the permitted levels.

Key-words: Secondary metabolites; food contamination; *Avena sativa*; *Triticum aestivum*.

2.1 INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi that may develop in an assortment of foodstuffs. When diets contaminated by mycotoxins are ingested by animals or humans, they may induce a toxic response (mycotoxicoses). Nevertheless, not all fungal growth results in toxins formation, and the existence of fungi does not imply in the presence of mycotoxins either (BINDER et al., 2007; FINK-GREMMELS, MALEKINEJAD, 2007; VOSS, SMITH & HASCHEK, 2007). *Fusarium*, *Aspergillus* and *Penicillium* are the fungal species of greatest occurrence which may contaminate, grow and produce these toxins in feed and food, before and after harvest or during storage (LUO, Y.; LIU, X.; LI, J., 2018). *Fusarium* is one of the complex genera, encompassing species which are adapted to a great variety of habitats. *Aspergillus* is one of the three most important and diverse genera which have a worldwide distribution (TANIWAKI, M.A.; PITT, I. J.; MAGAN N., 2018).

Another important genus is *Alternaria*. It includes fungi which are common pathogens of a diversity of cultures and also infect processed products (EFSA, 2011). *Alternaria alternata*, for instance, produces a great number of mycotoxins, such as: alternariol, alternariolmetileter, tentoxin and tenuazonic acid. These toxins have been shown to be carcinogenic in rats and mutagenic in *in vitro* tests (MAGAM & OLSEN, 2004).

For presenting elevated toxicity and having guidance levels, the most studied and assessed mycotoxins are aflatoxins, fumonisins, trichothecenes and zearalenone (ROCHA et al., 2014; CORRÊA et al., 2018). The levels of mycotoxins must be monitored in order to protect consumers and animals, so the cereal production sector has employed good agricultural practices in order to avoid mycotoxins development (KABAK, DOBSON & VAR, 2006).

Although an assortment of results has been reported in the literature, they are mostly restricted to the effect of a single mycotoxin; nonetheless, contamination by more than one mycotoxin is more likely to occur (ALI, et al., 1998; ESKOLA, PARIKKA & RIZZO, 2001; GONZALEZ et al., 1999; PRONK, SCHOTHORST & EGMOND, 2002). Furthermore, different lots are mixed up in the feed industry, which alters their individual contamination and generates a new risk profile (PENG, W.-X.; MARCHAL J.L.M.; VAN DER POEL A.F.B., 2019). As a result, some of the observed diseases or clinical signs may not always be connected to the effects of a single mycotoxin upon humans and animals. Combined effects of these toxins may be additive, synergistic or antagonistic (SPEIJERS & SPEIJERS, 2004).

Until recently, most of the studies involving mycotoxins in Brazil have approached a limited number of them. Considering that more than one mycotoxin may occur coincidently, and that maximum tolerable limits (MTL) have been established by the National Health Regulatory Agency (ANVISA), it is important to monitor a greater number of mycotoxins in order to identify co-occurring fungal metabolites in the samples (HUSSEIN & BRASEL, 2001).

Emerging mycotoxins are not commonly detected in routine analyses. Such toxins are not traditionally determined, nor have limits set by any regulation. Their occurrence and/or prevalence are not known either. These toxins have been evaluated in more recent studies. As far as Brazil is concerned, only a few studies relate to them. Their effects have not yet been completely elucidated due to the fact that they are not often investigated (SPEIJERS, G. J. A.; SPEIJERS, M. H. M., 2004), so more works are necessary in order to avoid being affected by them (BOLECHOVA et al., 2015). Therefore, investigation of the microbiology and of the mycotoxigenic species in grains is crucial to produce and maintain a quality product for trade and consumption (BINDER et al., 2007; COSTA & ZANELLA, 2012). Since one single fungal species may produce more than one mycotoxin, the determination methods should be able to detect and quantify the greatest number of mycotoxins possible in a sample (HUSSEIN & BRASEL, 2001).

Wheat is one the most important basic grains worldwide. Its economic and social meanings derive from its vast production and high capacity of generating several food items, such as bread, pasta, cake and biscuits. In Brazil, wheat production is concentrated in the South and Center-South, and the main producers are Paraná, Rio Grande do Sul (RS) and São Paulo states. The South region accounts for 90% of the national production (CONAB, 2018). However, such production corresponds to 50% of the domestic demand, thus requiring its import (TIBOLA et al., 2015).

Oat (*Avena sativa*) is specially sown in the South of Brazil; the regions' production accounts for 96% of the total national yield, *i.e.*, 2,1 t ha^{-1} (CONAB, 2018). Genetic advances allowed development and exploration, as they are adopted and simplified, to considerably boost oat production in the next decade. Because of that, it is crucial to monitor the mycotoxins which may be present in this cereal (STEWART & MCDOUGALL, 2014). Oat is a valuable raw material particularly used to make flakes, bran and oatmeal. It is a source of soluble dietary fiber in the form of β-glucan, a key-component which is responsible for the health benefits (CLEMENS & KLINKEN, 2014). Oat products are sources of very valuable

dietary components with nutritional and biological importance, *i.e.*, proteins, fats, fibers, carbohydrates, minerals and vitamins (PETERSON, 2001).

Nowadays, the Brazilian legislation regulates the maximum tolerable limits (MTL) for mycotoxins in food through resolution RDC No 7 of 18 February 2011 (ANVISA, 2011). This resolution has four annexes which list and classify the foods and establish the MTL for AF (AFB_1 + AFB_2 + AFG_1 + AFG_2 and AFM_1), ochratoxin A (OTA A), deoxynivalenol (DON), FB (FB_1 + FB_2), patuline and zearalenone; such levels are admissible in ready to eat foods and raw materials. Law enforcement is indispensable to protect public health and to defend the economic interests of the country. When the legislation is not adequately obeyed, or monitoring is not properly conducted, humans, animals and the economy may be harmed.

This investigation aimed at verifying possible contaminations by mycotoxins that oat and wheat may present, and at quantifying the occurrence and concentration of some regulated and emerging mycotoxins in samples of such cereals in 2016 and 2017. The method used for that is liquid chromatography coupled to mass spectrometry (LC-MS/MS). It is the first study to assess these toxins in oat and wheat in Brazil. Based on the results, strategies which may change this scenario and enhance the final product quality can be drawn.

2.2 MATERIAL AND METHODS

2.2.1 Samples

A total of 465 samples of oat and wheat were obtained from food industries in the South region of Brazil, in RS and Santa Catarina (SC) states, in 2016 and 2017. Forty-one samples of oat were analyzed in 2016 and 50 in 2017, whereas 115 samples of wheat were examined in 2016 and 259 in 2017.

2.2.2 Mycotoxin analysis

2.2.2.1. Chemicals and reagentes

Methanol (LC gradient grade) and glacial acetic acid (p.a.) were purchased from Merck (Darmstadt, Germany); acetonitrile (LC gradient grade) from VWR (Leuven, Belgium); and ammonium acetate (MS grade) from Sigma-Aldrich (Vienna, Austria). Standards formycotoxins were obtained from various research groups or from commercial sources. Water was purified successively by reverse osmosis with an Elga Purelab ultra analytic system from Veolia Water (Bucks, UK)

2.2.3 Samples preparation

Each sample was ground through a 1.5mm sieve (ZM 200; Retsch, Haan, Germany). The sample was then homogenized and weighed in a 50-mL polypropylene tube.

2.2.4 Samples extraction

Mycotoxins extraction was performed according to the methodology described by SULYOK et al. (2006), with adaptations. A volume of 10 mL of a solution containing ACN:H_2O:HAc (84:15:1, v/v/v) was added to the polypropylene tube containing 5 g of sample. The tube was then agitated in a stirring table for 90 min. After decantation of the sample by gravity, the extract was diluted in an acetonitrile:water solution (1:1, v/v). Quantification was performed by using an external calibration based on serial dilutions of a multi-analyte standard stock solution.

2.2.5. Chromatographic analysis

After dilution, the extract was subjected to separation by liquid chromatography, followed by detection via mass spectrometry. Methanol, water, ammonium acetate and acetic acid were used to compose the mobile phase, in a flux of 0.8 mL min^{-1}, with an injection volume of 40 µL. Chromatographic separation was performed with an Agilent Zorbax Eclipse C18 column (4.6 x 150 mm, 5 µm particle diameter).

Briefly, the equipment used was a 1290 Infinity HPLC system (Agilent Technologies) coupled to a Qtrap 5500 (ABSCIEX), equipped with an electrospray ionization source (ESI) in positive and negative mode. The mass spectrophotometer was operated in MRM mode (multiple reaction monitoring) and MS/MS optimized conditions. For external calibration, multi-analyte standard stock solutions were prepared and diluted.

2.2.6 Analytical Quality Assurance

The signal-to-noise ratios for the limit of quantification (LQ) and the limit of detection (LD) were set at 10/1 and 3/1, respectively, to assure analytical quality. Table 1 shows the limits for each analyte.

Table 1**.** Limits of detection and quantification for the metabolites.

Metabolites	LD (µg kg^{-1})	LQ (µg kg^{-1})

AME	0,8	2,5
AOH	0,8	2,5
TEN	1,7	5,0
NEO	0,2	0,5
NIV	33,3	100,0
WORT	0,8	2,5
3-DON	16,7	50,0
15-DON	16,7	50,0
DON	66,7	200,0
ZEA	6,7	20,0
AFB1	0,3	1,0
AFB_2	0,3	1,0
AFG_1	0,3	1,0
AFG_2	0,3	1,0
FB_1	41,7	125,0
FB_2	41,7	125,0
FB_3	41,7	125,0
OTA A	0,8	2,5
OTA α	0,8	2,5
T2	33,3	100,0
HT2	33,3	100,0
DAS	33,3	100,0
FUSA-x	1,3	4,0
CPA	33,3	100,0
GL	0,8	2,5
AGC	1,7	5,0
CITREO	0,7	2,0

<LQ=Below the limit of quantification. AME=alternariolmetileter, AOH=alternariol, TEN=tentoxin, NEO=neosolaniol, NIV=nivalenol, WORT=wortmannin, 3-DON=3-acetyl deoxynivalenol, 15-DON=15-acetyl deoxynivalenol, DON=deoxynivalenol, ZEA=zearalenone, AFB_1=aflatoxin B_1, AFB_2=aflatoxin B_2, AFG_1=aflatoxin G_1, AFG_2=aflatoxin G_2, FB_1=fumonisin B_1, FB_2=fumonisin B_2, FB_3=fumonisin B_3, OTA A=ochratoxin A, OTA α=ochratoxin α, T2=T-2 toxin, HT2=HT-2 toxin, DAS=diacetoxiscirpenol, FUSA-x=fusarenon x, CPA=cyclopiazonic acid, GL=gliotoxin, AGC=agroclavin, CITREO=citreoviridin.

To estimate the recovery of each analyte, seven spiked replicates were analyzed at different concentration levels for the analyte of interest. For that, a set of samples constituted of white fortified matrices with the adequate volume of the working analytes was prepared and analyzed. Next, the samples were left overnight to enable solvent evaporation and to establish equilibrium between the analytes and the matrix. The mycotoxins were fortified at concentration levels (DON: 200, 400, 1,601 and 6,005 μg kg^{-1}; ZEA: 20, 123, 493 and 1,232 μg kg^{-1}; AOH: 0.5, 2.5 and 25 μg kg^{-1} TEN: 5, 20 and 250 μg kg^{-1}). The mean concentrations of each level, the standard deviation and the coefficients of variation of the reproducibility of the samples were calculated, with all of the results being combined at each level. Recovery of

the method was determined based on the data obtained in the precision study. The criteria for recovery acceptance was established according to the Commission Regulation (EC) No 401/2006 of 23 February 2006.

By means of precision studies for the method quality assurance, it is possible to verify that the method is precise, since all of the coefficients of variation are below the limits according to criteria established by The Ministry of Agriculture, Livestock and Food Supply (MAPA) (2011) (Tables 2, 3 and 4).

Table 2. Data of the quality assurance study for four levels of deoxynivalenol and zearalenone, and three levels of alternariol and tentoxin.

Metabolites	Mean (µg kg^{-1})	σ	CV(%)
DON*	211.0	10.0	5.0
DON	403.3	14.0	3.0
DON	1,615.2	77.0	5.0
DON	6,292.3	213.3	3.0
ZEA*	15.8	1.0	6.0
ZEA	101.7	2.6	3.0
ZEA	404.9	12.4	3.0
ZEA	1,056.0	70.1	7.0
AOH	0.6	0.03	6.2
AOH	2.7	0.1	1.9
AOH	25.8	0.5	1.9
TEN	4.7	0.3	6.5
TEN	20.5	0.5	2.6
TEN	272.1	5.0	1.7

CV(%)=coefficient of variation was the parameter adopted as the acceptance criterion of the precision. MAPA (2011). *Toxins regulated by the Brazilian legislation. AOH=alternariol, TEN=tentoxin, DON=deoxynivalenol, ZEA=zearalenone.

Table 3. Recovery values for the levels of deoxynivalenol and zearalenone (µg kg^{-1}).

	Recovery (%)			
Metabolites	NI	NII	NIII	NIV
DON	105	101	101	105
ZEA	79	84	82	86

DON=deoxynivalenol, ZEA=zearalenone.

Table 4. Recovery values for the levels of alternariol and tentoxin (µg kg^{-1}).

	Recovery (%)		
Metabolites	NI	NII	NIII
AOH	109.4	106.1	103.4

TEN	94.4	82.1	108.9

AOH=alternariol, TEN=Tentoxin.

2.3 RESULTS AND DISCUSSION

Twelve metabolites were detected in oat and seven in wheat in the two assessed years, out of which at least 11 are included in the Brazilian legislation (ANVISA, 2011). Table 5 shows the results of the contamination of the samples collected in RS and SC in 2016 and 2017.

2.3.1 Mycotoxins present in oat samples

Twelve metabolites were detected in the analyzed oat samples; other 15 metabolites were under the limit of quantification (<LQ) (Table 5).

Table 5. Metabolites detected in the oat samples collected in Rio Grande do Sul and Santa Catarina states in 2016 and 2017.

Metabolites	2016		2017	
	Frequency (%)	Mean of positive samples (µg kg^{-1})	Frequency(%)	Mean of positive samples (µg kg^{-1})
AME	41 (100.00)	85.89	41 (85.42)	98.70
AOH	40 (97.56)	561.28	41 (85.42)	508.37
TEN	33 (80.49)	47.01	32 (66.67)	32.40
NEO	34 (82.93)	12.18	0 (0.00)	<LQ
NIV	29 (70.73)	373.68	31 (64.58)	550.68
WORT	0 (0.00)	<LQ	0 (0.00)	<LQ
3DON	1 (2.44)	21.90	3 (6.25)	680.76
15DON	0 (0.00)	<LQ	7 (14.58)	1,220.73
DON	19 (46.34)	200.00	12 (25.00)	1,272.32
ZEA	8 (19.51)	43.00	7 (14.58)	154.01
AFB_1	0 (0.00)	<LQ	3 (6.25)	3.70
AFB_2	0 (0.00)	<LQ	0 (0.00)	<LQ
AFG_1	0 (0.00)	<LQ	1 (2.08)	3.40
AFG_2	0 (0.00)	<LQ	0 (0.00)	<LQ
FB_1	1 (2.44)	197.00	1 (2.08)	178.70
FB_2	0 (0.00)	<LQ	0 (0.00)	<LQ
FB_3	0 (0.00)	<LQ	0 (0.00)	<LQ
OTA A	0 (0.00)	<LQ	0 (0.00)	<LQ
OTA α	0 (0.00)	<LQ	0 (0.00)	<LQ
T2	0 (0.00)	<LQ	0 (0.00)	<LQ
HT2	0 (0.00)	<LQ	0 (0.00)	<LQ
DAS	0 (0.00)	<LQ	0 (0.00)	<LQ

FUSA-x	0 (0.00)	<LQ	0 (0.00)	<LQ
CPA	0 (0.00)	<LQ	0 (0.00)	<LQ
GLI	0 (0.00)	<LQ	0 (0.00)	<LQ
AGC	0 (0.00)	<LQ	0 (0.00)	<LQ
CITREO	0 (0.00)	<LQ	0 (0.00)	<LQ

<LQ=Below the limit of quantification. AME=alternariolmetileter, AOH=alternariol, TEN=tentoxin, NEO=neosolaniol, NIV=nivalenol, WORT=wortmannin, 3DON=3-acetyl deoxynivalenol, 15DON=15-acetyl deoxynivalenol, DON=deoxynivalenol, ZEA=zearalenone, AFB_1=aflatoxin B_1, AFB_2=aflatoxin B_2, AFG_1=aflatoxin G_1, AFG_2=aflatoxin G_2, FB_1=fumonisin B_1, FB_2=fumonisin B_2, FB_3=fumonisin B_3, OTA A=ochratoxin A, OTA α=ochratoxin α, T2=T-2 toxin, HT2=HT-2 toxin, DAS=diacetoxiscirpenol, FUSA-x=fusarenon x, CPA=cyclopiazonic acid, GL=gliotoxin, AGC=agroclavin, CITREO=citreoviridin.

DON and ZEA, which are mycotoxins regulated by the Brazilian legislation, presented means within the guidance levels (ANVISA, 2011). Nonetheless, their concentrations in some of the analyzed samples were above the MTL in both years (Table 6). OTA A, whose limits are established by RDC 17/2011, was not detected in any of the analyzed samples (Table 5).

With respect to the metabolites without guidance levels in the Brazilian legislation, the one which had the greatest prevalence in oat samples was alternariolmetileter (AME), being detected in 100% of the samples from 2016 and in 85.42% of those from 2017. Another mycotoxin which is not included in the national law is alternariol (AOH). This metabolite was detected in most of the analyzed samples with a mean concentration of 561.28 and 508.37 μg kg^{-1} in 2016 and 2017, respectively. It should be highlighted that in some of the oat samples the concentration of AOH was higher than 4,700 μg kg^{-1} (Table 6). Co-occurrence of AME and AOH was confirmed in 97.56% and 85.42 of the oat samples in 2016 and 2017, respectively. At greater concentrations, AOH is able to modulate estradiol and progesterone production, besides altering gene expression (FRIZZELL et al., 2013).

Tentoxin (TEN) was also found in the majority of the oat samples: 80% in 2016 and 66% in 2017. No regulatory levels have been set for this mycotoxin in the Brazilian legislation and, according to the latest European Food Safety Authority report (EFSA, 2016), its isolated form does not pose a potential risk to human health.

WORT, AFB_2, AFG_2, FB_2, FB_3, OTA A, OTA α, T2, HT2, DAS, FUSA-x, CPA, GL, AGC and CITREO were not detected (Table 5). As stated by XU et al. (2013), detecting the occurrence of some mycotoxins in oat, as HT2 and T2, may not be possible because the agronomic and environmental factors do not allow the development of the pathogen.

FB_1 was found in one sample of each collection period (Table 5); there is an absence of specific limits for this mycotoxin in oat in the Brazilian legislation (ANVISA, 2011). A thorough search of the relevant literature has not yielded any articles related to the occurrence of FB in oat. The main items in which FB are found are maize and its by-products

(STANKOVIC et al., 2012; TANSAKUL et al., 2013; MENDES et al., 2015; CORRÊA et al., 2018). In extreme cases, the effects of FB may lead to esophageal cancer in humans (RHEEDER et al., 1992).

Table 6. Maximum concentration of the metabolites found in oat samples in 2016 and 2017.

Metabolites	Maximum concentration (µg kg^{-1})	
	2016	2017
AME	454.30	559.80
AOH	4,943.30	4,719.60
TEN	273.70	131.20
NEO	25.20	---
NIV	1,305.40	3,375.80
3DON	21.90	1,088.00
15DON	---	4,221.60
DON	5,802.90	3,688.00
ZEA	198.60	354.60
FB_1	197.00	178.70

AME=alternariolmetileter, AOH=alternariol, TEN=tentoxin, NEO=neosolaniol, NIV=nivalenol, 3DON=3-acetyl deoxynivalenol, 15DON=15-acetyl deoxynivalenol, DON=deoxynivalenol, ZEA=zearalenone, FB_1=fumonisin B_1.

Neither AFG_1 nor AFG_2 were found in the oat samples analyzed in 2016. In 2017, in turn, two samples were contaminated by these toxins. On the whole, such results evidence the low probability of AF contamination in oat. Environmental and agronomic factors are possible reasons for the occurrence of these mycotoxins in oat in different years. AFG_1 and AFG_2 have oncogenic and immunosuppressive properties, thus inducing infections (ROCHA et al., 2014). Consequently, they may contribute to raise the risk of liver cancer in those individuals chronically infected with hepatitis B (GROOPMAN et al., 2008).

For those mycotoxins within the standards of the Brazilian legislation, the greatest co-occurrence in oat samples was found for DON and ZEA (14.6%); a low frequency of co-occurrence was detected for AB_1 along with DON and ZEA (Table 7). Oliveira et al. (2017) obtained a coincident occurrence of DON and ZEA in around 35% of the maize samples collected in three Brazilian states; the highest co-occurrence frequency found in the study was for FB_1, FB_2, ZEA and DON.

Table 7. Co-occurrence of mycotoxins with guidance levels in the Brazilian legislation in oat samples[1].

Mycotoxins	2016 (n - 41)	2017 (n - 48)	Frequency of co-occurrence (%)
DON + ZEA and AB_1	0	1	1 (1.1)
DON and ZEA	6	7	13 (14.6)
DON and AB_1	0	1	1 (1.1)
ZEA and AB_1	0	1	1 (1.1)

[1]Besides the mycotoxins presented here, the Brazilian legislation also includes Ocratoxin A, but no levels of this toxin were detected in this assessment. n=number of analyzed samples.

2.3.2 Mycotoxins present in wheat samples

Comparing to oat, a smaller amount of metabolites was detected in wheat. Among the seven toxins found in the cereal, only DON, ZEA and AFB_1 have guidance levels in the Brazilian legislation. None of the mean concentrations quantified in the analyses exceeded the MTL set in the legislation (ANVISA, 2011) (Table 8).

AME and AOH were the toxins more often detected in the wheat samples: 87.83 and 86.09%, respectively. The maximum concentrations measured for such metabolites (Table 9) are almost 11 times greater than the mean levels measured in the positive samples (Table 8). Variations in the concentration of the mycotoxins in cereals may occur specially due to the collection site, climatic variation during flowering or harvesting, and the host resistance features; all of which may directly influence the total production of mycotoxins (XU et al., 2013).

Table 8. Metabolites detected in the wheat samples collected in Rio Grande do Sul and Santa Catarina states in 2016 and 2017.

Metabolites	2016		2017	
	Frequency (%)	Mean of positive samples (µg kg^{-1})	Frequency (%)	Mean of positive samples (µg kg^{-1})
AME	101 (87.83)	32.69	193 (74.23)	7.38
AOH	99 (86.09)	133.92	222 (85.38)	22.75
TEN	34 (29.56)	15.13	77 (29.61)	46.92
NEO	0 (0.0)	<LQ	0 (0.00)	<LQ
NIV	77 (66.96)	428.63	118 (45.38)	531.57
WORT	0 (0.00)	<LQ	0 (0.00)	<LQ
3DON	0 (0.00)	<LQ	0 (0.00)	<LQ
15DON	0 (0.00)	<LQ	0 (0.00)	<LQ
DON	75 (65.22)	511.38	188 (72.31)	973.17
ZEA	26 (22.61)	56.47	51 (19.62)	130.58
AFB_1	4 (3.48)	1.825	5 (1.92)	2.05
AFB_2	0 (0.00)	<LQ	0 (0.00)	<LQ
AFG_1	0 (0.00)	<LQ	0 (0.00)	<LQ

AFG_2	0 (0.00)	<LQ	0 (0.00)	<LQ
FB_1	0 (0.00)	<LQ	0 (0.00)	<LQ
FB_2	0 (0.00)	<LQ	0 (0.00)	<LQ
FB_3	0 (0.00)	<LQ	0 (0.00)	<LQ
OTA A	0 (0.00)	<LQ	0 (0.00)	<LQ
OTA α	0 (0.00)	<LQ	0 (0.00)	<LQ
T2	0 (0.00)	<LQ	0 (0.00)	<LQ
HT2	0 (0.00)	<LQ	0 (0.00)	<LQ
DAS	0 (0.00)	<LQ	0 (0.00)	<LQ
FUSA-x	0 (0.00)	<LQ	0 (0.00)	<LQ
CPA	0 (0.00)	<LQ	0 (0.00)	<LQ
GLI	0 (0.00)	<LQ	0 (0.00)	<LQ
AGC	0 (0.00)	<LQ	0 (0.00)	<LQ
CITREO	0 (0.00)	<LQ	0 (0.00)	<LQ

<LQ=Below the limit of quantification. AME=alternariolmetileter, AOH=alternariol, TEN=tentoxin, NEO=neosolaniol, NIV=nivalenol, WORT=wortmannin, 3DON=3-acetyl deoxynivalenol, 15DON=15-acetyl deoxynivalenol, DON=deoxynivalenol, ZEA=zearalenone, AFB_1=aflatoxin B_1, AFB_2=aflatoxin B_2, AFG_1=aflatoxin G_1, AFG_2=aflatoxin G_2, FB_1=fumonisin B_1, FB_2=fumonisin B_2, FB_3=fumonisin B_3, OTA A=ochratoxin A, OTA α=ochratoxin α, T2=T-2 toxin, HT2=HT-2 toxin, DAS=diacetoxiscirpenol, FUSA-x=fusarenon x, CPA=cyclopiazonic acid, GL=gliotoxin, AGC=agroclavin, CITREO=citreoviridin.

The concentrations of DON and ZEA stand out for being above the MTL set by the Brazilian legislation (Table 9). These are the most frequent and abundant mycotoxins found in wheat (XU et al., 2019). In China, the latter authors investigated 370 wheat samples and verified the presence of DON, NIV and ZEA in 100, 80 and 69% of them, respectively; the mean levels were 17,753.8 µg kg^{-1}, *i.e.*, 18 times greater than the levels tolerated by the Chinese legislation.

The highest concentration of DON found in the Canadian durum wheat in 2010 was 4,700 µg kg^{-1} (TITTLEMIER, 2013), while the current study detected mean concentrations of 511.38 and 973.17 µg kg^{-1} in 2016 and 2017, respectively. Also in Brazil, a 2009 study observed frequency of 86% and mean concentration of 1,046 µg kg^{-1} for this mycotoxin in wheat (DOMINGUES et al., 2016), results which are similar to the ones reported herein.

None of the analyzed samples presented FB or OTA, whose MTL have been set by the legislation. Wheat contamination by FB_1 is scarcely reported (STANKOVIC et al., 2012). MARIN et al. (1999) suggests that the little occurrence of this mycotoxin in winter cereals is a reflex of the presence of competing microbiota (which differs from the one present in corn); this may inhibit FB synthesis by *Fusarium* sp., or degrade the mycotoxin soon after it is produced. Furthermore, many nutritional components of wheat could act as inhibitors of FB biosynthesis.

Table 9. Maximum concentration of the metabolites found in wheat samples in 2016 and 2017.

Metabolites	Maximum concentration (µg kg^{-1})	
	2016	2017
AME	344.06	68.30
AOH	1,448.60	167.80
TEN	48.80	164.20
NIV	5,895.70	3,520.00
DON	1,655.50	4,523.70
ZEA	111.30	409.60
AFB_1	3.10	2.90

AME=alternariolmetileter, AOH=alternariol, TEN=tentoxin, NIV=nivalenol, DON=deoxynivalenol, ZEA=zearalenone, AFB_1=aflatoxin B_1.

Mycotoxins producing fungi which contaminate oat and wheat represent a worrying matter, since these toxins may be toxic to humans and animals. It is important to highlight that, considering the risk of each toxin on its own, co-occurrence may be a greater threat to the health of these individuals (MARIN et al., 2013; ROCHA et al., 2014).

In keeping with the oat findings, the greatest frequency of co-occurrence observed in wheat was for DON and ZEA; of the 374 samples collected in 2016 and 2017, 75 presented both mycotoxins. The remaining combinations had frequencies lower than 2% (Table 10).

Table 10. Co-occurrence of mycotoxins with guidance levels in the Brazilian legislation in wheat samples[1].

Mycotoxins	2016 (n - 41)	2017 (n - 48)	Frequency of co-occurrence (%)
DON + ZEA and AB1	1	2	3 (0.8)
DON and ZEA	26	49	75 (20.1)
DON and AB1	3	4	7 (1.12)
ZEA and AB1	1	2	3 (0.8)

[1]Besides the mycotoxins presented here, the Brazilian legislation also includes Ocratoxin A, but no levels of this toxin were detected in this assessment. n=number of analyzed samples.

2.4 CONCLUSIONS

Mycotoxins which are not regulated by the Brazilian legislation were detected in oat and wheat samples. AME, AOH, TEN and NIV were the most prevalent mycotoxins in these cereals, and their MTL have not yet been established.

Fungi and other contaminants in oat and wheat may produce mycotoxins which are toxic to humans and animals, thus representing a matter of great concern. Production of such contaminants is not exact and may reach levels above the MTL in favorable moments. Considering the potential risks associated to each mycotoxin in isolation, co-occurrence may

increase the probability of human and animal morbidity and mortality. Since little is known about the harm of the emerging mycotoxins in isolation, when combined they may have unknown effects. Identification of various combinations of mycotoxins would be useful to estimate any possible synergistic or additive effects in contaminated food or feed. The developed method is suitable for separation and quantification of 27 mycotoxins simultaneously. Further studies are necessary to expand the knowledge on the local occurrence of mycotoxins, aiming to reduce the contamination of these cereals. Good agricultural practices and proper storage systems may reduce the occurrence of these mycotoxins.

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3 MANUSCRITO II - SIMULTANEOUS DETERMINATION OF MYCOTOXINS IN PERUVIAN PURPLE MAIZE

AUTHOR: Natália Hoffmann Rossi
ADVISER: Carlos Augusto Mallmann

Abstract: Purple maize is an important foodstuff for the Peruvian people. Its unique nutritional and antioxidant characteristics makes it widely exported to other countries. However, when contaminated by fungi, it can trigger numerous health problems in the consumers. This study aimed at evaluating the presence of 27 mycotoxins in 63 samples of purple maize collected in Peru. Frequency of occurrence and mean concentration of the following mycotoxins were determined: alternariolmetileter (AME), alternariol (AOH), tentoxin, neosolaniol, nivalenol, wortmannin, deoxynivalenol, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, zearalenone, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, fumonisin B_1, fumonisin B_2, fumonisin B_3, ochratoxin A, ochratoxin α, T-2 toxin, HT-2 toxin, fusarenon x, cyclopiazonic acid, gliotoxin, agroclavin and citreoviridin. The main mycotoxins found in purple maize were AME and AOH, with a frequency of occurrence of 14.3 and 7.9%, and mean concentration of 23.3% and 1.8%, respectively. AME and AOH do not have guidance levels in the Brazilian legislation. Contrastingly, the levels of mycotoxins which are within the standards of the country's regulations were below the limit of quantification. The present results suggest that purple maize is a raw material with a great potential for the production and industrialization of special products.

Keywords: *Zea mays* L. alternariolmetileter. alternariol. secondary metabolites.

3.1 INTRODUCTION

There are several types of maize, which may present an assortment of colors: white, yellow, red, brown, green, blue and purple. The latter has been cultivated in Latin America, especially in Peru. Such culture and its beneficial health effects have been depicted in ceremonial pottery in Peru and other Hispanic countries (Inca and pre-Inca eras) (RAMOS-ESCUDERO et al., 2011).

The purple race of *Zea mays* L. is a native plant from America. It was one of the main foodstuffs of the many indigenous peoples in the pre-Columbian era, and several medicinal properties have been attributed to it; hence its industrial and export potential. In Peru, it is mostly consumed in the form of the refreshment chicha morada and the dessert mazamorra morada. Chicha morada is made with the water in which the purple maize was cooked. The chemical components of the purple maize are essences, salicylic acid, fats, resins, saponins, potassium and sodium salts, sulfur and phosphorus, and especially phenolic compounds (ARROYO et al., 2007).

The purple maize is an important source of anthocyanins, natural pigments which are widely distributed in the plant kingdom with peculiar characteristics regarding the color of the food plants (TSUDA, et al., 2003). The anthocyanins which have already been characterized in cobs and seeds of purple maize include cyanidin-3-glusoside, pelargonidin-3-glucoside, peonidin-3-glucoside and their respective malonyl derivatives (AOKI, KUZE, & KATO, 2002; PASCUAL-TERESA, SANTOS-BUELGA & RIVAS-GONZALO, 2002).

Anthocyanins have been reported to possess biological activities as antioxidant, anti-mutagenic, anti-carcinogenic and anti-obesity, and also to ameliorate hyperglycemia (HAGIWARA et al., 2001; CEVALLOS-CASALS & CISNEROS-ZEVALLOS, 2003; TSUDA et al., 2003). The purple maize produces several phenolic constituents as anthocyanins, which have been recognized as food colorants as well as healthy food material (PEDRESCHI & CISNEROS-ZEVALLOS, 2007).

Mycotoxins are toxic secondary metabolites which result from the contamination of the food, and are produced by a variety of fungi species. They may cause economic losses and affect human and animal health when contaminated food is ingested by them (HUSSEIN & BRASSEL, 2001). The Food and Agriculture Organization of the United Nations (FAO) has estimated that every year 25% of the food crops worldwide are contaminated by mycotoxins (BOUTRIF & CANET, 1998).

Food and feed contamination by mycotoxins is a risk factor to the population and the animals due to their harmful effects. Epidemiological evidence and studies carried out in animals indicate that these substances have a wide spectrum of toxicological effects, particularly those carcinogenic, teratogenic, neurotoxic and immunosuppressive ones. Severity of such effects depend on the type of mycotoxin, length of exposure, ingested dose, age and nutritional and health status of the subject. Constant exposure to several mycotoxins is always possible in a human diverse diet, especially at low concentrations. Besides, little is known about the effects of the interaction between these compounds in the organism, despite the fact that more than one mycotoxin is usually found in contaminated food (HUSSEIN e BRASEL, 2001; KUMAR et al., 2008).

The high incidence of mycotoxin contamination of maize demands more studies to be done so that only products having low concentrations of mycotoxins are used in the industry. Then, this study was aimed at investigating the concentrations of 27 mycotoxins in Peruvian purple maize through liquid chromatography coupled to mass spectrometry.

3.2 MATERIAL AND METHODS

3.2.1 Samples

Sixty-three samples of purple maize were obtained from commercial establishments in Peru from March to April 2017. Each sample was ground through a 1.5mm sieve (ZM 200; Retsch, Haan, Germany), homogenized and then weighed in a 50-mL polypropylene tube.

3.2.2 Extraction

Mycotoxins extraction was performed according to the methodology of SULYOK et al. (2006), with adaptations. A volume of 10 mL of a solution containing ACN:H_2O:HAc (84:15:1, v/v/v) was added to the polypropylene tube containing 5 g of sample, which was then agitated in a stirring table for 90 min. Dilution of the extract in an acetonitrile:water solution (1:1, v/v) was carried out after decantation of the sample by gravity. Quantification was performed by using an external calibration based on serial dilutions of a multi-analyte standard stock solution.

3.2.3 Chromatographic analysis

Briefly, the analyses were carried out with a LC-MS/MS QTrap 5500 System (ABSCIEX, Foster City, CA, EUA) equipped with an electrospray ionization source (ESI) in positive and negative mode (Turbo Ion Spray), and a 1290 Infinity HPLC System (Agilent, Waldbronn, Alemanha). Chromatographic separation was done at 30 °C with an Agilent Zorbax Eclipse C18 column (4.6 x 150 mm, 5 µm particle diameter). Methanol, water and ammonium acetate were used to compose the mobile phase, in a flux of 0.8 mL min^{-1}, with an injection volume of 40 µL.

The mass spectrophotometer was operated in MRM mode (multiple reaction monitoring), in positive and negative polarities, with two reactions of fragment per analyte being examined. The MRM detection window of each analyte was fixed to its expected retention time ±27 s and ±48 s in the positive and negative mode, respectively. Acquisition of two MRMs per analyte allowed confirmation of positive analyte identification. The LC retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30% relative, respectively.

3.2.4 Analytical Quality Assurance

To assure analytical quality, the signal-to-noise ratios for the limit of quantification (LQ) and the limit of detection (LD) were set at 10/1 and 3/1, respectively. Table 1 shows the limits for each analyte.

Table 1**.** Limits of detection and quantification for the metabolites.

Metabolites	LD (µg kg^{-1})	LQ (µg kg^{-1})
AME	0,8	2,5
AOH	0,8	2,5
TEN	1,7	5,0
NEO	0,2	0,5
NIV	33,3	100,0
WORT	0,8	2,5
3-DON	16,7	50,0
15-DON	16,7	50,0
DON	66,7	200,0
ZEA	6,7	20,0
AFB1	0,3	1,0
AFB_2	0,3	1,0

AFG_1	0,3	1,0
AFG_2	0,3	1,0
FB_1	41,7	125,0
FB_2	41,7	125,0
FB_3	41,7	125,0
OTA A	0,8	2,5
OTA α	0,8	2,5
T2	33,3	100,0
HT2	33,3	100,0
DAS	33,3	100,0
FUSA-x	1,3	4,0
CPA	33,3	100,0
GL	0,8	2,5
AGC	1,7	5,0
CITREO	0,7	2,0

<LQ=Below the limit of quantification. AME=alternariolmetileter, AOH=alternariol, TEN=tentoxin, NEO=neosolaniol, NIV=nivalenol, WORT=wortmannin, 3-DON=3-acetyl deoxynivalenol, 15-DON=15-acetyl deoxynivalenol, DON=deoxynivalenol, ZEA=zearalenone, AFB_1=aflatoxin B_1, AFB_2=aflatoxin B_2, AFG_1=aflatoxin G_1, AFG_2=aflatoxin G_2, FB_1=fumonisin B_1, FB_2=fumonisin B_2, FB_3=fumonisin B_3, OTA A=ochratoxin A, OTA α=ochratoxin α, T2=T-2 toxin, HT2=HT-2 toxin, DAS=diacetoxiscirpenol, FUSA-x=fusarenon x, CPA=cyclopiazonic acid, GL=gliotoxin, AGC=agroclavin, CITREO=citreoviridin.

Seven spiked replicates were analyzed at different concentration levels for the analyte of interest in order to estimate the recovery of each analyte. A set of samples constituted of white fortified matrices with the adequate volume of the working analytes was prepared and analyzed. The samples were then left overnight to allow solvent evaporation and to establish equilibrium between the analytes and the matrix. The white samples were fortified at concentration levels: alternariol - 0.5, 2.5 and 25 μg kg^{-1}, and tentoxin - 5, 20 and 250 μg kg^{-1}. Calculation of the mean concentrations of each level, the standard deviation and the coefficients of variation of the reproducibility of the samples was made, with all of the results being combined at each level.

The precision of the method is demonstrated by means of studies for the method quality assurance; all of the coefficients of variation are below the limits according to criteria established by The Ministry of Agriculture, Livestock and Food Supply (MAPA) (2011) (Table 2).

Table 2. Recovery values for the levels of alternariol (AOH) and tentoxin (TEN) (μg kg^{-1}).

	Recovery (%)		
Metabolites	NI	NII	NIII
AOH	109	105	103
TEN	93	81	107

3.3 RESULTS AND DISCUSSION

This is the first scientific report on the quantification of mycotoxins in purple maize. Of the 27 mycotoxins analyzed in the 63 samples of Peruvian purple maize, only two were detected: alternariolmetileter (AME) and alternariol (AOH). None of these metabolites is within the standards of the Brazilian legislation. Coincident occurrence of AME and AOH was found in 7.9% of the samples (data not shown). Co-occurrence of mycotoxins has been recurrently reported in maize samples, though not of AME and AOH (MORENO et al., 2009; SERRANO et al., 2012; KROUT-GREENBERG et al., 2013; JAMES et al., 2018). Furthermore, 54 samples did not present mycotoxins at detectable levels (within the LQ), and none of the analyzed metabolites which have guidance levels in the Brazilian legislation were detected in this assessment (BRASIL, 2011).

3.3.1 Alternariolmetileter

AME was detected in 12.7% of the samples, with a mean concentration of 23.3 μg kg^{-1}. It was the most frequent mycotoxin to be found in the analyzed samples (Table 3). It is present in a diversity of foodstuffs, as fruits, cereals and oilseeds (PATRIARCA, 2016). According to TIEMANN et al. (2009), ingestion of food contaminated with *Alternaria* toxin may affect the reproductive development of swine and other mammal species. Moreover, chronic exposure through the diet has shown to exceed the limit of toxicological warning for this mycotoxin (EFSA, 2011). Therefore, the present results indicate the need for additional toxicity data for AME in humans.

The greatest concentration found for AME was 86.8 μg kg^{-1} (Table 3). It must be highlighted that, along with AOH and all of the mycotoxins belonging to the genus *Alternaria*, AME does not have guidance levels (Hajnal et al., 2016). The latter authors stated that AME can be extracted from whole wheat flour by means of specific processes, thus resulting in a 94.5% extraction of the metabolite.

3.3.2 Alternariol

The highest concentration of AOH found in the purple maize samples was 3.75 μg kg^{-1} (Table 3). Since there are no standards for this mycotoxin within the legislation, it is not known whether this is a high or low amount. Nonetheless, it has been shown that AOH is

harmful to poultry health; synergistic estrogenic effects have been observed when binary combinations of AOH and zearalenone or α-zearalenol were tested (LEE *et al*., 2015; VEJDOVSZKY *et al*., 2017).

Present in 7.9% of the analyzed samples, AOH was less frequent than AME; both mycotoxins are produced by *Alternaria* fungi (LEE *et al*., 2015). These mycotoxins do not have guidance levels in any national or international legislations (HAJNAL *et al*., 2016), and this is likely to contribute to the fact that only a few scientific studies investigate their presence in maize, and none in purple maize.

3.3.3 Most frequent mycotoxins in yellow maize

None of the mycotoxins most frequently detected in yellow maize reached the LQ in the current study (Table 3). Deoxinivalenol, zearalenone, aflatoxinas B_1 and B_2 and fumonisins are the mycotoxins most often reported and also the ones to cause the greatest concerns in yellow maize (KROUT-GREENBERG *et al.*, 2013; ADEYEYE, 2019). Serious outcomes may be produced in humans and animals as a result of their ingestion (BELHASSEN *et al.*, 2015).

Table 3. Metabolites detected in the samples of purple maize collected in Peru from March to April 2017.

Metabolites	Frequency (%)	Mean concentration of positive samples (µg kg^{-1})	Maximum concentration (µg kg^{-1})
AME	9 (14.3)	23.3	86.78
AOH	5 (7.9)	1.8	3.75
TEN	0 (0.00)	<LQ	---
NEO	0 (0.00)	<LQ	---
NIV	0 (0.00)	<LQ	---
WORT	0 (0.00)	<LQ	---
3-DON	0 (0.00)	<LQ	---
15-DON	0 (0.00)	<LQ	---
DON	0 (0.00)	<LQ	---
ZEA	0 (0.00)	<LQ	---
AFB_1	0 (0.00)	<LQ	---
AFB_2	0 (0.00)	<LQ	---
AFG_1	0 (0.00)	<LQ	---
AFG_2	0 (0.00)	<LQ	---
FB_1	0 (0.00)	<LQ	---

FB_2	0 (0.00)	<LQ	---
FB_3	0 (0.00)	<LQ	---
OTA A	0 (0.00)	<LQ	---
OTA α	0 (0.00)	<LQ	---
T2	0 (0.00)	<LQ	---
HT2	0 (0.00)	<LQ	---
DAS	0 (0.00)	<LQ	---
FUSA-x	0 (0.00)	<LQ	---
CPA	0 (0.00)	<LQ	---
GL	0 (0.00)	<LQ	---
AGC	0 (0.00)	<LQ	---
CITREO	0 (0.00)	<LQ	---

<LQ=Below the limit of quantification. AME=alternariolmetileter, AOH=alternariol, TEN=tentoxin, NEO=neosolaniol, NIV=nivalenol, WORT=wortmannin, 3DON=3-acetyl deoxynivalenol, 15DON=15-acetyl deoxynivalenol, DON=deoxynivalenol, ZEA=zearalenone, AFB_1=aflatoxin B_1, AFB_2=aflatoxin B_2, AFG_1=aflatoxin G_1, AFG_2=aflatoxin G_2, FB_1=fumonisin B_1, FB_2=fumonisin B_2, FB_3=fumonisin B_3, OTA A=ochratoxin A, OTA α=ochratoxin α, T2=T-2 toxin, HT2=HT-2 toxin, DAS=diacetoxiscirpenol, FUSA-x=fusarenon x, CPA=cyclopiazonic acid, GL=gliotoxin, AGC=agroclavin, CITREO=citreoviridin.

With regard to the presence of toxins produced by fungi, the present findings allow to characterize the purple maize as an excellent raw material to be used in the food and feed industry. Moreover, the unprecedented data conveyed herein contributes to the development of strategies employing this special ingredient with extremely low levels of these noxious substances, which are so deleterious to human and animal health. Subsequent work on mycotoxins should be carried out in order to complement the data obtained in this investing, and thus feed the Peruvian authorities with scientific information to allow for decision making.

Further research should also be conducted to evidence the characteristics of purple maize which hold the capacity of inhibiting fungal proliferation and, consequently, mycotoxins development. Likewise, the nutritional properties of this culture should be better understood so that it could be used for the production of a greater diversity of items and thus expand its share in the market.

3.4 CONCLUSION

The methodology employed in the present work is effective to determine 27 mycotoxins simultaneously by using LC-MS/MS. No quantifiable levels of mycotoxins with guidance levels within the Brazilian legislation were detected. Of the 27 analyzed mycotoxins, only alternariolmetileter and alternariol were found. Thus, the Peruvian purple

maize may be considered a promising alternative to be used in the manufacturing of a variety of products.

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4 CONCLUSÃO GERAL

O método desenvolvido é efetivo para determinação de 27 micotoxinas simultaneamente. Os limites são inferiores ao da legislação brasileira, contemplam assim a mesma, e, além disso incluem micotoxinas que não são legisladas, chamadas emergentes. AME e AOH foram as mais prevalentes neste estudo. As concentrações máximas de AME e AOH obtidas em trigo foram 344,1 μg kg^{-1} e 1448,6 μg kg^{-1} respectivamente; em aveia, 559,8 μg kg^{-1} e 4943,3 μg kg^{-1}, respectivamente. Já em milho roxo as concentrações máximas obtidas de AME e AOH foram 86,8 μg kg^{-1} e 3,8 μg kg^{-1}, respectivamente. Este método pode ser utilizado em análises de rotina de micotoxinas, sendo relativamente mais rápido do que métodos que fazem a determinação de grupos de micotoxinas, o que facilita a tomada de decisões.

Por meio dos resultados obtidos no presente estudo é possível fornecer informações epidemiológicas para avaliação de risco e controle de doenças, incluindo controle químico e genético. Através de estratégias de controle será possível minimizar a produção das micotoxinas que não estão na legislação e diminuir a ocorrência daquelas que estão acima dos níveis tolerados para os cereais estudados.

A identificação da coocorrência das micotoxinas auxilia na antecipação de possíveis impactos para os consumidores. De qualquer forma, são necessários mais estudos das toxinas emergentes nessas matrizes e seus efeitos. No entanto, deve-se salientar que mais estudos devem ser feitos a respeito de contaminações sinérgicas, aditivas ou antagônicas, pois o efeito delas juntas será, possivelmente, mais deletério.

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