DETERMINATION OF PATULIN IN VEGETABLES AND EVALUATION OF ITS CYTOTOXICITY ON MAMMALIAN CELLS

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by

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Determination of patulin in vegetables and evaluation of its cytotoxicity on mammalian cells

I declare that this dissertation is my own work and has not been previously submitted for the purpose of obtaining a Master degree and that all sources that I used have been indicated and acknowledged by means of complete references.

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ABSTRACT

Monitoring mycotoxin contamination in foods and feeds is important in both human's and animals' health and it is of concern in the regulation of mycotoxins in foods and feeds worldwide. Out of all the contaminants found in food and feeds, mycotoxins are one of the extensively studied contaminants. Mycotoxins are secondary metabolites produced by a wide range of filamentous fungi. Mycotoxins are commonly found in agricultural commodities and derivate. Exposure to high concentrations of mycotoxins may have a toxicological effect on both animals and humans.

As mentioned, the major sources of mycotoxin contamination are agricultural commodities and derivative products. For this reason, an ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) method was optimised and validated for the simultaneous determination of patulin, aflatoxins B1, B2, G1, and G2 in vegetable samples. The optimised UHPLC-MS/MS method was validated by evaluating the method characteristics such as linearity, limits of detection (LODs), limits of quantification (LOQs), accuracy, and precision. The method exhibited good linearity and it was sensitive with good recoveries.

The prevalence and occurrence of fungal and mycotoxin contamination on vegetables from South Africa were determined. A total of hundred and twenty-one vegetable samples such as Carrots, cucumber, onions, potatoes, and sweet potatoes were collected from five South African provinces namely Limpopo Province (LP), Gauteng Province (GP), KwaZulu-Natal (KZN), Eastern Cape Province (EC) and Western Cape Province (WC). The isolated fungal species from the vegetable samples belonged to the *Aspergillus*, *Fusarium,* and *Penicillium* genera. The most predominant fungal isolate in the vegetable samples was *Aspergillus fumigatus*. Furthermore, the vegetable samples from KZN province were the most susceptible to fungal contamination.

Patulin was detected in carrots and peppers. It was found that the level of patulin in these samples was below the LOQ. The presence of patulin in vegetable samples demonstrated its prevalence in the food chain. Thus, it is important to set-

up guidelines for the monitoring of mycotoxins which can be achieved by the use of robust, sensitive, and simple analytical methods.

The cytotoxic effect of patulin on humans was evaluated. The results demonstrated that the decrease in the viable cancer cell is dependent on the concentration of patulin. Although the results of this study demonstrated that patulin has the potential to reduce cancer cell lines, prolonged consumption of patulin could have a negative impact on human health.

DEDICATION

I dedicate this dissertation to my family and my late father **James Masedi (RIP**), till we meet again.

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Conference presentations

1. Ratanang P.V. Mlaba, Thabo T.I. Nkambule, Bhekie B. Mamba, Titus A.M. Msagati, Hlengilizwe Nyoni, Method optimization and validation for the determination of patulin in vegetables using UHPLC system, 20 November 2019, Natural Organic Matter Indaba, Unisa, Florida. (Poster presentation).

Publications

1. Ratanang P.V. Mlaba, Thabo T.I. Nkambule, Bhekie B. Mamba, Titus A.M. Msagati, Hlengilizwe Nyoni, Determination of patulin and aflatoxins in spoiled vegetables from various climatic zones of South Africa: A combined use of QuEChERS and UHPLC-QTOF-MS techniques (submitted to Journal of Food Chemistry), February 2021.

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ABBRIVIATIONS

ATA	: Alimentary toxic aleukia
CAST	: Council for Agriculture Science and Technology
CHO-K1	: Chinese Hamster Ovary Cell Lines
CO_2	: Carbon Dioxide
DAD	: Diode array detector
DNA	: Deoxyribonucleic acid
dSPE	: Dispersive Solid Phase Extraction
EC	: Eastern Cape Province
ECD	: Electrons Capture Detector
EDTA	: Ethylenediaminetetraacetic acid
EFSA	: European Food Safety Authority
ESCs	: Embryonic stem cells
EU	: European Union
FA	: Formic acid
FAO	: Food and Agriculture Organisation
FAO	: Food and Agriculture Organization
FDA	: Food and Drug Administration
FHB	: *Fusarium* Head Blight
FLD	: Fluorescence light detector
FTA	: Trifluoroacetic acid
GC	: Gas chromatography
GP	: Gauteng Province
HCT-116	: Human Colon Career Cell Lines
HEK-293	: Human Embryonic Kidney Cell Lines
HLB	: Hydrophilic Lipophilic Balance
HMF	: Hydroxymethylfurfural
HPLC	: High-performance liquid chromatography
HPTLC	: High-performance thin-layer chromatography
IAC	: Immunoaffinity columns
IARC	: International Agency for Research on Cancer
iNanoWS	: Institute of Nanotechnology and Water Sustainability

iPSEs : Included pluripotent Stem Cells

ITS : Internal Transcribe Spacer

KZN : KwaZulu-Natal Province

LC-MS : Liquid chromatography coupled to mass spectroscopy

LD50 : Lethal Dose 50%

LEM : Leuko –Encephalomalacia

LLE : Liquid-Liquid Extraction

LOD : Limit of Detection

LOQ : Limit of Quantification

LP : Limpopo Province

m/z : Mass to-Change Ratio

MALDI-TOF-MS: Matric-Assisted Laser Desorption Ionization –Time-of-Flight Mass Spectrometry

MBT : MALDI Bio Typer

MIPs : Molecularly imprinted polymers

MS/MS : Tandem mass spectrometry

MTT : 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide

NPLC : Normal phase liquid chromatography

NRF : National Research Funds

NTDs : Neutral tube defects

O_2 : Oxygen

PCR : Polymerase chain reaction

qPCR : Quantitative polymerase chain reaction

QuEChERS : Quick, Easy, Cheap, Effective, Rugged, and Safe

rDNA : Ribosomal DNA

RNA : Ribonucleic acid

RP-LC : Reversed-phase liquid chromatography

rRNA : Ribosomal RNA

RSD : Relative Standard Deviation

SPE : Solid Phase Extraction

TLC : Thin layer chromatography

UHPLC-MS/MS: Ultra-high performance liquid chromatography coupled to tandem Mass Spectroscopy

UHT : Ultra – High temperature

Unisa : University of South Africa

UV : Ultraviolet light

W_a : Water Activity

WC : Western Cape Province

WHO : World Health Organisation

1 Chapter 1: Introduction

1.1 Background

Mycotoxin is a name that originated from the Greek word '*mykes*', which implies fungus, and the Latin word *'toxicum'*, which implies toxic (Turner *et al*., 2009). A toxin is a naturally occurring substance produced by a microorganism, plant species, or animal and is harmful to other living organisms. Mycotoxins are toxic secondary metabolites produced by various filamentous fungal species such as *Aspergillus, Fusarium Penicillium*, *Claviceps,* and *Alternaria* (Kokkonen, 2011).

Mycotoxins have low molecular weight, various chemical structures, and diverse biological effects. Due to their harmful biological effects, mycotoxins are hazardous to both animals and humans (Kokkonen, 2011). These effects differ from mild symptoms such as immunosuppression, nausea and weight loss to more acute symptoms such as teratogenicity, estrogenicity, mutagenicity, and carcinogenicity resulting from prolonged consumption of mycotoxins (Richard, 2007; Milićević *et al*., 2010). Recent studies suggest that the effects of mycotoxins on cells include plasma membrane disruption, Na^+ coupled amino acid transport inhibition, transcript and translation disruption, and DNA synthesis inhibition (Moake *et al*., 2005). The ingestion of mycotoxins by both animals and humans is usually through the consumption of products contaminated with mycotoxins or even mycotoxin-producing moulds.

The presence of mould that produces mycotoxins in foodstuff and feeds is influenced by factors such as temperature, moisture content, and/or storage conditions. The growth of the mould can occur at a temperature range of 10-40 °C, at a pH range of 4-8, and moisture content above 12-13% (Chilaka *et al.*, 2012; Shephard *et al.*, 2012). Therefore, depending on the type of fungal species, mycotoxins can be produced in low-temperature regions and tropical areas of the world.

Although mycotoxins are found mainly in agricultural commodities, they may also be carried over to processed and animal products through the consumption of contaminated feeds. Fruits, small-grains cereals, and maize are susceptible to

contamination by various mycotoxins during growth, storage, and processing through the invasion of fungal species (Berthiller *et al.*, 2005; Al-hazmi, 2010; Huang *et al.*, 2014). Mycotoxins occurrence in agricultural commodities is a serious threat to the international food and feed trade industries worldwide. In particular, post-harvest losses in developing countries have become severe because of poor storage and transportation (Sharma *et al.,* 2009). About 60% of grain supplies in African countries are at risk of being lost due to fungal and mycotoxin contamination (Spadaro and Gullino, 2004).

The extent of mycotoxin contamination started to be taken seriously during World War II when the consumption of mouldy grains resulted in skin necrosis, hemorrhage, organ failure (kidneys and lungs), and death in both animals and humans (Schuster *et al*., 1993). Although the causes and effects of contaminated food ingestion were established, no symptoms could be linked to secondary metabolites (mycotoxins). Furthermore, in 1960, more than 100 000 turkeys in England died from a disease known as “turkey X disease”. The disease was linked to contaminated peanuts in the turkey feeds (Asao *et al.*, 1963), and its cause was associated with secondary metabolites called aflatoxins, which are produced mainly by fungi called *Aspergillus flavus* and *Aspergillus parasiticus* (Papp *et al.*, 2002). It was only after this incident the hazardous properties of mycotoxin were considered as important. Furthermore, 125 people in 2004 died after eating corn contaminated with aflatoxins, and it was reported that this fatal incident that occurred in Kenya resulted from inadequate storage of the corn (Probst *et al*., 2007).

Patulin (see **Figure 1-1**) is an important mycotoxin that has a detrimental impact on both animals and humans health as well as the economies of many countries. This mycotoxin, which occurs in spoiled vegetables and fruits including their processed products, is produced by a wide range of moulds such as *Aspergillus, Penicillium,* and *Byssochlamys*. However, patulin commonly occurs in apples and apple juice (Spadaro *et a*l., 2007; Kharandi *et a*l., 2013; Zaied et al., 2013; Li *et al*., 2017). Also, the presence of patulin is employed as an indicator of the quality of the raw material used in the manufacturing of processed products.

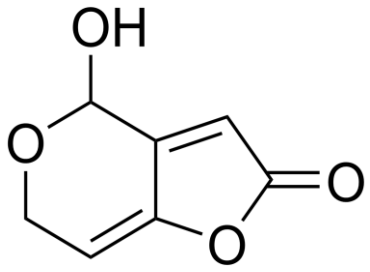

Figure 1-1: Molecular structure of patulin (Liu, 2006)

The prolonged exposure to high levels of patulin may pose serious health risks to both animals and humans. To protect consumers and monitor the level of mycotoxins, regulatory bodies have set a maximum allowable limit of several mycotoxins (Milićević *et al*., 2010). The World Health Organization (WHO) has set a maximum tolerable daily intake of 0.4 μgkg^{-1} of patulin and a maximum of 50 μgL^{-1} patulin in apple juice (Sajid *et al.*, 2018).

Both fruit and vegetables are consumed excessively as sources of vitamins, minerals, and other nutrients. Such consumption practices highlight the importance of food safety. Over the last decades, extensive research on patulin has been conducted, with particular attention being focused on patulin contamination in apples, apple juice, and apple-based baby products. On the other hand, the presence of patulin in vegetables has not been well studied, and this has created a knowledge gap on the contamination of vegetables by patulin. Van De Perre et al. (2014) have detected patulin in fresh moldy tomatoes; interestingly, patulin was not detected on tomatoes-based products due to the potential degradation of patulin during the production process. While ample evidence exists suggesting that patulin can be found in both fruit and vegetables, an even greater need exists to investigate the occurrence of patulin in vegetables such as cabbage, carrots, cucumbers, onions, peppers, potatoes, sweet potatoes, and others.

1.2 Problem statement

Contamination of food with mycotoxins is a worldwide concern due to the serious health consequences they bring in both humans (Bennett and Klich, 2003). Contamination of agricultural commodities with mycotoxins has an impact on both

the economy and the population's well-being. Food loss and low market value are two economic consequences of mycotoxins contamination on agricultural commodities, as well as a reduction in the supply of high-quality, safe food. Other economic repercussions include increased healthcare costs and the loss of farm animals (Akta et al., 2004; Jussara et al. 2007). As a result, continuous monitoring of these mycotoxins in agricultural commodities is required, as is determining the level of contamination of mycotoxins in agricultural commodities.

Although mycotoxins contamination of agricultural commodities has been extensively researched worldwide, there is a lack of knowledge on patulin and aflatoxins contamination on vegetables. Furthermore, no research has been done to evaluate if patulin and aflatoxins coexist. For these reasons, it is necessary to determine the levels of these mycotoxins in vegetables.

1.3 Justification

1.3.1 Mycotoxins - various mycotoxins and toxin-producing microbes

Despite being genotype-specific, mycotoxins can be produced by one or more fungal species depending on exposure to favorable conditions such as the presence of naturally occurring foodborne mycotoxin ochratoxin A (Turner *et al*., 2009). Ochratoxin A is produced by a variety of fungi including *Aspergillus ochraceus* and *Penicillium verrucosum* (Turner *et al*., 2009). The presence of recognized mycotoxigenic fungi does not certainly mean that the related mycotoxin will also be found, as many factors can influence the production of mycotoxins. Equally, the absence of visible mould is not an assurance of freedom from mycotoxins as the mould might have already died while leaving the toxin intact. Fungi generally tend to grow in isolated pockets and are not evenly distributed in stored commodities. Therefore, it is crucial to develop a procedure to ensure that a representative sample is taken for analysis.

Although more than 300 mycotoxins are known, the most commonly associated with health risk in both animals and humans include aflatoxins, patulin, ochratoxin A, trichothecenes, zearalenone, and fumonisins (Turner *et al*., 2009; Milićević *et al*., 2010; Stanciu *et al.*, 2019). Of the 300, only about a dozen mycotoxins including patulin have attracted the attention of researchers because their severe effects on human health and their occurrence in agricultural commodities.

Patulin was initially used as an antibiotic. However, although it has antiviral and antibacterial properties, patulin was found to be toxic and was therefore reclassified as a mycotoxin (Moake *et al*., 2005). Over the last decade, patulin has become an important factor to consider when guaranteeing the quality of products. The immunotoxicity levels of patulin are higher than those of ochratoxin A, and this has triggered increased interest in patulin research. Thereby, it is crucial to improve the analytical standard to monitor patulin.

1.3.2 Vegetables and regions chosen in the study

An increase in climatic variability associated with climatic change trends is expected to lead to high levels of mycotoxin contamination worldwide, which pose serious health and economic risks to the whole world. While several mycotoxins can be produced in both temperate and tropical regions depending on the fungal species, patulin is produced mainly in subtropical and temperate regions.

When compared with other countries in Sub-Saharan Africa, South Africa has a wider range of climatic zones. South African's climatic zones generally range from the Mediterranean in the south-western corner (Western Cape) to temperate in the central plateau (Free State and Gauteng) and subtropical in the northeast (Kwazulu-Natal). These different climatic zones may affect the levels of mycotoxin contamination in the various provinces as such environmental conditions may be favorable to the infestation of fungi. Kos *et al*. (2013) reported that in 2012, 137 maize sample were contaminated with aflatoxins with mean concentration of 36. 3 μgkg^{-1} due to the hot and dry weather. Similar aflatoxin contamination levels due to climatic change were reported in Hungary (Dobolyi and Varga, 2013). However, there is a lack of knowledge on how different climatic conditions may affect mycotoxin contamination in the Republic of South Africa.

Both fruit and vegetables are exposed to spoilage microbes by direct contact with the soil, dust and through handling during harvest and at postharvest. As a result, fruit and vegetables harbor a wide range of spoilage microbes including patulin-producing fungi. Different patulin-producing microbes grow under different climatic conditions. Studies on the determination of patulin in foods have been reported, with most of these studies being focussed on fruit, processed fruit products, and

fruit-based baby foods (Welke *et al*., 2009; Kinsella 2016; Hammami *et al*., 2017). In addition, despite the occurrence of patulin in different agricultural commodities, particularly in apple-based products, being established, very little research has been focused on vegetables (Cunha *et al.*, 2014). To safeguard animal and human health, it is important to regulate the concentration of various mycotoxins in both fruit and vegetables.

1.3.3 Measurement techniques: Quantification of the toxins - chromatographic techniques and sample preparation methods

Various types of mycotoxins including patulin are regulated, and several regulatory authorities globally have established a maximum allowable limit of mycotoxins in different foods, agricultural commodities, and feeds. To enforce these regulatory limits, the development of reliable analytical methods is necessary for the successful monitoring of the concentrations of mycotoxins in foods, agricultural commodities, and feeds. Research and monitoring of mycotoxins in foods and feeds is a challenging task because these compounds typically occur in complex sample matrices and at low concentrations (ppb) levels. Contamination of patulin in foods normally occurs at concentrations levels as low as < 10 μgkg^{-1} (Paíga *et al.*, 2012; Mwanza *et al.*, 2015; Ji *et al.*, 2017). Therefore, there is a need to develop effective analytical methods that are able of analyzing patulin occurring in matrices.

In general, sample preparation is often the most crucial part of the analysis of most mycotoxins, and it relies largely on both the physical and chemical properties of the contaminated agricultural commodities. Traditional methods for the analysis of patulin consist of a liquid-liquid extraction (LLE) step that is followed by clean-up, concentration, and separation steps. Some drawbacks associated with this method include tedious sample preparation; instability of patulin in alkaline conditions resulting from sodium carbonate clean-up; the need for extra clean-up step; or the development of a chromatographic separation process to prevent HMF and patulin from co-eluting. Therefore, a need exists for a rapid, simple, and robust sample preparation method for the analysis of patulin. To replace the liquid-liquid extraction step, Wang *et al.*, (2016) developed an SPE method that applies

to solid fruit and both clear and unclear fruit juices. Eisele & Gibson (2003) and Sigma Aldrich (2018) have also employed the SPE procedure for the analysis of patulin, and it was concluded that the SPE procedure yielded high accurate recovery rates with good reproducibility.

In the last decades, the detection and quantification of various mycotoxins have improved vastly; however, the sensitivity of the methods is still the limiting factor for the analysis of individual mycotoxins and the simultaneous analysis of mycotoxins (Köppen *et al.*, 2010). Liquid chromatography (LC) coupled to mass spectrometry (MS) or preferable tandem mass spectroscopy (MS/MS) is a good method for analyzing mycotoxins due to their high selectivity and sensitivity and reliable quantification and detection at low concentration levels. Mass spectrometric methods have recently been developed for the simultaneous analysis of mycotoxins (Sulyok *et al*., 2007; Alkadri *et al*., 2014). Various types of mycotoxins have different properties that make it difficult to purify a multi-mycotoxin system simultaneously. As a result, mycotoxins will show peaks of low intensity and repeatability due to insufficient matrix removal from food samples. The mass spectrometer has become a useful analytical method for the analysis of trace amounts of compounds in foods due to its high sensitivity, greater selectivity, and versatility as compared to previously reported detectors.

High-performance liquid chromatography (HPLC) has been extensively employed for the determination of mycotoxins (Spadaro *et al.*, 2007; Zouaoui *et al.*, 2015; Chromatography *et al.*, 2017). Recently, ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) (preferably UHPLC coupled to tandem MS (UHPLC-MS/MS) has found worldwide application in different fields including food safety because of its high sensitivity and selectivity and reliable quantification and detection of mycotoxins (Arroyo-Manzanares *et al*., 2013; Marsol-Vall *et al.*, 2014; Vaclavikova *et al.*, 2015). Christensen *et al.* (2009) developed a UPLC-MS/MS method for the simultaneous analysis of pesticides and patulin in apples. The same technique was applied for the determination of eight mycotoxins in fruits by Wang *et al.* (2016). In both cases, an SPE method was used for the purification of the sample. In this study, an LC-MS/MS-based method that allows the simultaneous detection patulin and other related mycotoxins in different vegetable samples (i.e. cabbages, carrots, cucumbers, peppers, and

potatoes) will be applied and validated. Although the UPLC based method has recently been used in the analysis of patulin and/or multi-mycotoxins found in different matrices, the analysis of patulin in vegetables selected for this study has not been reported. An LC-MS/MS system that employs different ionization techniques such as electrospray ionization (ESI) (Malysheva *et al.*, 2012; Iturat *et al.*, 2014), atmospheric pressure photoionization (APPI), and atmospheric pressure chemical ionization (APCI) were employed for the determination of patulin (Takino *et al*., 2003; Iturat *et al.*, 2014). Although an LC-MS/MS-based method is highly sensitive and selective in the analysis of mycotoxins, LC-MS/MS that uses an ESI mode and quadruple-based mass analyzer is much more reliable for quantifying patulin (Tuomi *et al*., 2001; Wang *et al*., 2016).

1.3.4 Identification of the microbes using MALDI Biotyper, Microscopic and Molecular methods

The first step when dealing with patulin-producing fungi involves accurate fungal quantification and identification. Generally, fungal species are identified through morphological characteristics and microscopic examinations. Although these traditional methods are commonly used, fungal identification by morphology is generally not as reliable and it provides inconsistent results (Alwakeel, 2013; Raja *et al.*, 2017). However, molecular techniques based on DNA sequencing have been used progressively for fungal identification.

Matrix-associated laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) biotyping was successfully used for the identification of bacteria, yeast, and higher eukaryotes including insects and fish (Boonzaaijer *et a*l., 2005; Kaufmann *et al*., 2011; Volta *et al*., 2012). Research has shown that fungal identification based on MALDI-TOF-MS provides more accurate results than morphologically based characterization techniques (Gautier *et al.*, 2014). The application of MALDI-TOF-MS has been extensively explored to give compelling evidence on the reliability of the technique (Kaufmann *et al.*, 2011; Volta *et al.*, 2012; Gautier *et al.*, 2014). To accurately identify the isolated fungal species, this

research study explored the molecular method using a polymerase chain reaction (PCR) and bio-typing method using MALDI-TOF/TOF.

1.3.5 Evaluation of cytotoxicity - MTT Assay and RTCA techniques

Several fungal species can contaminate foods and feeds at any given time, and most fungal strains can produce various mycotoxins simultaneously (Burmistrova et al. 2014; Wang et al. 2016). Therefore, the ingestion of mycotoxin-contaminated food can lead to an intake of a combination of mycotoxins. For this reason, a need exist for toxicological evaluation of mixed mycotoxins. The toxic effect of patulin on mammalian cell lines such as human embryonic kidney cell lines 293 (HEK-293) and human leukemia cell lines (HL-60) (Piqué *et al.*, 2013) has been evaluated, and patulin was found to induce apoptosis. Other studies have reported that patulin exerts mutagenic and genotoxic effects on Chinese hamster lung fibroblast V79 cells and mouse lymphoma L5178Y cells (Schumacher *et al.*, 2006). While extensive research studies based on the individual toxicological effect of patulin, very few research studies on the combined effect of patulin and other related mycotoxins have been undertaken. The co-occurrence of mycotoxins may have a greater toxicological effect as compared to the individual mycotoxins. Therefore, a need exists for the cytotoxic evaluation of the co-occurrence of patulin and other related mycotoxins.

Different cytotoxicity assays are commonly used for evaluating the cytotoxic effect of compounds. Over the years, cytotoxicity assays have been commonly undertaken because of their beneficial advantages such as high speed, cost-effectiveness, and non-requirement of the use of animals. The 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is one of the most frequently used colorimetric assays for cytotoxicity evaluation. The technique is extensively used for determining both the cell viability and cytotoxic effect at different doses of patulin, is easy to use, and is highly reproducible (Zouaoui et al. 2016; Abastabar et al. 2017).

In this study, mycotoxigenic fungi from spoilt vegetables (carrots, cucumbers, onions, peppers, potatoes, and sweet potatoes) and their processed products were isolated and identified. The patulin and other related mycotoxins were

extracted from vegetables collected from five South African provinces and quantified using UHPLC-ESI-QTOF-MS/MS, thereafter the cytotoxic effect of patulin on human cancer cell lines were evaluated.

1.4 Aim and Objectives

As part of an effort to undertake this research study, the aim and objectives of the study were formulated and outlined in the sub-sections that follow.

1.4.1 Aim

This research study aims to quantitatively determine patulin and other related mycotoxins, isolate and identify mycotoxigenic fungal species from various vegetables and their processed foods and evaluate the cytotoxic effect of patulin in mammalian cells.

1.4.2 Objectives

The aim of the research mentioned in the preceding sub-section cannot be tackled in isolation. In this regard, a series of specific research objectives were formulated to facilitate the attainment of the main aim of this research study. The specific objectives of this research study are therefore to:

i. Optimize and validate an analytical method for the determination of patulin in vegetables using UHPLC-ESI-QTOF-MS/MS;
ii. Quantitatively determine the presence of patulin in selected vegetables using UHPLC-ESI-QTO-MS/MS;
iii. Isolate and identify mycotoxigenic fungal species associated with vegetable spoilage in five different regions of the Republic of South Africa
iv. Evaluate the cytotoxic effect of patulin on mammalian cells by assessing the cell viability of the cultured mammalian cells.

1.5 Dissertation outline

This section gives a summary of the remaining chapters of this dissertation.

1.5.1 Chapter 2: Literature review

This chapter provides a general review of mycotoxins, their occurrence, and their effect on both animals and humans. The chapter also discusses the different methods used for the identification of mycotoxin-producing fungal species and the quantification of patulin and other related mycotoxins in various foods. The limits and shortcomings associated with the discussed methods are also highlighted in this chapter. Lastly, the value of evaluating cytotoxicity of patulin on mammalian cells is also discussed.

1.5.2 Chapter 3: Research Methodology

This chapter gives detailed experimental procedures followed to ensure that the aim and objectives of this research study are accomplished. All the materials, reagents, and instruments used in the study are listed. The sampling plan is also included.

1.5.3 Chapter 4: Validation of UHPLC-QTOF-MS method for the detection and quantification of mycotoxins in spoilt vegetables from five different climatic regions in South Africa

This chapter is based on the optimization and validation of the UHPLC-ESI-QTOF-MS/MS method and the applicability of the method in the detection and quantification of mycotoxins in vegetables. The specific experimental procedure relating to the optimization, validation, and method application is also outlined. Furthermore, the prevalence of mycotoxins in the five climatic regions was evaluated. After discussing optimization parameters for the adopted method, the results obtained following method validation were discussed before drawing some conclusions from the results obtained.

1.5.4 Chapter 5: Occurrence and the spatial distribution of mycotoxigenic fungi in spoilt vegetables from five different climatic regions in South Africa

In this chapter, results of the evaluation of the occurrence and spatial distribution of mycotoxigenic fungi in spoilt vegetables from five different climatic regions of South Africa are presented. Lastly, a conclusion is drawn from the results obtained.

1.5.5 Chapter 6: Cytotoxic evaluation of patulin on mammalian cells

This chapter is focused on the cytotoxic effects of patulin on mammalian cell lines. The specific experimental procedure for the evaluation of cytotoxicity and the results thereof are outlined.

1.5.6 Chapter 7: Conclusions and Recommendations

This chapter highlights some of the key findings generated from this research study and aligns them with the aim and objectives of the study. The chapter concludes with recommendations for future work.

1.5.7 Appendices

All the raw experimental data in the form of graphs, tables, spectra, and chromatographs are presented in the Appendix section.

1.6 References

Abastabar, M., *Akbari, A., Akhtari, K., Hedayati, M.T., Shokhoyi, T.* (2017) 'In vitro antitumor activity of patulin on cervical and colorectal cancer cell lines', *Current Medical Mycology*, 3(1), pp. 25–29.

Al-hazmi, A. (2010) 'Determination of Patulin and Ochratoxin A using HPLC in apple juice samples in Saudi Arabia', *Saudi Journal of Biological Sciences*, 17(4), pp. 353–359.

Alkadri, D., Rubert, J., Prodi, A., Pisi, A., Mañes, J., Soler, C. (2014) 'Natural co-

occurrence of mycotoxins in wheat grains from Italy and Syria', *Journal of Food Chemistry*, 157, pp. 111–118. Alwakeel, S. S. (2013) 'Molecular identification of isolated fungi from stored apples in Riyadh, Saudi Arabia', *Saudi Journal of Biological Sciences*, 20(4), pp. 311–317.

Arroyo-Manzanares, N., García-Campaña, A. M. and Gámiz-Gracia, L. (2013) 'Multiclass mycotoxin analysis in Silybum marianum by ultra-high-performance liquid chromatography-tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid-liquid microextraction', *Journal of Chromatography A, 1282, pp.11-19*.

Asao, T., Büch, G., Abdel-Kader, M. M., Chang, S. B., Wick, Emily L., Wogan, G. N. (1963) 'Aflatoxins B and G', *Journal of the American Chemical Society*, 85(11), pp. 1706–1707..

Bakhiva, N., Monien, B., Frank, H., Siedel, A., Glat, H. (2009) 'Renal organic anion transporters OAT1 and OAT3 mediate the cellular accumulation of 5-sulfooxymethylfurfural, a reactive, nephrotoxic metabolite of the Maillard product 5-hydroxymethylfurfural', Journal of *Biochemical Pharmacology*, 78(4), pp. 414–419.

Berthiller, F., Schuhmacher, R., Buttinger, G., Krska, R. (2005) 'Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high-performance liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*, 1062(2), pp. 209–216.

Boonzaaijer, G., Bobeldijk, I.,van Osenbruggen, W. A. (2005) 'Analysis of patulin in dutch food, an evaluation of a SPE based method', Journal of *Food Control*, 16(7), pp. 587–591.

Burmistrova, N. A., Rusanova, T. Yu., Yurasov, N. A., De Saeger, S., Goryacheva, I. Yu. (2014) 'Simultaneous determination of several mycotoxins by rapid immunofiltration assay', *Journal of Analytical Chemistry*, 69(6), pp. 525–534.

Capuano, E. and Fogliano, V. (2011) 'Acrylamide and 5-hydroxymethylfurfural (HMF): a review on metabolism, toxicity, occurrence in food and mitigation strategies', Journal of *LWT - Food Science and Technology*, 44(4), pp. 793–810.

Chilaka, C. A., de Kock., Phoku, J. Z., Mwanza, M., Mwanza, M., D, M. F. (2012) 'Fungal and mycotoxin contamination of South African commercial maize', *Journal of Food, Agriculture and Environment*, 10(2), pp. 296–303.

Christensen, H. B., Poulsen, M. E., Rasmussen, P. H., Christen, D. (2009)

‘Development of an LC-MS / MS method for the determination of pesticides and patulin in apples’, *Journal of Food Additives and Contaminants*, 26(7), pp. 1013–1023.

Chromatography, A., Arisoy, S., Tepe, T.K., Otağ, F.B. (2017) ‘High-Performance Liquid Chromatographic Method for the Determination of Patulin in Oil-based Products : Cheese Approach’, 4(1), pp. 1–4.

Cunha, S. C. *et al.* (2014) ‘Patulin assessment and fungi identification in organic and conventional fruits and derived products’, *Food Control*. Elsevier Ltd, 44, pp. 185–190.

Dobolyi, C. and Varga, J. (2013) ‘Occurrence of aflatoxin producing Aspergillus flavus isolates in maize kernel in Hungary’, *Acta Alimentaria*, 42(3), pp. 451–459.

Eisele, T. and Gibson, M. Z. (2003) ‘Syringe-cartridge solid phase extraction method for patulin in apple juice’, Journal of Food Chemical Contaminants, 86(6), pp. 1160–1163. Available at: file:///C:/O-drive/kfn/5866.pdf.

Gautier, M., Otağ, F.B., Normand, A., Becker, P., Packeu, A., Cassagne, C., Ollivier, C L., V., Hendrickx, M., Piarroux, R. (2014) ‘Matrix-assisted laser desorption ionization time-of-flight mass spectrometry : revolutionizing clinical laboratory diagnosis of mould infections’, Journal of *Clinical Microbiology and Infection*, 20(12), pp. 1366–1371.

Hammami, W. *et al.* (2017) ‘Patulin and patulin producing Penicillium spp. Occurrence in apples and apple-based products including baby food’, *Journal of Infection in Developing Countries*, 11(4), pp. 343–349.

Huang, L. C. *et al.* (2014) ‘Simultaneous determination of aflatoxin M 1, ochratoxin A, zearalenone and a -zearalenone in milk by UHPLC – MS / MS’, *Journal of food*, 146, pp. 242–249..

Iturat, B. *et al.* (2014) ‘Determination of patulin in apple derived products bt UHPLC-MS/MS. Study of matrix effects with atmospheric pressure ionization sources’, *Journal of Food Chemistry*, 142(Iso 690), pp. 400–407.

Ji, X. *et al.* (2017) ‘Occurrence of patulin in various fruit products and dietary exposure assessment for consumers in China’, *Food Control*. Elsevier Ltd, 78, pp. 100–107.

Jussara, S. *et al.* (2007) ‘Patulin in food: State-of-the-art and analytical trends ’, pp. 406–413.

Kaufmann, C. *et al.* (2011) ‘Evaluation of matrix-assisted laser desorption/ionization-time of flight mass spectrometry for characterization of Culicoides nubeculosus biting midges’, *Medical and Veterinary Entomology*, 25(1), pp. 32–38.

Kharandi, N., Babri, M. and Azad, J. (2013) ‘A novel method for determination of patulin in apple juices by GC – MS’, *Food Chemistry*. Elsevier Ltd, 141(3), pp. 1619–1623.

Kinsella, B. (2016) ‘Determination of Patulin in Processed Foods Using QuEChERS Extraction and UHPLC-MS-MS Analysis’, *Lc Gc Europe*, 29(7), p. 402.

Kokkonen, M. (2011) *The challenge of lc/ms/ms multi- mycotoxin analysis − heracles battling the hydra ?*

Köppen, R. *et al.* (2010) ‘Determination of mycotoxins in foods: Current state of analytical methods and limitations’, *Applied Microbiology and Biotechnology*, pp. 1595–1612.

Kos, J. *et al.* (2013) ‘Natural occurrence of aflatoxins in maize harvested in Serbia during 2009-2012’, *Food Control*, 34, pp. 31–34.

Li, Xianjiang *et al.* (2017) ‘Determination of trace patulin in apple-based food matrices’, *Food Chemistry*. Elsevier Ltd, pp. 290–301.

Liu, B.-H. (2006) ‘Mycotoxin Patulin Activates the p38 Kinase and JNK Signaling Pathways in Human Embryonic Kidney Cells’, *Toxicological Sciences*, 89(2), pp. 423–430.

Malysheva, S. V *et al.* (2012) ‘Improved positive electrospray ionization of patulin by adduct formation : Usefulness in liquid chromatography-tandem mass spectrometry multi-mycotoxin analysis’, *Journal of Chromatography A*. Elsevier B.V., 1270, pp. 334–339.

Marsol-Vall, A. *et al.* (2014) ‘A Fast and Reliable UHPLC-PDA Method for Determination of Patulin in Apple Food Products Using QuEChERS Extraction’, *Food Analytical Methods*, 7(2), pp. 465–471..

Milićević, D. R., Škrinjar, M. and Baltić, T. (2010) ‘Real and perceived risks for mycotoxin contamination in foods and feeds: Challenges for food safety control’, *Toxins*, 2(4), pp. 572–592.

Moake, M. M., Padilla-zakour, O. I. and Worobo, R. W. (2005) ‘Review of Patulin

Control Methods in Foods', 1.

Moake, M. M., Padilla-Zakour, O. I. and Worobo, R. W. (2005) 'Comprehensive review of patulin control methods in foods', *Comprehensive Reviews in Food Science and Food Safety*, pp. 8–21..

Mwanza, M. *et al.* (2015) 'Evaluation of analytical assays efficiency to detect aflatoxin M1 in milk from selected areas in Egypt and South Africa', *Journal of Dairy Science*. Elsevier, 98(10), pp. 6660–6667.

Paíga, P. *et al.* (2012) 'Extraction of ochratoxin A in bread samples by the QuEChERS methodology', *Food Chemistry*, 135(4), pp. 2522–2528..

Papp, E. *et al.* (2002) 'Liquid chromatographic determination of aflatoxins', *Microchemical Journal*, 73(1–2), pp. 39–46.

Van De Perre, E. *et al.* (2014) 'Screening for the presence of patulin in molded fresh produce and evaluation of its stability in the production of tomato products', *Journal of Agricultural and Food Chemistry*, 62(1), pp. 304–309.

Piqué, E. *et al.* (2013) 'Occurrence of patulin in organic and conventional apple-based food marketed in Catalonia and exposure assessment', *Food and Chemical Toxicology*, 60, pp. 199–204.

Probst, C., Njapau, H. and Cotty, P. J. (2007) 'Outbreak of an acute Aflatoxicosis in Kenya in 2004 : Identification of the Causal Agent □', *Applied and Environmental Microbiologyiology*, 73(8), pp. 2762–2764.

Raja, H. A. *et al.* (2017) 'Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community', *Journal of Natural Products*, 80(3), pp. 756–770. Richard, J. L. (2007) 'Some major mycotoxins and their mycotoxicoses-An overview', *International Journal of Food Microbiology*, 119(1–2), pp. 3–10.

Romanelli, A. M. *et al.* (2010) 'Sequence-based identification of filamentous basidiomycetous fungi from clinical specimens: A cautionary note', *Journal of Clinical Microbiology*, 48(3), pp. 741–752.

Sajid, M. *et al.* (2018) 'Effective adsorption of patulin from apple juice by using non-cytotoxic heat-inactivated cells and spores of Alicyclobacillus strains', *Toxins*, 10(9).

Schumacher, D. M. *et al.* (2006) 'DNA-DNA cross-links contribute to the mutagenic potential of the mycotoxin patulin', *Toxicology Letters*, 166(3), pp. 268–275.

Schuster, R., Marx, G. and Rothaupt, M. (1993) 'Analysis of Mycotoxins by HPLC with Automated Confirmation by Spectral Library', p. 16.

Severin, I. *et al.* (2010) 'Genotoxic activities of the food contaminant 5-hydroxymethylfurfural using different in vitro bioassays', *Toxicology Letters*, 192(2), pp. 182–194.

Sharma, R. R., Singh, D. and Singh, R. (2009) 'Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review', *Biological Control*. Elsevier Inc., 50(3), pp. 205–221.

Shephard, G. S. *et al.* (2012) 'Developments in mycotoxin analysis: An update for 2010-2011', *World Mycotoxin Journal*, 5(1), pp. 3–30.

Sigma Aldrich (2018) 'Selective Extraction and Cleanup of Patulin Mycotoxin from Apple Juice Samples | Sigma-Aldrich', pp. 14–15. Available at: https://www.sigmaaldrich.com/technical-documents/articles/analytical/food-beverage/patulin-apple-juice.html.

Spadaro, D. *et al.* (2007a) 'Incidence and level of patulin contamination in pure and mixed apple juices marketed in Italy', *Food Control*, 18(9), pp. 1098–1102.

Spadaro, D. *et al.* (2007b) 'Incidence and level of patulin contamination in pure and mixed apple juices marketed in Italy', *Food Control*, 18, pp. 1098–1102.

Spadaro, D. and Gullino, M. L. (2004) 'State of the art and future prospects of the biological control of postharvest fruit diseases', *International Journal of Food Microbiology*, 91(2), pp. 185–194.

Stanciu, O. *et al.* (2019) 'Study on trichothecene and zearalenone presence in Romanian wheat relative to weather conditions', *Toxins*, 11(3), pp. 1–17.

Sulyok, M., Krska, R. and Schuhmacher, R. (2007) 'A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples', *Analytical and Bioanalytical Chemistry*, 389(5), pp. 1505–1523.

Takino, M., Daishima, S. and Nakahara, T. (2003) 'Liquid chromatography / mass spectrometric determination of patulin in apple juice using atmospheric pressure photoionization', *Rapid communications in mass spectrometry*, 17, pp. 1965–1972.

Tuomi, T. *et al.* (2001) 'Detection of aflatoxins (G1-2, B1-2), sterigmatocystin, citrinin and ochratoxin A in samples contaminated by microbes', *Analyst*, 126(9),

pp. 1545–1550..

Turner, N. W., Subrahmanyam, S. and Piletsky, S. A. (2009) 'Analytical methods for determination of mycotoxins: A review', *Analytica Chimica Acta*, 632(2), pp. 168–180.

Vaclavikova, M. *et al.* (2015) 'Monitoring survey of patulin in a variety of fruit-based products using a sensitive UHPLC-MS/MS analytical procedure', *Food Control*. Elsevier Ltd, 47, pp. 577–584..

Volta, P. *et al.* (2012) 'Discrimination of freshwater fish species by Matrix-Assisted Laser Desorption/Ionization- Time Of Flight Mass Spectrometry (MALDI-TOF MS): A pilot study', *Journal of Limnology*, 71(1), pp. 164–169.

Wang, M. *et al.* (2016) 'A single-step solid-phase extraction for the simultaneous determination of 8 mycotoxins in fruits by ultra-high-performance liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*. Elsevier B.V., 1429, pp. 22–29.

Welke, J. E. *et al.* (2009) 'Quantitative analysis of patulin in apple juice by thin-layer chromatography using a charge-coupled device detector', *Food Additives & Contaminants: Part A*, 26(5), pp. 754–758.

Akta, A.H., Yilmazer, M., and Demirci, S. (2004) 'Determination of Patulin in Apple Juice Produced in Isparta, Turkey by HPLC with Diode Array Detection', *Journal of Food and Drug analysis*, 12(3), pp. 228–231.

Zaied, C. *et al.* (2013) 'Occurrence of patulin in apple-based-foods largely consumed in Tunisia', *Food Control*, 31(2), pp. 263–267.

Zouaoui, N. *et al.* (2015) 'Occurrence of patulin in various fruit juice marketed in Tunisia', *Food Control*. Elsevier Ltd, 51, pp. 356–360.

Zouaoui, N. *et al.* (2016) 'Cytotoxic effects induced by patulin, sterigmatocystin and beauvericin on CHO-K1 cells', *Food and Chemical Toxicology*, 89, pp. 92–103.

2 Chapter 2: Literature review

2.1 Patulin and other related mycotoxins in food: An Overview

This section provides an overview of patulin and other mycotoxins that occur in food. Specifically, the various types of microbes responsible for arising from fungal contaminated food, types of microbes accountable for food spoilage as well as environmental conditions that favor microbial growth and subsequent production of mycotoxins are reviewed in this section.

2.1.1 Various types of mycotoxins related to food contamination

The presence of mycotoxins in various food and feeds is a worldwide concern because they pose serious risks to food safety (Bennett and Klich, 2003). Mycotoxin contamination in various agricultural commodities occurs at any stage of processing, transportation, and storage (Ferrao *et al*., 2017). Mycotoxins are produced by a wide range of spoilage fungi, however, the growth of mycotoxin-producing fungi is affected by various factors such as temperature, humidity, the interaction between colonizing toxigenic fungal species, geographical location, and insect infestation (Megan *et al*., 2003; Tola and Kebede, 2016).

At the time of writing this dissertation, more than 300 mycotoxins had been reported, nonetheless, only a few of these mycotoxins have been found in foods and feeds. A list of important mycotoxins and their characteristics are summarized in **Table 2-1** and are discussed in more detail in the subsections that follow.

Table 2-1: Major mycotoxins, main sources, commonly contaminated commodities, and their toxic effects

Mycotoxin	**Fungal Species**	**Food commodity**	**Toxic effects**
Aflatoxins B1, B2, G1, G2 and M2	*Aspergillus flavus*, *Aspergillus parasiticus*	Maize, rice, wheat, milk, cheese, eggs, sorghum, peanut, figs, groundnuts, tree nuts pistachio, almond,	Carcinogenic, hepatotoxic, teratogenic, decreasing immune system, disrupting the DNA structure, hepatitis, bleeding, and kidney lesions

		cottonseed, spices	
Ochratoxin A	*Aspergillus carbonarius Aspergillus ochraceus*, *Penicillium verrucosum*	Cheese, cereals cocoa, dried vine fruits, grapes, wine	Kidney failure, liver damage, nausea, vomiting, loss of appetite, immunosuppression, and carcinogenic
Fumonisins B1, B2, and B3	*Fusarium proliferatum, Fusarium verticillioides*	Asparagus, sorghum, maize, and maize products	Encephalomalacia, neurotoxic, pulmonary edema, carcinogenic, heart liver , heart failure, and oesophageal cancer in human
Zearalenone	*Fusarium graminearum*	Wheat, maize, barley, cereals and cereal products	Carcinogenic, hormonal imbalance, reproductive problems, and teratogenic
Trichothecenes (deoxynivalenol, T-toxin, H2-toxin)	*Fusarium graminearum*, *Fusarium culmorum*	Cereals, cereal products, fruits and vegetables	Cytotoxicity, immune suppression, skin necrosis, anemia, hemorrhage and alimentary toxic aleukia (ATA)
Patulin	*Penicillium expansum*	Apples, and apple juice	Brain hemorrhage, neural syndromes, mutagenic, skin lesions, and antibacterial effect

2.1.1.1 Aflatoxins

Aflatoxins are natural contaminants mainly produced by *Aspergillus* species such as *Aspergillus flavus* and *Aspergillus parasiticus* (Kurtzman and Horn, 1987).

Recently, Campagnollo *et al*., (2016) showed that *Aspergillus nomius*, *Aspergillus sergii*, *Aspergillus pseudocaelatus*, *Aspergillus speudotamari,* and *Aspergillus ochraceoroseus* have aflatoxigenic properties; however, these species naturally occur at low levels. The fungal growth and subsequent production of aflatoxins are affected by factors such as high environmental temperature, moisture, relative humidity, carbon dioxide (CO_2) and oxygen (O_2), insect infestation, pesticides, fungicides, and sporulation (Bryden, 2012). Among these, temperature and relative humidity have the biggest effect on the production of aflatoxins. *Aspergillus flavus* was reported to optimally grow at temperatures from 29 to 35 °C while high levels of aflatoxin B1 were obtained at 24 °C and relative humidity of less than 70 % (Campagnollo *et al.*, 2016).

Ever since their discovery in Britain in 1960 as a causative agent for Turkey X disease, aflatoxins have been the topic of research and are now regarded as the most frequently studied mycotoxins (Pereira *et al*., 2014). The most important and dangerous aflatoxins include Aflatoxins B1, B2, G1, G2, and M1 (see **Figure 2-1**) (Campagnollo *et al.*, 2016). Aflatoxin M1 is the metabolite of aflatoxin B1 and is commonly found in milk. Aflatoxins have been reported to have carcinogenic, teratogenic, hepatotoxic, and immunosuppressive effects on both animals and humans, and can affect organs such as the liver (Bennett and Klich, 2003). Aflatoxins B1 and M1 have been respectively classified as group 1 and 2B carcinogens by the International Agency for Research on Cancer (IACR) (Ostry *et al.*, 2017).

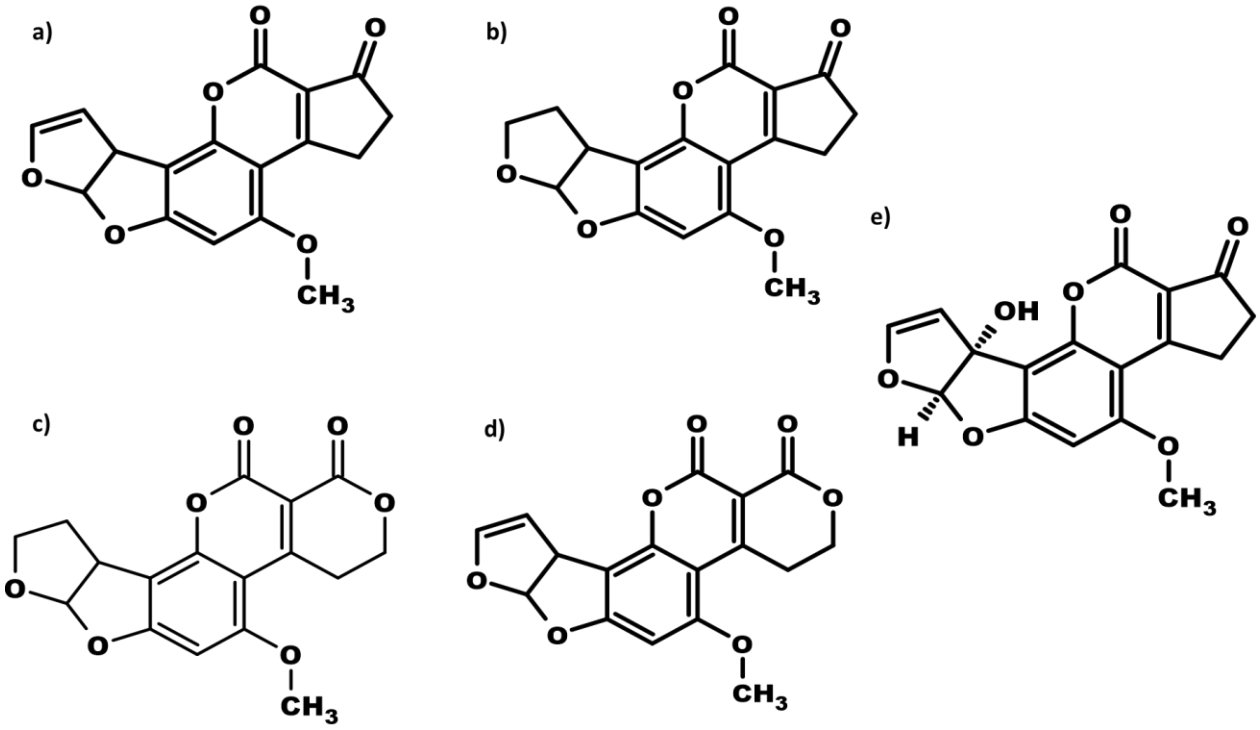

Figure 2-1: Molecular structures of the most important and studied aflatoxins (a) Aflatoxin B1, (b) Aflatoxin B2, (c) Aflatoxin G1, (d) Aflatoxin G2 and Aflatoxin M1

2.1.1.2 Ochratoxin A

Ochratoxins are secondary metabolites discovered in South Africa in 1995 and are produced by various species of *Aspergillus* and *Penicillium* (Wilson *et al*, 2002; Bennett and Klich, 2003). Although other types of ochratoxins are known, ochratoxin A (see **Figure 2-2**) owing to its prevalence and toxicity it is the most important amongst the ochratoxins. It causes both acute and chronic effects and is both carcinogenic and teratogenic (JECFA, 2001). Furthermore, ochratoxin A is classified by the IARC as a group 2B carcinogen (Ostry *et al.*, 2017).

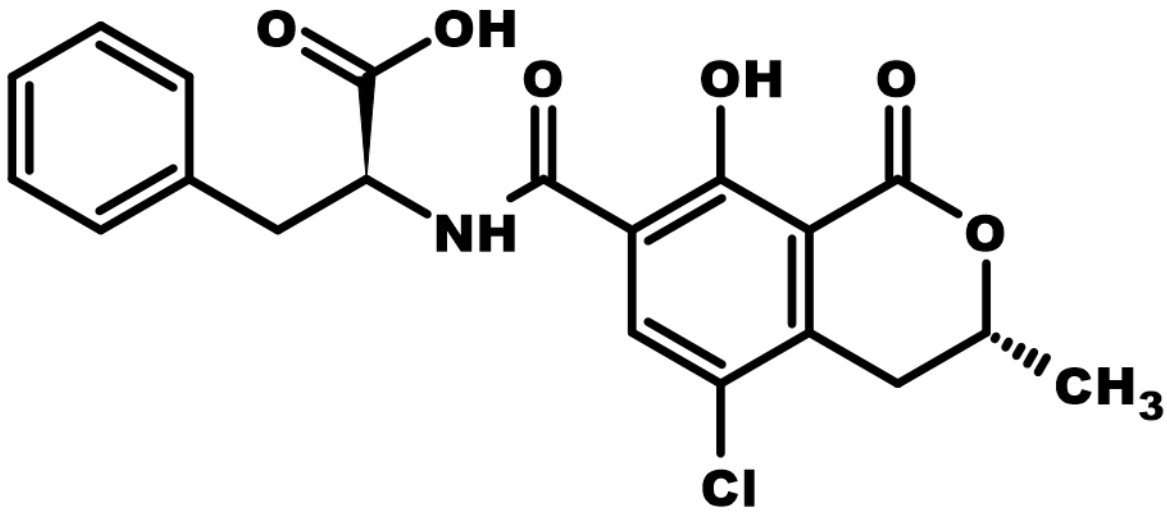

Figure 2-2: Molecular structure of mycotoxin ochratoxin A, the most important ochratoxin because of its prevalence and toxicity

2.1.1.3 Trichothecenes

Over 150 forms of trichothecenes have been identified to date, however, only a few of them are of agriculturally important (Yazar and Omurtag, 2008). Of all the mycotoxins, trichothecenes are the most chemically diverse (Bennett and Klich, 2003). Trichothecenes with relevance to food safety are deoxynivalenol, nivalenol, HT-2 toxin, and T-2 toxins (see **Figure 2-3**), which their presence/occurrence are regulated in cereals (Zachariasova *et al.*, 2010). Despite being the least toxic, deoxynivalenol is the most common and well-studied trichothecenes (Bennett and Klich, 2003). Nevertheless, trichothecenes are still less toxic than aflatoxins and ochratoxin A. Trichothecenes are produced by various species of *Fusarium* fungi such as *Fusarium graminearum* and *Fusarium culmorum*, which cause *Fusarium* Head Blight (also known as FHB) (Bottalico and Perrone, 2002). Species such as *Acremonium* (*Cephalosporium*), *Cylindrocarpron*, *Dendrodochium*, *Myrothecium*, *Trichoderma,* and *Stachybotrys* are also known to produce trichothecenes (Bottalico and Perrone, 2002).

Not only do trichothecenes cause acute adverse effects such as vomiting, diarrhea, bleeding, and skin inflammation in humans, they also have chronic effects such as gastrointestinal dysfunction and immunodeficiency.

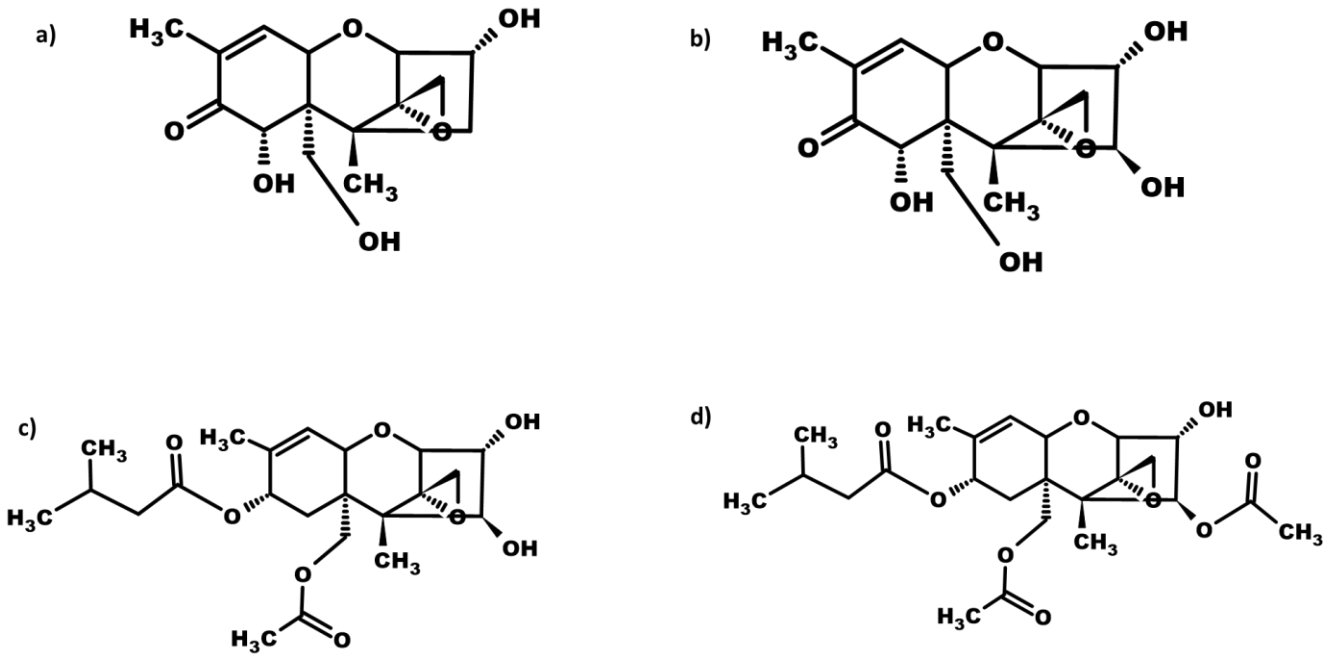

Figure 2-3: Molecular structures of a) deoxynivalenol b) nivalenol c) HT-2 toxins and d) T-2 toxins, types of Trichothecenes that are relevant to food safety.

2.1.1.3.1 Deoxynivalenol and nivalenol

Deoxynivalenol, also called vomitoxin, is the most frequently found trichothecenes in agricultural commodities such as corn, oats, wheat, rye and barley, and corn; it is, however, less commonly found in rice and sorghum (Spanjer *et al*., 2008). The prevalence of nivalenol in cereals have been found to be generally lower than of deoxynivalenol; however, in Japan, its prevalence in barley and wheat is similar to that of deoxynivalenol (Hu *et al.*, 2016). The respect values of lethal dose 50% (LD_{50}) for deoxynivalenol and nivalenol in mice are 78 and 79 mg/kg (Koesukwiwat *et a*l., 2014). Similar to other trichothecenes, deoxynivalenol and nivalenol inhibit protein, DNA, and RNA synthesis (Koesukwiwat *et a*l., 2014). Furthermore, these mycotoxins have an effect on cell division and mitochondrial functions (Creppy *et al.*, 2004; Richard, 2007; Ismaiel and Papenbrock, 2015). Both mycotoxins give rise to symptoms such as diarrhea, vomiting, throat inflammations, abdominal discomfort, and weight loss (De Ruyck *et al.*, 2015).

2.1.1.4 Fumonisins

Discovered in 1998, fumonisins consist of a group of hydrophilic non-fluorescent mycotoxins that are structurally different from the majority of mycotoxins (Bennett and Klich, 2003). They are produced by *Fusarium* fungal species such as *Fusarium proliferatum* and *Fusarium verticillioides*. *Fusarium verticillioides* was isolated in South Africa in 1970 from corn that was related to an outbreak of leuko-encephalomalacia (LEM) in the equine. In addition, it was reported that pigs suffered from pulmonary edema after feeding on fumonisin contaminated corns (Yazar and Omurtag, 2008).

Currently, more than 28 fumonisins have been identified and categorised into groups A, B, C, and P (Rheeder *et al*., 2002). The most prevalent fumonisins in food, fumonisins B1 and B2 (see **Figure 2-4**), are classified as carcinogens of the group 2B by the IARC (Ostry *et al.*, 2017). Fumonisins have a structure similar to sphinganine, and fumonisins B1 is known to disrupt the metabolism of sphingolipids (Yazar and Omurtag, 2008). Fumonisins mainly target the liver and the kidney and thus have severe toxic effects on animals (Mazzoni *et al.*, 2011). Owing to their hydrophilicity, fumonisins are introduced into milk in cattle; low concentrations of fumonisins also accumulate in edible tissues (Richard, 2007). World Health Organization (WHO) has established a tolerable daily intake of fumonisins of 2 µg/kg body weight (Yazar and Omurtag, 2008). While the Food and Drug Administration (FDA) has set a maximum regulated levels of 2-4 mg/L of fumonisins food commodities such as corn and corn-based derived products intended for human consumption and, the recommended maximum levels of 5-100 mg/L are set for different animal feeds. According to the WHO, with good agricultural and manufacturing practices, these levels are achievable.

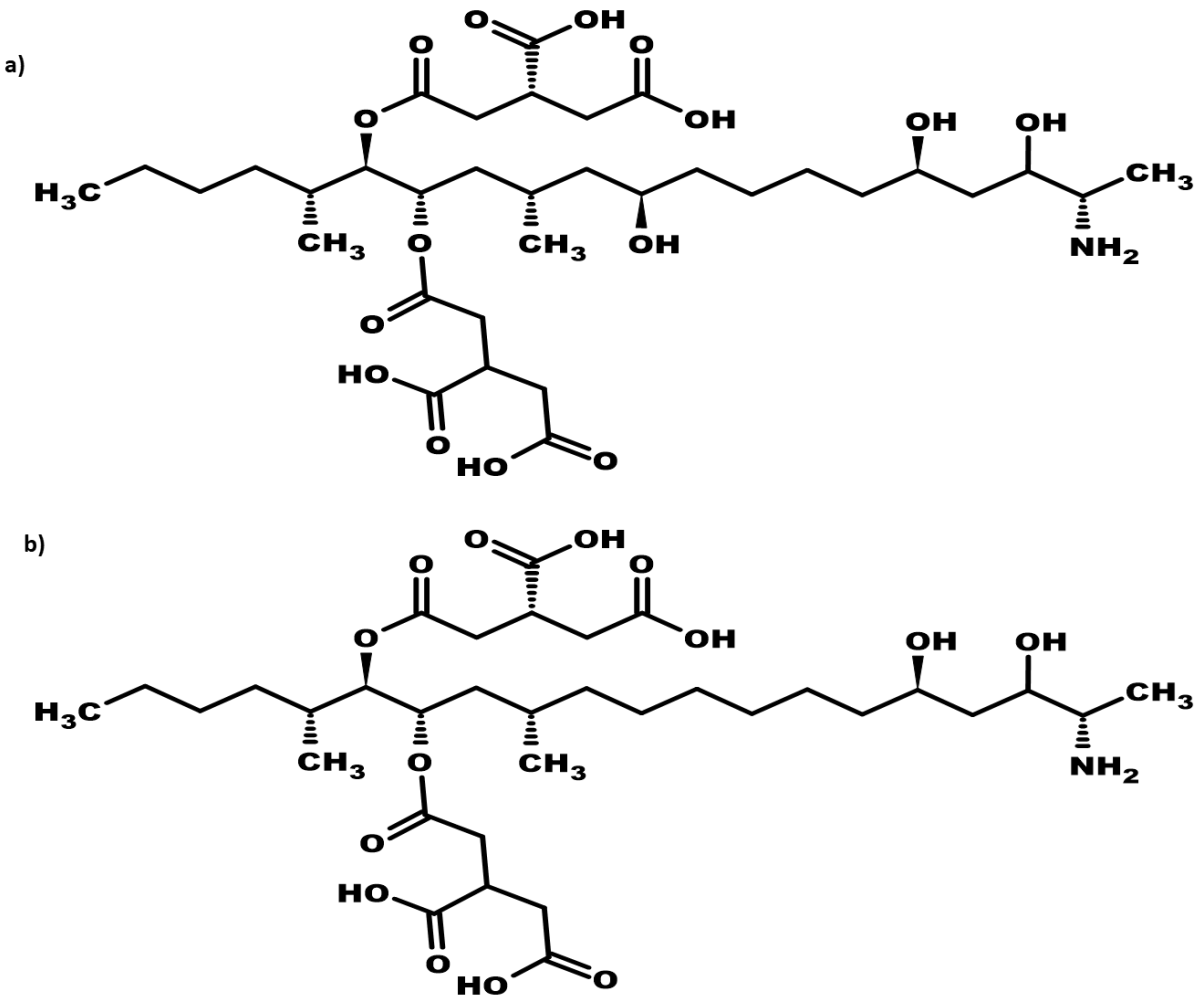

Figure 2-4: Molecular structure of the most prevalent fumonisins in food commodities a) fumonisin B1 and b) fumonisin B2

2.1.1.5 Zearalenone

Zearalenone (see **Figure 2-5**) is known as an estrogenic mycotoxin produced by *Fusarium* species and *Aspergillus niger* (Bhatnagar, Yu and Ehrlich, 2002). Zearalenone production is favored by low temperature and high humidity conditions. Zearalenone occurs simultaneously with deoxynivalenol and less commonly with aflatoxins. It is stable under cooking temperature, however, it can be removed under high temperatures (Castelo *et al*, 1998).

Zearalenone is classified as a Group 3 carcinogen by the IARC. Health concerns over zearalenone emanate from its strong estrogenic activity. To date, there are no recommended levels of zearalenone established by the US FDA. Nonetheless, the European Union (EU) regulates the maximum allowable levels of zearalenone to range between 20 and 100 mg/L in various food commodities (Richard, 2007).

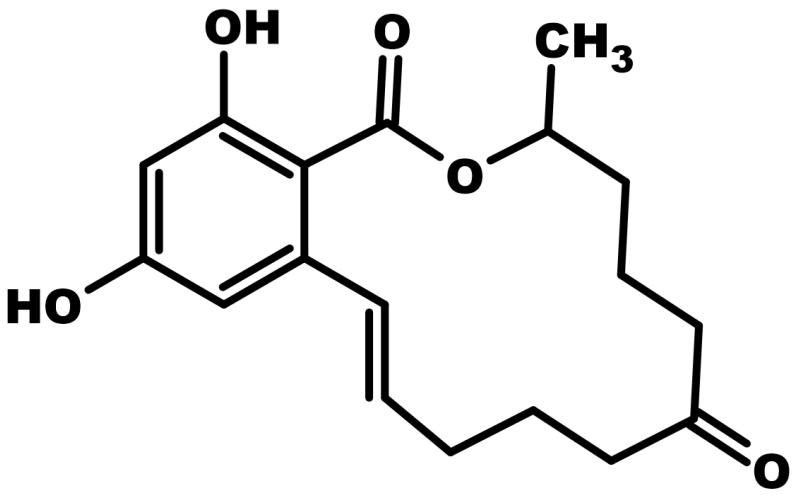

Figure 2-5: Molecular structure of mycotoxin zearalenone

2.1.1.6 Patulin

Patulin (also called 4-hydro-4,6-dihydrofuro[3.2-c]pyran-2one) was discovered in 1943. It is an unsaturated heterocyclic compound (see the structure of patulin in **Figure 1-1**) that is soluble in water and other organic compounds. It is also colorless and crystalline and has a melting point of 110 °C, a molecular weight of 154.12 g/mol, and a molecular formula $C_7H_6O_4$.

Initially, patulin was studied as a potential antibiotic. For instance, Chalmers and Clarke, (2004) tested patulin as a spray for both throat and nose for treatment of common cold also as an ointment for treating skin infections caused by fungi. Even so, In 1950s and 1960s, it became evident that additionally the antiviral, antibacterial, and antiprotozoal properties patulin, it found to be harmful to both animals and plants thus preventing its use as an antibiotic. Therefore it was reclassified as a mycotoxin (Bennett and Klich, 2003). Research has demonstrated that its toxic effects on humans include nausea, vomiting, ulceration, and hemorrhage (Richard, 2007; Puel *et al*., 2010). The LD_{50} of patulin in rodents ranged between 29 - 55 mg/kg body weight (Puel *et al*., 2010). Although the IARC has expressed concerns regarding the likely carcinogenicity of patulin, it has classified patulin as a Group 3 carcinogen (Abnet, 2007). The EU has set a maximum allowable level of 50 µg/L patulin in fruit juices and concentrated fruit

juices, 25 µg/L for solid apple products, and 10 µg/L for juices and foods meant for the consumption of babies and infants.

2.1.2 Types of microbial species associated with food spoilage

Microbial contamination in foods and feeds poses an economic challenge in the food industry. About 25% of food spoilage in North America is due to microbial contamination, and microbes filamentous fungi are mostly responsible for food spoilage (Snyder and Worobo, 2018; Criado *et al.*, 2005). Furthermore, in developing countries, fungal spoilage occurs in about 5-10 % of food commodities (Pitt and Hocking, 2009).

In terms of fungal spoilage on food, food can be classified into fresh or perishable foods and processed foods. Fresh foods can be further classified into foods made up of living cells such as fruits and vegetables and non-living cells such as meat, milk, and fruit juice. However, the spoilage of different classes occurs in various ways and is caused by different fungal species.

2.1.2.1 Fruits and vegetables

Fruits and vegetables consist of varying pH, which drives microbial spoilage. For instance, acidic (pH range 1.8-5.0) fruits such as lemons, tomatoes, and figs are resistant to bacterial invasion, while vegetables of neutral pH are more prone to both bacterial and fungal invasion (Tournas, 2008). Also, microbial spoilage in fruits and fruit-derived products is caused by fungi (Sulyok *et al*., 2007). Both bacterial and fungal spoilage on food is important, however, this dissertation is focused on fungal spoilage.

Solanaceous fruits such as tomatoes, peppers, and eggplant are acidic with pH ranging from 4.2 to 4.5 and can be affected by the *Alternaria* species (Moss, 2008). The invasion of the *Alternaria* species can result from mechanical injury which is cracking due to excessive moisture during growing. The *Alternaria* rots appear as dark brown to black lesions with a firm texture on the stem-end of the fruit, and it can only grow to several centimeters in diameter (Ghosh, 2009; Samuel and Orji, 2015). *Alternaria solani* is known to cause an early blight rot on tomatoes but not on other solanaceous fruits.

Potatoes are mostly invaded by *Fusarium* species, most commonly *Fusarium solani,* and *Fusarium sulphureum*. Spoilage on potatoes appears as brown lesions and as the spoilage progresses the potatoes become wrinkled. However, fungal spoilage on sweet potatoes is caused by the invasion of *Ceratocystis Fimbriata* (Olaoye, 2015; Oduola *et al*., 2018). The fungal invasion occurs at harvest or during storage and appears as a dry, firm, dark-colored rot that does not extend into the cortex of the sweet potato (Nelson, 2000). Spoilage in carrots has been reported to be caused by *Rhizopus* species (Onitsha *et al.*, 2016). *Rhizopus stolonifer* and *Rhizopus oryzae* produce a soft watery rot with little color change. Furthermore, the sour rot on carrots is caused by *Geotrichum candidum* (Hudecová *et al*., 2009) and the root rot that appears as a black or brown decaying ring on the top of carrots at the point where the leaves are attached is caused by *Fusarium avenacceum* and *Fusarium solani.*

Citrus fruits are susceptible to blue and green rots that are caused by the invasion of *Penicillium italicum* and *Penicillium digitatum*; however, rots caused by *Penicillium italicum* can be distinguished by the formation of coremia on the fruits (Bashir *et al*., 2020). In addition, *Penicillium ulaiense* can also cause spoilage in citrus fruits-derived products*.* Volume (2009) has reported that the black-center rot on oranges is caused by *Alternaria citri*. *Penicillium ulaiense* is resistant to commonly-used preservatives and it gives rise to problems in citrus-packaging plants in the majority of the countries. Fruits can be contaminated with these fungal species at any stage after harvesting. Fungal invasion can spread rapidly through contact with one fruit with another.

2.1.2.2 Dairy products

Ultra-high temperature (UHT) processed dairy products are susceptible to fungal spoilage resulting from post-processing contamination with, for instance, *Geotrichum candidum* (Garnier, 2017). Although *Fusarium* species such as *Fusarium oxysporu*m is not heat resistant, it can grow at very low oxygen pressure and cause fermentative spoilage. *Fusarium oxysporu*m has been reported to causative agent for spoilage in UHT-flavoured milk drinks.

Penicillium species such as *Penicillium glabrum*, *Penicillium commune,* and *Penicillium chrysogenum* are associated with spoilage in sour cream (Snyder *et al*., 2019). Moreira *et al.* (2001) have isolated *Penicillium glabrum* and *Penicillium chrysogenum* including *Aspergillus sydowii* from spoiled sour cream yogurt based desert.

Low-salt magerines are more susceptible to fungal invasion as compared to those with a normal amount of salt. The common spoilage fungi in Australia are *Penicillium chrysogenum* and *Cladosporium* species, with *Cladosporium cladosporioides* being the most frequently isolated (Tournas, 2005) In addition to being lipolytic, these fungal species are responsible for off-flavor spoilage, specifically earthy taints from the production of 2-methylisoborneol and egoism.

2.1.2.3 Meats

Although of great importance, very few studies have been conducted on mould spoilage of meats in the last 50 years. Mould spoilage only occurs on meat if the conditions for spoilage do not favor bacterial growth; however, very little data has been collected on the duration and temperature conditions required for the development and growth of visible mould. Spoilage moulds on meat compete with bacteria only at storage temperatures below 0 °C or when the meat surface dries out and achieves water activity (a_w) that favors fungal growth. It has been reported that fungal spoilage on chilled or frozen meat is usually caused by *Mucorales*, particularly *Thamnidium elegans* and *Muco*r species that grow as “whiskers on stored”.

2.1.2.4 Cereals, Nuts, and oilseeds

Fungal species that grow on crops that are eventually dried are classified as a field- and storage-fungal species. Field-fungal species are microbes that invade growing crops before harvest. They hardly play a substantial role in fungal spoilage after harvest.

From a comprehensive 3 years survey of mycotoxins of Scottish oats, wheat, and barley Yoshizawa (1991) reported similar field contamination for all these cereal crops. More than 85 % of kernels were found to be infected with *Altenaria alternate*. Rots caused by *Alternaria alternate* appear as a grey discoloration on

cereals (Milani, 2013). Furthermore, it was observed that spoilage in barley and oats was commonly caused by *Cladosporium* species. Other fungal species that occur in barley and oats include *Epicoccum nigrum* and *Penicillium* species.

The ears of corn are covered by a strong husk that protects it from fungal invasion. However, *Fusarium* species such as *Fusarium verticillioides, Fusarium graminearum*, and *Fusarium sublglutinans* commonly cause spoilage in corns (Desjardins *et al*., 2006; Mazzoni *et al.*, 2011; Chilaka *et al.*, 2012). The rot caused by *Fusarium graminearum* usually appears as a reddish discoloration on the corn and pinkish to red discoloration on the husk. The invasion of the *Fusarium* species results from damage induced by insects (Miller *et al*., 2007). *Fusarium verticillioides* and *Fusarium proliferatum* appear to be commensals and they are commonly isolated from maize in most parts of the world, however, they are not related to spoilage in corns (Summerell *et al*., 2003; Desjardins *et al*., 2006).

Griffin and Garren (1976) have reported that the *Aspergillus* species, particularly *Aspergillus niger* and *Aspergillus flavus*, have been frequently isolated from peanut kernels. Out of 109 samples, 91% were contaminated with *Aspergillus flavus*.

2.1.3 Environmental conditions and mycotoxin-producing microbial species

Fungal growth and production of mycotoxins on food commodities are highly dependent on environmental factors such as temperature and relative humidity, with pre- and post-harvest temperatures being the most important (see **Table 2-2**).

Table 2-2: Optimum temperatures that favor the growth of mycotoxigenic fungal species and subsequent production of mycotoxins

Fungal species	Mycotoxin	Temperature °C	Water activity (a_w)
Aspergillus flavus	Aflatoxin	33	0.99
Fusarium verticillioides	Fumonisins	15-30	0.9-0.955
Fusarium Proliferatum and Fusarium culmorum	Deoxynivalenol	26-30	0.995
Aspergillus ochraceus	Ochratoxin A	25-30	0.98
Penicillium expansum	Patulin	20-25	0.95-0.99
Penicillium verrucosum	Zearalenone	25	0.96

Fungal growth is favored different climatic conditions, which makes mycotoxin production prevalent worldwide (Pitt and Hocking, 2009; Snyder and Worobo, 2018; Saleh and Al-thani, 2019).

2.1.3.1 Aflatoxin

Climate change affects the growth of fungal species, which in turn has an impact on the worldwide occurrence of mycotoxins (Cotty and Jaime-Garcia, 2007; Paterson and Lima, 2010; Tirado *et al.*, 2010; Nam, Kim, and Lee, 2018) For instance, rain in warm regions promotes the occurrence of high concentrations of aflatoxins in many crops. Generally, crops are resistant to aflatoxin contamination. However, a change in environmental conditions that favor the growth of aflatoxin-producing fungal species leads to a significant increase in the concentration/level of aflatoxins to an extent that this resistance is dismissed or even annihilated thus leading to increased concentration of aflatoxins in crops. Optimum conditions for the liberation of aflatoxins by *Aspergillus flavus* and *Aspergillus parasiticus are* 33 °C and 0.95 a_w.

Aspergillus flavus and *Aspergillus parasiticus* contamination in maize ears and subsequent production of aflatoxins are favored by warm and humid subtropical and tropical climatic conditions (Udoh *et al.*, 2015; Paterson and Lima, 2017). In addition, drought can lead to the invasion of *Aspergillus flavus* and *Aspergillus parasiticus* and subsequent production of aflatoxins in nuts.

2.1.3.2 Ochratoxin A

Fungal species that produce ochratoxin A, that is *Penicillium verrucosum, Aspergillus ochraceus, and Aspergillus carbonarius* (Samson *et al.*, 2002), grow in conditions of relatively high humidity during storage (Birzele *et al*., 2000). In warm climates, such as those experienced in West and Central Africa, the production of ochratoxin A is more related with *Aspergillus ochraceus* than it is with *Penicillium verrucosum.*

Cereal crops are mainly contaminated with *Penicillium verrucosum* in cool and temperate climatic regions (JECFA, 2001). *Aspergillus ochraceus* is the most common fungal species that produce ochratoxins. It can grow in barley grains at a

temperature ranging from 8 to 37 °C; also, it is typically present at low concentrations but is not associated with spoilage (JECFA, 2001). Urbano *et al.* (2001) assert that the highest level of ochratoxin A in cereal products, nuts, spices, and coffee is obtained at optimum a_w and a temperature range of 25-30 °C.

2.1.3.3 Fumonisins

Fumonisins have been reported to occur in maize and maize-based products in tropical and subtropical regions owing to the invasion by *Fusarium verticillioides* and *Fusarium proliferatum* (Royer *et al*., 2004; Meyer *et al.*, 2019). These fungal species can produce fumonisins in maize crops harvested under high moisture content conditions (Sospedra *et al.*, 2010; Chilaka *et al.*, 2012). Fumonisins can accumulate in grains before a_w decrease to levels favorable for the growth of fungal species that produce them. Sanchis and Magan (2004) have reported that fumonisins are optimally produced at temperatures of 15-30 °C and a_w of 0.9-0.995

2.1.3.4 Zearalenone

Accumulation of zearalenone in crops is influenced by several factors such as temperature, fungal growth duration, substrate, and strains of fungal species have an effect on the levels of accumulated zearalenone in crops (Scudamore and Patel, 2000; De-Saeger *et al*., 2003). In the last few years, research studies have shown that, in the central Europe climatic zones, mycotoxins produced *Fusarium* species, most particularly zearalenone, play an important role in spoilage of food, or feed (Conkova *et al.*, 2003). The highest level of zearalenone produced by *Fusarium* was obtained at <25°C daily temperature and 16% humidity (Zwierzchowski *et al.*, 2005).

Inhibition of fungal growth and mycotoxin production has proven to be the best approach to decrease risk, for example, by harvesting grains at maturity and low moisture content, and storing them in cold and dry conditions. However, this is challenging in countries with a warm and humid climate.

2.1.3.5 Patulin

Accumulation of patulin in apples can be driven by environmental changes such as temperature, gas composition, and pH (Sanchis *et al*., 2004; Zaied *et al.*, 2013b; Aiko and Mehta, 2015; Avsaroglu *et al.*, 2015). The storage temperature affects the growth of blue-mould decay, which is directly related to the liberation of patulin in decayed apples. Most essentially, the accumulated patulin in these contaminated apples is likely to be carried over to apple-derived products if toxins are not removed during the production process. Patulin is commonly produced in apples contaminated by *Penicillium expansum*. The growth and colonial morphology of *Penicillium expansum* are affected by room temperature, as a result, the production of patulin could also be affected (Gougouli and Koutsoumanis, 2010). Although *Penicillium expansum* can optimally grow temperature below 0 °C, however, faster sporulation and mycelia growth was achieved at storage temperature range 20–25 °C (Prata *et al.*, 2010; Paíga *et al.*, 2012; Tannous *et al.*, 2015). The highest amount of patulin was found in apples and apple-cider samples stored at >20 °C (McCallum, 2002; Salomão *et al.*, 2009; Spadaro *et al.*, 2013). The use of poor-quality apples can result in apple-based products contaminated with patulin.

Environmental factors have an influence on the production of patulin by *Penicillium expansum*, *Penicillium patulum*, and *Aspergillus clavatus*. Moss, (2008) reported that fungal growth and production of patulin respectively occurred at temperature range 0-24, 4-31, and 12-24 °C and minimum a_w of 0.99, 0.95, and 0.99. For apples, the minimum temperature range for the production of patulin by *Penicillium expansum* was 1-4 °C, interestingly it was dependent on the variety of apples. Interestingly, the *Penicillium patulum* was not able to invade apple tissues. In consideration of the diversity of *Penicillium expansum* isolates and apple cultivars, it is required to assess the effect of the interaction between the fungal strains and apples on the production of patulin

2.2 The occurrence of patulin and other related mycotoxins in food

The type of food associated with mycotoxin contamination and as well as their prevalence in different food commodities are discussed in this section.

2.2.1 Types of foods associated with patulin and other related mycotoxins

Mycotoxin contamination occurs in a various food commodities globally, with a high prevalence in agricultural commodities. Mycotoxin contamination of food and feedstuff occurs pre- or post-harvest and during manufacturing process, packaging, and storage of food commodities (Pereira *et al*, 2014). Mycotoxins such as aflatoxins, ochratoxin A, fumonisins, zearalenone, and trichothecenes commonly occur in grains, cereals, maize, and their processed products (see **Table 2-1**). Other agricultural commodities such as nuts and oilseeds may be contaminated with different types of mycotoxins, such as aflatoxins, zearalenone, nivalenol, deoxynivalenol, and fumonisins (Logrieco *et al*., 2009; Stoev *et al.*, 2010). Furthermore, spices and dried fruits are susceptible to aflatoxin and ochratoxin contamination (Bircan, 2009). Fresh vegetables and their products are also susceptible to mycotoxin contamination. For instance, patulin is commonly detected in apples and apple juice, grape, and wine are mainly contaminated with ochratoxins (Blesa *et al.*, 2006; Valero *et al.*, 2008). Also, coffee, tea, and cocoa are commonly contaminated with ochratoxin A (Miraglia *et al.*, 2002).

Patulin-producing fungal species have been isolated from a wide range of fruits and vegetables including apples, plums, peaches, and pears (Piqué *et al.*, 2013). Patulin is stable in cereals, and apple and grape juice, however, it decomposes during the production of cider (Iha and Sabino, 2008; Guo *et al.*, 2013; Zaied *et al.*, 2013b; Lee *et al.*, 2014a). Patulin residues can pose serious food safety issues in apple and citrus fruits based products (Berreta, 2000). Patulin persists in heated juices, and its presence in apple-based products can be used in assessing the quality of the fruits used the production (Jackson and Dombrink-Kurtzman, 2006).

Trichothecenes contaminate mainly occur in wheat barley, oats, rye, maize, and rice (Yazar and Omurtag, 2008). However, they may also occur in Sunflower seeds, potatoes, soybeans, bananas, and peanuts and have been detected in some processed products derived from cereals (Bennett and Klich, 2003). Deoxynivalenol is the most broadly scattered *Fusarium* mycotoxin and has been detected in cereals in Japan, Korea, South Africa, and Australia (Yoshizawa, 1991). Zearalenone contamination commonly occurs in corn, wheat, barley,

barley, sorghum, and rye, with a higher prevalence in corn and wheat (Hussein and Brasel, 2001).

Certain mycotoxins may be carried over through the ingestion of contaminated feeds to animal products such as milk, eggs, and meat (Marin *et al.*, 2013). Aflatoxin M1, a metabolite of aflatoxin B1 can be found in milk as well (Bennett and Klich, 2003). Aflatoxin M1 has also been detected in dairy products such as cheese; however, the aflatoxin M1 concentration in cheese was found to be similar to those in raw milk since aflatoxin M1 stable under high temperatures, binds well to casein, and is not affected by processes involved in cheese making (Barbiroli *et al.*, 2007). In addition, although zearalenone, ochratoxin A and fumonisins can be transmitted into milk low levels of these mycotoxins have been found in milk (Boudra *et al.*, 2007; Gazzotti *et al.*, 2009). Eggs were reported to be contaminated with µg/kg or lower levels of aflatoxins, deoxynivalenol, and zearalenone (Sypecka *et al*., 2004; Valenta and Dänicke, 2005; Garrido Frenich *et al.*, 2011). In addition, ochratoxin A may be transferred to an animal's internal organs, Milicevic *et al.*, (2009) detected low levels of this mycotoxin in pig kidneys and liver.

Several mycotoxins can simultaneously occur in the same product because one fungal species can produce several toxins or several species that produce various toxins can be occur concurrently. Even though the sample may have low levels of mycotoxins, the co-occurrence of mycotoxins is of concern because of its synergistic effects of combined toxins. Mycotoxin co-occurrence is frequently reported in cereals. For example, it has been reported that trichothecenes and zearalenone simultaneously occur in small grain cereals (Eskola *et al*., 2001). Typically, maize is often simultaneously contaminated with both aflatoxins and fumonisins as well as deoxynivalenol, nivalenol, and zearalenone (Sangare-Tigori *et al.*, 2006; Stoev *et al.*, 2010; Ndleve and Njobeh, 2018). Comparably, rice, nuts, and dried fruits and spices can be simultaneously contaminated with numerous mycotoxins (Sangare-Tigori *et al.*, 2006; Bircan, 2009; Stoev *et al.*, 2010).

2.2.2 Prevalence of mycotoxins

A significant number of studies worldwide have studied the extent to which crops, cereals, fruits and vegetables, and other products have been contaminated by

mycotoxins. Several research studies have been carried out on the prevalence of fumonisins in South Africa, Malawi, and Zimbabwe, and high levels of fumonisins were reported in maize (Shephard *et al*., 2012; Matumba *et al*, 2014; Hove *et al.*, 2016). A maximum level of 11.624 µg/kg of fumonisin in maize was reported in South Africa (Boutigny *et al.*, 2012). Probst *et al*. (2012) reported that 53% of maize samples were found to be contaminated by fumonisins with levels greater than 100 µg/kg. Mwalwayo and Thole (2016) have also reported high concentrations of fumonisin in Malawian sorghum beer. Recently Murashiki *et al.* (2017) reported that 100 % of the 388 maize samples collected from rural households in the Shamva and Makoni districts of Zimbabwe were contaminated with fumonisins at concentration levels below FDA's and EC's regulatory limits of 2000 µg/kg and 1000 µg/kg, respectively.

Previous studies suggest that patulin food contamination is a worldwide problem. Funes and Resnik (2009) reported that 21.6% of 51 analysed samples were found to contain 17–221 μgkg^{-1} of patulin. However, about 50% of the contaminated samples were apple puree samples. In Michigan, patulin was detected in cider mill (*n* = 493) samples at concentration levels exceeding 4 μgL^{-1} with only 18.7% contaminated samples, and 2.2% samples were found to contain patulin concentrations higher than 50 μgL^{-1}. For samples (*n* = 159) collected from Michigan retail grocery stores, patulin was detected in apple juice and cider samples, with 11.3% samples containing levels of patulin higher than 50 μgL^{-1} (Harris *et al.*, 2009).

In Malaysia, patulin ranging from 13.1 to 33.7 μgL^{-1} was detected in 3 of the 56 fruit (i.e., apple, pineapple, guava, litchi, mango, tamarind, and mixed fruit) samples that were analysed (Lee *et al.*, 2014b). In China, 95 apple-based product samples such as apple juice, apple-baby food, apple juice concentrate, and mixed juice were analysed for patulin (Yuan *et al.*, 2010). The concentrations of patulin in 15 apple products were found to be higher than 50 μgkg^{-1}, and only 12 of the samples contained patulin levels that were lower than 50 μgkg^{-1} (Yuan et al., 2010). In Italy, patulin levels ranging from 1.58 to 55.41 μgkg^{-1} were detected in 34.8 % of mixed apple juices (n = 82) and pure apple juices (n = 53) (Spadaro et al., 2007). In Africa, only 35% of the 85 apple-product samples collected from Tunisia were contaminated with patulin (Zaied et al., 2013b).

2.3 Toxic effects of patulin and other related mycotoxins

Mycotoxin exposure occurs through consumption, inhalation, and dermal contact. Most cases of mycotoxicosis in animals and humans result from the ingestion of contaminated foods or feeds (Council for Agricultural Science and Technology (CAST), 2003). Since mycotoxins have adverse effects on both human and animal health, mycotoxin-contaminated food and feed cause serious health risks to animals and humans. The various acute and chronic effects of mycotoxins are dependent on the mycotoxin-producing fungal species, however, the impact, especially in humans, is influenced by sex, age, weight, exposure to the contaminated products, and the presence of other mycotoxins (Zain, 2011).

Many mycotoxins are toxic to vertebrates and other animal groups (Edite Bezerra da Rocha *et al.*, 2014). Even at low levels, some of these mycotoxins are mutagenic, teratogenic, and carcinogenic. Also, they can cause autoimmune diseases, cause allergic reactions and exhibit hormonal activity (Edite Bezerra da Rocha *et al.*, 2014). In addition, mycotoxins may cause skin irradiation, have an effect on the gastrointestinal system, have hematological effects, and inhibit growth. The toxic effects exerted by some of the most common mycotoxins were summarised in **Table 2-1**.

Most of the mycotoxins are grouped, by their toxic activity; in this regard, they can be classified further in terms of their carcinogenic, mutagenic, or teratogenic effects in humans. Grouping by their site of action cause hemo-, hepato-, nephron-, dermato-, neuro-, or immunotoxins (Niessen, 2007). Fumonisins and aflatoxins have been reported to possess carcinogenic effects (Mazzoni *et al.*, 2011). Aflatoxins can bind to DNA cells, whereby it affects the synthesis of proteins and causes immunosuppression in humans. Several studies on poultry, pig, and rodents have shown that exposure to aflatoxins causes' immunosuppression (Niessen, 2007). Aflatoxins are lipophilic and can penetrate the placenta. Furthermore, Aflatoxin B1 is associated with liver cancer (Wild and Gong, 2010).

The carcinogenic properties of fumonisins do not include DNA interaction (CAST, 2003). The similarity of fumonisins with sphingosine, however, indicates a possible intervention in the biosynthesis of sphingolipids. Inhibition of the biosynthesis of sphingolipid results in serious problems associated with cell function, as these substances are important for the composition of the membrane. Animal-based

studies have shown that exposure to Fumonisin B1 can cause neural-tube defects (also known as NTDs) creating further concerns that this mycotoxin may cause similar effects in humans (Sherif *et al*., 2009).

Ochratoxin A has various effects on animals such as nephrotoxicity, hepatotoxicity, neurotoxic, and teratogenicity (Bennett and Klich, 2003). This mycotoxin is related to human nephropathy and is thought to be the causative agent of Tunisian Nephropathy and human Balkan Endemic Nephropathy. Acute symptoms such as headache, abdominal pains, vomiting, diarrhea, and fever have been reported to be caused by human exposure to deoxynivalenol-contaminated grains. (Bennett and Klich, 2003).

Patulin cause immune suppression and inhibits DNA synthesis (Wu *et al.*, 2005). Bosco and Mollea, (2012) demonstrated exposure to patulin animal models caused nephropathy and gastrointestinal tract failure. Depending on substrate availability and environmental conditions, a combination of mycotoxins can occur simultaneously. Therefore, it can be anticipated that both animals and humans can be exposed to a combination rather than an individual toxin (Milićević *et al*, 2010).

2.3.1 Considerations for assessing toxicological risks

Mycotoxin health risk assessment is a big challenge because it depends on toxicity assessment and exposure assessment. However, evaluating the toxicological risks associated with mycotoxins is important in food safety. The information on the risks related with mycotoxins will assist in improving a realistic assessment of mycotoxins.

Generally, a complete diet would usually include a variety of food commodities that could be contaminated with various mycotoxins (EFSA, 2010, 2013). Hence, there is rising concern about the risks of mycotoxins. Depending on the absorption rates of the various mycotoxins, the ingestion of mycotoxin-contaminated commodities will lead to a cumulative intake. Toxicity data from the mycotoxin mixture is therefore needed and will provide more accurate risk assessments. Determining the level of exposure to multiple mycotoxins, the effects related to cumulative exposure, and the degree to which mycotoxins interact is a major challenge in risk assessment. Many studies are based on the interaction of single mycotoxins, but few studies have focused on the cumulative effects of

mycotoxins. There is significantly little knowledge on the relationship between mycotoxins that occur simultaneously and combined toxic effects to the degree that their effects on food safety evaluation are still generally unknown (Bouaziz *et al.*, 2013). Hence, the evidence of a possible combined interaction is a cause for concern.

2.4 Techniques for the simultaneous determination of patulin and related mycotoxins in foods

Owing to the toxicity of mycotoxins and their occurrence in food and feeds there is a need for a highly selective analytical method for accurate identification and quantification of mycotoxins in foods and feeds. Various methods have been developed to simultaneously analyze a wide range of mycotoxins co-occurring in different matrices (Biselli and Hummert, 2007; Christensen *et al.*, 2009; Romero-González *et al.*, 2009; Sun *et al.*, 2019). This section gives a brief discussion on the different preparatory methods as well as qualitative and quantitative methods employed in the determination of mycotoxins are briefly discussed.

2.4.1 Sample collection, handling, and preservation

It is important to collect and preserve samples in a manner that prevents or minimizes degradation and contamination. Proper sample handling is critical for accurate mould and mycotoxin analysis. When transporting samples, eliminating oxygen and cooling the samples assists in conserving the integrity of the sample. Vacuum sealing is highly recommended for the mycotoxin samples; however, the Ziploc sealing system works well for vacuum sealing of samples meant for moulds and mycotoxins analysis. Ziploc bags are easy to use and inexpensive. The samples should be kept under cool conditions upon transportation these conditions provide additional protection for multiple day's transportations. Ice packs are highly recommended but should not be in direct contact with the samples. During storage of food samples, moisture condensation or leaks may cause a portion of the sample lot to mould and contain varying concentrations of mycotoxins. This would, therefore, lead to inaccurate quantification of mycotoxins. Thus, the samples need to be preserved until needed for analysis.

Prevention of mould growth on food samples (post-sampling) to be used in mycotoxin analysis is very difficult for studies involving a large number of samples from widely dispersed locations. However, keeping samples dry or storage at ≤0 °C prevents mould growth on samples. The following procedure could minimize the growth of moulds and production of mycotoxins post-sampling:

(1) Samples should be stored at ≤0 °C in vacuum-sealed bags, and the sample can be cooled by dry ice, liquid nitrogen, or refrigeration. Acetic acid or propionic acid can be added to the sample before cooling. The majority of mycotoxins such as patulin are stable under acidic conditions (Cunha *et al*, 2009; Jakovac-Strajn and Tavar-Kalcher, 2012; Diao *et al.*, 2018).

(2) Closely packed piles of samples may not cool rapidly enough. Therefore, the samples should be spread until they have been cooled. The mass of the samples may also hinder the cooling of the samples; therefore, samples should be stored in large bags so that they can spread evenly within the bag.

Thawing of the sample can result in condensation and high moisture levels; therefore, samples stored by refrigeration should be analysed immediately upon removal from the refrigerator or other special efforts should be taken to prevent condensation on the sample (e.g., keeping samples in moisture-proof containers on removal from refrigeration until samples reach ambient temperatures).

2.4.2 Sample preparation

The analysis of the presence of mycotoxins in various food commodities contributes towards improving food safety. The general procedure determining mycotoxins involves the extraction of the analyte, detection, and lastly, quantification using various analytical techniques (Capriotti *et al.*, 2012; Arroyo *et al.*, 2013). Because of the toxicity and occurrence of various mycotoxins at low concentration levels, the use of reliable and sensitive analytical methods is important for the effective monitoring of mycotoxin contamination in various food commodities (Boonzaaijer *et al*., 2005; Cunha *et al.*, 2009; Sun *et al.*, 2019).

However, because of the heterogeneity and complexity of food samples, treatments such as grinding, homogenization, and sifting are required before the

extraction. An efficient homogenization step is essential in obtaining reliable results because mycotoxins generally do not evenly distribute in the sample.

In addition, the preparation step of the sample depends mostly on the physiochemical properties of mycotoxin-contaminated commodities as well as on mycotoxins. Consequently, for the quantitative determination of mycotoxins in vegetables, an adequate extraction and clean-up step is often requirement because the vegetable sample consists of ingredients such as sugar, sorbitol, organic acid, phenolic acid, and up to 85 % glucose (Markowski *et al.*, 2015), which may hinder the analysis. Therefore, the first step in preparing the sample is to reduce the interference from the various components of the matrix.

2.4.2.1 Extraction methods

The choice of the extraction method to be used is influenced by the matrix, the analyte of interest, and the purpose of the extraction. For instance, if the purpose of the extraction is to monitor the presence of mycotoxins at high concentrations, simple extraction methods are to be used. In contrast, if the purpose is to develop a more sensitive and selective method for the analysis of mycotoxins, a more exhaustive extraction method is required.

Owing to the occurrence of mycotoxins at low concentrations, various extraction methods such as Solid-Phase Extraction (SPE), liquid-liquid extraction (LLE) and the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method have been used for the extraction of mycotoxins. These methods efficiently extract the analyte of interest while also extracting a high percentage of interfering compounds. If the extract is subsequently analysed by Liquid Chromatography coupled with Mass Spectrometry (LC-MS) after extraction, the interfering compounds may cause a matrix effect on the signal intensity. Thus, the need exists to develop a more selective extraction technique or an additional clean-up step after extraction.

Although the extraction techniques used for the analysis of mycotoxins are generally tedious, costly, use high-solvent volumes, and require more time, they are an important part of the determination of mycotoxin. Therefore, a careful selection of the extraction method is crucial for reducing performance errors. The current principle in sample extraction is the use of less solvent volume. Therefore,

following this principle, an SPE method was adopted in this research study for purposes of sample preparation of mycotoxins in vegetables.

2.4.2.1.1 Liquid-Liquid Extraction

LLE is an extraction method in which a compound is separated based on its solubility in two immiscible solvents. The use of LLE for the extraction of mycotoxin is based on the varying solubility of mycotoxins in aqueous and organic phases. Non-polar solvents such as n-hexane, cyclohexane, and toluene are commonly used for extracting non-polar mycotoxins (Turner *et al.*, 2009). LLE method has proven to be effective for a few toxins but works better on small-scale sample preparations. The disadvantages of LLE include the use of high volumes of toxic solvents, it is time-consuming, and is dependent solely on the complexity of the analyte. Other drawbacks include sample loss through adsorption on the surface of the glassware and possible sample contamination by the solvent (Turner *et al.,* 2009).

Liquid-liquid extraction using ethyl acetate, acetonitrile or n-hexane is an extensively-used method worldwide for the analysis of mycotoxins, which has been validated for collaborative studies based on the analysis of patulin in clear- and cloudy-apple juices and apple puree (MacDonald *et al.*, 2000; Spadaro *et al.*, 2007; Moukas, Panagiotopoulou, and Markaki, 2008). The method consists of three steps, namely: extraction with ethyl acetate, sample neutralization with Na_2CO_3, and sample drying with Na_2SO_4. The neutralization with Na_2CO_3 lowers the interference from the sample matrices and the drying with Na_2SO_4 removes excess moisture. The most encountered drawbacks of this technique are that patulin is unstable in alkaline Na_2CO_3 solution; therefore, the neutralization step needs to be carried out rapidly to avoid losses. Welke *et al.* (2009a) and Farhath and Jayashree (2015) have extracted patulin through the LLE method using ethyl acetate, however, poor recoveries and limits of detection were experienced. Apart from the mentioned disadvantages of the method, one major drawback associated with this technique is that it is time-consuming.

2.4.2.1.2 Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS)

Initially Quick, Easy, Cheap, Effective, Rugged and Safe **(**QuEChERS) method was developed for the extraction of pesticides from fruits and vegetables and has been broadly used for the determination of mycotoxins in various food matrices (Anastassiades and Lehotay, 2003; Lehotay, 2011). The QuEChERS method involves the separation of the sample matrix using solvent mixtures induced by adding inorganic salts. Whereas the organic solvent extracts the analytes, the greater polar impurities remain in the aqueous layer. Residual carbohydrates, fatty acids, and other co-extractable H-bonding matrices are removed from the organic solution by incorporating primary and secondary amine (PSA) accompanied by dispersive solid-phase extraction (dSPE).

During extraction, CH_3CO_2Na (sodium acetate) and $MgSO_4$ are added to the supernatural liquid to adsorb water and remove impurities from the sample. Sometimes, extraction without any clean-up step is enough for analysis. However, to avoid any carryovers, a clean-up step is required. A C18 sorbent can also be used in the QuEChERS method. C18 and PSA have different characteristics related to retention time and selectivity.

An important factor to consider when developing the QuEChERS extraction method is the selection of the extracting solvent (Sospedra *et al.*, 2010).

2.4.2.1.3 Solid-phase extraction

SPE is a separation technology based on liquid chromatography theory, and it has been extensively used in the separation of organic and inorganic compounds. In SPE, the analyte of interest is adsorbed onto the surface of the solid phase then eluted with an organic solvent (Zheng *et al.*, 2006). Generally, the common goal of all extraction methods before analysis is efficient clean-up, concentration, and solvent exchange (e.g., aqueous to organic). Therefore, in SPE, these goals are achieved in the following four simple steps:

1 Conditioning: This is achieved by passing a volume of a solvent, similar to the sample matrix, through the column; for example, sequential passing of methanol (MeOH), and water through a C18 cartridge before extraction of the sample matrix.

2 Sample loading: The sample is loaded into a pre-conditioned cartridge. The analyte is retained to the surface of the sorbent through chemical interactions such as van der Waals forces or non-polar interaction between the hydrocarbon chains of an analyte and the hydrocarbons of a C18-bonded phase.

3 Washing: The solvent is allowed to pass through the cartridge to remove interfering compounds while the target analyte is retained within the sorbent bed. Water is commonly used as a solvent for a non-polar extraction using C18 sorbent.

4 Elution: This involves passing an appropriate extraction solvent, which results in the selective elution of the target analyte. MeOH is an example of an organic solvent that is strong enough to disrupt the interaction between the target analyte and a C18 bonded phase.

Reversed-phase (also known as RP) such as C8, C18, cation or anion exchangers, or polymeric materials with incorporated properties are used in traditional SPE procedures. Multifunctional MycoSep uses modern clean-up processes (Berthiller *et al.*, 2005; Biselli and Hummert, 2007; Ren *et al.*, 2007) or immunoaffinity columns (IAC) (Mwanza *et al.*, 2015; Kim *et al.*, 2017; Woo *et al.*, 2019). These techniques, however, are highly costly than traditional clean-up methods. , The MycoSep columns are made of a combination of charcoal, ion-exchange resins, and other materials and are satisfactory for the extraction of aflatoxin, trichothecenes, zearalenone, ochratoxins, and patulin. (Romero-González *et al.*, 2009). Zaied *et al.* (2013) have assessed the efficiency of the SPE method for the extraction of patulin from apple products using a MycoSep® 228 AflaPat column. The use of MycoSep was found to give good recoveries. The average recovery of the extraction was 75%, and the method was rapid, reliable, and rugged. Maragos (2015) also used a MycoSep® 228 AflaPat column for the extraction of patulin from apples, and mean recovery rates ranging from 77.3-110.6% were obtained.

Molecularly imprinted polymers (MIPs) specifically for mycotoxins are also regarded as a possible and inexpensive clean-up, are not hindered by organic solvent storage constraints and stability issues as compared to IACs. MIPs have been developed for many mycotoxins, including deoxynivalenol, zearalenone,

ochratoxin A and patulin, with recognition properties (De Smet *et al.*, 2011; Weiss et al., 2003).

A polymeric reversed-phase Oasis® hydrophilic-lipophilic balance (HLB) was developed by Waters for the extraction of a variety of compounds from different matrices. The Oasis HLB sorbent provides cleaner samples in reduced time and with less effort (Seo *et al*., 2015; Wang *et al.*, 2016). This sorbent is designed in a way such that it is suitable for the nature and number of samples to be analysed. The Oasis HLB sorbent was employed in several studies involving the analysis of patulin. For instance, Arslan *et al.* (2015) employed the Oasis HLB cartridge to clean up the analyte after extraction with ethyl acetate; therefore, high sensitivity and LOD of 9.66 ng/kg were obtained. The Oasis HLB cartridge and other sorbents were used by Eisele and Gibson (2003) to evaluate the clean-up efficiency of eight mycotoxins including patulin. The clean-up method using HLB resulted in a short preparation time with significantly high recoveries for all mycotoxins and maximum matrix effect as compared to conventional methods.

Once again, Eisele and Gibson (2003) evaluated the effectiveness of the SPE method through the use of Oasis HLB extraction cartridge that uses a mixture of acetonitrile and ethyl ether (2:98, v/v). The analysis of the extracted patulin was achieved by using LC/MS. The separation of the analyte was achieved using the C18reversed-phase column. The average recoveries of patulin ranged from 93-104% with an RSD of between 0.9 and 13.4% (Trucksess and Tang, 2001). These studies have demonstrated that the SPE method is rapid, uses less volumes of organic solvents, yields high recovery rates, and is reliable. These advantages were corroborated by Katerere *et al*. (2008) in a comparative study, where three solid SPE adsorbents (HLB microporous copolymer, MycoSep® 228 AflaPat, and silica gel) were compared with the LLE method.

In most studies, co-elution of interfering compounds with mycotoxins is observed. Therefore, to limit any interferences, the extracts from the SPE are cleaned-up before analysis (Maragos, 2015), thus increasing the sample extraction time. Thus, the use of combined polar and non-polar sorbent materials is preferred for SPE because they are an effective clean-up technique (Anastassiades and Lehotay, 2003). However, for the efficacy of an analytical method, careful selection of the clean-up technique is important. SPE cartridges can retain co-eluting

interferences from the matrix while allowing the analyte of interest to pass through; this technique is, therefore, more rapid and convenient. For this reason, an HLB cartridge was used for the sample clean-up in this work.

2.4.3 Instrumental techniques for identification and quantification

Separation and detection techniques are also regarded as important in the determination of mycotoxins. Liquid chromatography (LC) is the most commonly used and preferred technique for detecting mycotoxins. Other techniques employed in the determination of mycotoxins include gas chromatography (GC) and thin-layer chromatography (TLC). Tandem mass spectrometry (MS-MS) is the most commonly used technique for the detection of mycotoxins and is recognized as the main method for both experimental research and routine analysis (Biselli and Hummert, 2007; Christensen *et al.*, 2009; Beltrán *et al.*, 2014). The choice of one technique over the other depends on the polarity, volatility, and thermal stability of the analyte of interest.

2.4.3.1 Thin layer Chromatography

TLC is one of the conventional methods for the detection of mycotoxins. Other than being cost-effective, this technique enables the screening of a great number of samples and easy identification of the target analyte. A silica gel layer is the most frequently used stationary phase in TLC; however, phenyl non-polar bonded, silanized and polyamide have also been used (Turner *et al*., 2009; Welke *et al.*, 2009a, 2009b). Mycotoxins are visually detected on the TLC plate through spraying chemicals that with mycotoxins to boost the fluorescence of the analyte or generate colored analytes under ultra-violet (UV) light. Naturally, aflatoxins, citrinin, and ochratoxins are fluorescent compounds; they can therefore be easily identified by means of their fluorescent properties. For instance, the aflatoxins B and G are distinguished by their respective blue and green fluorescent colors, while citrinin is distinguished by its yellow fluorescent color. Aflatoxins have been identified by spraying trifluoroacetic acid and sulphuric acid on the TLC plate (Chilaka, Suretha de Kock, *et al.*, 2012). In high-performance thin-layer chromatography (also known as HPTLC), the TLC technique has been enhanced to improve resolution and accuracy. HPTLC has been used for the identification of

aflatoxins in peanut products and has demonstrated to have precision, accuracy, and sensitivity equivalent to LC.

2.4.3.2 Liquid chromatography-mass spectrometry

The most frequent method for separating, detecting, and quantifying mycotoxins in food is LC. The LC analytical methods may be either normal-phase liquid chromatography (also known as NPLC) or reverse-phase liquid chromatography (also known as RPLC) coupled to various detector systems for the analysis, purification, and separation of mycotoxins in foods. Different detectors provide different selectivities when combined with LC techniques. Compared to GC, there is no derivatization step required for LC and high sensitivity can be obtained. The selection of the stationary and the mobile phases is the most important factor influencing the separation of mycotoxins.

The selection of the stationary phase for use in chromatographic separation is dependent on the physiochemical properties of the analyte of concern. For instance, a reversed-phase column such as C18 is used for more polar mycotoxins such as patulin. A less polar stationary phase will slow down the elution of polar mycotoxins. The mobile-phase composition is dependent on the target analyte and the polarity mode used for the TLC. Acetonitrile and MeOH are two of the most commonly used organic solvents. The mobile phase is composed mainly of a mixture of an aqueous and a small percentage of the organic phase. At the same time, the mobile phases can be modified by adding a small concentration of salts and a small percentage of acids to improve the ionization efficiency of the analyte. Commonly used salts include ammonium acetate $CH_3CO_2NH_4$ (NH4Ac) and ammonium formate (NH_4HCO_2) and commonly used acid are HCO_2H and CH_3CO_2H. These salts and acids are compatible with MS ionization and are, in most cases, added to one of the mobile phases. However, the additives can be added to both mobile phases to obtain reproducible results.

MS is an analytical method employed in the quantification and identification of an unknown analyte found in a sample, and it can also be used for the interpretation of structural and chemical properties of different molecules. MS ionizes the molecules found in a sample, separates them based on their mass-to-charge (m/z) ratio, and thereafter detects the relative abundance of each ion. The accessibility

of a wide range of ionization sources and different types of mass analyzers has made the MS a useful tool for controlling targeted analytes and identifying less studied mycotoxins. Recently, the hyphenated LC-MS technique was mainly used for the detection and quantification of mycotoxins following chromatographic separation (Kim *et al.*, 2017; Malachová *et al.*, 2018). However, other techniques used over the last two decades for the detection of mycotoxins include fluorescence detector (also known as FLD), UV, and diode array detector (DAD) (Hernández *et al.*, 2006; Farhadi and Maleki, 2011; Zdzieblo and Reuter, 2015). The use of MS detection is increasing due to its high sensitivity, high output, and its ability to confirm the identity of the compounds in a complex sample.

Since the detection of the co-occurrence of various mycotoxins and their associated synergistic toxic effects (Creppy *et al.*, 2004; Biomin, 2018), an additional effort have been put in place to improve analytical methods for the simultaneous quantitative and qualitative analysis of various groups of mycotoxins using LC-MS/MS. Furthermore, it would be advantageous to focus on the mycotoxins addressed by Commission Regulation 1881/ 2006 (fumonisins B1 and B2, aflatoxins B1, B2, G1, G2 and M1, deoxynivalenol, ochratoxin A, HT-2, T-2 toxins, and patulin) with a single method as this improves the sample output and reduces the costs. Due to the wide chemical variety of mycotoxins, specific sacrifices have to be made when choosing an extraction solvent and mobile phases (Guidetti *et al.*, 2015).

Recently, mass spectrometry-based methods have focused on identifying mould species based on their metabolite profile (Adetunjwe *et al.*, 2019). Other efforts were directed towards the simultaneous identification of mycotoxins produced by *Aspergillus* and *Penicillium* species in raw building materials (Tuomi *et al.*, 2001) and artificial food matrix (Rundberget and Wilkins, 2002). Although poor recovery of some analytes was experienced with the first approach, excellent precision and reliability were established and the procedure was thereafter applied in the simultaneous identification of aflatoxins following a slight adjustment of the extraction solvent (Kokkonen, 2011).

A quantitative method using an SPE procedure for the analysis of deoxynivalenol and Fumonisin B1 in maize was subsequently (Royer *et al*, 2004). New methods have emerged since the introduction of MycoSep ® columns for the clean-up of

the raw extracts. For instance, (Berthiller *et al.*, 2005) developed a method for the simultaneous analysis of type-A and B trichothecenes. Biselli and Hummert (2007) have also developed a method for the simultaneous analysis of *Fusarium* toxins in cereals and cereals-based products using LC/MS. A signal suppression was detected for some matrices. Therefore, the matrix effects were resolved by further dilution of the sample extract and using a matrix-matched calibration.

Recently, the ultrapure-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) based method was used for the simultaneous analysis of multi-mycotoxin contamination in foods and feeds, and the method included a direct SPE-based clean-up of crude extracts (Tanaka *et al.*, 2006; Ren *et al.*, 2007). As a result, a decreased analysis time was achieved. In both cases, 70% of the recovery for all the analytes was obtained and no matrix effect was reported. However, the drawback is that these techniques cannot be used reliably for all mycotoxins; for example, these methods cannot be used for the analysis of fumonisins and ergot alkaloids, because the conditions for extraction and/or clean-up are not compatible.

To resolve this problem, some techniques established the injection of extract crudes into the LC/MS, which is, excluding sample clean-up. Using this method, 22 mycotoxins, as well as the ergot alkaloid ergotamine, were detected in various food matrices (Spanjer *et al*., 2008). Samples were extracted using a mixture of acetonitrile and water and reconstituted with water before injection. For every analyte, the matrix effects were investigated and the validation results demonstrated that the analysis of dilute raw extracts is attainable and at the same time highly sensitive for analyzing the majority of mycotoxins at levels set in the legislation. Furthermore, Sulyok *et al.* (2007) achieved a simultaneous analysis of 87 mycotoxins based on the expansion of a previously reported method that involves LC-ESI-MS/MS.

2.4.3.4 Gas chromatography

GC has been used extensively for the detection of mycotoxins, especially trichothecenes in food samples. GC is suitable for compounds that are thermally stable, non-polar, semi-polar, volatile, and semi-volatile. Derivatization methods are always employed to increase the volatility of mycotoxins and improve their

responses to the GC detection system (Turner *et al.*, 2009). The most common detectors include electron capture detection (also known as ECD), flame ionization, and MS. Patulin is a polar compound, with low molecular weight and low polarity that restricts its direct analysis by GC. Despite this limitation, several GC-MS-based studies for the analysis of patulin have been reported. For example, Cunha *et al.* (2009) have developed a GC-MS method for the detection of apple and apple-based products as well as for juice, cider, and baby food. Another example is by Kharandi *et al* (2013), which involves the development of robust GC-MS methods for the detection of patulin in grain using the QuEChERS protocol. Over and above the examples mentioned above, GC has also been used for the detection of patulin in fruits and fruit derivatives.

2.5 Analysis of microbial species associated with food spoilage

This section describes the methods and conditions appropriate for isolation and identification of fungal isolates associated with food spoilage as well as also the preservation method used for fungal isolates.

2.5.1 Sample preparation methods

Several methods are used to prepare samples for microbial analysis. However, this sub-section focuses on preparatory methods for fungal analysis.

2.5.1.1 Direct plating

Direct plating involves the transfer of mould from a natural sample onto a fresh agar plate using an inoculating needle. The needle needs to be sterilized through heating until red hot and thereafter cooled before use. Once the transfer of the mould has been made, the plate can be incubated for several days until the colonies form. In most cases, the sample should be disinfected before plating to remove any contamination that may arise from the dust and other sources.

The sample is disinfected by immersing it for two minutes in a 4-5% chlorine solution while stirring occasionally; thereafter, the solution is drained. The nature of chlorine solutions are change by organic matter rapidly; therefore, it is crucial to use an excess chlorine solution that is 10 times the total volume of the sample and the solution can only be used once. After the chlorine solution is decanted, the

sample can be rinsed with sterile water. Following disinfection and rinsing, the rotten tissues of the sample are directly plated onto a solidified agar using a disinfected inoculating needle.

Surface disinfection gives rise to an effective analysis of the intrinsic mycological quantity and allows the evaluation of the presence of mycotoxins. This method does not offers direct indication of the degree of fungal contamination on each sample. However, it is fair to presume that a high percentage of fungal contamination is associated with widespread fungal invasion on the samples and possible occurrence of mycotoxins.

2.5.1.2 Dilution plating method

An appropriate technique for fungal-based study on powdered and liquid food samples is dilution plating. Furthermore, it acceptable for grains intended for the production of flour and other circumstances in which total fungal contamination is necessary. (Czaban and Wróblewska, 2006). In this method, 1 g (ground up if necessary) of the sample is dispersed in 9 mL of sterile water. 1 mL of this solution is transferred to a second tube with 9 mL of sterile water, allowing the mass of the initial sample to be diluted by 0.01. The procedure is repeated to achieve dilutions of 0.001, 0.0001, and 0.00001 or, even further if needed. 1 mL of each dilution is pipetted into Petri dishes separately after dilution, and a pre-cooled, agar medium is then poured over it. The solution can alternatively be distributed uniformly on the surface of a solidified plate of agar. Colonies can appear in different densities after a few days of incubation, depending on the amount of dilution from the original sample.

The accuracy of this technique is low when only one plate is used to count the colonies. Various factors, however, are known to contribute to the inaccuracy of the method, such as improper spore dispersion during dilution, failure to break up spore masses, or mutual growth inhibition by certain fungi. More accuracy is achieved by multiple dilution plates, perhaps ten or more, at the most suitable dilution.

2.5.1.3 Incubation conditions

A typical incubation temperature for fungi is 30 °C, and cultures should be incubated for 21 days in a humidified environment. The cultures should be inspected daily for at least a week, and at least three times weekly thereafter. Some fungi are very slow and may need longer incubation times. Fungal incubation at 42 °C prevents the growth of the *Candida* species thus allowing the growth of the *Aspergillus* species.

2.5.2 Fungal isolation techniques

Fungal cultures isolated from rotten tissues usually grow into a mixture of different colonies, and this is difficult to identify the fungal species responsible for the spoilage (Pitt and Hocking, 2009; Snyder, Churey and Worobo, 2019). Generally, to identify fungal species, fungal isolation is required in which pure cultures free from contaminations are used Filamentous fungi are usually isolated by picking a small sample of hyphae or spore and inoculating it on a fresh agar plate, preferably in the center of the plate to allow colony growth and sporulation.

The purity of fungal culture is judge after incubation based on the uniform macroscopic morphology of the colony. The easiest starting point for fungal isolation is a culture plate with well-separated colonies. A sterilized inoculating needle should be used. After heating, the hot needle should be cooled down by immersing it in a cold agar. Using the tip of the cooled needle, a few spores of mycelium just enough to be visible should be picked off and inoculated on an agar plate. For mixed cultures resulting from direct plating or dilution plating methods, the same procedure can be applied. Notes on the appearance of the sampled colony area should be recorded, as this will show whether the growing culture is the same as the one to be isolated.

Generally, it is simple to isolate that grow fast from those with very low growth rates. Normally, the outermost hyphal tips are free from contamination. Sometimes, the reverse process is even difficult. It also takes expertise and patience to isolate slow-growing fungal species in the presence of fast-growing fungi. It is advisable to monitor the point inoculum regularly for at least several days, because certain stages in the lifecycle may provide advantages. The slow-

growing colony may germinate faster or may have the needle-accessible area, or more freely spores.

2.5.3 Fungal preservation techniques

The methods for the preparation and isolation of microbes are time-consuming and costly, and it is thus necessary to preserve the specimen so that the isolation processes are not repeated. The importance of the preservation of culture plates arises from the need to have organism or specimen available at any time for either experimental purposes or routine work (Milićević *et al.*, 2010). The use of simple and efficient preservation techniques is of significant importance as far as research in microorganisms is concerned.

Fungal culture preservation is an important element in any study that involves fungi. To keep the viability and genetic integrity of cultures over time, cultivation and preservation methods are necessary. However, the cost and convenience of each method should be considered.

2.5.3.1 Short-term fungal preservation

Short-term preservation effectively maintains cultures for up to one year. A short-term preservation method that is widely used for most fungal cultures is the serial transfer method. The method is simple and cost-efficient. However, some of the disadvantages of the method include its time-consuming nature and extensive labor. In addition, most fungal cultures can be contaminated during the preservation period. Therefore, the method requires that the cultures be frequently monitored for mites or other contaminants and for drying. The morphology and physiology of a fungal culture may change over time. Particularly, the ability to sporulate may be lost after repeated transfer. Generally, this technique is not suitable for the long-term preservation of fungal culture exceeding one year.

The use of agar plates for the storage of fungal isolates that are routinely used is highly recommended and satisfactory. In addition, fungal species that easily sporulate can be preserved for months and sometimes longer when stored on media such as Czapeck Yeast extract Agar (CYA) at 1-4 °C. Vigorous, sporulating cultures can also be tightly closed-up and stored in a freezer at -18 to -20 °C to improve survival and increase the interval between the required transfers. One of

the disadvantages of storing fungal isolates at these temperatures is contamination with psychrophilic *Penicillium* or *Cladosporium* species. However, most filamentous fungal species can survive at least 1-2 years at 4 °C.

2.5.3.2 Long-term fungal preservation

Several methods such as storage in liquid nitrogen, lyophilization, freezing, and immersion in distilled water can be used for long-term preservation of fungal culture (Guimarães *et al.*, 2014).

Liquid nitrogen storage: This is an effective method for the preservation of most of the microbes including those that cannot undergo lyophilization. This method is generally more expensive than lyophilization because liquid nitrogen needs to be refilled every few days. This method is suitable for fungal species that do not produce spores in culture and also fungi that have larger or delicate spores and cannot withstand freeze-drying. The major advantages of this method include reduced labor, prevention of losing fungal cultures, and assurance of long-term availability of the cultures.

Lyophilization: This technique, also known as freeze freeze-drying, is a process of dehydration at low temperatures that involves freezing the product, reducing the pressure, and then removing the ice by sublimation. Although cost-efficient, this method is not suitable for all fungi. Lyophilisation is primarily used for the preservation of fungal species that can form large numbers of spores (10 µm or less). In addition, spores of appropriate size can also be killed by forming ice crystals during the freezing process. Thus, it is required that each isolate must have many visible spores initially. The formation of crystal ice is minimized by the addition of a solvent and rapid freezing. The most commonly used solvents are non-fat milk powder solution (5 or 10%) and filter-sterile bovine serum.

Freezing: Most fungal cultures stored at -20 to -80 °C retain their viability. Direct storage in a freezer is most suitable for culture grown in screw-capped test tube containing agar (American Type Culture Collection (ATCC, 2011; Guimarães *et al.*, 2014). Generally, vigorously growing and sporulating cultures survive freezing better as compare to less vigorous strains. Repeated freezing and thawing is not recommended because it significantly reduces the viability of the cultures.

Immersing in distilled water: This is an inexpensive and low-maintenance method for the long-term storage of fungal cultures. Water suppresses the morphological changes in most fungi. Small blocks of 7-10 mm^2 are cut from the actively growing inoculum and placed in distilled water in a glass test tube with a rubber-lined test tube, and thereafter stored at 1-10 °C. Cultures may be revived by removing the block and place it in a suitable growth medium. The use of this technique has shown that the fungal cultures retain their characteristics and remain viable for up to seven years.

2.5.4 Instrumental techniques for characterization and identification of various microbial species

The identification of unknown fungal isolates is crucial because a name enables additional information to be found in the literature. Information such as the fungal growth conditions, heat or preservative resistance, and production of mycotoxins can be obtained based on the identified fungal isolate and this information can affect decision making. Several techniques such as morphological, molecular, and bio-typing techniques, which can be used for fungal identification, are discussed below (Romanelli *et al.*, 2010; Kaufmann *et al.*, 2011; Sarubbi *et al.*, 2016; Hammami *et al.*, 2017).

2.5.4.1 Morphological techniques

Fungal identification using morphological characteristic include a macroscopic and microscopic examination. The macroscopic examination involves the observation with a naked eye the colonial features such as color, shape size, and hyphae (Liam, 2001). The microscopic examination involves the examination of the shapes forming from the arrangement of spores under a microscope (Gaddeyya *et al.*, 2012). The use of morphological characteristics in fungal identification is very important to understand the development of morphological characters. However, morphology-based approaches may not perform well for identification to species level *(Wang* et al*., 2016)*. Moreover, identification of fungal species based solely on morphology can be difficult, particularly when non experienced people are handling fungal cultures as there is an inadequate number of morphological characters that can be employed for identification. For instance, *Fusarium* species

provides no phenotypic characters that can be used for identification to species level. As a result, DNA sequence-based techniques have emerged for identifying fungal isolate to species level (Romanelli *et al.*, 2010; Chilaka, de Kock, *et al.*, 2012; Raja *et al.*, 2017).

2.5.4.2 Molecular identification techniques

The use of molecular techniques for fungal identification is rapid, sufficient, and reproducible. In addition, it can give high specificity to discriminate between the species and subspecies of fungi as compared to morphological techniques (Liu *et al.*, 2000). A specific barcode for determining and identifying various fungal isolates at the species level is provided by molecular identification techniques based on total fungal DNA extraction (Landeweert *et al.*, 2003). Fungal identification by molecular techniques is carried out by DNA amplification using polymerization chain reaction-based methods (Liu *et al.*, 2000; Tannous *et al.*, 2015). Filamentous fungi can be accurately identified to species level by the use of molecular methods such as polymerase chain reaction (PCR), 18S ribosomal RNA (rRNA) gene sequencing, or ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequence analysis (Romanelli *et al.*, 2010). However, not only are these methods time-consuming and expensive, but they also require expertise.

2.5.4.2.1 DNA extraction

DNA extraction from fungal isolates may be challenging even from pure cultures. This becomes more challenging when the extraction is performed from fungal species isolated from complex matrices such as food. The extraction method affects the outcome of the molecular identification, thus the need to optimize the method for each food sample. Some of the challenges faced during DNA extraction include obtaining a pure and sufficient amount of DNA. Fungal growth is often spread heterogeneously through a sample and, similar to culture-based techniques, a sufficient sample size should be analysed (Geisen, 2007). Furthermore, molecular techniques usually require a small number of food samples that might result in a low copy number in the DNA extract. Fungal species are structurally diverse and this could affect the reproducibility of the extraction method. For instance, the *Penicillium* and *Aspergillus* species can produce single-

celled structures, while the *Alternaria* and *Fusarium* species produce multi-celled structures and hyphae. These structures can either be thin-walled, thick-walled, and/or melanized. *Cladosporium* and *Alternaria* species produce resistant, melanized cell walls, and breaking these cells is challenging even by bead beating. Thus, each type of cell type is expected to have different DNA recovery (Summerbell, Corr and Scott, 2011).

2.5.2.2.2 PCR amplification and sequence-based method

Conventional PCR and real-time or quantitative PCR (qPCR) based methods can be used as an alternative method for fungal identification. The qPCR-based methods are routinely used for fungal identification of mostly mycotoxin-producing fungi (Mayer *et al.*, 2003; Nicolaisen *et al.*, 2009). However, the sensitivity of PCR-based methods is relatively low and is dependent on the quality of the DNA extract, PCR conditions, and primers used. For the evaluation of the presence of fungi, conventional PCR is mostly used without knowledge about the copy number in the sample. In a qPCR assay, the copy number can be quantified using an (internal) standard, and a quantification option is an interesting tool if detection alone is not sufficient. Results obtained from conventional PCR are displayed at the end of the reaction, while for qPCR they are displayed after each cycle.

Previously, it was reported that fungal species isolated from food are identified based on their phenotypic characteristics (Bandh *et al*., 2011; Gautam and Bhadauria, 2012). However, phenotypic-based identification can be time-consuming and it is susceptible to identification errors when compared with molecular identification. Molecular identification based on PCR amplification requires one or two genes or loci to be sequenced.

The ITS rDNA region is an official DNA barcode for fungi (Schoch *et al.*, 2012), however, its resolution is not sufficient for fungal identification at species level particularly *for Aspergillus*, *Cladosporium*, *Fusarium,* and *Penicillium* (Samson *et al.*, 2004). The gene to be targeted is dependent on the genus of the unknown fungal isolate. For instance, when identifying an unknown *Aspergillus* isolate, it is recommended that a part of the calmodulin gene is sequenced; the amplification and sequencing of the b-tubulin gene are recommended for the identification of the *Penicillium* isolate (Samson *et al.*, 2004).

It is highly recommended that the ITS region be sequenced first for the identification of unknown isolates. However, there are public databases such as GenBank that are stocked with ITS sequences that can be used to compare database sequences against the generated ITS sequences of the unknown isolate. From this, the genus and the species may be revealed. Generally, a secondary barcode is required where ITS sequencing is not sufficient for fungal identification at the species level.

The results on sequence-based fungal identification are highly dependent on the quality of the database. Houbraken and Samson (2017) have reported a 20 % error in fungal identification using GenBank. However, the use of sequence-based and morphological-based techniques are for accurate fungal identification.

The 16S rDNA sequencing is one of the commonly used methods in the PCR-based methods *(*Cherkaoui *et al., 2010)*. However, these methods are costly and time-consuming because DNA extraction, target amplification, and sequencing need to be done to obtain the sequence to be compared in the reference.

2.5.4.3 MALDI bio-typing

The matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a commonly used technique for the identification of bacteria, yeasts, and filamentous fungi. MALDI-TOF MS is robust, reliable, and has a short turnaround time. To date, MALDI-TOF MS has frequently been used in the clinical diagnosis and identification of bacteria. In addition, MALDI-TOF MS has been used with success for the routine identification of filamentous fungi (Marklein *et al.*, 2009; Kolecka *et al.*, 2013, 2014; Gautier *et al.*, 2014). On the other hand, identification of filamentous fungi using MALDI-TOF MS is more challenging and is not yet popular with foodborne fungal identification.

The comparison of the generated spectrum against the reference database with the mass spectra is an important step in the identification process using MALDI-TOF MS. Quality and diversity of species or strains within the database are essential for correct identification (Vallejo *et al.*, 2013). In addition, the sample preparation method is crucial because it can negatively affect the quality of the spectra and fungal identification (Lima and Santos, 2017).

MALDI Bio Typer (also known as MBT) is rather a new system, established by Bruker Daltonics, integrating MALDI-TOF MS analysis with a designated fungal identification database. Even though it was initially intended for clinical use, it has recently been used in other aspects of microbiology. However, its applicability in the identification of environmental isolates is still scarce, particularly in environments where the lack of availability of a comprehensive reference spectra database further restricts its use.

2.6 Cytotoxicity evaluation of patulin and related mycotoxins on mammalian cells

Food and feeds can be contaminated by several fungal species at once and most fungi can simultaneously produce different mycotoxins as secondary metabolites (Rundberget and Wilkins, 2002; Biselli and Hummert, 2007; Capriotti *et al.*, 2012). For this reason and the fact that the human diet comprises a variety of different commodities, the possibility of human co-exposure to several mycotoxins is very likely. Although some predictions about the toxic effects of combinations of mycotoxins can be made based on their toxicities, experimental data are still limited to allow a reliable hazard assessment. Thus, more studies are needed to allow new risk assessment strategies that take into consideration the toxicological interactions of mycotoxins in food and feeds.

Cytotoxicity is one of the most crucial measurement for the biological assessment of in vitro studies. Various mycotoxins may have toxic effects on cells through various mechanisms such as cell membrane destruction, protein synthesis inhibition, enzymatic reaction, and irreversible binding to receptors (Aslantürk, 2018). Therefore, a need exists for the development of a reliable, cheap, and reproducible method for evaluating the cytotoxic effect and cell death caused by these mechanisms.

Apoptosis (known as programmed cell death) is a process for reducing cells homeostasis of multicellular organisms (Grütter, 2000). Some studies have reported that patulin have caused apoptosis on colon cancer cells (Kwon *et al.*, 2012; Katsuyama *et al.*, 2014) kidney cells (Zhang *et al.*, 2015), and skin cells (Saxena *et al.*, 2009).

Beuvericin was reported to cause oxidative stress and depletion of antioxidant cellular mechanism; it has also been associated with DNA damage and causes apoptosis in human cell lines (Dornetshuber *et al.*, 2007; Ferrer *et al.*, 2009). Similar effects were liberated by patulin (Alves *et al.*, 2000; Liu *et al.*, 2007; Ferrer *et al.*, 2009).

Patulin cell treatment inhibits RNA and protein synthesis and also disrupts the activity of several enzymes. Patulin is an electrophilic molecule that exerts its cytotoxic effect by covalently bonding to vital sulfhydryl groups in proteins and amino acids. Adducts of patulin and sulfhydryl compounds are less toxic than patulin. However, there is very little knowledge on the specific mechanisms or molecular basis for the toxicity of patulin to human cells.

2.6.1 Handling and maintenance of cell lines

Cell lines (cell cultures) are generally used in biomedical and biomedical research laboratories. Even though cell lines do not inherently pose a risk to laboratory workers, due to their potential to harbor pathogenic organisms, specific safety-handling precautions are needed. Generally, cell lines may be contaminated with fungi, bacteria, fungi, viruses, mycoplasma, and prions. Care should therefore be taken not to expose cell lines to inappropriate conditions (for example extended periods of incubation). Key equipment items, including, laminar airflow, incubators, and microbiological safety cabinets, and cryo-storage systems, must be properly designed and used. Aseptic techniques, where necessary, should be applied rigorously. The growth of mammalian cells is dependent on various parameters such as temperature, atmospheric gas, pH, and trypsinization.

2.6.1.1 Temperature

The optimum temperature is dependent on the type of cell line used. Insect cell lines generally grow optimally at low temperatures compared to mammalian cells, and their growth characteristics may be modified at temperatures 28 °C and above. Exposure of mammalian cells to temperatures above 39 °C may induce cell death, while growth below 35 °C may slow replication but may also increase the expression of certain cellular proteins.

2.6.1.2 Atmosphere

Oxygen and carbon dioxide are considered to be important to cell growth, and changes in the levels of these gasses can affect cell cultures. For instance, high levels of both gasses are toxic to cells and inhibition of cell growth and cell death occur at low levels. Levels of oxygen may require to be optimised for specific purposes, such as the promotion of growth in large-scale cultures in bioreactors. For several cell cultures, an atmosphere containing 5% v/v carbon dioxide is most suitable, but the optimal level of carbon dioxide would be dependent on the medium being used, the cells being cultured, and possibly other considerations.

2.6.1.3 pH

Mammalian cells optimally grow at pH 7.2-7 and insect cells at pH 6.0. Variability outside a relatively narrow pH range can have substantial effects on cell phenotype, development, and viability.

2.6.1.4 Cell detachment and subculture

Disassociation solutions such as trypsin/EDTA may have significant effects on cells if they are not used in particular circumstances. Residual disassociation solutions may have detrimental effects and thus should be removed after cell dissociation. Most cell lines are sub-cultured before they reach confluence (for example Caco-2 cells). The frequent passage of some cell lines after they have reached full confluence may lead to the loss of desirable traits. For instance, the subculture regime may affect the evident productivity of recombinant cell lines and the variability capacity of Caco-2 cells.

2.6.2 Preservation of cell lines

Freezing has been proven to be the most effective long-term preservation method for various cell types. However, during the freezing process cell may be damaged by environmental conditions. Factors such as the preservation methods, freezing temperature, biomaterials, and preservation solution influence the viability of cells cultured preservation and thawing (Miyamoto *et al.*, 2018). . Preservation solutions such as glycerol, ethylene glycol, dimethyl sulfoxide (DMSO) are commonly used and are most effective because they have a high cell penetration rate. In addition,

starch and oligosaccharides such as trehalose and maltose have been reported to be effective in suppressing damage to the cells (Miyamoto *et al.*, 2018).

Vitrification is an effective preservation method of induced pluripotent stem cells (also known as iPSCs) and embryonic stem cells (also known as ESCs) but an increase in osmotic pressure damages the cells. However long-term storage of cells in liquid nitrogen carries risks of mycoplasma infection and bacterial and viral agents. Thus, it is required to consider more effective techniques for freezing and storage of different types of cells.

When cryo-preserved cells are needed for the study, it is essential to properly thaw the cells to maintain the viability of the culture and allow the culture to recover quickly. Some cryo-protective compounds, such as DMSO, are toxic at temperatures above 4 °C; therefore, cultures must be rapidly thawed and diluted in a culture medium to reduce the toxic effect.

2.6.3 In vitro cytotoxicity methods

Several *in vitro* cell viability and cytotoxicity assays have been widely used for investigating cytotoxicity and drug delivery. The selection of an appropriate method to be used for cytotoxicity and cell viability should be based on the test compound, detection mechanism, sensitivity, and selectivity of the methods (Aslantürk, 2018). In this section, the *in vitro* cytotoxicity and cell viability assays are briefly discussed.

Dye exclusion assays: Dye exclusion methods are the most widely used and simplest methods for the evaluation of cell viability. These methods can also be employed in the determination of membrane integrity. In the dye exclusion method, viable cells are stained while dead cells are not. The staining procedure is easy, however, it is more challenging and time-consuming and the use of more samples (Aslantürk, 2018). A wide range of dyes has been used including trypan blue, Congo red, erythro-sine B, and eosin, with trypan blue being the most extensively used dye (Aslantürk, 2018).

When using the dye exclusion method, the following must be taken into consideration: (i) more days are needed for dead cells to lose the integrity of the membrane; (ii) living cells may continue to grow rapidly during the culture period and (iii) some of the dead cells may not seem to be stained with the dye at the end

of the culturing period since cell disintegration may occur at an early stage (Lee *et al.*, 2005; Son *et al.*, 2003).

Some of the advantages of the dye exclusion methods include the requirement of a small number of cells, they are simple, they can detect cell kill in non-dividing cells (Aslantürk, 2018). However, these dyes are not recommended for monolayer cell cultures, however, they are preferred to be used in suspension, so far, it is required that monolayers be trypsinized first (Aslantürk, 2018).

Colorimetric assay: In colorimetric assays, the metabolic activity of the cells is evaluated by measuring the biochemical makers. Reagents being employed in colorimetric assays change color in regards to cell viability, enabling colorimetric measurement of viable cells through the use of a spectrophotometer. Colorimetric assays can be applied in suspended and adhered cell lines, they are simple to conduct and are comparatively economical (Präbst *et al.*, 2017). Several companies have kits for colorimetric assays available and the standard operating procedures are usually included in kit packages. This technique is far better compared to the dye exclusion methods because it is easy to use, safe, highly reproducible, and commonly used for the evaluation of both cell viability and cytotoxicity.

Fluorometric assays: Evaluation of cell viability and cytotoxicity through the use of fluorometric assays are easy to perform when using a fluorometer, fluorescence microscope, and flow cytometer or fluorescence microplate reader. This method is more advantageous as compared to commercial dye exclusion and colorimetric assays. Fluorometric assays can also be used for suspended or adherent cell lines, are simple to use, and are highly sensitive (Aslantürk, 2018). Similar to colorimetric assays, commercial fluorometric assay kits are available from a number of companies, and standard operating procedures are usually included in kit packages.

Luminometric assays: These assays provide rapid and easy detection of cell proliferation and cytotoxicity in mammalian cells. They can be conducted in a suitable 96-well and 384-well microliter plate and the detection can be achieved by a luminometric microplate reader (Mueller *et al.*, 2004; Duellman *et al.*, 2015). In luminometric assays, after the addition of the reagent, a constant glow signal is produced. This characteristic can be used to monitor both cell viability and

cytotoxicity from the same well (Niles et al., 2009). Several companies have luminometric assay kits available and the standard operating procedures are usually included in the kit packages.

When all the methods discussed above are compared with one another, the colorimetric assay using 3-4,5- dimethylthiazol-2yl, 2,5-diphenyltetrazolium bromide (MTT) assay has been commonly used for the evaluation of cytotoxicity and cell viability of various mycotoxins on mammalian cell lines (Aslantürk, 2018). MTT assay evaluates cell viability through the determination of mitochondrial functions of cells by measuring mitochondrial enzyme activity such as succinate dehydrogenase (Stone *et al*., 2009). This assay has been employed to evaluate the *in vitro* cytotoxicity of patulin on human epithelial colorectal adenocarcinoma cell lines (Caco-2) (Assunção *et al.*, 2016), and it was reported that the cell viability of Caco-2 cell line decreased with increasing patulin concentration. Furthermore, Zouaoui *et al.* (2016) evaluated the toxicity of beuvericin, patulin, and sterigmatocystin individually and combined on Chinese hamster ovary cell lines (CHO-K1). The results showed that combined mycotoxins exerted a higher additive effect on the cells. In another study, the effect of individual and combined mycotoxins was evaluated using the dye exclusion method using neutral red dye (Creppy *et al.*, 2004). It was revealed that the dye exclusion method may be suitable for the *in vitro* cytotoxicity of individual and combined mycotoxins occurring in foodstuffs (Creppy *et al.*, 2004). The cell viability of patulin-induced human colon cancer cell lines (HCT116) and human embryonic kidney cell lines (HEK-293) was evaluated using flow cytometry and it was reported that the cell of the cell lines decreased with increasing patulin concentration (Boussabbeh *et al.*, 2015).

2.7 Reference

Abnet, C. (2007) ‘Carcinogenic Food Contaminants’, *Cancer Investigation*, 25, pp. 189–196.

Adetunji, M. C. *et al.* (2019) ‘Fungal metabolite and mycotoxins profile of cashew nut from selected locations in two African countries’, *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*. Taylor and Francis Ltd.

Aiko, V. and Mehta, A. (2015) ‘Occurrence, detection and detoxification of mycotoxins’, *Journal of Biosciences*, 40(5), pp. 943–954.

Alves, I. *et al.* (2000) ‘Induction of micronuclei and chromosomal aberrations by the mycotoxin patulin in mammalian cells: role of ascorbic acid as a modulator of patulin clastogenicity’, *Journal of Mutagenesis*, 15, pp. 229–234.

American Type Culture Collection (ATCC) (2011) ‘Preservation and recovery of filamentous fungi’, *The Essentials of Life science Research*, (1), pp. 1–4. Available at: www.atcc.org.%0Awww.atcc.org.

Anastassiades, M. and Lehotay, S. (2003) *Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and ‘Dispersive Solid-Phase Extraction’ for the Determination of Pesticide Residues in Produce*, *JOURNAL OF AOAC INTERNATIONAL*.

Arroyo-Manzanares, N., García-Campaña, A. M. and Gámiz-Gracia, L. (2013) ‘Multiclass mycotoxin analysis in Silybum marianum by ultra-high-performance liquid chromatography-tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid-liquid microextraction’, *Journal of Chromatography A*.

Arslan Karakose, Senem Sanli, N. S. (2015) ‘Evaluation of Patulin in Commercial Baby Foods by Solid Phase Extraction and Liquid Chromatography PDA Detection’, 33(1), pp. 52–57. Aslantürk, Ö. S. (2018) ‘In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages’, in *Genotoxicity - A Predictable Risk to Our Actual World*. InTech.

Assunção, R. *et al.* (2016) ‘Characterization of in vitro effects of patulin on intestinal epithelial and immune cells’, *Toxicology Letters*. Elsevier Ireland Ltd, 250–251, pp. 47–56.

Avsaroglu, M. D. *et al.* (2015) ‘Use of pulsed-high hydrostatic pressure treatment to decrease patulin in apple juice’, *High Pressure Research*. Taylor and Francis Ltd., 35(2), pp. 214–222.

Bandh, S., Kamili, A. and Ganai, B. A. (2011) ‘Identification of some Penicillium species by traditional approach of morphological observation and culture’, (October).

Barbiroli, A. *et al.* (2007) ‘Binding of Aflatoxin M1 to Different Protein Fractions in Ovine and Caprine Milk’, *Journal of Dairy Science*, 90, pp. 532–540.

Bashir, M., Hamza, F. A. and Pukuma, M. S. (2020) 'Postharvest Fungal Spoilage of Some Citrus Fruits', (April), pp. 10–15.

Beltrán, E. *et al.* (2014) 'Determination of patulin in apple and derived products by uhplc-ms/ms. Study of matrix effects with atmospheric pressure ionizat

Bennett, J. W. and Klich, M. (2003) 'Mycotoxins', *Journal of Clinical Microbiology*, 16, pp. 497–516.

Berreta, B. (2000) 'Patulin in Apple-based foods: Occurence and safety evaluation.', *Journal of Food Additives and Contaminants*, 17(5), pp. 399–406.

Berthiller, F. *et al.* (2005) 'Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high-performance liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*, 1062(2), pp. 209–216.

Bhatnagar, D., Yu, J. and Ehrlich, K. C. (2002) 'Toxins of filamenteous fungi', *Journal of Chemistry and Immuno*, 81, pp. 167–206.

Biomin.net (2018) 'Mycotoxins Definition', *Mycotoxins.Info*. Available at: http://www.mycotoxins.info/mycotoxins/mycotoxins-definition/ (Accessed: 9 December 2019).

Bircan, C. (2009) 'Incidence of ochratoxin A in dried fruits and co-occurrence with aflatoxins in dried figs', *Journal of Food and Chemical Toxicology*, 47, pp. 1996-2001.

Birzele, B., Prange, A. and Kramer, J. (2000) 'Deoxynivalenol and ochratoxin A in German wheat and changes of level in relation to storage parameters', *Journal of Food Additives and Contaminants*, 17, pp. 1027–1035.

Biselli, S. and Hummert, C. (2007) 'Development of a multicomponent method for Fusarium toxins using LC-MS/MS and its application during a survey for the content of T-2 toxin and deoxynivalenol in various feed and food samples'.

Blesa, J. *et al.* (2006) 'Factors affecting the presence of ochratoxin A in wines.', *Journal of Food Science and Nutrition*, 46, pp. 473–478.

Boonzaaijer, G., Bobeldijk, I. and van Osenbruggen, W. A. (2005) 'Analysis of patulin in dutch food, an evaluation of a SPE based method', *Food Control*, 16(7), pp. 587–591.

Bosco, F. and Mollea, C. (2012) 'Mycotoxins in Food', in *Industrial Processes-Methods and Equipment*, pp. 169–200.

Bottalico, A. and Perrone, G. (2002) ‘Toxigenic Fusarium species and mycotoxin associated with head blight in small grain cereals in Europe’, *Journal of Plant Pathology*, 108, pp. 611–624.

Bouaziz, C. *et al.* (2013) ‘The in vitro effects of zearalenone and T-2 toxins on Vero cells’, *Journal of Toxicology and Pathology*, 65, p. 497501.

Boudra, H. *et al.* (2007) ‘Aflatoxin M1 and ochratoxin A in raw bulk milk from french dairy herds. :.’, *Journal of Dairy Science*, 90, pp. 3197–3201.

Bouslimi, A. *et al.* (2008) ‘Individual and combined effects of ochratoxin A and citrinin on viability and DNA fragmentation in cultured Vero cells and on chromosome aberrations in mice bone marrow cells. Toxicology 251’, *Journal of Toxicology*, 251, pp. 1–7.

Boussabbeh, M. *et al.* (2015) ‘Patulin induces apoptosis through ROS-mediated endoplasmic reticulum stress pathway’, *Toxicological Sciences*, 144(2), pp. 328–337.

Boutigny, A., I. *et al.* (2012) ‘Quantitative detection of Fusarium pathogens and their mycotoxins in South African maize’, *Journal of Plant Pathology*, 61, pp. 522–31.

Bryden, W. L. (2012) ‘Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security’, *Journal of Animal Feed Science and Technology*, 173, pp. 134–158.

Campagnollo, F. B. *et al.* (2016) ‘The occurrence and effect of unit operations for dairy products processing on the fate of aflatoxin M1: A review’, *Journal of Food Control*, 68, pp. 310–329.

Capriotti, A. L. *et al.* (2012) ‘Multiclass screening method based on solvent extraction and liquid chromatography-tandem mass spectrometry for the determination of antimicrobials and mycotoxins in egg’, *Journal of Chromatography A*. Elsevier B.V., 1268, pp. 84–90.

Castelo, M. M., Summer, S. S. and Bullerman, L. B. (1998) ‘Stability of fumonisins in Thermally processed coern products’, *Journal of Food Protection*, 161, pp. 1030–1033.

Chalmers, I. and Clarke, M. (2004) ‘The 1944 patulin trial: The first properly controlled multicentre trial conducted under the aegis of the British Medical Research Council’, *International Journal in Epidemiology*, 33(3), pp. 253–260.

Cherkaoui, A. *et al.* (2010) ‘Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level’, *Journal of Clinical Microbiology*, 48(4), pp. 1169–1175.

Chilaka, C. A., de Kock, Suretha., *et al.* (2012) ‘Fungal and mycotoxin contamination of South African commercial maize’, *Journal of Food, Agriculture and Environment*, 10(2), pp. 296–303.

Chilaka, C. A., de Kock, Suretha, *et al.* (2012) ‘Fungal and mycotoxin contamination of South African commercial maize’, *Journal of Food, Agriculture and Environment*, 10(2), pp. 296–303.

Christensen, H. B. *et al.* (2009) ‘Development of an LC-MS/MS method for the determination of pesticides and patulin in apples’, *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 26(7), pp. 1013–1023.

Christensen, H B *et al.* (2009) ‘Development of an LC-MS / MS method for the determination of pesticides and patulin in apples’, *Journal of Food Additives and Contaminants*, 26(7), pp. 1013–1023.

Conkova, E. *et al.* (2003) ‘Fusarial toxins and their role in animal diseases’, *Veterinary Journal*, 165, pp. 214–220.

Cotty, P. J. and Jaime-Garcia, R. (2007) ‘Influences of climate on aflatoxin producing fungi and aflatoxin contamination’, *International Journal of Food Microbiology*, (119), pp. 109–115.

Council for Agricultural Science and Technology (CAST) (2003) *Mycotoxins: Risk in Plants, Animals and Humans*.

Creppy, E. E. *et al.* (2004) ‘Synergistic effects of fumonisin B1 and ochratoxin A: Are in vitro cytotoxicity data predictive of in vivo acute toxicity?’, *Toxicology*, 201(1–3), pp. 115–123.

Cunha, S. C., Faria, M. A. and Fernandes, J. O. (2009) ‘Determination of patulin in apple and quince products by GC-MS using 13C5-7 patulin as internal standard’, *Food Chemistry*. Elsevier Ltd, 115(1), pp. 352–359.

Czaban, J. and Wróblewska, B. (2006) ‘A Simple, Direct Plating Method, Alternative to Dilution Plating, for Estimation of the Abundance of Penicillium verrucosum on Incubated Cereal Grain’, 55(3), pp. 237–241.

Davies, A. P. *et al.* (2012) 'Identification of clinical isolates of α-hemolytic streptococci by 16S rRNA gene sequencing, matrix-assisted laser desorption ionization-time of flight mass spectrometry using MALDI Biotyper, and conventional phenotypic methods', *Journal of Clinical Microbiology*, 50(12), pp. 4087–4090.

De-Saeger, S., Sibanda, L. and Van-Peteghem, C. (2003) 'Analysis of zearalenone and α-zearalenone in animal feed using high-performance liquid chromatography', *Journal of Analitica Chimica Acta*, 487, pp. 137–143.

Desjardins, A. E., Maragos, C. M. and Proctor, R. H. (2006) 'Maize ear rot and moniliformin contamination by cryptic species of Fusarium subglutinans', *Journal of Agricultural and Food Chemistry*, 54, pp. 7383–7390.

Diao, E. *et al.* (2018) 'Improving the safety of apple juice by UV irradiation', *Journal of Food Measurement and Characterization*. Springer Verlag, 12(3), pp. 2005–2011.

Dornetshuber, R. *et al.* (2007) 'Enniatin exerts p53-dependent cytostatic and p53-independent cytotoxic activities against human cancer cells', *Journal of Chemistry Research and Toxicology*, 20, pp. 465–473.

Duellman, S. J. *et al.* (2015) 'Bioluminescent, nonlytic, real-time cell viability assay and use in inhibitor screening', *Journal of Assays Drug Development Technologies*, 13(8).

Edite Bezerra da Rocha, M. *et al.* (2014) 'Mycotoxins and their effects on human and animal health', *Food Control*, pp. 159–165.

Eisele, T. and Gibson, M. Z. (2003) 'Syringe-cartridge solid phase extraction method from patulin in apple juice', *Journal of Food Chemical Contaminants*, 86(6), pp. 1160–1163. Available at: file:///C:/O-drive/kfn/5866.pdf.

Eskola, M., Rizzo, A. and Soupas, L. (2001) 'Occurrence and amounts of some Fusarium toxins in Finnish cereal samples in 1998', *Journal of Plant and Soil Science*, 50, pp. 183–186.

European Food Safety Authority (EFSA) (2010) 'Management of left-censored data in dietary exposure assessment of chemical substances', *Journal of European Food Safety Authority*, 8(3), p. 1557.

European Food Safety Authority (EFSA) (2013) 'International frameworks dealing with human risk assessment of combined exposure to multiple chemicals', *Journal*

of European Food Safety Authority, 11(7), p. 3313.

Farhadi, K. and Maleki, R. (2011) 'Dispersive liquid-liquid microextraction followed by HPLC-DAD as an efficient and sensitive technique for the determination of patulin from apple juice and concentrate samples', *Journal of the Chinese Chemical Society*. Chinese Chemical Society Taiwan, 58(3), pp. 340–345.

Farhath, K. and Jayashree, G. (2015) 'Patulin contamination in pure and mixed apple products marketed in Mysore city (India)', *International Journal of Current Research*, 7(12), pp. 23508–23511.

Ferrao, J. *et al.* (2017) 'Mycotoxins, food and health mycotoxins, food and health', *Journal of Nutritional Health and Food*, 5(7), pp. 1–10.

Ferrer, E. *et al.* (2009) 'Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells', *Journal of Toxicology and Vitro*, 23, pp. 1504–1509.

Funes, G. J. and Resnik, S. L. (2009) 'Determination of patulin in solid and semisolid apple and pear products marketed in Argentina', *Food Control*, 20, pp. 277–280.

Gaddeyya, G. *et al.* (2012) 'Isolation and identification of soil mycoflora in different crop fields at Salur Mandal', *Journal of Advanced Application of Science Research*, 3, pp. 2020–2026.

Garnier, L. (2017) 'Diversity and Control of Spoilage Fungi in Dairy Products : An Update', pp. 1–33.

Garrido Frenich, A. *et al.* (2011) 'Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry', *Journal of Chromatography A*, 1218, pp. 4349–4356.

Gautam, A. K. and Bhadauria, R. (2012) 'Characterization of Aspergillus species associated with commercially stored triphala powder', 11(104), pp. 16814–16823.

Gautier, M. *et al.* (2014) 'Matrix-assisted laser desorption ionization time-of-flight mass spectrometry : revolutionizing clinical laboratory diagnosis of mould infections', *Clinical Microbiology and Infection*. European Society of Clinical Infectious Diseases, 20(12), pp. 1366–1371.

Gazzotti, T. *et al.* (2009) 'Determination of fumonisin B1 in bovine milk by LC-MS/MS', *Journal of Food Control*, 20, pp. 1171–1174.

Geisen, R. (2007) ‘Molecular detection and monitoring’, in *Food Mycology - a Multifaceted Approach to Fungi and Food*, pp. 255–278.

Ghosh, A. (2009) ‘Identification of microorganisms responsible for spoilage of tomato (lLycopersicon esculentum) fruit’, 1(6), pp. 414–416.

Gougouli, M. and Koutsoumanis, K. P. (2010) ‘Modelling growth of Penicillium expansum and Aspergillus niger at constant and fluctuating temperature conditions’, *International Journal of Food and Microbiology*, 140, pp. 254–262.

Griffin, G. J. and Garren, K. H. (1976) ‘Colonization of Rye Green Manure and Peanut Fruit Debris by Aspergillus flavus and Aspergillus niger Group in Field Soils1’, 32(1), pp. 28–32.

Grütter, M. G. (2000) ‘key players in programmed cell death’, 10(6), pp. 649–655.

Guidetti, R. *et al.* (2015) ‘Mycotoxins: Are They Perceived as a Serious Threat for the Human Health?’, in *Chemical Engineering transactions*.

Guimarães, L. C. *et al.* (2014) ‘Methods to preserve potentially toxigenic fungi’, 47, pp. 43–47.

Guo, Y. *et al.* (2013) ‘Survey of patulin in apple juice concentrates in Shaanxi (China) and its dietary intake’, *Food Control*, 34(2), pp. 570–573.

Hammami, W. *et al.* (2017) ‘Patulin and patulin producing Penicillium spp. Occurrence in apples and apple-based products including baby food’, *Journal of Infection in Developing Countries*, 11(4), pp. 343–349.

Harris, K. L. *et al.* (2009) *Patulin Surveillance in Apple Cider and Juice Marketed in Michigan*, *Journal of Food Protection*.

Hernández, M. J. *et al.* (2006) ‘Validation of two analytical methods for the determination of ochratoxin A by reversed-phased high-performance liquid chromatography coupled to fluorescence detection in musts and sweet wines from Andalusia’, *Analytica Chimica Acta*, 566(1), pp. 117–121.

Houbraken, J. and Samson, R. A. (2017) ‘Current taxonomy and identification of foodborne fungi.’, *Journal of Food Science and Technology*, 17, pp. 84–88.

Hove, M. *et al.* (2016) ‘Review on the natural co-occurrence of AFB1 and FB1 in maize and the combined toxicity of AFB1 and FB1.’, *Journal of Food Control*, 59(6), pp. 75–82.

Hu, X. *et al.* (2016) ‘Development of a multiple immunoaffinity column for simultaneous determination of multiple mycotoxins in feeds using UPLC-MS/MS’,

Journal of Analytical and Bioanalytical Chemistry, 408, pp. 6027–6036.

Hudecová, A., Valík, Ľ. and Liptáková, D. (2009) 'Quantification of Geotrichum candidum Growth in Co-Culture with Lactic Acid Bacteria', 27(2).

Hussein, H. S. and Brasel, J. M. (2001) 'Toxicity, metabolism, and impact of mycotoxins on animals and humans', *Journal of Toxicology*, 167, pp. 101–134.

Iha, M. H. and Sabino, M. (2008) 'Incidence of patulin in Brazilian apple-based drinks', *Food Control*, 19(4), pp. 417–422.

Ismaiel, A. A. and Papenbrock, J. (2015) 'Mycotoxins: Producing Fungi and Mechanisms of Phytotoxicity', pp. 492–537.

Jackson, L. and Dombrink-Kurtzman, M. A. (2006) *Patulin in microbiology of fruits and vegetables*.

Jakovac-Strajn, B. and Tavar-Kalcher, G. (2012) 'A Method Using Gas Chromatography-Mass Spectrometry for the Detection of Mycotoxins from Trichothecene Groups A and B in Grains', *Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications*.

JECFA (2001) 'Safety evaluation of certain mycotoxins in food. FAO Food and Nutrition Paper 74/WHO', *Journal of Food Additives & Contaminants*, 47, pp. 281–415.

Katerere, D. R., Stockenström, S. and Shephard, G. S. (2008) 'HPLC-DAD method for the determination of patulin in dried apple rings', *Food Control*, 19(4), pp. 389–392..

Katsuyama, A. *et al.* (2014) 'The mycotoxin patulin decreases expression of density-enhanced phosphatase-1 by down-regulating PPARγ in human colon cancer cells', *The Tohoku journal of experimental medicine*, 233(4), pp. 265–274.

Kaufmann, C. *et al.* (2011) 'Evaluation of matrix-assisted laser desorption/ionization-time of flight mass spectrometry for characterization of Culicoides nubeculosus biting midges', *Medical and Veterinary Entomology*, 25(1), pp. 32–38.

Kharandi, N., Babri, M. and Azad, J. (2013) 'A novel method for determination of patulin in apple juices by GC – MS', *Food Chemistry*. Elsevier Ltd, 141(3), pp. 1619–1623.

Kim, D. H. *et al.* (2017) 'Simultaneous determination of multi-mycotoxins in cereal grains collected from South Korea by LC/MS/MS', *Toxins*, 9(3), pp. 1–13.

Koesukwiwat, U., Sanguankaew, K. and Leepipatpiboon, N. (2014) 'Evaluation of a modified QuEChERS methods for analysis of mycotoxins in rice', *Journal of Food Chemistry*, 153, pp. 44–51.

Kokkonen, M. (2011) *The challenge of LC-MS/MS multi- mycotoxin analysis − heracles battling the hydra ?*

Kolecka, A. *et al.* (2013) 'Identification of medically relevant species of arthroconidial yeasts by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry', *Journal of Clinical Microbiology*, 51, pp. 2491–2500.

Kolecka, A. *et al.* (2014) 'Efficient identification of Malassezia yeasts by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)', *Journal of Dermatology*, 70, pp. 332–341.

Kurtzman, C. W. and Horn, B, W. (1987) 'Aspergillus nomius, a new aflatoxin-producing species related to Aspergillus flavus and Aspergillus tamarrii', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 53(3), pp. 147–158.

Kwon, O. *et al.* (2012) 'Patulin induces colorectal cancer cells apoptosis through EGR-1 dependent ATF3 up-regulation. Cell. Signal.', 24, pp. 943–950.

Landeweert, R. *et al.* (2003) 'Molecular identification of ectomycorrhizal mycelium in soil horizons', *Applied and Environmental Microbiologyiology*, 39, pp. 327–333.

Lee, J. C. *et al.* (2005) 'Plant-originated glycoprotein, G-120, inhibits the growth of MCF-7 cells and induces their apoptosis', *Journal of Food and Chemical Toxicology*, 6(43), pp. 961–968.

Lee, T. P. *et al.* (2014a) 'High-performance liquid chromatography method for the determination of patulin and 5-hydroxymethylfurfural in fruit juices marketed in Malaysia', *Food Control*, 38(1), pp. 142–149.

Lee, T. P. *et al.* (2014b) 'High-performance liquid chromatography method for the determination of patulin and 5-hydroxymethylfurfural in fruit juices marketed in Malaysia', *Food Control*, 38, pp. 142–149..

Lehotay, S. J. (2011) 'QuEChERS sample preparation approach for mass spectrometric analysis of pesticide residues in foods', *Methods in Molecular Biology*, 747, pp. 65–91.

Liam, R. F. (2001) 'Viability, morphological characteristics and dimorphic ability of fungi preserved by different methods', *Review of Mycotoxins*, 18, pp. 191–196.

Lima, N. and Santos, C. (2017) 'MALDI-TOF MS for identification of food spoilage filamentous fungi', *Journal of Food Science and Technology*, 13, pp. 26–30.

Liu, B. H. *et al.* (2007) 'Oxidative stress response by the mycotoxin patulin in mammalian cells', *Journal of Toxicology and Science*, 95, pp. 340–347.

Liu, D. *et al.* (2000) 'Application of PCR to the identification of dermatophyte fungi.', *Journal of Medical Microbiology*, 49, pp. 493–497.

Logrieco, A., Moretti, A. and Solfrizzo, M. (2009) 'Alternaria toxins and plant diseases: An overview of origin, occurrence and risks', *World Mycotoxin Journal*, 2, pp. 129–140.

MacDonald, S. *et al.* (2000) 'Liquid chromatographic method for determination of patulin in clear and cloudy apple juices and apple puree: Collaborative study', *Journal of AOAC International*, 83(6), pp. 1387–1394.

Malachová, A. *et al.* (2018) 'Advanced LC–MS-based methods to study the co-occurrence and metabolization of multiple mycotoxins in cereals and cereal-based food', *Analytical and Bioanalytical Chemistry*. Springer Verlag, pp. 801–825.

Maragos, C. M. (2015) 'Quantification of patulin in fruit leathers by ultra-high-performance liquid chromatography-photodiode array (UPLC-PDA)', (June).

Marin, S. *et al.* (2013) 'Mycotoxins: Occurrence, toxicology, and exposure assessment.', *Journal of Food Chemistry and Toxicology*, 60, pp. 218–237.

Marklein, G. *et al.* (2009) 'Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates', *Journal of Clinical Microbiology*, 47, pp. 2912–2917.

Markowski, J. *et al.* (2015) 'Composition of clear and cloudy juices from French and Polish apples in relation to processing technology', *LWT - Food Science and Technology*, (62), pp. 813–820.

Matumba, L., C. Van Poucke, T. and Biswick, M. (2014) 'A limited survey of mycotoxins in traditional maize-based opaque beers in Malawi', *Journal of Food Control*, 36(1), pp. 53–56.

Mayer, Z. *et al.* (2003) 'Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of Aspergillus flavus in foods', *Journal of Food Microbiology*, 82, pp. 143–151.

Mazzoni, E. *et al.* (2011) 'Field control of Fusarium ear rot, Ostrinianubilalis (Hubner), and fumonisins in maize kernels', *Journal of Pest Management and*

Science, 67, pp. 458–465.

McCallum, J., Tsao, R. and Zhou, T. (2002) 'Factors affecting patulin production by Penicillium expansum', *Journal of Food Protection*, 65, pp. 1937–1942.

Megan, N. H., Cairns, V. and Alded, D. (2003) 'Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in-store grain.', *Journal of Plant Pathology*, 109(7), pp. 723–730.

Meyer, H. *et al.* (2019) 'Long term monitoring (2014–2018) of multi-mycotoxins in South African commercial Maize and wheat with a locally developed and validated LC-MS/MS method', *Toxins*, 11(5).

Milani, J. M. (2013) 'Ecological conditions affecting mycotoxin production in cereals : a review', 2013(8), pp. 405–411.

Milicevic, D. R. *et al.* (2009) 'Residue of ochratoxin A in swine tissues - Risk assessment.', *Journal of Oncology*, 17, pp. 56-60.

Milićević, D. R., Škrinjar, M. and Baltić, T. (2010) 'Real and perceived risks for mycotoxin contamination in foods and feeds: Challenges for food safety control', *Toxins*, 2(4), pp. 572–592.

Miller, S. S., Reid, L. M. and Harris, L. J. (no date) 'Colonization of maize silks by Fusarium graminearum, the causative organism of gibberella ear rot', *2007*, 85, pp. 369–376.

Miraglia, M. *et al.* (2002) *Assessment of dietary intake of Ochratoxin A by the population of EU Member States.*

Miyamoto, Y. *et al.* (2018) 'Long-term Cryopreservation of Human and other Mammalian Cells at 80 C for 8 Years', *Journal of Cell Medicine*, 10, pp. 1–7.

Moreira, S. R. *et al.* (2001) 'isolation and identification of yeasts and filamentous', pp. 117–122.

Moss, M. O. (2008) 'Fungi, quality and safety issues in fresh fruits and vegetables', *Journal of Applied Microbiology*, 104(5), pp. 1239–1243.

Moukas, A., Panagiotopoulou, V. and Markaki, P. (2008) 'Determination of patulin in fruit juices using HPLC-DAD and GC-MSD techniques', *Food Chemistry*, 109(4), pp. 860–867.

Mueller, H., Kassack, M. U. and Wiese, M. (2004) 'Comparison of the usefulness of the MTT, ATP and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines', *Journal of Biomolecular Screening*, 9, pp. 506–

515.

Murashiki, T. C. *et al.* (2017) ‘Levels and daily intake estimates of aflatoxin B1 and fumonisin B1 in maize consumed by rural households in Shamva and Makoni districts of Zimbabwe’, *Journal of Food Control*, 72, pp. 105–109.

Mwalwayo, D. S. and Thole, B. (2016) ‘Prevalence of aflatoxin and fumonisins (B1 C B2) in maize consumed in rural Malawi.’, *Toxicology Reports*, 3, pp. 173–179.

Mwanza, M. *et al.* (2015) ‘Evaluation of analytical assays efficiency to detect aflatoxin M1 in milk from selected areas in Egypt and South Africa’, *Journal of Dairy Science*. Elsevier, 98(10), pp. 6660–6667.

Nam, H. L., Kim, Y. and Lee, H. (2018) ‘Prediction of mycotoxin risks due to climate change in Korea’, *Applied Biological Chemistry*. Springer Netherlands, 61(4), pp. 389–396.

Ndleve, K. and Njobeh, P. (2018) ‘Multi-Mycotoxin Occurrence in Dairy Cattle Feeds from the Gauteng Province of South Africa : A Pilot Study Using UHPLC-QTOF-MS / MS’.

Nelson, S. C. (no date) ‘Black Rot of Sweet Potato Disease Cycle and Management Outline of Presentation • Black rot pathogen : Ceratocystis fimbriata’.

Nicolaisen, M. *et al.* (2009) ‘Real-time PCR for quantification of eleven individual Fusarium species in cereals’, *Journal of Microbiological Methods*, (76), pp. 234–240.

Niessen, L. (2007) ‘PCR-based diagnosis and quantification of mycotoxin producing fungi, Int. J. Food Microbiol’, *International Journal of Food Microbiology*, 119, pp. 38–46.

Niles, A. L., Moravec, R. A. and Riss, T. (2009) ‘In vitro viability and cytotoxicity testing and same- well multi-parametric combinations for high throughput screening.’, *Journal of Chemical Genomics*, 33, pp. 33–41.

Oduola, A. A., Awojobi, K. O. and Adeyemo, S. M. (2018) ‘Microorganisms Associated with Post Harvest Spoilage of Sweet Potatoes in Ile-Ife , Nigeria’, 8(1), pp. 1–8.

Olaoye, O. (2015) ‘Isolation and Identification of bacteria associated with spoilage of sweet potatoes during post-harvest storage ISSN 2249-8516 Original Article Isolation and Identification of bacteria associated with spoilage of sweet potatoes during post-harvest storage’, (May).

Onitsha, O. M. *et al.* (2016) ‘Isolation, Characterization and Identification of Microorganisms from Spoilt Carrots Obtained from’, 4(1), pp. 6–9.

Ostry, V. *et al.* (2017) ‘Mycotoxins as human carcinogen-the IARC Monographs classification’, *Journal of Mycotoxin Res*, 33, pp. 818–824.

Paíga, P. *et al.* (2012) ‘Extraction of ochratoxin A in bread samples by the QuEChERS methodology’, *Food Chemistry*, 135(4), pp. 2522–2528.

Paterson, R. R. M. and Lima, N. (2010) ‘How will climate change affect mycotoxins in food?’, *Food Research International*. Elsevier Ltd, 43(7), pp. 1902–1914.

Paterson, R. R. M. and Lima, N. (2017) ‘Thermophilic Fungi to Dominate Aflatoxigenic / Mycotoxigenic Fungi on Food under Global Warming’, pp. 1–11.

Pereira, V. L., Fernandes, J. O. and Cunha, S. C. (2014) ‘Mycotoxins in Cereals and related foodstuffs: A review on occurrence and recent methods of analysis’, *Journal of Food Science and Technology*, 36, pp. 96–136.

Piqué, E. *et al.* (2013) ‘Occurrence of patulin in organic and conventional apple-based food marketed in Catalonia and exposure assessment’, *Food and Chemical Toxicology*, 60, pp. 199–204.

Pitt, J. I. and Hocking, A. D. (2009) ‘The ecology of fungal food spoilage’, in *Fungi and Food Spoilage*, pp. 3–9.

Präbst, K. *et al.* (2017) ‘Basic colorimetric proliferation assays: MTT, WST and Resazurin’, in *Cell Viability Assays, Methods and Protocols*, pp. 1–17.

Prata, M. B. *et al.* (2010) ‘Fructooligosaccharide production by Penicillium expansum’, *Journal of Biotechnology*, 32, pp. 837–840.

Probst, C., Bandyopadhyay, P. J. and Cotty., P. J. (2012) ‘Diversity of aflatoxin-producing fungi and their impact on food safety in sub-Saharan Africa Microbiology’, *International Journal of Food Microbiology*, 174, pp. 113–122.

Puel, O., Galtier, P. and Oswald, I. P. (2010) ‘Biosynthesis and toxicological effects of patulin’, *Journal of Food Additives and Contaminants: part B*, 2(4), pp. 613–631.

Raja, H. A. *et al.* (2017) ‘Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community’, *Journal of Natural Products*, 80(3), pp. 756–770.

Ren, Y. *et al.* (2007) ‘Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography-

tandem mass spectrometry', *Journal of Chromatography A*, 1143(1–2), pp. 48–64.
Rheeder, J. p., Marasas, W. F. and Vismer, H. F. (2002) 'Production of fumonisin analog by Fusariam species', *Journal of Applied Microbiology*, 68, pp. 2101–2105.
Richard, J. L. (2007) 'Some major mycotoxins and their mycotoxicoses-An overview', *International Journal of Food Microbiology*, 119(1–2), pp. 3–10.
Romanelli, A. M. *et al.* (2010) 'Sequence-based identification of filamentous basidiomycetous fungi from clinical specimens: A cautionary note', *Journal of Clinical Microbiology*, 48(3), pp. 741–752.
Romero-González, R. *et al.* (2009) 'Application of conventional solid-phase extraction for multimycotoxin analysis in beers by ultrahigh-performance liquid chromatography-tandem mass spectrometry', *Journal of Agricultural and Food Chemistry*, 57(20), pp. 9385–9392.
Royer, D., Humpf, H. U. and Guy, P. A. (2004) 'Quantitative analysis of Fusarium mycotoxins in maize using accelerated solvent extraction before liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry', *Food Additives and Contaminants*, 21(7), pp. 678–692.
Rundberget, T. and Wilkins, A. L. (2002) 'Determination of Penicillium mycotoxins in foods and feeds using liquid chromatography-mass spectrometry', *Journal of Chromatography A*, 964(1–2), pp. 189–197..
De Ruyck, K. *et al.* (2015) 'Dietary mycotoxins, co-exposure, and carcinogenesis in humans: Short review', *Mutation Research - Reviews in Mutation Research*. Elsevier B.V., pp. 32–41.
Saleh, I. and Al-thani, R. (2019) 'Fungal food spoilage of supermarkets ' displayed fruits', 12.
Salomão, B. C. *et al.* (2009) 'Influence of storage temperature and apple variety on patulin production by Penicillium expansum', *Journal of Food Protection*, 72, pp. 1030–1036.
Samson, R. A. *et al.* (2002) 'Mycotoxin production by common filamentous fungi', in *Introduction to Food and Airborne Fungi*, p. 383.
Samson, R. A. *et al.* (2004) 'Methods for the detection, isolation and characterization of food-borne fungi', in *Introduction of Food- and Airborne Fungi, sixth ed*, pp. 283–305.
Samuel, O. and Orji, M. U. (2015) 'Fungi Associated with the Spoilage of Post-

harvest Tomato Fruits Sold in Major Markets in Awka, Nigeria', 3(2), pp. 11–16.

Sanchis, V., Lleida, U. De and Magan, N. (2004) 'Environmental conditions affecting mycotoxins', (April 2020).

Sanchis, V. and Magan, N. (2004) 'Environmental conditions affecting mycotoxins', in *Mycotoxins in Food: Detection and Control*, p. 496.

Sangare-Tigori, B. *et al.* (2006) 'Co-occurrence of afl atoxin B1, fumonisin B1, ochratoxin A and zearalenone in cereals and peanuts from Côte d'Ivoire', *Journal of Food Additives & Contaminants*, 23, pp. 1000–1007.

Sarubbi, F. *et al.* (2016) 'Patulin in homogenized fruit's and tomato products', *Food Control*. Elsevier Ltd, 59, pp. 420–423.

Saxena, N. *et al.* (2009) 'Patulin causes DNA damage leading to cell cycle arrest and apoptosis through modulation of Bax, p53 and p21/WAF1 proteins in skin of mice', *Journal of Toxicology and Applied Phamarcology*, 234, pp. 192–201.

Schoch, C. L. *et al.* (2012) 'Nuclear ribosomal internal transcribed spacer (ITS) region as a universal Smith, DNA barcode marker for Fungi', *Proceedings of the National Academy of Sciences*, 109, pp. 6241–6246.

Scudamore, K. A. and Patel, S. (2000) 'Survey for aflatoxins, ochratoxin A, zearalenone and fumonisins in maize imported into the United Kingdom.', *Journal of Food Additives and Contaminants*, 17, pp. 407–416.

Seo, M., Kim, B. and Baek, S. Y. (2015) 'An optimised method for the accurate determination of patulin in apple products by isotope dilution-liquid chromatography/mass spectrometry', *Analytical and Bioanalytical Chemistry*. Springer Verlag, 407(18), pp. 5433–5442.

Shephard, G. S., Rheeder, J. P. and van der Westhuizen, L. (2012) 'Effect of the traditional cooking practice on fumonisin content of maize porridge consumed in the former Transkei region of South Africa', *World Mycotoxin Journal*, 5(4), pp. 405–7.

Sherif, O. S., Salama, E. E. and Abdel-Wahhab, M. A. (2009) 'Mycotoxins and child health: The need for health risk assessment', *International Journal of Hygiene and Environmental Health*, 212, pp. 347–368.

De Smet, D. *et al.* (2011) 'Development of a molecularly imprinted polymer for patulin in apple juice', *World Mycotoxin Journal*. Wageningen Academic Publishers, 4(4), pp. 375–383..

Snyder, A. B., Churey, J. J. and Worobo, R. W. (2019) ‘Association of fungal genera from spoiled processed foods with physicochemical food properties and processing conditions’, *Journal Of Food Microbiology*. Elsevier Ltd, 83(August 2018), pp. 211–218.

Snyder, A. B. and Worobo, R. W. (2018) ‘Fungal spoilage in food processing’, *Journal of Food Protection*, 81, pp. 1035–1040.

Son, Y. O. *et al.* (2003) ‘Ripe fruits of Solanım nigrum L. inhibits cell growth and induces apoptosis in MCF-7 cells’, *Journal of Food and Chemical Toxicology*, 41, pp. 1421–1428.

Sospedra, I. *et al.* (2010) ‘Use of the modified quick easy cheap effective rugged and safe sample preparation approach for the simultaneous analysis of type A- and B-trichothecenes in wheat flour’, *Journal of Chromatography A*.

Spadaro, D. *et al.* (2007) ‘Incidence and level of patulin contamination in pure and mixed apple juices marketed in Italy’, *Food Control*, 18, pp. 1098–1102..

Spadaro, D. *et al.* (2013) ‘A new strain of Metschnikowia fructicola for postharvest control of Penicillium expansum and patulin accumulation on four cultivars of apple’, *Journal of Postharvest Biology and Technology*, 75, pp. 1–8.

Spanjer, M. C., Rensen, P. M. and Scholten, J. M. (2008) ‘LC-MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs’, *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 25(4), pp. 472–489.

Stoev, S. *et al.* (2010) ‘Simultaneous occurrence of mycotoxins in human food commodities from Cameroon’, *Journal of Mycotoxin Research*, 26, pp. 47–57.

Stone, V., Johnston, H. and Schins, R. P. F. (2009) ‘Development of in vitro systems for nanotoxicology: Methodological considerations’, *Critical Reviews in ToxicologyReviews in Toxicology*, 7(39), pp. 613–626.

Sulyok, M., Krska, R. and Schuhmacher, R. (2007) ‘A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples’, *Analytical and Bioanalytical Chemistry*, 389(5), pp. 1505–1523.

Summerbell, R. C. G., B.J., Corr, D. and Scott, J. A. (2011) ‘Molecular methods for

bioaerosol characterization', in *Microorganisms in Home and Indoor Work Environments*, pp. 247–263.

Summerell, B. A., Salleh, B. and Leslie, J. F. (2003) 'A utilitarian approach to Fusarium identification', *Journal of Plant Discovery*, 87, pp. 117–128.

Sun, D. *et al.* (2019) 'Development of Sensitive and Reliable UPLC-MS / MS Methods for Food Analysis of Emerging Mycotoxins in China Total Diet Study', *Toxins*, 166(11), pp. 1–16.

Sypecka, Z., Kelly, M. and Brereton, P. (2004) 'Deoxynivalenol and zearalenone residues in eggs of laying hens fed with a naturally contaminated diet: Effects on egg production and estimation of transmission rates from feed to eggs', *Journal of Agricultural and Food ChemistryFood Chemistry*, 52, pp. 5463–5471.

Tanaka, H. *et al.* (2006) 'Development of a liquid chromatography/time-of-flight mass spectrometric method for the simultaneous determination of trichothecenes, zearalenone and aflatoxins in foodstuffs', *Rapid Communications in Mass Spectrometry*, 20(9), pp. 1422–1428.

Tannous, J. *et al.* (2015) 'Development of a real-time PCR assay for Penicillium expansum quantification and patulin estimation in apples', *Food Microbiology*. Academic Press, 50, pp. 28–37.

Tirado, M. C. *et al.* (2010) 'Climate change and food safety : A review', *Food Research International*. Elsevier Ltd, 43(7), pp. 1745–1765.

Tola, M. and Kebede, B. (2016) 'Occurrence of important and control of mycotoxins: A review', *Journal of Food Science and Technology*, 2, p. 1191103.

Tournas, V. H. (2005) 'Spoilage of vegetable crops by bacteria and fungi and related health hazards', *Critical Reviews in Microbiology*, 31(1), pp. 33–44.

Trucksess, M. W. and Tang, Y. (2001) 'Solid-phase extraction method for patulin in apple juice and unfiltered apple juice.', *Methods in molecular biology (Clifton, N.J.)*, 157(October), pp. 205–213.

Tuomi, T. *et al.* (2001) 'Detection of aflatoxins (G1-2, B1-2), sterigmatocystin, citrinin and ochratoxin A in samples contaminated by microbes', *Analyst*, 126(9), pp. 1545–1550.

Turner, N. W., Subrahmanyam, S. and Piletsky, S. A. (2009) 'Analytical methods for determination of mycotoxins: A review', *Analytica Chimica Acta*, 632(2), pp. 168–180.

Udoh, I.P., et al. (2015) Studies on Fungi Responsible for the Spoilage/Deterioration of Some Edible Fruits and Vegetables. Advances in Microbiology, 5, pp. 285-290..

Urbano, G. R. *et al.* (2001) 'Occurrence of ochratoxin A producing fungi in raw Brazilian coffee', *Journal of Food Protection*, 64, pp. 1226–1230.

Valenta, H. and Dänicke, S. (2005) 'Study on the transmission of deoxynivalenol and deepoxy-deoxynivalenol into eggs of laying hens using a high-performance liquid chromatography-ultraviolet method with clean-up by immunoaffinity columns', *Journal of Molecular Nutrition and Food Research*, 49, pp. 779–785.

Valero, A. *et al.* (2008) 'Survey: Ochratoxin A in European special wines', *Journal of Food Chemistry*, (108), pp. 593–599.

Vallejo, J. A. *et al.* (2013) 'A typical yeasts identified as Saccharomyces cerevisiae by MALDI-TOF MS and gene sequencing are the main responsible of fermentation of chicha, a traditional beverage from Peru.', *Journal of Applied Microbiology*, 36, pp. 560–564.

Volume, B. (2009) 'Isolation and identification of postharvest spoilage fungi associated with sweet oranges (citrus sinensis) traded in kano metropolis', 2(1), pp. 122–124.

Wang, M. *et al.* (2016) 'A single-step solid-phase extraction for the simultaneous determination of 8 mycotoxins in fruits by ultra-high performance liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*. Elsevier B.V., 1429, pp. 22–29.

Wang, Z. *et al.* (2016) *Biology of Microfungi*, *Springer*.

Welke, J. E. *et al.* (2009a) 'Quantitative analysis of patulin in apple juice by thin-layer chromatography using a charge-coupled device detector', *Food Additives & Contaminants: Part A*, 26(5), pp. 754–758.

Welke, J. E. *et al.* (2009b) 'Quantitative analysis of patulin in apple juice by thin-layer chromatography using a charge-coupled device detector', *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 26(5), pp. 754–758.

Wild, C. P. and Gong, Y. Y. (2010) 'Mycotoxins and human disease: a largely ignored global health issue', *Journal of Carcinogenesis*, 31, pp. 71–82.

Wilson, D. M., Mubatanhema, W. and Jurjevic, Z. (2002) 'Biology and ecology of

mycotoxigenic Aspergillus species as related to economic and health concerns.', *Journal of Advances in Experimental Medicine and Biology*, 504, pp. 3–17.

Woo, S. Y. *et al.* (2019) 'Simultaneous Determination of Twenty Mycotoxins in the Korean Soybean Paste Doenjang by LC-MS / MS with Immunoaffinity Cleanup'.

Wu, T. S. *et al.* (2005) 'Activation of ERK mitogen-activated protein kinase in human cells by the mycotoxin patulin', *Toxicology and Applied Pharmacology*, 207(2), pp. 103–111.

Yazar, S. and Omurtag, G. Z. (2008) 'Fumonisins, trichothecenes and zearalenone in cereals', 9, pp. 2062–2090.

Yoshizawa, T. (1991) . 'Natural occurrence of mycotoxins in small grain cereals (wheat, barley, rye, oats, sorghum, millet, rice). In ; Smith, J.E., Henderson, R.S., Eds.; CRC: Boca Raton, FL, USA, 1991', *Journal of mycotoxins and Animal Foods*, pp. 301–324.

Yuan, Y. *et al.* (2010) 'Patulin content in apple products marketed in Northeast China', *Food Control*, 21, pp. 1488–1491.

Zachariasova, M. *et al.* (2010) 'Analysis of multiple mycotoxins in beer employing (ultra)-high-resolution mass spectrometry', *Journal of Mass Spectrometry*, 24, pp. 3357–3367.

Zaied, C. *et al.* (2013a) 'Occurrence of patulin in apple-based-foods largely consumed in Tunisia', *Food Control*, 31, pp. 263–267.

Zaied, C. *et al.* (2013b) 'Occurrence of patulin in apple-based-foods largely consumed in Tunisia', *Food Control*, 31(2), pp. 263–267.

Zain, M. E. (2011) 'Impact of mycotoxins on human and animals', *Journal of Saudi Chemival Society*, 15, pp. 129–144.

Zdzieblo, A. P. and Reuter, W. M. (2015) 'Analysis of Patulin in Apple Juice by UHPLC with UV Detection', *PerkinElmer*, pp. 1–7.

Zhang, B. *et al.* (2015) 'Oxidative stress is involved in Patulin induced apoptosis in HEK293 cells', *Journal of Toxicology*, 94, pp. 1–7.

Zheng, M. Z., Richard, J. L. and Binder, J. (2006) 'A review of rapid methods for the analysis of mycotoxins', *Journal of Mycopathologia*, 161, pp. 261–273.

Zouaoui, N. *et al.* (2016) 'Cytotoxic effects induced by patulin, sterigmatocystin and beauvericin on CHO-K1 cells', *Food and Chemical Toxicology*, 89, pp. 92–103.

Zwierzchowski, W. *et al.* (2005) ‘Level of zearalenone in blood serum and lesions in ovarian follicles of sexually immature gilts in the course of zearalenone micotoxicosis’, *Polish Journal of Veterinary Science*, 8, pp. 209–218.

3 Chapter 3: Methodology

3.1 Materials and reagents

All reagents and standards used were of analytical grade. Standards of Patulin (100.2 µg/mL in acetonitrile), aflatoxins mixture (B1, B2, G1, and G2 254, 251, 254, and 250 ng/mL respectively in acetonitrile were purchased from Industrial Analytical (Pty) Ltd (Midrand, South Africa). Methanol (CH_3OH, ≥99.9%) LC/MS CHROMASOLV® grade, acetonitrile (C_2H_3N, ≥99.9%) LC-MS CHROMASOLV® grade and formic acid (FA) (CH_2O_2, ≥99.0%) LC-MS grade, absolute ethanol, Trifluoroacetic acid (TFA), 5-(hydroxymethyl) Furfural (HMF) (100 mg solid), Sabouraud dextrose agar (SDA) with chloramphenicol, grade I 25% Glutaraldyhyde (for electron microscope) and 4% osmium tetroxide (for electron microscope) were purchased from Sigma Aldrich (MO, USA). The matrix α-cyano-4hydrocannamic acid (HCCA) was purchased from Bruker Daltonik (Bremen, Germany). The Dulbecco's Modified Eagles Medium (DMEM) high glucose was purchase from Sigma Aldrich (MO, USA). Phosphate Buffer Saline (PBS) from Bio Whittaker, Fetal Bovine Serum (FBS) from Hyclone, and Penicillium-streptomycin were purchased from Biowest (France). QuEChERS extraction kit was purchased from Restek (Bellefonte, USA). The ultrapure water for UHPLC and LC-MS used for preparing all the solutions was purified by a Milli-Q system with an LC (Bio-Pak) polisher (Massachusetts, USA).

Materials used during the analysis included disposable gloves, centrifuge tubes, different kinds of glassware, micro-pipets and Eppendorf pipet tips, extraction tubes, parafilm, Petri dishes, vials, disposable inoculating loop, autoclaving tape, MALDI target plate, etc. The Oasis® HLB cartridge (6 mL) was purchased from Waters (South Africa). Acquity UHPLC® BEH C_{18} (1.7 µm 2.1 × 100 mm) column used was purchased from MICROSEP (Johannesburg, South Africa).

3.2 Climatic region and sampling plan

South Africa has a wide variety of climatic conditions (see **Figure 3-1**). The South Africa climate varies from the Mediterranean in the southwestern corner of South Africa to temperature in the plateau and subtropical in the northwest. The various sampling points were selected based on the different climatic conditions of the province.

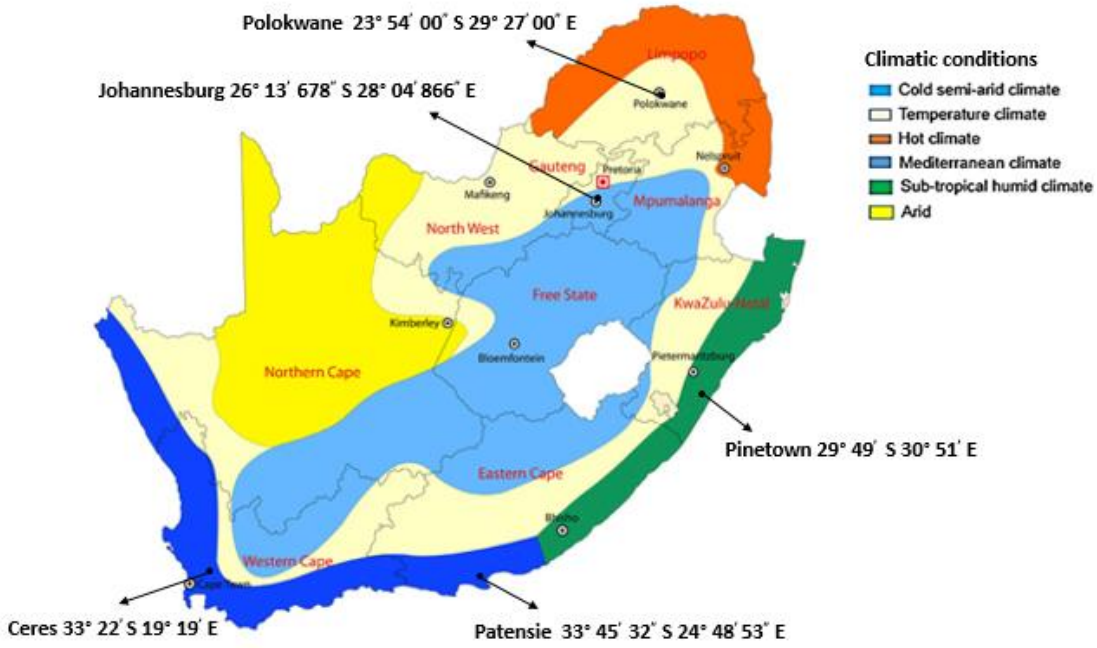

Figure 3-1: South African map showing the different climatic zones in South Africa and the sampling points for this study (fyssouthafrica, 2012)

Table 3-1: A brief description of the cities and towns located within the five South African provinces where the vegetable samples were collected from.

SAMPLING SITE	DESCRIPTION
CERES	This is the largest town located in the Witzenberg Local Municipality in the Western Cape province, South Africa. Ceres is characterized by a Mediterranean climate. The town experiences warmer temperatures in summer, with frequent rainfall, meanwhile, winter days are cool to cold and wet, with

	frequent snowfall.
PANTENSIE	This town is located in the Sarah Baartman District Municipality in the Eastern Cape Province, South Africa. The town is the center of citrus, tobacco, and vegetable farming in the area. It has the same climatic conditions as Ceres, Eastern Cape. It is located between the Western Cape and KwaZulu –Natal and its climate is a bit of a mixture of the two, Mediterranean and subtropical.
PINETOWN	This small city is located in eThekwini Metropolitan Municipality in Durban KwaZulu-Natal, South Africa. The town experiences a sub-tropical and very humid climate, with hot summers with rainfall and very mild winters.
POLOKWANE	The capital city of the Limpopo Province of South Africa has hot climatic conditions with low rainfall patterns averaging between 200-500 mm.
TARLTON	This town is located in the West Rand District Municipality in Gauteng Province, South Africa. It has a delightfully mild climate that is neither humid nor too hot.

3.3 Sample collection, handling, and preservation

A total of 111 Spoiled vegetable samples (see **Table 3-2** and **Figure 3-2**) (carrots, cucumber, onions potatoes, peppers, and sweet potatoes) were collected and analysed for fungal and mycotoxin contamination. The samples were collected from sites located in five provinces, namely Gauteng (GP), Limpopo (LP), KwaZulu-Natal (KZN), Western Cape (WC), and Eastern Cape (EC) (see **Figure 3-1** and **Table 3-1**). The samples were collected from farms, supermarkets, and wholesalers. The sampling was conducted in 2019 during winter and 1 kg of each sample was collected to obtain a representative sample.

Vegetable processed products consisting of two vegetable-based baby puree, one ready-to-eat vegetable salad, one pickled vegetable, and one vegetable sauce (see **Figure 3-3**) were bought from a supermarket.

Table 3-2: Number of vegetable samples collected from each province in South Africa

PROVINCES	VEGETABLE SAMPLES					
	Potato	Sweet potato	Carrot	Pepper	Onion	Cucumber
GP	-	3	10	4	2	3
LP	3	3	5	3	3	5
KZN	-	5	-	4	5	-
WC	5	5	6	4	2	3
EC	6	4	8	4	3	3

Figure 3-2: vegetable samples showing signs of spoilage or visible moulds were collected from five provinces and analysed for fungal and mycotoxin contamination

Figure 3-3: Processed vegetable products were bought from the supermarket and analysed for mycotoxin contamination

The samples were collected in polyethylene Ziplock bags and placed in an airtight cooler that contains ice. The bags were labeled with labels containing the following legible information sample name and sampling area. Thereafter, the samples were transported to the Institute of Nanotechnology and Water Sustainability laboratories, University of South Africa. On arrival, the samples were culture within two days.

The remaining samples were homogenized using a blender and stored in a glass bottle at -22 °C they were analysed. The homogenized vegetable samples were stored at -22 °C to prevent further growth of moulds before analysis and to ensure that mycotoxins levels reflect mycotoxin levels when the samples were collected. The samples were quickly put back into the freezer after a subsample has been taken.

Toxigenic fungal species such as *Aspergillus* and *Penicillium* pose a health risk, furthermore, the mycotoxins produced by these fungal species are among the most known carcinogens. Therefore, it is essential to follow guidelines when handing them. The precautions for handling fungal cultures and mycotoxins outlined by Harvard University, (2009) were followed. Briefly, the disposable latex and nitrile gloves for fungal isolates and mycotoxins respectively, and proper

personal protective equipment (PPE) were used at all times. The inoculation or transferring of fungal isolates was performed under a laminar flow hood which was surface disinfected with 70 % ethanol before working. All the glassware used for the preparation of fungal isolates were autoclaved before use and thoroughly washed with 30% bleach and 70 % ethanol after use.

The fungal isolates were preserved using the agar slant culture method. Briefly, the isolates were inoculated on a fresh agar and incubated for 24 hours, and then stored in a refrigerator. The Petri dishes were closed with a parafilm to prevent the culture plate from drying out. The cultures were periodically transferred after two months to maintain their viability.

3.4 Sample preparation

3.4.1 Solid-phase extraction of patulin and other related mycotoxins

In this study, the extraction and clean-up were carried out using Oasis HLB cartridges. A manual solid-phase extraction setup was used, the cartridges were placed on a manifold connected to a vacuum pump (see **Figure 3-4**).

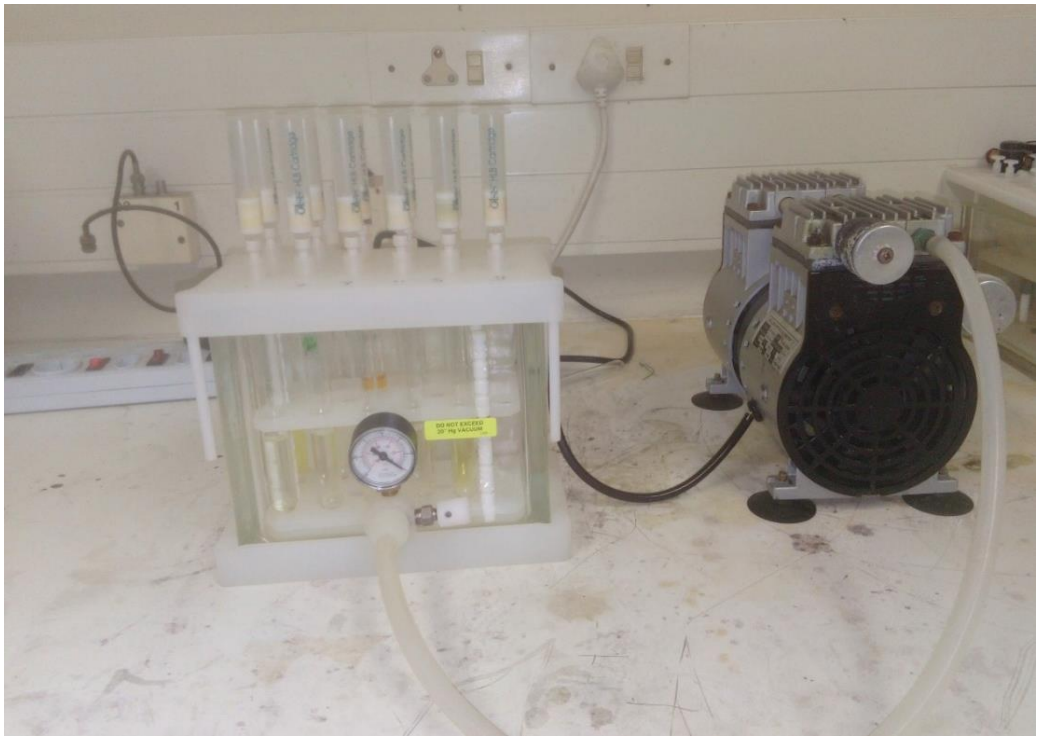

Figure 3-4: Manual solid-phase extraction setup used for the extraction of mycotoxins

Mycotoxins were extracted as described by Wang *et al.*, (2016), with slight modifications. Briefly, homogenized vegetable samples (10 g) were separately weighed into a centrifuge tube. Then 20 mL of 80% acetonitrile solution (v/v) was added, sonicated for 10 min, and centrifuged for 30 min at 3500 rpm. The supernatant liquid was extracted using water Oasis HLB 100 mg cartridges as outlined in **Figure 3-5**.

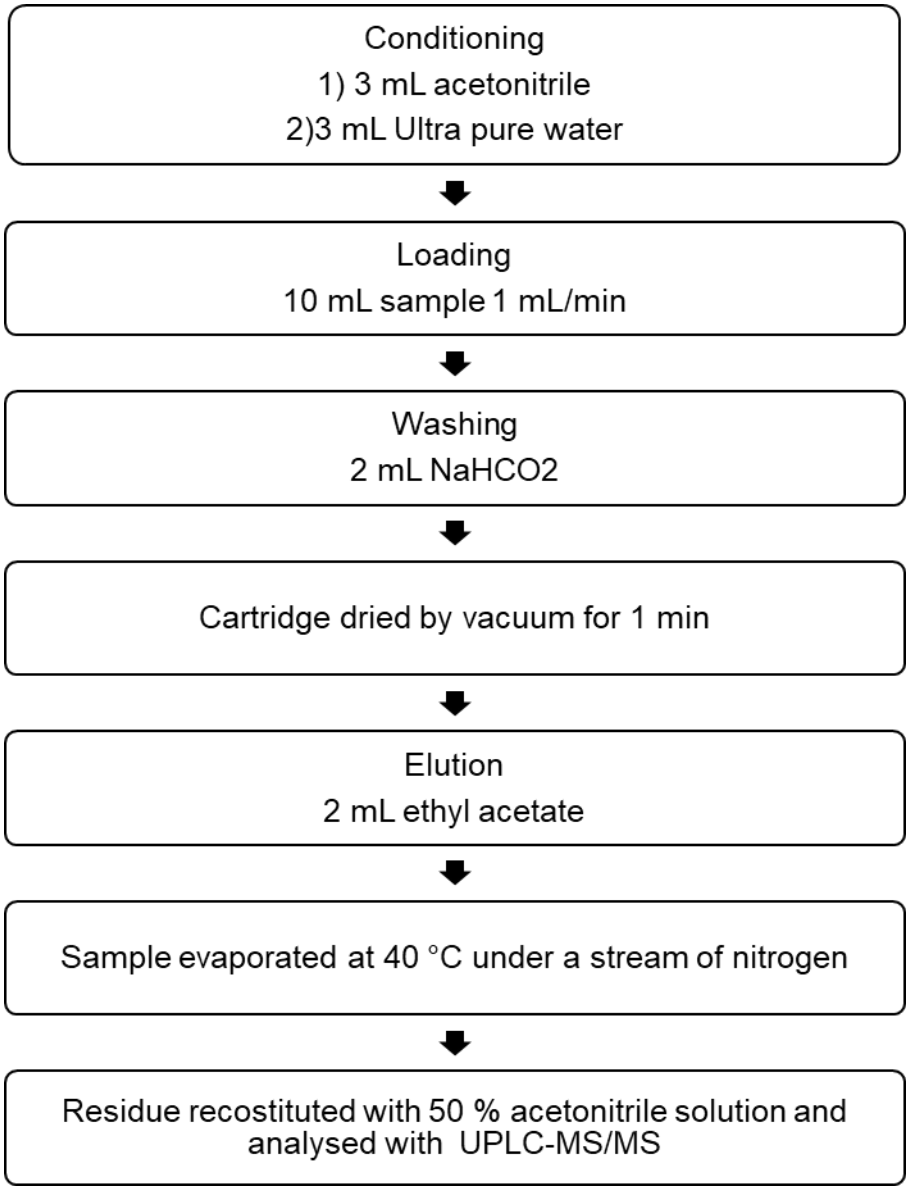

Figure 3-5: Outline of the solid phase extraction used for extraction of mycotoxins from vegetable samples

3.5 QuEChERS

QuECHERS (quick, easy, cheap, effective, rugged, and safe techniques is a solid phase extraction method used in the analysis of mycotoxin and pesticide residues in foods (Capriotti *et al*., 2012; Sospedra *et al*, 2010). This technique involves the sample extraction using acetonitrile, followed by liquid-liquid partitioning using salts such as magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) and followed by clean-up step using dispersive solid-phase extraction (dSPE) before analyzing the samples (see **Figure 3-6 a-b**).

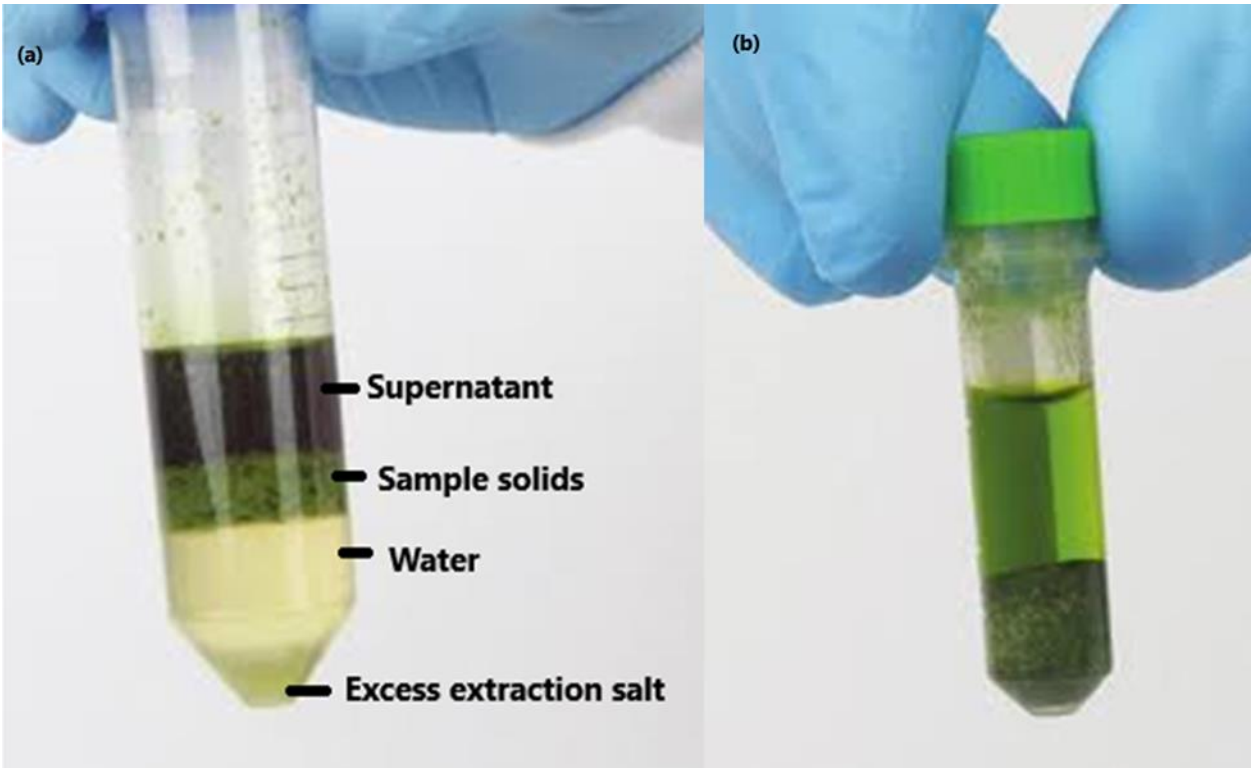

Figure 3-6: Two stages of the QuECHERS method (a) liquid-liquid partitioning step and b) the dispersive solid-phase extraction step (Restek, 2018)

The mycotoxins in this study were extracted from vegetables using the so-called QuEChERS method as described by (Anastassiades and Lehotay, 2003) with modifications. Homogenized vegetable samples (15 mg) were separately weighed into 50 mL centrifuge tubes. The 1% acetic acid solution in acetonitrile (v/v) (15 mL) was added to the samples and vortexed for 1 min. The entire content of the extraction salt packet (6 g anhydrous $MgSO_4$ and 1.5 g CH_3CO_2Na) was added to the mixture, vortexed again for 1 min, and centrifuged for 5 min at 4500 rpm.

Following centrifugation, 1 mL of the supernatant liquid was transferred in a 1.5 mL dispersive solid-phase extraction (dSPE) tube containing 25 mg PSA sorbent and 150 mg anhydrous $MgSO_4$, vortexed for 1 min, and centrifuged for 2 min. after centrifuging the supernatant liquid was transferred to an LC vial and analysed using UPLC-MS.

The extraction salts improve the extraction efficiency by allowing the separation of the organic solvent from water in the sample. Vigorously shaking and centrifuging of the samples also makes the separation of organic phase from aqueous solution and solids easier. In the case of fortification, the samples are spiked with an unknown concentration of mycotoxins before adding the salt.

The dSPE media is used for further clean-up and separation of the subsample from the supernatant liquid. The dSPE step reduces the sugars, fatty acids, pigments, lipids, and other potential interferences in the samples.

3.6 LC-MS analysis of patulin and other related mycotoxins

In liquid chromatography-mass spectrometry (LC-MS) different components in a mixture are separated and ionized based on their mass-to-charge ratio (m/z). In this study, the LC/MS method used for the analysis of mycotoxins optimised and validated, thereafter applied to real-life samples as discussed below.

3.6.1 UHPLC-MS/MS method optimization

Dionex Ultimate 3000 ultrahigh-performance liquid chromatography (UPLC) system equipped with a binary pump, an online degasser, column oven, and an autosampler coupled to Bruker Impact-II Quadrupole time-of-flight (QTOF) tandem mass spectrometer (MS/MS) with electrospray ionization (ESI) was used to analyze all extracts in this study. Analytes were separated on an Acquity UPLC® BEH C18 1.7 µm (2.1 × 100 mm) column at a flow rate of 0.3 $minL^{-1}$ and 30 ˚C. The mobile phases consisted of 0.1 % FA with water (A) and 0.1 % formic acid with acetonitrile (B). The injection volume was 5 µL for a total run time of 14 min.

For reliable identification and quantification the retention times, the accurate masses of extracted ion chromatograms, and the fragmentation patterns of each analyte in the samples were compared to those of the standards. The mass spectrometer was operated in the ESI interface in both positive and negative modes. The parameters were as follows; capillary voltage 4.5 kV, the drying gas was 8.0 L/min, drying gas temperature 220 ˚C, and nebulizer gas (N_2) 1.8 bar. The mass range of 50-1500 was fully scanned and the MS was calibrated using a sodium formate cluster. The infusion of the calibrant took place at the front of each sample run and it was used to recalibrate and calculate the accuracy of the mass.

3.6.2 Method validation

To evaluate the linearity of the method different concentrations range of 1-10 µg/mL (for patulin) and 1-10 ng/mL (for the aflatoxins) were injected into the UPLC system in triplicate, all the concentrations were injected under the same conditions. The calibration curve was constructed using the area of the mycotoxins standards as a function of concentration.

The limits of detection (LOD) and Limit of quantification (LOQ) were determined using the standard deviation of the linear regression and the slope of the calibration curve as shown in equations 1 and 2, respectively.

$$\mathrm{LOD} = \frac{3.3\mathrm{Sy}}{\mathrm{S}} \qquad 1$$

$$\mathrm{LOQ} = \frac{10\mathrm{sy}}{\mathrm{S}} \qquad 2$$

Where:

Sy: standard error of the y-intercept,

S: is the slope of the regression line.

A recovery experiment to evaluate the extraction efficiency was performed. Briefly, vegetable samples spiked with 5 µg/ mL of patulin and 5 ng/mL of aflatoxins B1, B2, G1, and G2 and detected using the optimised UHPLC-QTOF-MS/MS. The samples were spiked in triplicates. The concentration of each analyte was calculated using the calibration equation and the measured peak area of each analyte. The accuracy and precision were investigated by the recoveries of each analyte in the fortified samples and intra-day and inter-day Relative Standard Deviation (RSD) for six replicates within three consecutive days. The recovery was calculated as follows:

$$\text{Recovery}\ (\%) = \frac{\text{Measure Conc for spiked sample}}{\text{Theoretical spiked Conc}} \times 100 \qquad 3$$

3.7 Identification of microbial species

Fungal identification requires cultured pure fungal isolates. In this study, fungal isolates were identified using macroscopic, microscopic, and Biotyping techniques. These techniques are individually discussed in the following subsection.

3.7.1 Culturing and isolation

The Sabouraud dextrose agar (SDA) containing chloramphenicol was used for fungal growth and isolation. The SDA was prepared according to the manufacturer's instructions. The medium was weighed (63.5 g) and suspended in 1L ultrapure water in a glass bottle and autoclaved at 121 °C for 15 min. After sterilization, the media were aseptically dispensed in 20 mL sterile Petri dishes and were allowed to set on a flat surface. The Petri dishes were labeled and stored in the refrigerator for later use.

In this study, a direct plating method was used for fungal isolation. The isolation was carried as described in a method by Yahaya *et al.* (2016). Briefly, the vegetable samples were firstly surface disinfected with ultrapure water to remove contamination arising from soil and other sources. Subsequently, a small portion of

the rotten tissues was directly plated on the solidified agar incubated for 3 days at 28 °C. The plates were monitored for fungal growth. Subsequently, these fungal isolates were continually transferred to fresh agar plates to obtain pure isolates. The resulting pure fungal cultures were used for further analysis

The SDA contains peptones that provide a nutrition source of amino acids and nitrogenous compounds for fungal growth. Dextrose is used as a source of energy and carbon. Agar is the solidifying agent. The presence of chloramphenicol prevent bacterial growth.

3.7.2 Microscopic analysis

A scanning electron microscope (SEM) is a useful tool for studying fungi. The SEM is commonly used for studying the morphology of the specimen. Most SEM specimen are imaged in a high vacuum (ca. 10^{-8} Torr) after being chemically fixed, dehydrated, and coated with gold to avoid charge build-up from the electron beam.

In this study, SEM was used to study the morphological characteristics of fungal species isolated from spoiled vegetables. The fungal isolates were prepared as described by (Assress *et al.*, 2020). The fungal species isolated from 3-7-day-old culture were with a phosphate-buffered solution (PBS) three times. The specimen was fixed with 2% Glutaraldehyde for 2 hours and post-fixed with 150 µL of 1% osmium tetroxide for 2 hours. The specimen was then dehydrated with ethanol at 10-90% for 10 minutes and followed with absolute alcohol for 20 minutes. Dried specimen were mounted on aluminum stubs, coated with gold using Q1 50R sputter (Quorum technologies, UK) before observation and imaging on a tungsten scanning electron microscope. The SEM images were taken using the JOEL IT 300 scanning electron microscope (JOEL, UK).

3.7.3 Biotyping – sample preparation and MALDI analysis

The samples were prepared using direct transfer and formic acid extraction methods as outlined in.

Direct transfer (see **Figure 3-7a**) the colonies were picked using a toothpick and smeared onto a target plate. Then 1 µL the matrix (alpha-cyano-4-hydrocinnamic acid (HCCA) dissolved in a solution of ACN and TFA) is added on each spot and allowed to dry. However, the filamentous fungal isolates required the use of the formic acid extraction preparatory method to generate spectra of sufficient quality to enable microbial identification.

Formic acid extraction (see **Figure 3-7b**): The actively growing mycelium from a 3 days old culture was picked using an inoculating loop and placed in an Eppendorf microcentrifuge tube with 300 µL ultrapure water and mixed by pipetting. Subsequently, 900 µL 70 ethanol was added and mixed thoroughly by pipetting and centrifuged for 2 min at 15000 rpm. The supernatant liquid was decanted without disturbing the pellet and the ethanol was allowed to dry at room temperature. The pellet was then re-suspended with 100 µL solution containing an equal volume of 70 % formic acid and acetonitrile. The resulting suspension was then centrifuged at 15000 rpm for 2 min. Thereafter, 1 µL was pipetted onto a MALDI 96-well target plate, dried at room temperature, and overlaid with 1 µL of HCCA matrix.

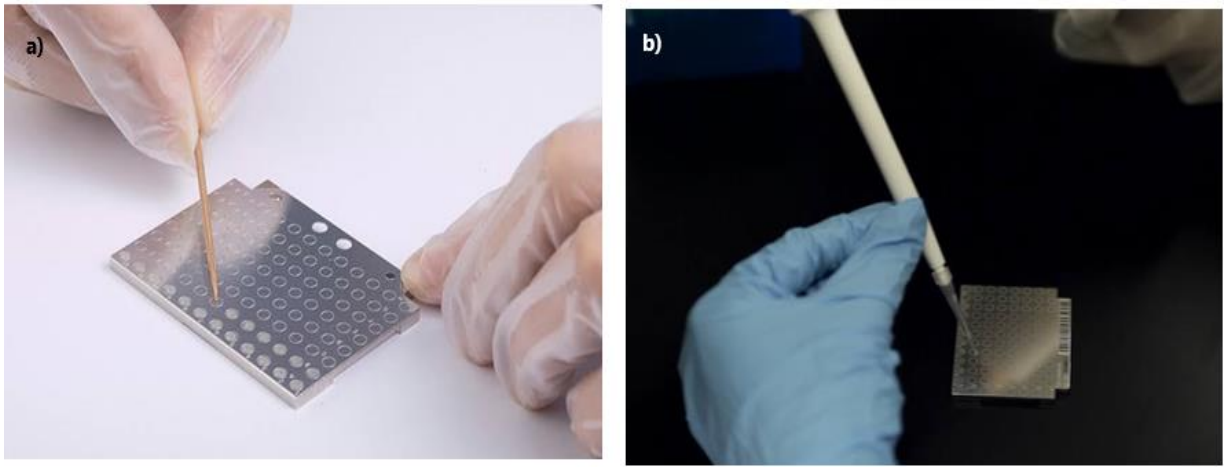

Figure 3-7: a) direct transfer (Chirus, 2020) and b) formic acid extraction (Zhang *et al.*, 2015) preparatory method used for MALDI biotyping analysis

MALDI-TOF/TOF MS (Bruker, Germany) equipped with Flexcontrol system v3.4 software (Bruker, Germany) and Biotyper Compass v4.1.14 software (Bruker, Germany) was used for the biotyping. A filamentous fungi library containing 127

fungi was used for spectral comparison. Spectral information was recorded in the mass range of 2000 – 15000 Da with a laser intensity of 60-80 %. The data were processed using Flexanalysis software v3.4 (Bruker, Bermen, Germany). The matrix co-crystalizes the sample.

Matrix association laser desorption ionization (MALDI) is a two-phase process (see **Figure 3-8**): ionization and time of flight (TOF) phase, which are discussed briefly below.

Ionization phase: A laser beam is bombarded on a crystalline matrix fixed sample. The matrix absorbs the energy from the laser releasing it into the sample as heat. Subsequently, molecules of the sample vaporizes into a vacuum chamber and simultaneously ionized without fragmenting or decomposing. High voltage is then applied to accelerate the charged particles.

TOF phase: following ionization the ions are separated by their mass-to-charge ratio (m/z) and the m/z is determined by the duration to reach the detector. In the flight tube, light-weight ions travel faster and are detected early than heavy ions. A mass spectrum is generated by plotting the m/z against the signal intensity. Only proteins with high abundance are detected. The mass spectral fingerprint is compared with large library spectra. Accurate identification of various microorganisms at the genus and species level is possible because each microorganism has unique fingerprints.

In this study, the MALDI biotyping was used to identify fungal isolates. The MALDI-TOF/TOF mass spectrometry biotyping was performed on a MALDI Ultra-flex II mass spectrometer (Daltonik, Bremen, Germany).

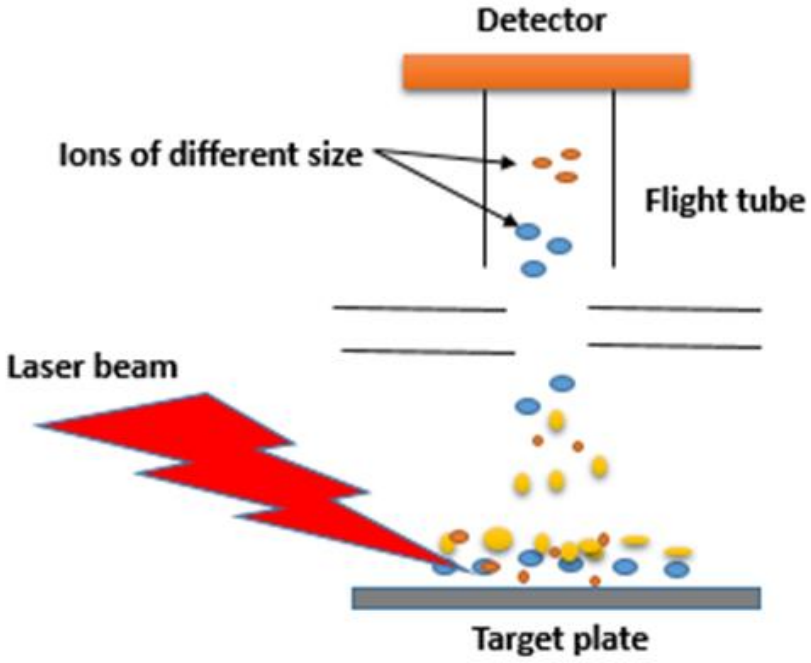

Figure 3-8: Schematic diagram of the principle of MALDI-TOF/MS

3.8 Cytotoxicity evaluation

The health of the cells is indicated by the level of viable cells. Patulin has adverse toxic effects on cells and these effects include cell membrane disruption, protein synthesis inhibition, polydeoxynucleotide elongation inhibition, and enzymatic reaction *(*Assunção *et al., 2016;* Boussabbeh *et al*., 2015; Wu *et al*., 2005). Therefore, there is a need for using cost-effective, reliable, and reproducible cytotoxicity and cell viability assays.

An ethical clearance (2019/SSR-ERC/002) was granted by the Ethics Review Committee of the University of South Africa in January 2019 before the cytotoxicity assay since human cancer cell lines were to be used.

3.8.1 Cell viability assay

Human breast carcinoma cells (MCF-7) were cultured using Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin. The cells were seeded after trypsinization in a 96-well plate at a final concentration of 5.0 x 10^3 cells per well and incubated at 37° C for

approximately 24 hours to allow cell growth at an exponential phase before drug treatment. After 24 hours, patulin (concentration range 0.05-5 µm) was added in triplicates for each concentration. The plate was then incubated for 24 hours post-treatment. Following incubation, resazurin dye was added to the wells using a multi-pipette (10ul reagent per 50ul media per well), therefore Incubated for 4 hours to allow the complete and homogenous reduction of resazurin to resorufin. The fluorescence at 560/590 nm was measured using an ELISA plate reader. Lastly, the viable cells were quantified by subtraction of the background signal of untreated control cells.

3.9 References

Anastassiades, M. and Lehotay, S. (2003) *Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and 'Dispersive Solid-Phase Extraction' for the Determination of Pesticide Residues in Produce*, *JOURNAL OF AOAC INTERNATIONAL*.

Assress, H. A. *et al.* (2020) 'Azole antifungal resistance in fungal isolates from wastewater treatment plant effluents'. Environmental Science and Pollution Research.

Assunção, R. *et al.* (2016) 'Characterization of in vitro effects of patulin on intestinal epithelial and immune cells', *Toxicology Letters*.

Boussabbeh, M. *et al.* (2015) 'Patulin induces apoptosis through ROS-mediated endoplasmic reticulum stress pathway', *Toxicological Sciences*, 144(2), pp. 328–337.

Capriotti, A. L. *et al.* (2012) 'Multiclass screening method based on solvent extraction and liquid chromatography-tandem mass spectrometry for the determination of antimicrobials and mycotoxins in egg', *Journal of Chromatography A*. Elsevier B.V., 1268, pp. 84–90.

Chirus (2020) *Autobio Autof ms1000 MALDI-TOF/ Diagnostic system/ Chirus*. Available at: https://chirus.com/autobio-autof-ms1000-maldi-tof/ (Accessed: 1 December 2020).

fyssouthafrica (2012) *Climate / South Africa climate change*. Available at: https://fyssouthafrica.wordpress.com/climate/ (Accessed: 1 December 2020).

Harvard University (2009) *Laboratory safety guideline*.

Restek (2018) *QuEChERS-Sample-Preparation-Procedures.pdf*. Available at: https://blog.restek.com/wp-content/uploads/2020/05/QuEChERS-Sample-Preparation-Procedures.pdf (Accessed: 1 December 2020).

Sospedra, I. *et al.* (2010) 'Use of the modified quick easy cheap effective rugged and safe sample preparation approach for the simultaneous analysis of type A- and B-trichothecenes in wheat flour', *Journal of Chromatography A*.

Wang, M. *et al.* (2016) 'A single-step solid-phase extraction for the simultaneous determination of 8 mycotoxins in fruits by ultra-high performance liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*. Elsevier B.V., 1429, pp. 22–29.

Wu, T. S. *et al.* (2005) 'Activation of ERK mitogen-activated protein kinase in human cells by the mycotoxin patulin', *Toxicology and Applied Pharmacology*, 207(2), pp. 103–111.

Yahaya, S. M. *et al.* (2016) 'Isolation and Identification of Pathogenic Fungi Causing Deterioration of Lettuce Plant (Lactuca sativa) A Case Study of Yankaba and Sharada Vegetables Markets Research article', *Journal of Plant Science and Research*, 3(1), pp. 35–38.

Zhang, L. *et al.* (2015) 'Use of MALDI-TOF Mass Spectrometry and a Custom Database to Characterize Bacteria Indigenous to a Unique Cave Environment (Kartchner Caverns, AZ, USA)', *Journal of Visual experiment*. MyJoVE Corp, (95), p. 52064..

4 Chapter 4: Validation of UHPLC-QTOF-MS method for the detection and quantification of mycotoxins in

spoiled vegetables from five different climatic regions in South Africa.

4.1 Introduction

Continuous food safety problems caused by fungi are a common problem globally. The major fungi that are the source of recurring mycotoxin contamination in food commodities belong to the genus *Aspergillus, Fusarium,* and *Penicillium* (Frisvad *et al.*, 2011; Zouaoui *et al.*, 2015; Beccari *et al.*, 2016). More than 300 mycotoxins are known, and the six mycotoxins that commonly occur in food include aflatoxins, trichothecenes, zearalenone, fumonisins, ochratoxins, and patulin (Bennett and Klich, 2003; Iqbal *et al.*, 2018).

Mycotoxigenic fungi can invade a wide-ranging agricultural commodities and they produce one or more groups of mycotoxins that have additional or even synergistic toxic effects that negatively affect the economy (Pamel *et al.*, 2011). For this reason, the simultaneous analysis of co-occurring mycotoxin in foods and feeds is important for purposes of saving time and cost. In addition, such an analysis is important from the health, economic, and legislative perspectives. However, accurate, reliable, and sensitive analytical methods are required for the enforcement of mycotoxin regulations. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the most frequently used method for the simultaneous analysis of mycotoxins because of its high sensitivity, accurate quantification, and an absence of need for sample derivatization (Kokkonen *et al*., 2005; Capriotti *et al.*, 2012; Kim *et al.*, 2017)

Vegetables form part of our daily diet, however, very little research on the contaminations of vegetables by mycotoxins has been conducted in South Africa. Therefore this study aimed at optimizing and validating the UHPLC-ESI-QTOF-MS/MS method for simultaneous quantitative analysis of patulin, Aflatoxins B1, B2, G1, and G2 in vegetables collected from five provinces in South Africa. The performance of the method was assessed using linearity LOD, LOQ, accuracy, and precision. The applicability of the method was also evaluated.

4.2 Experimental

This section provides a systematic summary of the experimental work undertaken to achieve the aim of this study.

4.2.1 Sample collection

The vegetable samples were collected from vegetable farms, markets, and wholesalers from five South African provinces namely WC, EC, GP, LP, and KZN. These provinces are characterized by varying climatic conditions. A detailed map of the sampling areas is provided in Section 3.2. Vegetable samples were collected separately in Ziploc bags, placed in a cooler box, and thereafter transported to the Institute for Nanotechnology Water Sustainability (iNanoWS) laboratories, University of South Africa (UNISA), South Africa. On arrival and prior to analysis, the vegetable samples were homogenized and stored at -22 °C. The blender was sterilised after the blending of each sample.

4.2.2 Materials and reagents

All the materials and reagents used in this study are listed in Chapter 3 section 3.1.

4.2.3 Preparations of standards

A stock solution of HMF was prepared by weighing 1 mg of the pure HMF solid dissolved in 1 mL acetonitrile and stored at 20 °C. The stock solutions of patulin and aflatoxins B1, B2, G1, and G2 were prepared in acetonitrile and stored at -18 °C. The stock solutions of each analyte were diluted to the appropriate concentration using a solution of acetonitrile and water (50/50, v/v).

4.2.4 Sample preparation

The extraction of mycotoxins from vegetable samples and processed products was carried out using the QuEChERS and SPE as discussed in Section 3.4.

4.2.5 UHPLC-MS/MS method optimization

To improve the chromatographic separation and increase the MS response, two mobile phase solvents (i.e., acetonitrile and methanol) that contain 0.1 FA were evaluated. An ESI mode that yields the highest sensitivity was selected after evaluating both the positive and negative ESI modes. The precursor ions and the fragments of the analytes were also determined.

UHPLC (Thermo scientific, Massachusetts, United States) equipped with a binary pump, an online degasser, column oven, and an autosampler coupled to Impact II Quadrupole Time of Flight (QTOF) tandem mass spectrometer (Bruker, Germany) with electrospray ionization (ESI) was used for the detection of the 5 analytes. Separation of the analytes was carried out on an Acquity UHPLC® BEH C_{18} (1.7 µm 2.1 × 100 mm) column; (MICROSEP, Johannesburg, South Africa). The flow rate was 0.3 mL/min with an injection volume of 5 µL.

4.2.6 Method validation

The optimised method for the simultaneous determination of the targeted mycotoxins was validated and the method performance parameters such as linearity, LOD, LOQ, accuracy, and precision were evaluated as described in Section 3.5.2. These parameters were evaluated following the guidelines described in European Commission Decision 2002/657/EC.

4.2.7 Method application

The validated UHPLC-ESI-QTOF-MS/MS method was used for the simultaneous analysis of patulin and aflatoxins B1, B2, G1, and G2. The quantification of

mycotoxins was achieved using the linear equation of the calibration curves (y=mx + c) and the measured peak area at retention times of the mycotoxins.

4.3 Results and discussion

Ultra-high-pure-performance liquid chromatography-tandem mass spectroscopy (UHPLC-MS/MS) has been recently used for the detection and identification of mycotoxins (Tanaka *et al.*, 2006; Ren *et al.*, 2007). This analytical technique has been confirmed to be the most effective technique for the detection of various mycotoxins and it has been widely used for the analysis of mycotoxins (Arroyo-Manzanares *et al.*, 2013; Marsol-Vall *et al.*, 2014; Vaclavikova *et al.*, 2015). Due to the diversity of the analytes, the development, and optimization of a simple and rapid UHPLC-MS/MS method for the simultaneous determination of mycotoxins in various matrices poses a significant number of analytical challenges. In this study, a sensitive and accurate LC-MS/MS method was optimised and validated for the simultaneous detection of patulin, and Aflatoxins B1, B2, G1, and G2 in vegetables collected from five provinces in South Africa.

4.3.1 Optimization of MS/MS parameters

To achieve high sensitivity in this research study, MS/MS detection was optimized for each analyte. The molecular ion of each mycotoxin was observed using MS/MS full scan over a range of 50-1600 m/z. Both positive and negative polarity modes were used, however for patulin, a high signal response was obtained under the positive ionization mode, it is, noteworthy that, no signal was obtained for aflatoxins under the negative mode. Previous studies have shown that patulin can ionize into protonated and deprotonated molecular ions under both positive and negative ionization modes respectively (Sulyok *et al*., 2007; Seo *et al*., 2015). However, very few studies have optimised the positive mode for the detection of patulin. All the aflatoxins including patulin were ionized in the form $[M+H]^+$. The obtained results are consistent with previous studies that demonstrated the ability of aflatoxins to form protonated molecular ions *(*Sulyok *et al*., 2007; Capriotti *et al.*, 2012; Arroyo-Manzanares *et al*., 2013a).

The MS/MS spectrum (see **Figure 4-1**) shows a protonated molecular ion of patulin at m/z 155.0339. The fragments at m/z 81.0334 and 109.0287 correspond to the ions $[C_5H_4O]+H$ and $[C_6H_4O_2]+H$ respectively which were assigned to the loss of carbon monoxide (CO) and carbon dioxide (CO_2) or acetaldehyde (CH_3CHO) respectively. A similar patulin fragmentation pattern was reported (Rupp and Turnipseed, 2000; Malysheva *et al.*, 2012).

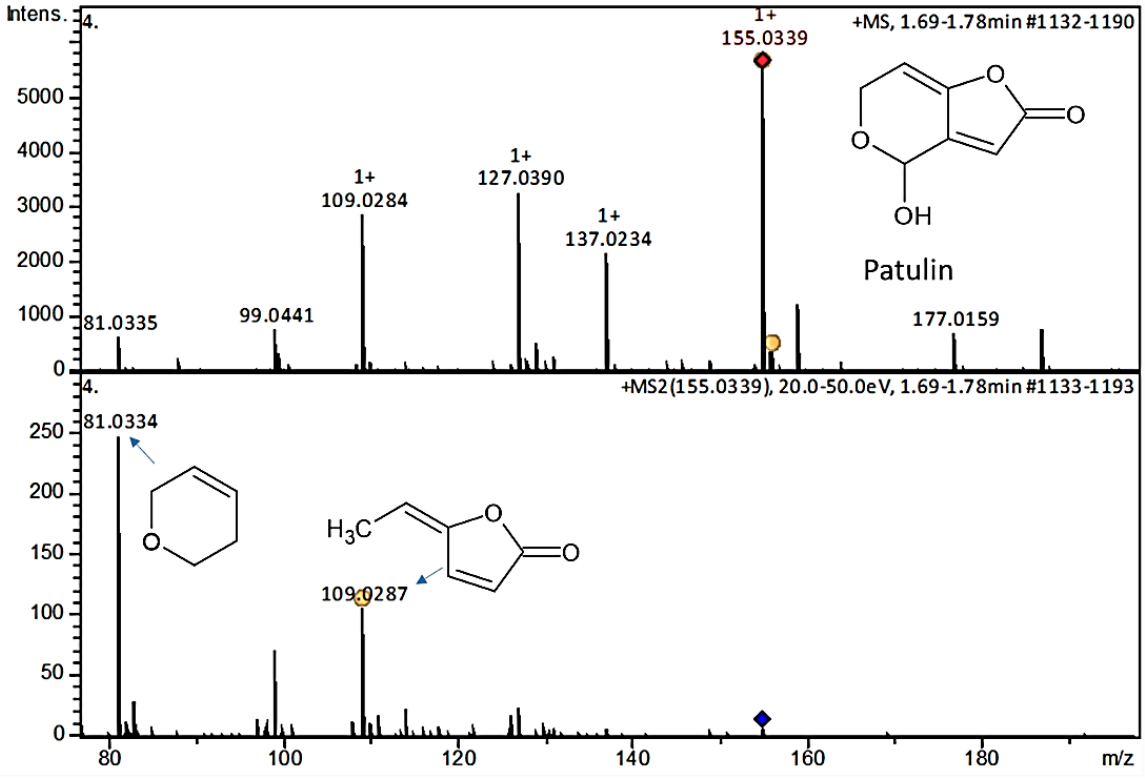

Figure 4-1: MS and MS2 spectra showing the protonated molecule patulin and fragments under ESI positive mode

Figure 4-2 shows the protonated molecular ions of Aflatoxins B1 (m/z 313.0704), B2 (m/z 315.0866), G1 (m/z 329.0659), and G2 (m/z 331.0817). The MS/MS spectra show a distinct fragment of each analyte at m/z 241.0495, 243.0662, 311.0555, and 189.0546 corresponding to $[C_{14}H_9O_4]^+$, $[C_{14}H_9O_4+H]+H$, $[C_{17}H_{12}O_6-H]+H$ and $[C_{11}H_{10}O_3-H]+H$ respectively. As reported by Cervino *et al.* (2008) the fragment ions were assigned to the loss of CO, CO_2, H_2O, and other small molecule ions.

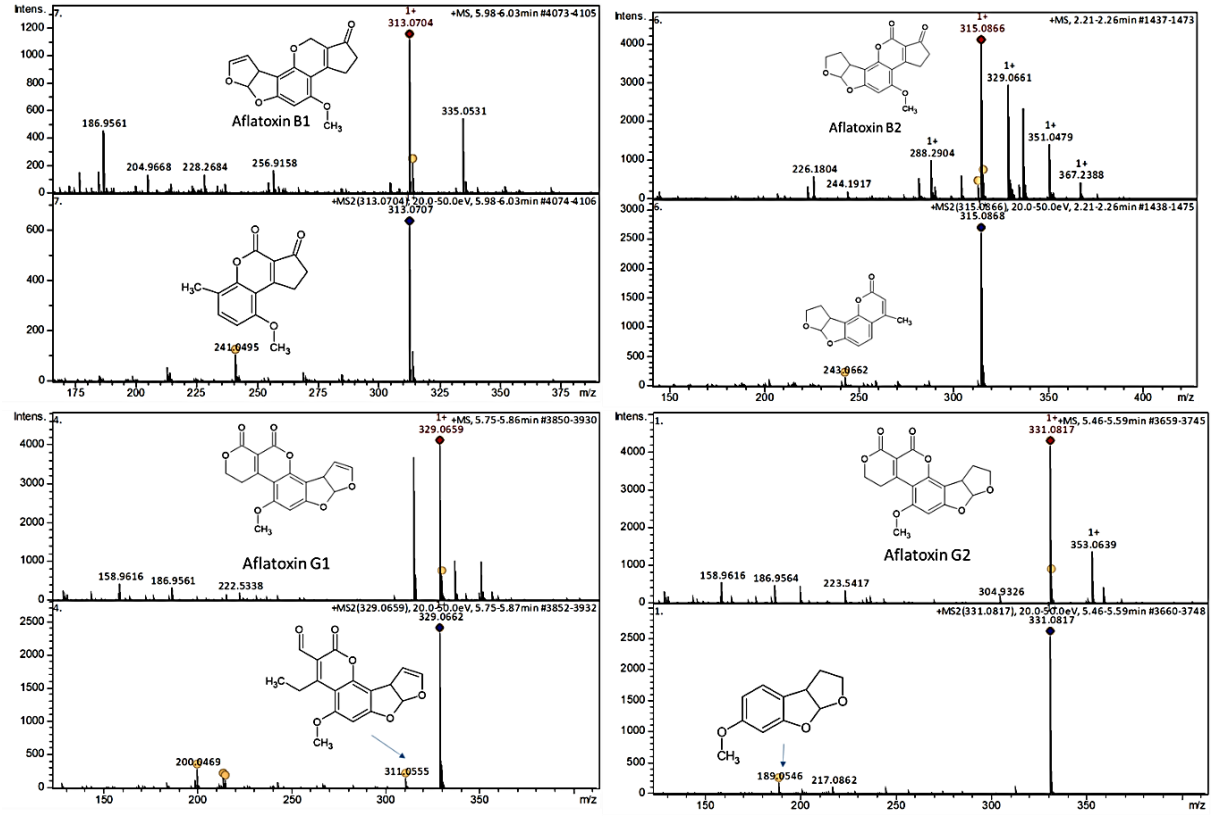

Figure 4-2: MS and MS2 spectra showing the protonated molecules of Aflatoxins B1, B2, G1, and G2 and fragments under ESI positive mode

4.3.2 Chromatographic separation

The UHPLC separation of the analytes was achieved using the Acquity UHPLC® BEH C_{18} (1.7 µm 2.1 × 100 mm) column. The decision to use this type of column was based on previous work (Ren *et al.*, 2007; Rosinská *et al.*, 2009) which showed that reverse-phase liquid chromatography yields superior peak shapes enhanced sensitivity and increased precision times

The UHPLC analysis of the patulin, and Aflatoxins B1, B2, G1, and G2 showed that the analytes were eluted in the order: patulin, Aflatoxins G2, B2, G1, and B1 (see **Figure 4-3**). For a reverse-phase liquid chromatography, the elution order of analytes depends on factors such as polarity, solubility, and molecular weight of the analyte. However, polarity affects the retention of analytes in the column more than any other factors to an extent that the more polar analyte would elute first. The elution order of the 5 target analytes suggests that patulin was the most polar analyte and Aflatoxin B1 is the least polar of all the analytes.

In this study, the composition and location of functional groups play an important role when assigning the polarity of each of the analysed mycotoxins. Patulin contains three oxygen sites at well as one hydroxyl (OH) group (see **Figure 1-1**). Of all the elements in the periodic table, oxygen is the most electronegative, with an electronegativity value of 3.5. Therefore, oxygen strongly attracts electrons towards itself thus creating a partial negative charge on the lone pairs; this results in an increased overall polarity of the molecule. Owing to the presence of a highly electronegative oxygen atom, the presence of the OH functional group is an additional contributor to the polarity of the patulin molecule.

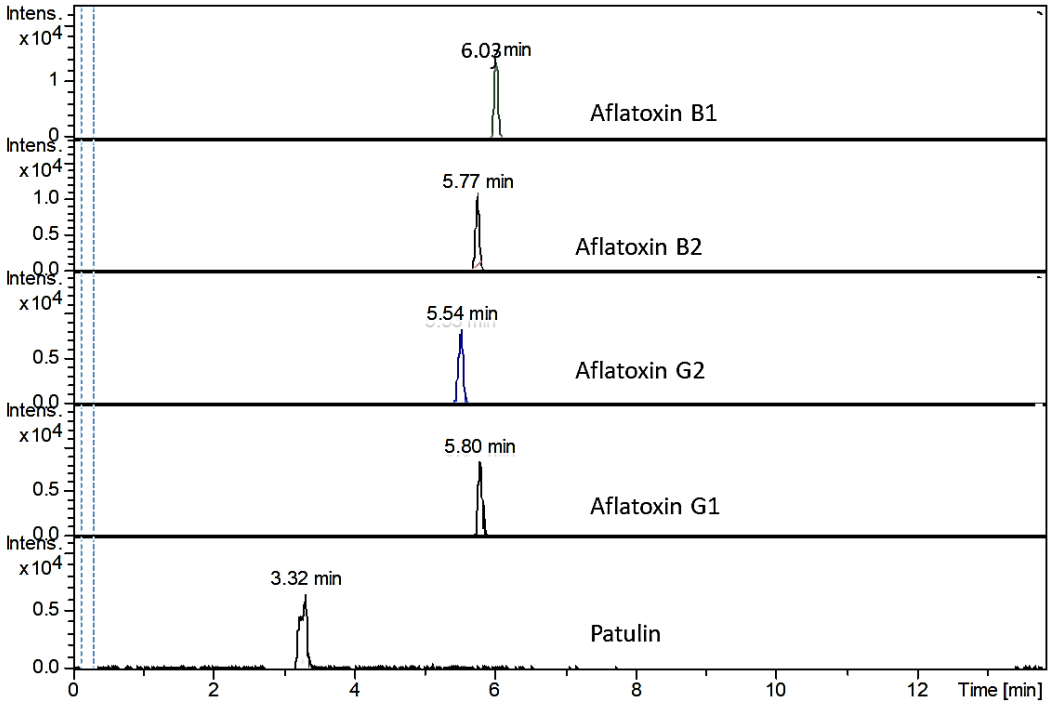

Figure 4-3: Extracted ion chromatogram of a mixed standard of patulin, Aflatoxins B1, B2, G1, and G2 separated using Acquity UHPLC® BEH C_{18} (1.7 µm 2.1 × 100 mm) column.

Patulin and HMF show similar chromatographic properties. Therefore, HMF is an interfering compound during the liquid chromatographic analysis of patulin. As shown in **Figure 4-4** no co-elution between patulin and HMF could be detected because patulin and HMF were eluted separately at 3.32 and 3.11 min respectively. This has demonstrated that the presence of HMF did not interfere with the detection of patulin.

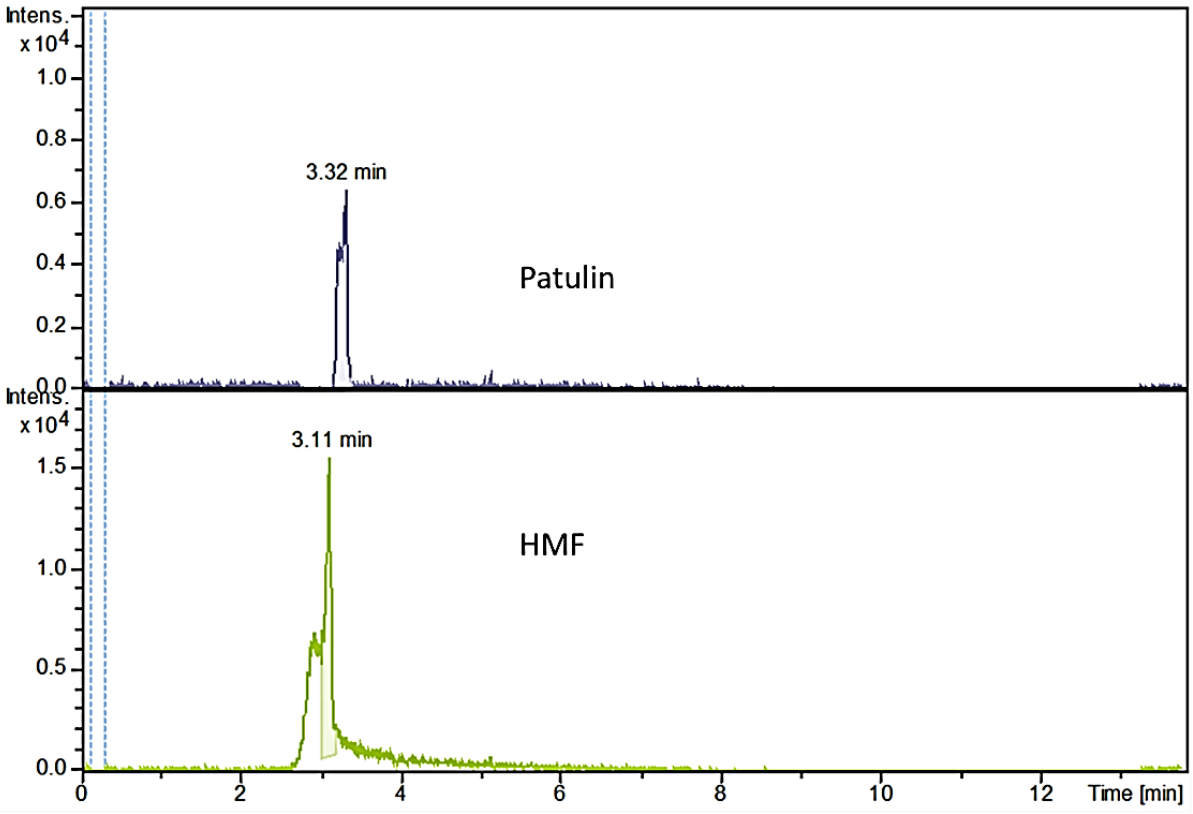

Figure 4-4: Extracted ion chromatogram of a mixed standard of patulin and HMF separated using Acquity UHPLC® BEH C_{18} (1.7 µm 2.1 × 100 mm) column

The careful selection of the mobile phase is important to improve the chromatographic separation of the analytes and improve the ionisation efficiency of the analyte. Various mobile phases such as water-acetonitrile and water-methanol with formic acid (Capriotti *et al*., 2012; Sospedra *et al*, 2010), ammonium acetate (Berthiller *et al*., 2005; Paíga et al., 2012), and ammonium formate (Arroyo *et al*., 2013) with gradient elution on the HPLC-MS/MS determination of mycotoxins have been reported. In this study, two mobile phase solvents, acetonitrile and methanol containing 0.1% formic acid were investigated. The results demonstrated that the use of methanol-formic acid mobile phase gave a poor signal response (peak area), and the highest signal response was obtained when acetonitrile-formic acid mobile phase was used (see **Figure 4-5**). As reported by Nakhjavan (2020) the use of acetonitrile led to an improved signal response.

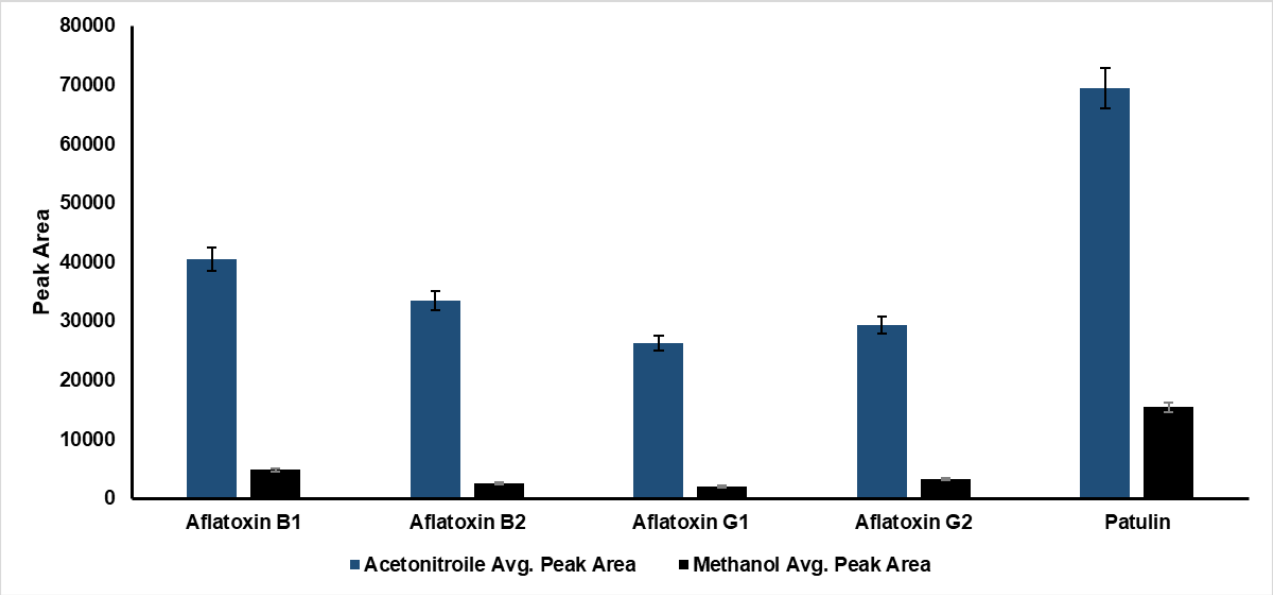

Figure 4-5: The effect of mobile phase solvent acetonitrile and methanol on the LC peak area ofeach analyte.

4.3.3 Sample preparation

The sample preparation step in the analysis of multianalyte is the most important step, most especially when food commodities with complex matrices are analysed. However, various extraction and clean-up methods were successfully used for mycotoxins extraction from various food commodities (Sospedra *et al.*, 2010; Antonia *et al.*, 2011; Zhao *et al.*, 2011; Jesus *et al.*, 2014; Katharina *et al.*, 2017).

The analysis of mycotoxins in vegetables is challenging because vegetables are complex matrix that requires an extraction method /or clean-up step to remove matrix interferences with a view to yielding acceptable recoveries of all the target analytes. In this study, an SPE using an HLB cartridge and a QuEChERS clean-up step were evaluated based on the recovery of patulin and Aflatoxins B1, B2, G1, and G2. **Figure 4-6 shows** the percentage recoveries of the 5 analytes (i.e., patulin and Aflatoxins B1, B2, G1, and G2) obtained using the SPE HLB cartridge and the QuEChERS method. The QuEChERS method yielded high recoveries for all the analytes ranged between 63.01 – 105.06%. These results corroborate the results of the studies by Sospedra *et al.* (2010), Malachová *et al.* (2018), and Bessaire *et al.* (2019), which reported high recoveries for multianalytes when the

QuEChERS method was used. Furthermore, the results demonstrated that SPE using HLB cartridge yielded poor recoveries ranging between 12.3 - 50.8 % for all analytes. The poor recoveries are attributed to the loss of the analyte during the purification process. In contrast, previous studies have reported high recoveries for the simultaneous extraction of analytes when using HLB cartridges (Rigi *et al.*, 2015; Seo, Kim and Baek, 2015). Owing to its highest recovery rate of all analytes when compared with the SPE method using HLB cartridge, the QuEChERS method was selected as a suitable clean-up technique for the simultaneous extraction of patulin and Aflatoxins B1, B2, G1, and G2.

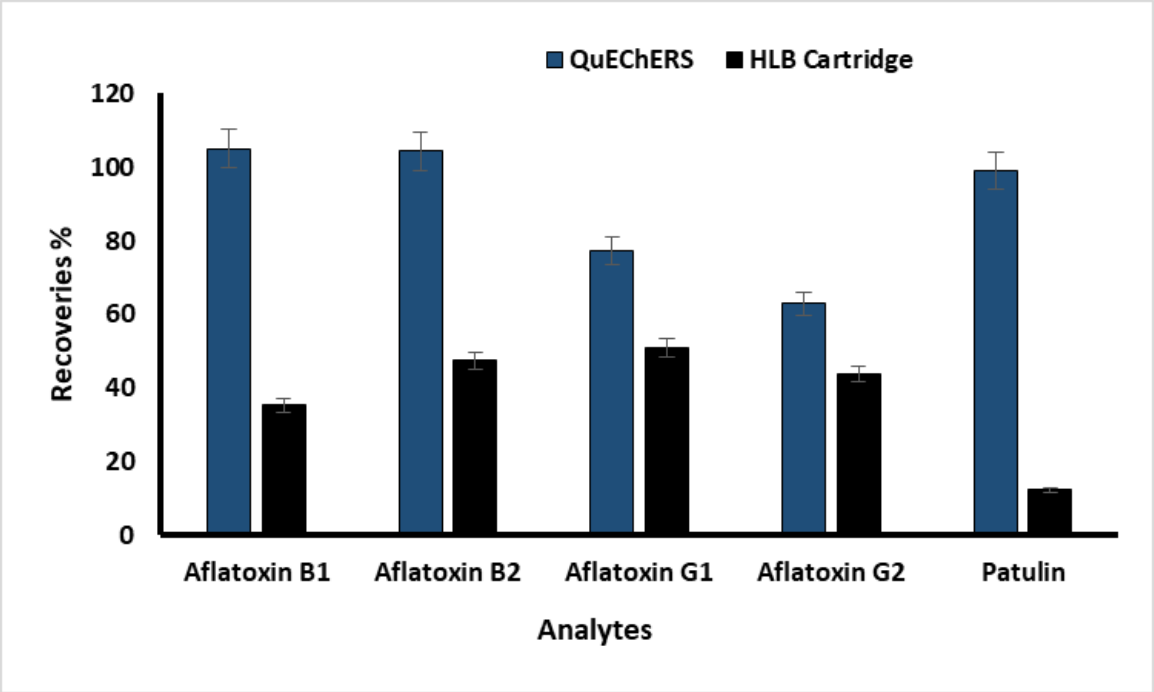

Figure 4-6: Mycotoxins recoveries (%) from sweet potato spiked with mycotoxin standards, extracted using QuECHERS and SPE using HLB cartridge

4.3.4 Validation studies

The method performance characteristics (i.e., linearity, LOD, and LOQ) were evaluated by analysing the individual standard solution of the mycotoxins and the results are summarized in **Table 4-1.**

The linearity of an analytical method is referred to as its ability to show results that are correlated to the concentration of the analyte in a sample within a specific range. To obtain the calibration curve, the mean peak area obtained from the UHPLC was plotted against the corresponding concentration of each analyte. The calibration curves of ten points in the range 1-10 µg/mL (for patulin) and 1-10 ng/mL (for the aflatoxins) were employed investigate the linearity of the method using linear regression analysis. The correlation coefficient ranging between 0.90-0.99 was obtained for all the 5 mycotoxins (i.e., patulin and Aflatoxin B1, B2, G1, and G2). Based on these results, it was concluded that these analytes exhibited good linearity within the concentration ranges.

The LODs and LOQs were found to be 2.28 and 6.92 µg/mL for patulin and ranged between 0.551-1.04 and 1.67-3.155 ng/mL for aflatoxins respectively, which were acceptable because they were within the acceptable maximum level of mycotoxins. The LOD and LOQ for the patulin were significantly lower than those reported by Catană *et al.* (2011) (2.91 and 9.79 µg/L), but slightly higher than those reported by (Desmarchelier *et al.*, 2011). However, the LOD and LOD figures obtained for aflatoxins are slightly higher than those reported in previous studies (Capriotti *et al.*, 2012; Kim *et al.*, 2017; Nakhjavan *et al*., 2020).

Table 4-1: Method performance characteristics: Linearity, LOD, and LOQ for the validation of UHPLC-MS/MS method for the simultaneous determination patulin, aflatoxin B1, B2, G1, and G2.

Analyte	Calibration curve			R^2	LOD	LOQ
	Calibration Range	Slope	intercept			
Aflatoxin B1	1-10 ng/mL	-1474	4×10^6	0.9797	1.04	3.15
Aflatoxin B2	1-10 ng/mL	1650.6	2×10^6	0.9915	0.669	2.02
Aflatoxin G1	1-10 ng/mL	1090	2×10^6	0.9916	0.872	2.64
Aflatoxin G2	1-10 ng/mL	1138.6	3×10^6	0.9942	0.551	1.67
Patulin	1-10 µg/mL	1304.3	502.5	0.9095	0.00228	0.00692

The method's accuracy and precision were assessed using recoveries of each analyte in the fortified sample. Each of the spiked sample was analysed in six

replicates (n=6) within a day and for three consecutive days. The mean recoveries for all the analytes ranged between 77.3-105.06% with an intra-day RSD and inter-day RSD less than 7.11% 13.52 respectively being achieved (see **Table 4-2**). The recovery rates of the mycotoxins in this study are higher than those reported by Arroyo-Manzanares *et al*. (2013b) (62.3-98.9%), however slightly lower than those reported by Sulyok *et al*. (2007), (75– 108%). In addition, the obtained recoveries and RSDs are within satisfactory levels recommended by the European Commission regulation (EC 401/2006) and are in line with SANCO/12571/2013 guidelines for the recovery of analytes, which range between 70-110%.

Table 4-2: Accuracy and precision of the QuEChERS method used for the extraction of patulin and Aflatoxins B1, B2, G1, and G2.

Analyte	Mean recovery (%)	Intra-day RSD (%)	Inter-day RSD (%)
Aflatoxin B1	105.06	6.109	6.67
Aflatoxin B2	104.03	5.17	4.46
Aflatoxin G1	77.3	4.45	13.20
Aflatoxin G2	63.01	9.63	7.15
Patulin	99.14	7.11	13.52

4.3.5 Method application

To demonstrate the applicability of the optimised and validated UHPLC-QTOF-MS/MS method, mouldy vegetable samples (carrots, cucumber, peppers, potatoes, and sweet potatoes, and vegetable products) were analysed for the presence of patulin and Aflatoxins B1, B2, G1, and G2. The contamination of food commodities by patulin and aflatoxins was assessed extensively, and patulin is frequently detected in apple and apple-based products (Harris *et al*, 2009; Desmarchelier *et al.*, 2011; Piqué *et al.*, 2013; Iturat *et al.*, 2014) while aflatoxins were commonly found in maize (Papp *et al.*, 2002; Arroyo-Manzanares *et al*, 2013; Raiola A, 2015; Kim *et al.*, 2017). This study is aimed at determining the contamination levels of these mycotoxins in vegetables and vegetable processed products. To the best of our knowledge, very few studies on the determination of

patulin and aflatoxins in vegetables and vegetable-processed products have been undertaken.

According to the results presented in **Table 4-3** patulin was detected in peppers and carrots, however, no patulin contamination was established in onions, cucumbers, potatoes, and sweet potatoes. However, the presence of patulin in moulded peppers (16.1%) has been reported (Van De Perre *et al.*, 2014)

However, in this study, the levels of patulin in both carrots and peppers were lower than the LOQ. The LOQ was considerably lower than the allowable level of patulin in apple juice 50µg/kg. Furthermore, none of the analysed vegetable samples were found to be contaminated with aflatoxins. Similar results were obtained when cucumbers, onions potatoes, and carrots were analysed for aflatoxins (Sahar *et al.*, 2009).

The vegetable-processed products were also analysed for mycotoxin contamination. Although the carrots and peppers were tested positive for patulin contamination, none of the corresponding processed-products were found to be contaminated with mycotoxins. The absence of any mycotoxins in the processed products suggests that the vegetables used for preparing these processed products were in good condition. The quality of raw materials used for the preparation of processed products can be determined during pre-harvest, harvest, and post-harvest stages. However, poor agricultural and manufacturing practices can also contribute to the increase in mycotoxin in processed products (Welke *et al.*, 2009). Accordingly, damaged raw materials should be removed before the manufacturing stage of the processed food as they may be susceptible to fungal proliferation and mycotoxin contamination. However, Laidou (2001), has pointed out that the severance of the damaged part or rots from fruits and vegetables cannot protect consumers from consuming mycotoxins because up to 25 % of mycotoxins can be diffused into the sound tissues around the damaged portion.

Table 4-3: Mycotoxin analysis of vegetable samples and derived products using ultra-high-performance liquid chromatography

Samples	Mycotoxins				
	Patulin	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2
Carrots	Detected (<LOQ)	ND	ND	ND	ND
Carrot salad	ND	ND	ND	ND	ND
Cucumbers	ND	ND	ND	ND	ND
Pickled cucumbers	ND	ND	ND	ND	ND
Peppers	Detected (<LOQ)	ND	ND	ND	ND
Pepper sauce	ND	ND	ND	ND	ND
Sweet potato	ND	ND	ND	ND	ND
Sweet potato-based baby puree	ND	ND	ND	ND	ND
Potatoes	ND	ND	ND	ND	ND
Potato-based baby puree	ND	ND	ND	ND	ND
Onions	ND	ND	ND	ND	ND

ND: Not detected

4.3.6 The occurrence of mycotoxins in five South African provinces

Research interest in mycotoxin contamination has increased drastically globally over the last few decades. Mycotoxin contamination studies have been conducted in various countries such as Brazil (Iha and Sabino, 2008), Turkey (Akta *et al*.,, 2004), Japan (Kamei and Watanabe, 2005), South Africa (Leggott and Shephard, 2001; Al-hazmi, 2010), Iran (Cheraghali *et al.*, 2005), the Netherlands (Boonzaaijer *et al*, 2005), Italy (Spadaro *et al.*, 2007), Argentina (Funes and Resnik, 2009), and China (Guo *et al.*, 2013). In addition, the effect of climatic conditions on the occurrence of mycotoxins has been evaluated within these countries. Levels of mycotoxins varies between seasons and years, depending on environmental

conditions and agricultural management practice of countries where agricultural commodities are produced (Milani, 2013; Alberts *et al.*, 2019; Christiane Gruber-Dorninger, Jenkins, 2019; Meyer *et al.*, 2019).

The distribution of food and feed commodities across the world, including mycotoxin contaminated products, has demonstrated the significance of studies based on determining the occurrence of mycotoxins worldwide, in a country as well as in a specific region. Studies have proven that both the occurrence and level of mycotoxins in various food commodities vary from one food matrix to another, the production region, and the year of production (Leggott and Shephard, 2001; Iqbal *et al.*, 2018; Adetunji *et al.*, 2019). A quantitative analysis of mycotoxin in agricultural commodities is important for the development of meaningful practices minimizing contamination of mycotoxins in the food chain.

The occurrence of mycotoxins in vegetable samples collected from EC, GP, KZN LP, and WC was investigated. These five provinces are characterized by different climatic conditions. According to the results presented in **Figure 4-7**, patulin was detected in 12.5% and 10.4% vegetable samples collected from EC and WC provinces respectively. However, none of the vegetable samples collected from KZN, GP, and LP were found to be contaminated with patulin. As a potent carcinogenic (Mahfoud *et al.*, 2002; Bennett and Klich, 2003), the absence of patulin in commercially grown vegetables in these provinces gives vegetable producers in these provinces a huge advantage.

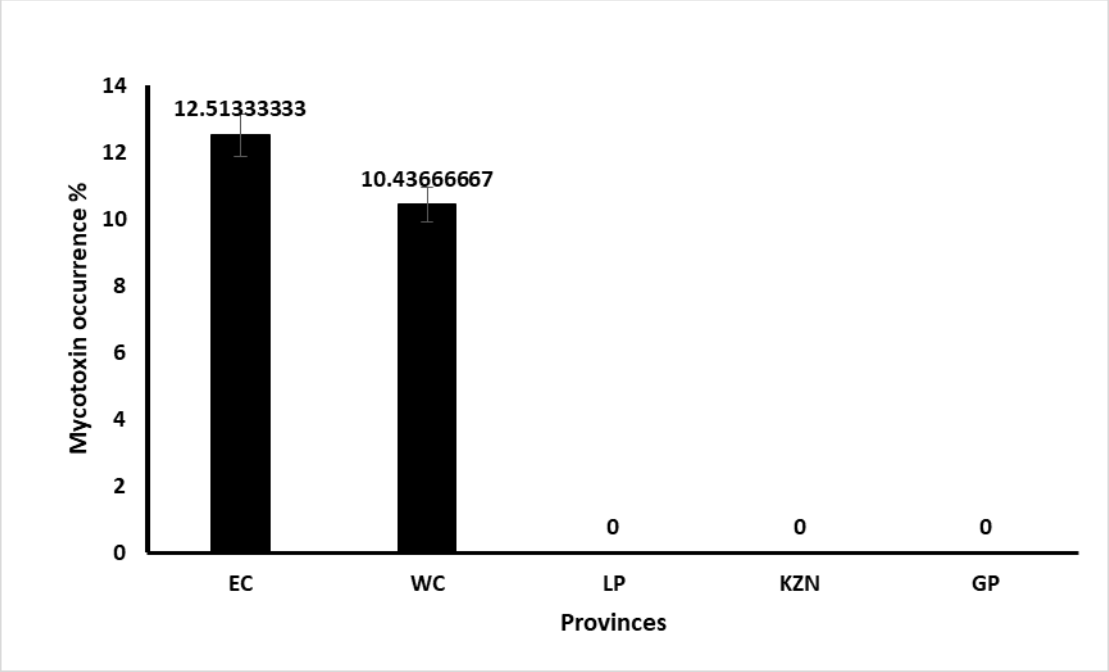

Figure 4-7: Occurrence of patulin in vegetable samples collected from EC, GP, LP, KZN, and WC provinces of South Africa**.**

The major factors that influence the level of mycotoxins agricultural commodities include varying climatic conditions and poor agricultural practices (Drusch and Ragab, 2003, McCallum *et al*., 2002). Not only do climate conditions influence the levels of mycotoxins present, they also influence the type of mycotoxin produced (Paterson and Lima, 2011). Agricultural commodities grown in tropical and subtropical areas are typically affected by high temperature and high humidity, and are, therefore susceptible to fungal infection and accompanied by the production of mycotoxins. *Aspergillus* species optimally grow in tropical and subtropical climates and, mycotoxins such as aflatoxin, therefore, pose a major challenge in these regions. *Fusarium* and *Penicillium* species grow optically under the temperature climatic conditions and *Fusarium* toxin such as trichothecenes occur mainly in the temperature climate areas (Magan *et al*., 2011; Paterson and Lima, 2011).

The EC province is on the other hand located between the KZN and WC and its climate is a bit of a mixture of the Mediterranean and subtropical climate. On the other hand the WC province has a Mediterranean climate characterized by mild and wet conditions. These two provinces have a prevalence of patulin

contamination. Patulin is mainly produced by *Penicillium expansum*, and it commonly occur in apples and apple-based products. *Penicillium expansum* optimally grows at 25 °C and the optimum temperature of the production of patulin is 23-25 °C (Baert *et al.*, 2007; Tannous *et al.*, 2016; Zbyňovská *et al.*, 2016). The WC and EC provinces are some of the coldest provinces in South Africa with an average temperature of 24 °C. The temperatures in these two provinces, therefore, favor both the growth of *Penicillium expansum* and the production of patulin. Besides the climatic temperature, poor vegetable storage could also contribute to the production of patulin in these provinces. The importation from the other parts of the country of vegetables that were already contaminated with patulin cannot be ruled out. Furthermore, poor agricultural practices in the respective provinces could have also contributed to the contamination of vegetables with patulin.

The absence of patulin or any other mycotoxin in the non-contaminated vegetable samples does not necessarily mean mycotoxin production will be inhibited. At a later stage and under favorable conditions, mycotoxigenic fungi may can produce mycotoxins. The poor agricultural practices contribute to the increase in fungal contamination and mycotoxin contamination in agricultural commodities. Therefore, the adoption and implementation of good agricultural practices and post-harvest control strategies are necessary to reduce mycotoxin contamination. Generally, proper storage and picking of sound fruits and vegetables lead to less spoilage and mycotoxin contaminations (Sydenham *et al.*, 1995).

4.4 Conclusion

A simple, rapid, and sensitive analytical method has been optimised and validated for the simultaneous determination of patulin and Aflatoxins B1, B2, G1, and G2. UHPLC-QTOF-MS is a much more accurate technique of creating structural information of each analyte with limited sample treatment and without derivatization. The sensitivity of the method was improved by optimising the chromatographic and mass spectrometric conditions. The optimised method exhibited good linearity, low LODs and LOQs, and good recovery rates of the analytes. The method performance parameters indicates that this method is

acceptable to be used for routine analysis of mycotoxins in vegetables and processed products. The presence of patulin in vegetables necessitates the need for monitoring and regulating mycotoxins in vegetables. However, the results generated herein suggest that mycotoxin regulation measures should be established by taking into account the regional conditions, for example, regulation measures applied in EC and WC should be stringent than those of the other provinces because of the high prevalence of patulin in these two provinces. Furthermore, the study has demonstrated the need for the implementation of good agricultural practices when handling vegetables.

4.5 Acknowledgments

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4.6 References

A new approach in sample treatment combined with UHPLC-MS/MS for the determination of multiclass mycotoxins in edible nuts and seeds | INC - International Nut and Dried Fruit Council (no date). Available at: https://www.nutfruit.org/index.php/industry/scientific-studies/a-new-approach-in-sample-treatment-combined-with-uhplc-msms-for-the-determination-of-multiclass-mycotoxins-in-edible-nuts-and-seeds-2 (Accessed: 14 November 2019).

Adetunji, M. C. *et al.* (2019) 'Fungal metabolite and mycotoxins profile of cashew nut from selected locations in two African countries', *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*. Taylor and Francis Ltd.

Al-hazmi, A. (2010) 'Determination of Patulin and Ochratoxin A using HPLC in apple juice samples in Saudi Arabia', *Saudi Journal of Biological Sciences*. King Saud University, 17(4), pp. 353–359.

Alberts, J. *et al.* (2019) ‘Rural subsistence maize farming in South Africa: Risk assessment and intervention models for reduction of exposure to fumonisin mycotoxins’, *Toxins*, 11(6), pp. .

Antonia, G. F. *et al.* (2011) ‘Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry’, *Journal of Chromatography.A*, 1218, pp. 4349–4356.

Arroyo-Manzanares, N., García-Campaña, A. M. and Gámiz-Gracia, L. (2013a) ‘Multiclass mycotoxin analysis in Silybum marianum by ultra-high-performance liquid chromatography-tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid-liquid microextraction’, *Journal of Chromatography A*, 1282, pp. 11–19.

Arroyo-Manzanares, N., García-Campaña, A. M. and Gámiz-Gracia, L. (2013b) ‘Multiclass mycotoxin analysis in Silybum marianum by ultra high-performance liquid chromatography-tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid-liquid microextraction’, *Journal of Chromatography A*.

Baert, K. *et al.* (2007) ‘Influence of starage conditions of apple on growth and patulin production by Penicillum expansum’, *Journal of Food Microbiology*, 119, pp. 170–181.

Beccari, G. *et al.* (2016) ‘Presence of Fusarium Species and Other Toxigenic Fungi in Malting Barley and Multi-Mycotoxin Analysis by Liquid Chromatography–High-Resolution Mass Spectrometry’.

Bennett, J. W. and Klich, M. (2003) ‘Mycotoxins’, *Journal of Clinical Microbiology*, 16, pp. 497–516.

Berthiller, F. *et al.* (2005) ‘Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high-performance liquid chromatography-tandem mass spectrometry’, *Journal of Chromatography A*, 1062(2), pp. 209–216.

Bessaire, T. *et al.* (2019) ‘Multiple Mycotoxins Determination in Food by LC-

MS/MS: An International Collaborative Study', *Toxins*, 11(11)..

Boonzaaijer, G., Bobeldijk, I. and van Osenbruggen, W. A. (2005) 'Analysis of patulin in dutch food, an evaluation of a SPE based method', *Food Control*, 16(7), pp. 587–591.

Capriotti, A. L. *et al.* (2012) 'Multiclass screening method based on solvent extraction and liquid chromatography-tandem mass spectrometry for the determination of antimicrobials and mycotoxins in egg', *Journal of Chromatography A*. Elsevier B.V., 1268, pp. 84–90.

Catană, M. *et al.* (2011) 'Determination of patulin in apple juice', 1(1), pp. 65–69.

Cervino, C. *et al.* (2008) 'Use of isotope-labeled aflatoxins for LC-MS/MS stable isotope dilution analysis of foods', *Journal of Agricultural and Food Chemistry*, 56(6), pp. 1873–1879..

Christiane Gruber-Dorninger, T. Jenkins, G. S. (2019) 'Global Mycotoxin Occurrence in Feed ':, *Toxins*, 11(375), pp. 1–25.

Desmarchelier, A. *et al.* (2011) 'Analysis of patulin in pear- and apple-based foodstuffs by liquid chromatography-electrospray ionization tandem mass spectrometry', in *Journal of Agricultural and Food Chemistry*, pp. 7659–7665.

Drusch, S. and Ragab, W. (2003) 'Mycotoxins in fruits, fruit juices, and dried fruits', *Journal of Food Protection*, 66, pp. 1514–1527.

Frisvad, J. C. *et al.* (2011) 'Fumonisin and ochratoxin production in industrial Aspergillus niger strains', *PLoS ONE*, 6(8), pp. 2–7.

Funes, G. J. and Resnik, S. L. (2009) 'Determination of patulin in solid and semisolid apple and pear products marketed in Argentina', *Food Control*, 20(3), pp. 277–280.

Guo, Y. *et al.* (2013) 'Survey of patulin in apple juice concentrates in Shaanxi (China) and its dietary intake', *Journal of Food Control*, 34, pp. 570–573.

Harris, K. L., Bobe, G. and Bourquin, L. D. (2009) 'Patulin surveillance in apple cider and juice marketed in Michigan', *Journal of Food Protection*. International Association for Food Protection, 72(6), pp. 1255–1261..

Iha, M. H. and Sabino, M. (2008) ‘Incidence of patulin in Brazilian apple-based drinks’, *Food Control*, 19(4), pp. 417–422.

Iqbal, S. Z. *et al.* (2018) ‘Natural occurrence of patulin in different fruits, juices and smoothies and evaluation of dietary intake in Punjab, Pakistan’, *Food Control*. Elsevier Ltd, 84, pp. 370–374.

Iturat, B. *et al.* (2014) ‘Determination of patulin in apple derived products bt UHPLC-MS/MS. Study of matrix effects with atmospheric pressure ionization sources’, *Journal of Food Chemistry*, 142(Iso 690), pp. 400–407.

Jesus, B. *et al.* (2014) ‘Simultaneous determination of Fusarium mycotoxins in wheat grain from Morocco by liquid chromatography coupled to triple quadrupole mass spectrometry’, *Journal of Food Control*, 46, pp. 1–5.

Kamei, K. and Watanabe, A. (2005) ‘Aspergillus mycotoxins and their effect on the host’, *Medical Mycology*, 43(SUPPL.1), pp. 95–99. Katharina, H. *et al.* (2017) ‘Multi-mycotoxin stable isotope dilution LC-MS/MS method for Fusarium toxins in beer’, *Journal of Food Chemistry*, 218, pp. 447–454.

Kim, D. H. *et al.* (2017) ‘Simultaneous determination of multi-mycotoxins in cereal grains collected from South Korea by LC/MS/MS’, *Toxins*, 9(3), pp. 1–13.

Kokkonen, M., Jestoi, M. and Rizzo, A. (2005) ‘Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled tandem with mass spectrometry’, *Food Additives and Contaminants*, 22(5), pp. 449–456.

Laidou, A., Thanassoulopoulos, C. C. and Liakopoulou-Kyriakides, M. (2001) ‘Diffusion of patulin in the 290 flesh of pears inoculated with four post-harvest pathogens’, *Journal of Phytopathology*, 149, pp. 457–461.

Leggott, N. L. and Shephard, G. S. (2001) ‘Patulin in South African commercial apple products’, *Food Control*, 12(2), pp. 73–76. Magan, N., Medina, A. and Aldred, D. (2011) ‘Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest’, *Journal of Plant Pathology*, 60, pp. 150– 163.

Mahfoud, R. *et al.* (2002) ‘The mycotoxin patulin alters the barrier function of the intestinal epithelium: Mechanism of action of the toxin and protective effects of

glutathione', *Toxicology and Applied Pharmacology*, 181, pp. 209–218.

Majid Cheraghali, A. *et al.* (2005) 'Incidence of patulin contamination in apple juice produced in Iran', *Food Control*, 16(2), pp. 165–167.

Malachová, A. *et al.* (2018) 'Advanced LC–MS-based methods to study the co-occurrence and metabolization of multiple mycotoxins in cereals and cereal-based food', *Analytical and Bioanalytical Chemistry*. Springer Verlag, pp. 801–825.

Malysheva, S. V. *et al.* (2012) 'Improved positive electrospray ionization of patulin by adduct formation: Usefulness in liquid chromatography-tandem mass spectrometry multi-mycotoxin analysis', *Journal of Chromatography A*, 1270, pp. 334–339.

Meyer, H. *et al.* (2019) 'Long term monitoring (2014–2018) of multi-mycotoxins in South African commercial Maize and wheat with a locally developed and validated LC-MS/MS method', *Toxins*, 11(5).

Milani, J. M. (2013) 'Ecological conditions affecting mycotoxin production in cereals : a review', 2013(8), pp. 405–411.

Nakhjavan, B., Ahmed, N. S. and Khosravifard, M. (2020) 'Development of an improved method of sample extraction and quantitation of multi-mycotoxin in feed by LC-MS/MS', *Toxins*, 12(7).

Paíga, P. *et al.* (2012) 'Extraction of ochratoxin A in bread samples by the QuEChERS methodology', *Food Chemistry*, 135(4), pp. 2522–2528.

Pamel, E. V. *et al.* (2011) 'Mycotoxin production by pure fungal isolates analysed by means of an uhplc-ms/ms multi-mycotoxin method with possible pitfalls and solutions for patulin-producing isolates', *Mycotoxin Research*, 27, pp. 37–47.

Papp, E. *et al.* (2002) 'Liquid chromatographic determination of aflatoxins', *Microchemical Journal*, 73(1–2), pp. 39–46.

Paterson, R. R. M. and Lima, N. (2011) 'Further mycotoxin effects from climate change', *Food Research International*. Elsevier Ltd, 44(9), pp. 2555–2566.

Van De Perre, E. *et al.* (2014) 'Screening for the presence of patulin in molded fresh produce and evaluation of its stability in the production of tomato products',

Journal of Agricultural and Food Chemistry, 62(1), pp. 304–309.

Piqué, E. *et al.* (2013) 'Occurrence of patulin in organic and conventional apple-based food marketed in Catalonia and exposure assessment', *Food and Chemical Toxicology*, 60, pp. 199–204.

Raiola A, S. A. (2015) 'Aflatoxins, Ochratoxins, Trichothecenes, Patulin, Fumonisins and Beauvericin in Finished Products for Human Consumption', *Journal of Clinical Toxicology*, 05(04).

Ren, Y. *et al.* (2007) 'Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*, 1143(1–2), pp. 48–64.

Rigi, M. R., Farahbakhsh, M. and Rezaei, K. (2015) 'Use of solid-phase extraction with hydrophilic-lipophilic balance (HLB) cartridge as the appropriate option for metribuzin extraction from contaminated soils', *Journal of the Brazilian Chemical Society*, 26(1), pp. 156–164.

Rosinská, D., Vieriková, M. and Lehotay, J. (2009) 'Determination of patulin in apple products using HPLC with photodiode array detector and ultra-performance liquid chromatography with electrospray-tandem mass spectrometry', *Journal of Liquid Chromatography and Related Technologies*, 32(4), pp. 500–511.

Rupp, H. S. and Turnipseed, S. B. (2000) 'Confirmation of patulin and 5-hydroxymethylfurfural in apple juice by gas chromatography/mass spectrometry', *Journal of AOAC International*, 83(3), pp. 612–620.

Sahar, N. *et al.* (2009) 'Screening of mycotoxins in wheat, fruits and vegetables grown in Sindh ', (February).

Seo, M., Kim, B. and Baek, S. Y. (2015) 'An optimised method for the accurate determination of patulin in apple products by isotope dilution-liquid chromatography/mass spectrometry', *Analytical and Bioanalytical Chemistry*. Springer Verlag, 407(18), pp. 5433–5442.

Sospedra, I. *et al.* (2010) 'Use of the modified quick easy cheap effective rugged and safe sample preparation approach for the simultaneous analysis of type A-

and B-trichothecenes in wheat flour', *Journal of Chromatography A*..

Spadaro, D. *et al.* (2007) 'Incidence and level of patulin contamination in pure and mixed apple juices marketed in Italy', *Food Control*, 18, pp. 1098–1102.

Sulyok, M., Krska, R. and Schuhmacher, R. (2007) 'A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples', *Analytical and Bioanalytical Chemistry*, 389(5), pp. 1505–1523.

Sydenham, E. W. *et al.* (1995) 'Reduction of patulin in apple juice samples influence of initial processing.', *Journal of Food Control*, 6, pp. 195–200.

Tannous, J. *et al.* (2016) 'A study on the physicochemical parameters for Penicillium expansum growth and patulin production: Effect of temperature, pH, and water activity', *Journal of Food Science and Nutrition*, 4, pp. 611–622.

Welke, J. E. *et al.* (2009) 'Effect of processing stages of apple juice concentrate on patulin levels. Food Control 20', *Journal of Food Control*, 20, pp. 48–52.

Wu, R. N. *et al.* (2009) 'Analysis of patulin in apple products by liquid-liquid extraction, solid-phase extraction and matrix solid-phase dispersion methods: A comparative study', *European Food Research and Technology*, 228(6), pp. 1009–1014.

Akta, A.H., Yilmazer, M., and Demirci, S. (2004) 'Determination of Patulin in Apple Juice Produced in Isparta, Turkey by HPLC with Diode Array Detection', *Journal of Food and Drug analysis*, 12(3), pp. 228–231.

Zbyňovská, K. *et al.* (2016) 'Patulin - a contaminant of Food and Feed : A review', *Acta fytotechnica et zootechnica*. Slovak University of Agriculture in Nitra, 19(02), pp. 64–67.

Zhao, D. *et al.* (2011) 'Preparation and characterization of a molecularly imprinted polymer by grafting on silica supports: A selective sorbent for patulin toxin', *Analytical and Bioanalytical Chemistry*, 401(7), pp. 2259–2273.

Zouaoui, N. *et al.* (2015) 'Occurrence of patulin in various fruit juice marketed in

Tunisia', *Food Control*. Elsevier Ltd, 51, pp. 356–360.

5 Chapter 5: Occurrence and spatial distribution of mycotoxigenic fungi in spoilt vegetables from five different climatic regions

5.1 Introduction

Fungal growth on crops and subsequent production of mycotoxins is a worldwide problem. Not only does it have an impact on the economy, it also poses health risks to both animals and humans. While fungal and mycotoxin contamination can occur before the harvesting stage, it can also be carried over to the post-harvest stage through poor management and improper post-harvest (Yeun *et al.*, 2011). Fungal growth is favoured by various environmental conditions such as high temperatures, humidity and hurricanes, unseasonal rains and floods (Paterson and Lima, 2010; Edite *et al.*, 2014). In addition, poor harvesting and storage practices as well as improper transportation can also contribute to fungal growth. These factors are attributes of most African countries, and high incidences of mycotoxins have been reported in crops such as maize, which is susceptible to toxigenic fungi and subsequent production of mycotoxins (Lewis *et al.*, 2005). The presence of mycotoxin-producing fungal species and their mycotoxins in agricultural commodities usually results in the loss of these commodities and subsequent reduced income for farmers.

Over and above its effect on the economy, the effect of mycotoxins on the health of animals and humans has necessitated the need for the development of strategies for the management of mycotoxin contamination. These strategies include good agricultural practices during production, harvest, and storage and they typically involve proper drying, removal of damaged crops, crop rotation, pest control, and irrigation on a regular basis (Muthomi *et al.*, 2008; Bryden, 2009).

Despite literature being replete with information on fungal growth, there is lack of information on the distribution of mycotoxigenic fungi in farms located across the different climatic regions of South Africa. Since storage and handling practices vary from one region to another, there is a greater likelihood that difference in the

fungal population can occur within the various climatic regions. The present study, therefore, aims to investigate the occurrence and the distributions of fungal isolates on vegetable samples collected from 5 different climatic regions of South Africa.

5.2 Experimental

This section provides a systematic summary of the experimental work done to achieve the aim of this study.

5.2.1 Reagents and materials

All the reagents and materials used in this study are described in Section 3.1.

5.2.2 Sample collections and fungal isolation

Spoiled vegetables (carrots, cucumbers, onions, peppers, potatoes, sweet potatoes) were collected from 5 different climatic regions of South Africa, namely: Gauteng Province (GP), Limpopo province (LP), KwaZulu-Natal (KZN) Eastern Cape, and Western Cape. A detailed map of the sampling areas is given in Section 3.2. The samples were collected separately in a Ziploc bag and transported to the laboratory in a cooler box with ice packs and cultured within 2 days following transportation.

The samples were surface sterilized by rinsing with ultrapure water to remove contamination emerging from the dust and other sources. The rotten part of the vegetables was directly plated onto solidified SDA and incubated at 28 °C for 3-5 days. The fungal cultures were observed for colony growth. Each distinct colony was subcultured on a fresh SDA media to obtain pure colonies. A mixed culture containing more than one fungal species was obtained, therefore, a small sample from each fungal species was inoculated into a fresh agar plate. This step was repeated until pure fungal isolates containing one fungal species were obtained. All the experiments were carried out under a laminar air flow and sterile conditions.

Each of the fresh vegetables was used as a control. The vegetable samples were surface sterilized with 70 % ethanol. Thereafter, a small piece was cut with a sterile blade, placed on fresh agar plates, and incubated for 5 days at 28.

5.2.3 Fungal identification

The identification of all fungal isolates was performed by SEM imaging and MALDI biotyping. A brief description of the preparation method for both techniques is outlined in Section 3.6

Samples for SEM imaging were prepared using 3-7 day old cultures. The specimen were washed with PBS, fixed with 2% glutaraldehyde, post-fixed with 150 µL osmium tetroxide, and dehydrated sequentially with 10-90% ethanol and absolute ethanol. Thereafter, the dried specimen was mounted on a stub and gold-coated using Q150R sputter (Quorum technologies, UK) before imaging on a JOEL IT 300 scanning electron microscope (JOEL, UK).

The samples for MALI Biotyping analysis were prepared according to the Bruker MALDI Biotyper Standard operating procedure (Revision 4 June 2015) (Bruker, 2015). The biotyping was performed using a MALDI-TOF/TOF MS (Bruker, Germany) equipped with a Flex control system v3.4 (Bruker, Germany) and MALDI-Biotyper compass v3.4 software (Bruker, Germany).

5.2.4 Determining the mycotoxigenic potential of the identified species

To determine the toxigenic potential of the fungal isolates, the plug agar method, described by Adetunji *et al.* (2014) was used with slight modification. Briefly, 15 g of the culture medium was cut out (near the centre) and placed in a 50 mL centrifuge tube before adding 15 mL of acetonitrile. The resulting mixture was then homogenized. The extraction of toxins was achieved using the QEuChERS methods as discussed in Section 3.4.1. The UPLC-QTOF-MS was used for the analysis of mycotoxin. The LC/MS conditions were outlined in Section 3.5.

5.3 Results and discussion

5.3.1 Isolation and identification of fungal isolates

The present study ranks as one of the few studies that have evaluated fungal contamination on vegetable samples. It has been reported that fugal contamination is responsible for the loss of vegetables due to spoilage (Ray and Misra, 1995; Minna *et al*., 2013; Udoh *et al.*, 2015). Spoilage is associated with any change in color, texture, flavor, texture, and shape in food. These changes make food unpleasant or even toxic to consume

In this study vegetable samples (carrots, cucumbers, onions, peppers, potatoes, and sweet potatoes) were collected and analysed for fungal contamination. A total of 90 fungal species were isolated from the collected spoilt vegetable samples and were identified based on their macroscopic characteristics such as color, texture, and growth of the colony (see **Figure 5-1**). No fungal growth was observed on the control.

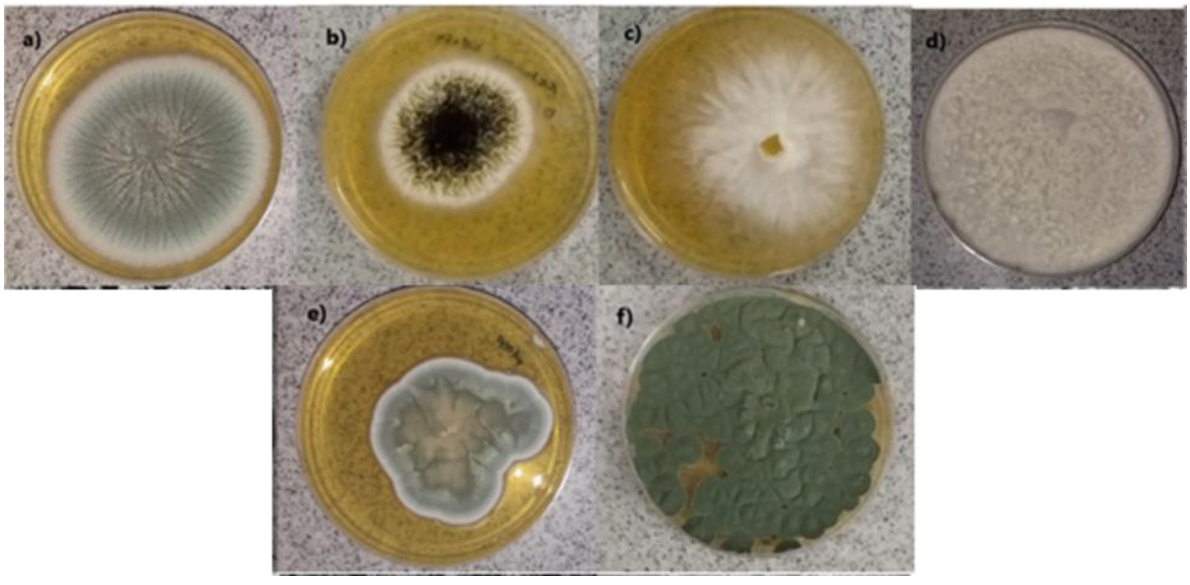

Figure 5-1: Top view of culture plates showing Macroscopic characteristics of fungal isolates: a) *Aspergillus fumigatus*; b) *Aspergillus niger*; c) *Fusarium proliferatum*; d) *Fusarium verticillioides* e) *Penicillium commune* and f) *Penicillium discolor*

Based on their macroscopic characteristics, the isolated species were found to belong to 3 fungal genera and 6 fungal species. The isolated fungal species were *Aspergillus fumigatus*, *Aspergillus niger, Fusarium proliferatum, Fusarium verticillioides, Penicillium commune, and Penicillium discolor*. The *Aspergillus fumigatus* isolates colonies were grey-green with narrow white borders with a velvety texture. After two weeks of further incubation at room temperature, the color became darker. The *Aspergillus niger* isolates were initially pale yellow but changed to black after 2 weeks of incubation. The *Fusarium proliferatum* appeared to have white colonies with light purple pigments. The rest of the isolates were grouped based on their appearance. However, the fungal identification was further complemented with SEM imaging and MALDI biotyping.

MALDI biotyping and SEM are useful tools for the identification of microorganisms (Kaminskyj and Dahms, 2007; Lima *et al*., 2010; Assress *et al.*, 2020). MALDI was used to identify all the following fungal isolates: *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium proliferatum*, *Fusarium verticillioides*, *Penicillium commune,* and *Penicillium discolor*. The MALDI were corroborated by results of microscopic analysis. The *Aspergillus fumigatus* recorded a score value of 2.54 while isolates of *Aspergillus niger*, *Fusarium proliferatum*, *Fusarium verticillioides*, *Penicillium commune,* and *Penicillium discolor* score values of 2.35, 2.01, 2.35, 2.35, and 2.54, respectively. The score value is a measure of the likelihood that an isolate is accurately identified when compared with the fungal species in the reference library. A score value between of 0.000-1.699 means the identification was not possible, while a score value of between 1.700-1.999 is only reliable at the genus level. A score of ranging from 2.00 to 3.00 is considered accurate identification.

The morphology of the isolates was examined and the isolates were found to possess distinguishing morphological features (see **Figure 5-2**). *Aspergillius* species showed conidial heads that are compact, columnar, and biseriate. Conidia shape are globose to ellipsoidal and conidiophore hyaline with vesicle to flask. The *Fusarium* species showed microconidia in club shape, with false head in short monophiliades and abundant uni-or bicellular microconidia. Furthermore, the *Penicillium* species showed a globose to elongated sausage-shaped conidial head. The morphological characteristics of the fungal isolates demonstrated in the

SEM images further corroborated the identification of the isolates as *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium proliferatum*, *Fusarium verticillioides*, *Penicillium commune* and *Penicillium discolor.*

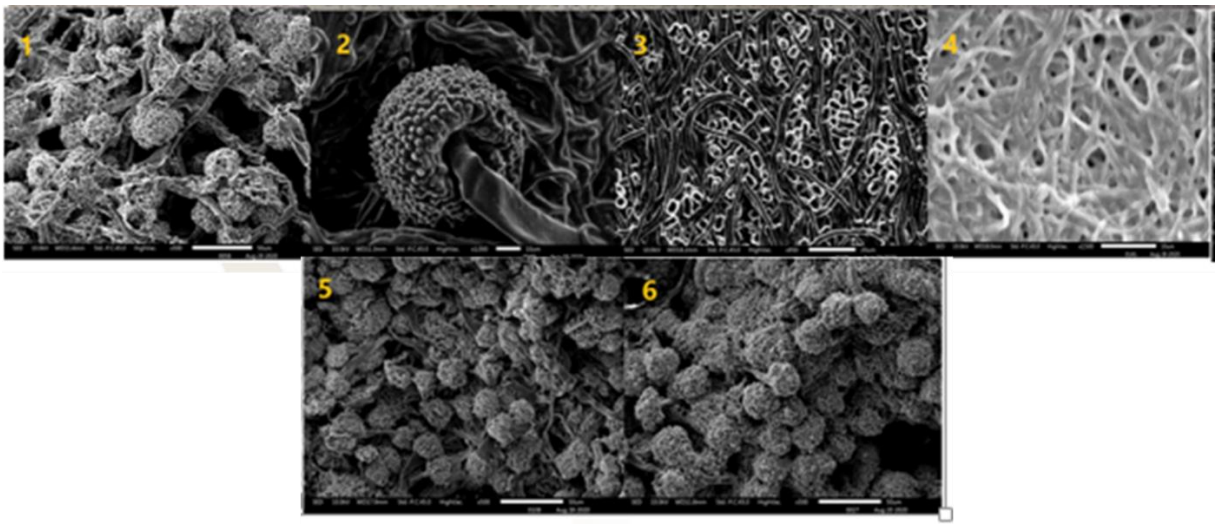

Figure 5-2: Scanning electron microscope images showing morphological characteristics of fungal isolates: 1) Aspergillus fumigatus; 2) Aspergillus niger 3) Fusarium proliferatum; 4) Fusarium verticillioides; 5) Penicillium commune and 6) Penicillium discolor

Of all the *Aspergillus* genus, the *Aspergillus fumigatus* was predominant in all vegetable samples, while the *Aspergillus niger* was isolated from onions only (see **Table 5-1**). The *Fusarium proliferatum* was found to occur in onions, peppers, potatoes, and sweet potatoes, while *Fusarium verticillioides* occurred in carrots and onions only. Furthermore, it was observed that the most dominant fungal species among the *Penicillium* genus was the *Penicillium discolor*, which occurred in carrots, peppers, potatoes, and sweet potatoes. The *Penicillium commune* was, however, isolated from peppers and sweet potatoes only.

This study has demonstrated that species from the genera *Aspergillus, Fusarium Penicillium* can contaminate a wide range of vegetables. However, these species can be regarded as causative agents of the spoilage on the vegetables, which is in agreement with previously reported studies. Oduola *et al*. (2018) reported that the *Fusarium* species were isolated from sweet potatoes and they were responsible for the soft rots on sweet potatoes. Furthermore, Ibrahim *et al.* (2014) has reported

that the *Aspergillus* and *Fusarium* species were isolated from potatoes and that the *Fusarium* species was responsible for the dry rot in potatoes. Vico *et al.* (2014) reported that the *Penicillium* species were responsible for the blue mould on fruits. Frimpong *et al.* (2019) recently isolated *Aspergillus*, *Fusarium,* and *Penicillium* species from peppers, which further confirms that these fungal species are responsible for vegetable spoilage.

The control experiment proved the non-occurrence of mycotoxigenic fungi on fresh vegetables. This indicates that the isolated fungi from the rotten vegetables were introduced at the post-harvesting stage. The fungal isolates on the spoilt vegetables may be due to poor handling of fresh vegetables, and this resulted in the growth of spoilage fungi.

Table 5-1: Fungal species isolated from the collected vegetable samples, the identification was based on macroscopic characteristics, SEM imaging, and MALDI Biotyping

Genus	**Species**	**Vegetable samples**					
		Carrots	Cucumbers	Onions	Peppers	Potatoes	Sweet potatoes
Aspergillus	*A.Fumigatus*	+	+	+	+	+	+
	A.Niger	-	-	+	-	-	-
Fusarium	*F.Proliferatum*	-	-	+	+	+	+
	F.Verticillioides	+	-	+	-	-	-
Penicillium	*P.Commune*	-	-	+	-	-	+
	P.Discolor	+	-	-	+	+	+

5.3.2 The occurrence of mycotoxigenic fungi on vegetable samples

In terms of prevalence, species of the Aspergillus genus were the most prevalent *Penicillium* and *Fusarium* genera (see **Figure 5-3**). Although high-frequency rates (98 %) of the A*spergillus* genus were observed in onions, its occurrence in sweet potatoes was low. In addition, a significantly high occurrence of *Penicillium* was found in carrots compared to cucumbers. The *Penicillium* species was found to be

dominant in peppers with an occurrence rate of 56 %; no occurrence of the *Penicillium* species was recorded in potatoes. The occurrence of the *Fusarium* species was lower than the *Aspergillus* and *Fusarium* species in all vegetable samples except onion where it occurred at a rate of 69 %.

Regarding the specific species, this study showed that the *Aspergillus fumigatus* was highly prevalent among all vegetables, while the *Aspergillus niger, Fusarium proliferatum, Fusarium verticillioides*, *Penicillium commune,* and *Penicillium discolor* had moderate to low occurrences. These results concur with studies by Adebayo-Tayo *et al.* (2012) and Tafinta *et al.* (2013), which reported the *Rhizopus stolinofer* and the *Aspergillus fumigatus* as the most predominant species while the *Aspergillus niger* was the less dominant species in the vegetable samples. In contrast, Mailafia *et al.* (2017) have reported a high prevalence rate (70%) of the *Aspergillus niger* in fruit samples. Fruit and vegetables can get damaged during harvest, storage, packaging, and transportation, and this can lead to post-harvest loss and possible fungal contamination. In addition, poor management of vegetables from the farm can also contribute to the problem of loss of agricultural commodities.

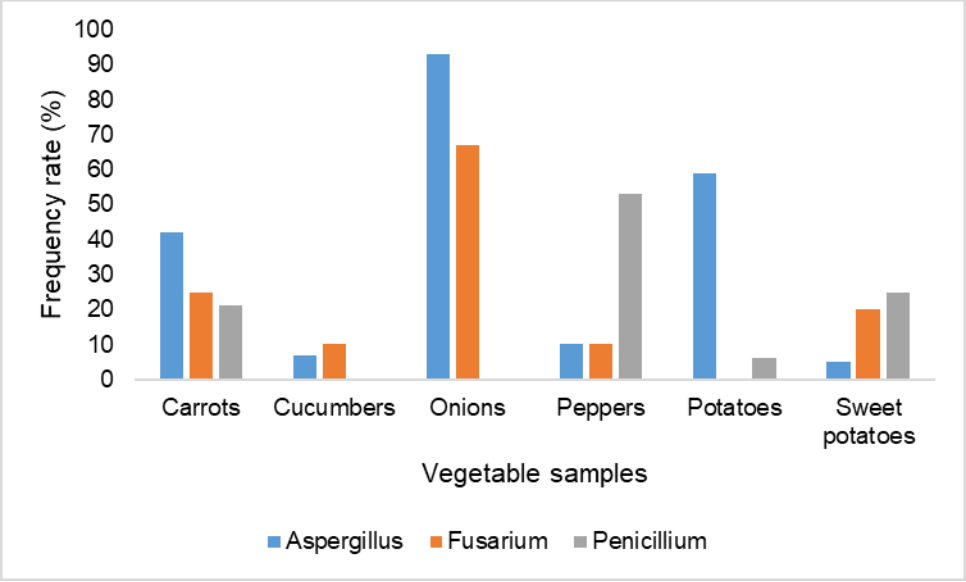

Figure 5-3: The occurrence frequency (%) of *Aspergillus, Fusarium*, and *Penicillium* genera in spoilt carrots cucumber, onions, peppers, potatoes, and sweet potatoes

5.3.3 Distribution of mycotoxigenic fungi in the five climatic regions

The vegetable samples analysed in this study were collected from vegetable farms, markets and wholesalers from 5 different province in South Africa (Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, and Western Cape) characterized by different climatic zones. The distribution of the fungal contamination on the vegetable samples collected from these provinces was determined (see **Figure 5-4**). All the provinces were found to possess significantly varying fungal infection rates. The KZN province had a high fungal infection rate of the mycotoxigenic fungi (38%) followed by the GP with a 20% infection rate. Infection with *Aspergillus fumigatus*, *Aspergillus Niger,* and *Fusarium verticillioides* was not observed in the WC. In addition, *Penicillium commune* was isolated from vegetables collected from KZN only. *Fusarium proliferatum* was the only fungal species occurring in all the 5 provinces.

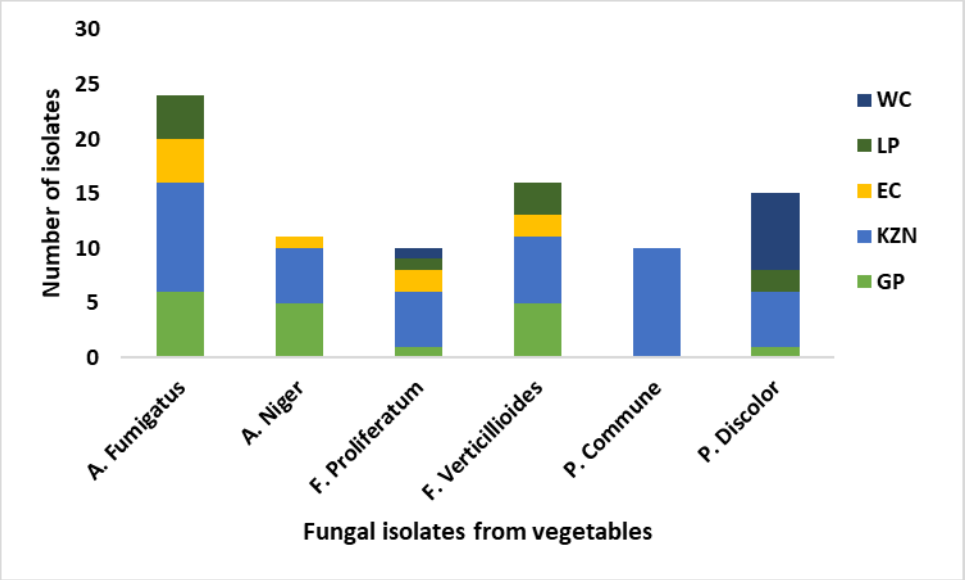

Figure 5-4: Distribution of isolated mycotoxigenic fungi isolated from vegetables collected from five provinces in South Africa characterized by different climatic regions

In this study, the occurrence of fungal species associated with vegetables in South Africa was found to vary with climatic conditions. As the climate conditions varies from one province to another, so does the occurrence of mycotoxigenic species in each province. A high prevalence of fungal contamination was found in KZN province, a province with a sub-tropical and humid climate. KZN was followed by

GP, which is characterized by subtropical climate. The average humidity in KZN province is 79.1 % and temperature range between 16 and 32 °C. GP has an average humidity of 59.0% and a temperature range of 16–30 °C. Furthermore, low prevalence of fungal contamination was observed in the WC province which has a Mediterranean climate characterized by cold and wet conditions. High fungal occurrence in both KZN and GP observed in this study demonstrated that fungal growth was favored by high humidity and warm temperatures and less favored by the cold and wet climatic conditions of the WC province. These results concur with previous studies that reported that mycotoxigenic fungi compete poorly under cool temperatures (Mshelia *et al.*, 2020). Climate is one of the crucial factors that can influence the occurrence of a wide range of fungi (Paterson and Lima, 2017; Perrone *et al.*, 2020). According to Talley *et al.* (2002), climatic factors that influence the distribution of mycotoxigenic fungal species include temperature, rainfall, and seasons. Furthermore, it has been reported that warm temperature and high humidity are favorable conditions for the growth of mycotoxigenic fungi and the production of mycotoxins (Rao *et al.*, 2011). Therefore, crops produced in warm regions have are susceptible to fungal contamination, and in some regions, contamination will occur when temperature increases and drought sets in (Bock *et al*., 2004; Paterson and Lima, 2010).

5.3.4 The toxigenic potential of fungal isolates

The identified fungal isolates from vegetables were analysed for their ability or the potential to produce mycotoxins. Out of all the isolated fungal species only *Fusarium verticillioides* produced mycotoxins, namely fumonisin B1 and B2.

Generally, fungal species that caused food spoilage are regarded as toxigenic or pathogenic (Al-hindi *et al*., 2011). However, when these fungi invade and colonize crops, they utilize the available nutrients thus deterioration or spoilage of crops. The fungi may further produce mycotoxins in the food commodities is reliant on the potential of fungal species to produce mycotoxins, and the storage duration and conditions for commodities (Tournas and Stack, 2001; Al-hindi *et a*l., 2011).

Results obtained in this study showed that *Fusarium verticillioides* was the only fungal species that was able to produce fumonisins. Both Fusarium verticillioides and Fusarium proliferatum have been reported to be potential producers of fumonisins (Rheeder *et al*., 2002; Chilaka, de Kock, *et al.*, 2012; Kim *et al.*, 2017). However, strains of the *Fusarium proliferatum* isolated in this study were not able to produce fumonisins. This may be because strains of *Fusarium proliferatum* can degrade fumonisins rapidly (Keller and Sullivan, 1998). On the other hand fungal isolate *Aspergillus Fumigatus*, *Aspergillus niger*, *Penicillium commune* except for *Penicillium discolor* have been reported as potential producers of mycotoxins gliotoxin (Kamei and Watanabe, 2005), ochratoxin A (Frisvad *et al.*, 2011), cyclopiazonic acid (Taniwaki *et al.*, 2001) respectively. However, in the present study, these strains did not produce any toxins. This may be because the strains isolated lack the gene required for the biosynthesis of these toxins. Another reason could be because the laboratory conditions in this study were not optimum for mycotoxin production and also because of the synthetic medium (SDA) used in this study. It has been reported that the fungal metabolic profile depends on the medium and condition (Kokkonen *et al*, 2005). In addition, it has been reported that among the *Penicillium* species, the *Penicillium expansum* is the common producer of patulin (Harris *et al.*, 2009; Hammami *et al.*, 2017), unfortunately, none of the isolated fungi in this study were reported to produce patulin, which was also observed in this study.

5.4 Conclusion

This study has demonstrated that a wide range of mycotoxigenic fungi are responsible for vegetable spoilage in South Africa. The distribution of mycotoxigenic fungi in South African provinces is attributed to the climatic and environmental conditions of the country. The control experiment revealed that spoilage of vegetables occurred during the post-harvest stage. Vegetables in South Africa are infected by a wide range of mycotoxin-producing fungi, and this poses risk of getting exposed to mycotoxins since the environmental and storage conditions in Sub-Saharan in Africa favors the production of mycotoxins. These

results demonstrated that the isolated *Fusarium verticillioides* had the potential of producing mycotoxins in culture.

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5.6 References

Adebayo-Tayo, B. C. *et al.* (2012) 'Microorganisms Associated With Spoilage Of Stored Vegetables In Uyo Metropolis, Akwa Ibom State, Nigeria', *Journal of Nature and Science*, 10(3), pp. 23–32.

Adetunji, C. *et al.* (2014) 'The distribution and mycotoxigenicity of fungal isolates of stored maize grains from five agro-ecological zones of Nigeria', *Mycotoxicology*, 1(September 2018), pp. 19–28.

Assress, H. A. *et al.* (2020) 'Azole antifungal resistance in fungal isolates from wastewater treatment plant effluents'. Environmental Science and Pollution Research.

Bock, C. H., Mackey, B. and Cotty, P. J. (2004) 'Population dynamics of Aspergillus flavus in the air of an intensively cultivated region of south-west Arizona', *Journal of Plant Pathology*, 53(4), pp. 422–433.

Bruker (2015) *Standard operation procedure, cultivation and sample preparation for filamenteous fungi, Revision 4*.

Bryden, L. W. (2009) 'Mycotoxins and mycotoxicosis, significance, occurrence and mitigation in the food chain.', *Journal of Applied and System Technology*, 3, p. 6.

Edite Bezerra da Rocha, M. *et al.* (2014) 'Mycotoxins and their effects on human and animal health', *Food Control*, pp. 159–165.

Frimpong, G. K. *et al.* (2019) ‘Identification and toxigenic potential of fungi isolated from capsicum peppers’, *Microorganisms*, 7(9), pp. 1–10. Ibrahim, M. *et al.* (2014) ‘Identification of fungi associated with storage rots of Irish Potato (Solanum Tuberosum l .) tubers in Sokoto Metropolis Available online at http://abiosci.com/archive.html’, *Journal of Biological Science*, 2(2), pp. 1–4.

Kaminskyj, S. G. W. and Dahms, T. E. S. (2007) ‘High spatial resolution surface imaging and analysis of fungal cells using SEM and AFM’, pp. 1–13.

Lewis, L. *et al.* (2005) ‘Contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya’, *Journal of Environmental Health Perspectives*, pp. 1763–1767.

Lima, N., Santos, C. and Vena, A. (2010) ‘Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry’, 108, pp. 375–385.

Mailafia, S. *et al.* (2017) ‘Isolation and identification of fungi associated with spoilt fruits vended in Gwagwalada market, Abuja, Nigeria’, 10, pp. 393–397.

Minna, A., Tsado, E. K. and Aghotor, P. (2013) ‘Fungi associated with spoilage of some edible vegetables in and’, 2(2), pp. 110–113.

Mshelia, L. P. *et al.* (2020) ‘Effect of temperature, water activity and carbon dioxide on fungal growth and mycotoxin production of acclimatized isolates of fusarium verticillioides and F. Graminearum’, *Toxins*, 12(8), pp. 1–14.

Muthomi, J. W. *et al.* (2008) ‘The occurrence of Fusarium species and mycotoxins in Kenyan wheat.’, *Journal of Crop Protection*, 27(8), pp. 1215–1219.

Oduola, A. A., Awojobi, K. O. and Adeyemo, S. M. (2018) ‘Microorganisms Associated with Post Harvest Spoilage of Sweet Potatoes in Ile-Ife, Nigeria’, 8(1), pp. 1–8.

Paterson, R. R. M. and Lima, N. (2010) ‘How will climate change affect mycotoxins in food?’, *Food Research International*. Elsevier Ltd, 43(7), pp. 1902–1914.

Paterson, R. R. M. and Lima, N. (2017) ‘Thermophilic Fungi to Dominate Aflatoxigenic / Mycotoxigenic Fungi on Food under Global Warming’, pp. 1–11..

Perrone, G. *et al.* (2020) ‘Toxigenic fungi and mycotoxins in a climate change scenario: Ecology, genomics, distribution, prediction and prevention of the risk’, *Microorganisms*, 8(10), pp. 1–20.

Rao, V. K. *et al.* (2011) ‘Incidence of mycotoxigenic penicillium in feeds of Andhra Pradesh, India’, *Molecular Biology*, 2(February), pp. 46–50.

Ray, R. C. and Misra, R. S. (1995) ‘Spoilage of sweet potato tubers in tropics. I. Microorganisms associated’, *Advances in Horticultural Science*, 9(1), pp. 19–22.

Tafinta, I. Y. *et al.* (2013) ‘Isolation and identification of fungi associated with the spoilage of sweet orange (Citrus sinen- sis) fruits in Sokoto State. Niger.’, *Journal of Basic Applied Science*, 21(3), pp. 193–196.

Talley, S. M., Coley, P. D. and Kursar, T. A. (2002) ‘The effects of weather on fungal abundance and richness among 25 communities in the Intermountain West’, *BMC Ecology*, 2, pp. 7–10.

Udoh, I.P., et al. (2015) Studies on Fungi Responsible for the Spoilage/Deterioration of Some Edible Fruits and Vegetables. Advances in Microbiology, 5, pp. 285-290.

Vico, I. *et al.* (2014) ‘causing postharvest blue mold decay of apple fruit’, 29(4), pp. 257–266.

Yeun, J. *et al.* (2011) ‘Molecular and Morphological Identification of Fungal Species Isolated from Bealmijang Meju’, 21(September), pp. 1270–1279.

6 Chapter 6: Cytotoxic evaluation of patulin on mammalian cells

6.1 Introduction

Mycotoxin exposure poses serious health risks to both animals and humans (Zaki, 2012; Song *et al.*, 2014; Abastabar *et al.*, 2017). Mycotoxins are chemically diverse and are therefore likely to exert varying toxic effects on both animals and humans (Tanya, 2010). The extent to which these effects are exhibited is not only determined by the type and level of toxin contaminated the consumed commodity, but also the duration of exposure (IFT, 2006; James and Marion, 2007). Several studies have investigated the toxic effects of mycotoxin on animals and humans. For example, Whites and Rees (1981) have conducted *in vivo* studies on model animals such as rats. Futhermore, Maenetje *et al*. (2008) and Makun *et al.* (2011) have conducted an *in vitro* study using human lymphocytes. The study by Maenetje *et al*. (2008) has shown that cell viability decreased at high concentrations of Fumonisin B1 after 2 hours of exposure.

This study was aimed at evaluating the effect of patulin on the cell viability of human cancer cell lines.

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6.2 Experimental

This section provides a systematic summary of the experimental work done to achieve the aim of this study.

6.2.1 Reagent and materials

All the reagents and materials used in this study are listed in Section 3.1.

6.2.2 Cell culture and cell viability assay

Human breast carcinoma cells (MCF-7) were cultured using Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin. The cells were seeded after trypsinisation in a 96-well plate at a final concentration of $5.0X10^3$ cells per well. The plate was incubated at 37° C for approximately 24 hours to allow cell growth at an exponential phase before drug treatment. After 24 hours, patulin (concentration range 0.05-5 µm) was added in triplicates for each concentration. The plate was then incubated for 24 hours post-treatment. After incubation, resazurin dye was added to the wells using a multi-pipette (10ul reagent per 50ul media per well), being incubated for 4 hours to allow the complete and homogenous reduction of resazurin to resorufin. The fluorescence at 560/590 nm was measured using an ELISA plate reader. Lastly, the viable cells were quantified by subtraction of the background signal of untreated control cells.

6.3 Results and discussion

To evaluate the growth effect of patulin on human breast carcinoma cells (MCF-7), the cell lines were incubated with different patulin concentrations (0.05-5 µm) for 24 hours. After incubation, the cell viability was measured using resazurin reduction assay and it was recorded as % cell viability. **Figure 6-1a** shows that the resazurin fluorescence measured after 24 hours of patulin exposure decreases as the concentration of patulin increased. **Figure 6-1b** shows that the cell viability of MCF-7 decreases with increasing concentration of patulin.

Breast cancer is the most common cancer in women globally. It is the fifth most common cause of mortality in women, with estimated 522 000 deaths (6.4% of total deaths) (Siegel *et al*, 2016). Despite recent advancements in the treatment of cancer, therapeutic approaches have effects such as side effects, incompliancy of patients as well as the metastasis and resistance to various drugs, which may be challenging to specialists (Nicolay *et a*l., 2009; Remesh, 2012). Therefore, it is essential to develop efficient compounds that target cancer.

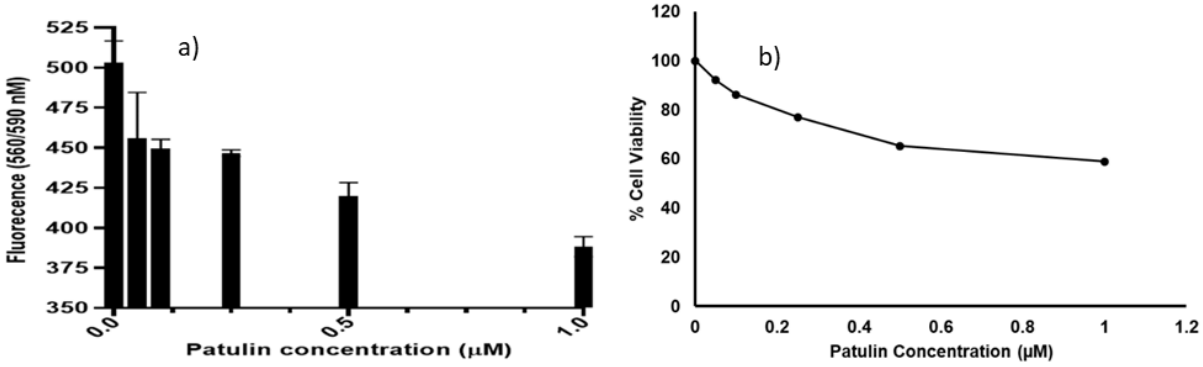

Figure 6-1: a) The fluorescence absorbance and b) the cell viability of MCF-7 breast cancer cells, following treatment with Patulin at concentrations of 0.05, 0.1, 0.25, 0.5, 1, and 5 µM

Patulin is a toxic metabolite produced by various fungal species with anticancer activity, however, its specific biological target remains unnoticeable (Puel *et al*., 2010). Few studies on the effect of patulin on mammalian cells, especially cancer cell lines, have been conducted. In this study, the toxic effect of patulin on MCF-7 was evaluated. Cells of a total concentration of 5000 were plated on a 96 well plate and the fluorescence was measured. The observed decrease in fluorescence (**Figure 6-1a**) indicates a high degree of reduction of resazurin to resarufin as the concentration of patulin increases. Reduction of resazurin to resorufin measures the metabolic activity of cells as a function of cell viability. Owing the fluorescent property of resarufin, viable cells are able to reduce resazurin to resorufin. Non-viable cells rapidly lose their metabolic capacity resulting in a decrease in fluorescence signal and cell viability.

Resazurin dye (7-hydroxy-3H-phonoxazin-3-one-10-oxide) is a reliable indicator of cell viability in proliferation and cytotoxic assays (Zouaoui *et al.*, 2016). Other than resazurin dye, 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) has been used to evaluate the cell viability, however, the resazurin assays have been reported to be more sensitive than the MTT assay (Abastabar *et al.,* 2017).

Previously studies indicates that patulin induces toxic effects on human cells such as human embryonic kidney 293 (HEK293) (Liu, 2006) and human immortalized keratinocyte as well as Chinese hamster ovary (CHO-K1) (Ferrer *et al*., 2009). Low cell viability was reported by Liu (2006) for human leukemia cell lines (HL-60)

that are exposed to 2 µm of patulin, Furthermore, Abastabar *et al.* (2017) have reported that, at a concentration level of 4 µm, patulin reduced the cell viability to 55 and 65 % on colonic adenocarcinoma cell lines (SW-48 cells) and human cervical cancer cell lines (HeLa), respectively. In this study, patulin reduced the cell viability of MCF-7 to 80 % at a concentration of 5 µm. However, this study demonstrated that the cell viability of human cancer cell lines decreases with increasing patulin concentration, which is in accordance with reported studies (Caiias and Aranda, 1996; Boussabbeh *et al.*, 2015; Zouaoui *et al.*, 2016) that reported that patulin induces cell death on human normal and cancer cell lines.

6.4 Conclusion

This study has demonstrated that patulin induces cell death on human cancer cell lines. Owing to its ability to decrease the growth of the cancer cell lines, patulin can be used as an anti-cancer agent.

6.5 Acknowledgments

The student would like to thank the Institute of Nanotechnology and Water Sustainability, the University of South Africa for the opportunity to undertake this research. The College of Agriculture and Environmental Science is duly acknowledged for its resources and the National Research Fund (MND190624450318) for the financial support.

6.6 References

Abastabar, M. *et al.* (2017) ‘In vitro antitumor activity of patulin on cervical and colorectal cancer cell lines’, *Current Medical Mycology*, 3(1), pp. 25–29.

Boussabbeh, M. *et al.* (2015) ‘Patulin induces apoptosis through ROS-mediated endoplasmic reticulum stress pathway’, *Toxicological Sciences*, 144(2), pp. 328–337..

Caiias, P. and Aranda, M. (1996) ‘Decontamination and Inhibition of Patulin-Induced Cytotoxicity’, 11(1 996), pp. 249–253.

Ferrer, E. *et al.* (2009) ‘Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells’, *Journal of Toxicology and Vitro*, 23(8), pp. 1504–1509.

IFT, I. of F. T. (2006) ‘Food mycotoxins’, *Journal of Food Science*, 71, pp. 1750–3841.

James, M. S. and Marion, J. F. (2007) ‘Health effects of molds in children’, *Pediatric Clinics of North America*, 54, pp. 309–333.

Liu, B.-H. (2006) ‘Mycotoxin Patulin Activates the p38 Kinase and JNK Signaling Pathways in Human Embryonic Kidney Cells’, *Toxicological Sciences*, 89(2), pp. 423–430.

Maenetje, P. W., De Villiers, N. and Dutton, M. F. (2008) ‘The use of isolated human lymphocytes in mycotoxin cytotoxicity testing’, *International Journal of Molecular Science*, 9, pp. 1515–1526.

Makun, H. A. *et al.* (2011) ‘Mycotoxins profiling of the culture material of Fusarium verticillioides (Sacc). Nirenberg culture (CABI-IMI 292668) isolated from rice in Niger State, Nigeria and its cytotoxic effect on human lymphocytes comparatively to those of some mycotoxin standard’, *African Journal of Biotechnology*, 10, pp. 12031–13038.

Nicolay, N. H., Berry, D. P. and Sharma, R. A. (2009) ‘Liver metastases from colorectal cancer: radioembolization with systemic therapy’, *Journal of National Review Clinical Oncology*, 6(12), pp. 687–697.

Puel, O., Galtier, P. and Oswald, I. P. (2010) ‘Biosynthesis and toxicological effects of patulin’, *Journal of Food Additives and Contaminants: part B*, 2(4), pp. 613–631.

Remesh, A. (2012) ‘Toxicities of anticancer drugs and its management’, *International Journal of Basic Clinical Pharmaceuticals*, 1(1), pp. 2–12.

Siegel, R. L., Miller, K. D. and Jemal, A. (2016) ‘Cancer statistics’, *Journal of*

Clinical Toxicology, 1, pp. 7–30.

Song, E. *et al.* (2014) 'Hepatotoxicity and genotoxicity of patulin in mice, and its modulation by green tea polyphenols administration', *Food and Chemical Toxicology*. Elsevier Ltd, 71, pp. 122–127.

Tanya, T. (2010) 'Carcinogenic crops: analyzing the effects of aflatoxin o global liver cancer rates', *Environmental Health perspective*, 118, pp. 258–258.

Whites, J. S. and Rees, K. R. (1981) 'Modification of the cytotoxicity of aflatoxin B1 in rats liver cells by steriods', *Journal of Chemical and Biological Interactions*, 1, pp. 85–93.

Zaki, M. (2012) 'Mycotoxins in animals: Occurrence, effects, prevention and management', *Journal of Toxicology and Environmental Health Sciences*, 4(1), pp. 13–28.

Zouaoui, N. *et al.* (2016) 'Cytotoxic effects induced by patulin, sterigmatocystin and beauvericin on CHO-K1 cells', *Food and Chemical Toxicology*, 89, pp. 92–103..

7 Chapter 7: Conclusion and recommendations

7.1 Conclusion

Due to the negative effects of mycotoxin contamination on human health, animal productivity, and trade, mycotoxin contamination has gained worldwide attention. This study aimed at investigating the effects of fungal and mycotoxin contamination on vegetable samples acquired from 5 provinces in South Africa and the cytotoxic effect of patulin on humans. This study was carried out with a view to contribute and expand knowledge on the occurrence of mycotoxins in various vegetables in South Africa. Although current literature is replete with studies on fungal and mycotoxin contamination of agricultural commodities, a need exists for further research to be undertaken on mycotoxin contamination in South African produced vegetables. Most of the work appears to have been focused on patulin contamination in apple and apple-based products both in South Africa and worldwide. Also, although enough information is available on the contamination of maize and cereals by aflatoxins, very little information on the contamination of vegetables exists. Hence there a need exists for the analysis of the co-occurrence of patulin and aflatoxins in vegetables.

Results emanating from this study have shown that mycotoxigenic fungal species isolated from the vegetable samples belong to the genera of *Aspergillus*, *Penicillium,* and *Fusarium*. Of the isolated fungi, *Aspergillus fumigatus* was the most dominated species found in vegetable samples. The results have also shown that the occurrence of patulin at levels lower than LOD in carrots and peppers. Furthermore, it was demonstrated that, under favorable laboratory conditions, the *Fusarium verticillioides* is capable of simultaneously producing Fumonisins B1 and B2. The occurrence of fungal species was, however, distributed across all 5 South African provinces. The occurrence of *Aspergillus fumigatus* in all the provinces suggests that the varying climatic conditions of the various provinces did not have a significant impact on the fungal contamination of vegetables.

Unfortunately, a lack of awareness exists amongst many South Africans on the effect of consuming contaminated products because very little attention has been given to mycotoxin-related research in the country. In rural areas, lack of knowledge and other socio-economic factors contribute to the problem of monitoring fungal and mycotoxin contamination because farmers often reserve the contaminated food commodities for their consumption while reserving high-quality food commodities for sale. The presence of patulin in the vegetable samples investigated in this study gives warrants the implementation of strategies for controlling fungal and mycotoxins contamination in vegetables from South Africa. Effective monitoring of fungal and mycotoxin contamination include safe handling of crops in the field, during transportation and post-harvesting stage by:

- Implementing proper agricultural and management practices to limit the damage of the crops by insects;
- Removing any visibly unhealthy crops to protect remaining healthy crops; and
- Storing agricultural commodities under appropriate conditions to avoid the accumulation of moisture and heat to minimize fungal infection and the concomitant production of mycotoxins.

Toxicity studies of patulin on human breast cancer cells using resazurin-based cell viability assay showed a reduction in viable cells. Although the results of this study demonstrated that patulin has the potential to induce cell death on breast cancer cell lines its consumption on a daily basis even at low levels could negatively affect human health. Thus there is the need to test the cytotoxicity of patulin on normal cell lines.

Due to the presence of patulin in vegetable samples, improved safe handling of South African vegetables in the field, during transportation, and at the post-harvest stage is important.

7.2 Recommendations and future work

Even though this work may be regarded to be effective, it leaves the need for further investigation. Some of the recommendations for future work include the

evaluation of fungal and mycotoxin contaminations on vegetable samples collected from Free State, Mpumalanga, Northern Cape, and North West province in South Africa. Other studies that can be done include the investigation of the effect of seasonal variation on the prevalence of patulin in South African vegetables. Furthermore, the cytotoxicity of patulin extracts on normal human cell lines using MTT assay and RTCA should be evaluated. The normal cell lines can be used as control.

8 Appendices

Appendix I: Raw data for the Ultra-High performance Liquid Chromatography analysis

Table AI-1: Mean peak area of each analyte at different concentration levels.

Concentration	**Mean peak area**				
	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2	Patulin
1.00	1353.011	3398.146	3350.659	4255.643	4255.643
2.00	5349.433	6376.024	6147.562	8177.154	8177.154
3.00	9193.201	8681.408	7500.111	11127.81	11127.81
4.00	14344.82	11291.81	10179.56	14356.72	14356.72
5.00	18578.56	13190.18	10964.21	16568.41	16568.41
6.00	23728.54	16156.17	13890.25	21537.14	21537.14
7.00	26859.27	17577.36	14183.88	22869.02	22869.02
8.00	31267.56	20684.33	17127.02	25506.24	25506.24
9.00	32778.82	23590.22	18810.6	28873.52	28873.52
10.0	33615.03	23594.41	19643.05	29979.76	29979.76

The concentration range for patulin was 1-10 µg/mL and Aflatoxins 1-10 ng/mL

Table AI-2: A representative of ANOVA analysis

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	20830151.7	20830152	80.44376	1.9E-05
Residual	8	2071524.435	258940.6		
Total	9	22901676.14			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	1304.34668	347.618937	3.752231	0.005606	502.736	2105.957	502.736	2105.957
X Variable 1	502.4805342	56.02386443	8.969045	1.9E-05	373.2893	631.6718	373.2893	631.6718

Df: Degree of freedom; SS: Sum of square; MS: Mean square; F: F-value

Table AI-3: The evaluation of the effect of mobile phase solvents on the peak areas of each analyte using Acetonitile as mobile phase solvent.

ANALYTES	PEAK 1	PEAK 2	PEAK 3	AVERAGE	STD DEV
AFLATOXIN B1	40468	40470	40470	40469.33333	0.942809042
AFLATOXIN B2	33467	33467	33469	33467.66667	0.942809042
AFLATOXIN G1	26284	33466	26284	28678	3385.627268
AFLATOXIN G2	29364	29365	29360	29363	2.160246899
PATULIN	69426.49	69426.31	69426.89	69426.56333	0.242395453

Table AI-4: The evaluation of the effect of mobile phase solvents on the peak areas of each analyte using methanol as mobile phase solvent.

ANALYTES	PEAK 1	PEAK 2	PEAK 3	AVERAGE	STD DEV
AFLATOXIN B1	4832.15	4832.89	4832.65	4832.563333	0.37753587
AFLATOXIN B2	2572.13	2572.13	2572.2	2572.153333	0.040414519
AFLATOXIN G1	2020.35	2020.56	2020.35	2020.42	0.121243557
AFLATOXIN G2	3341.9	3341.85	3341.89	3341.88	0.026457513
PATULIN	15440.13	15440.22	15440.22	15440.19	0.051961524

Table AI-5: recoveries obtained when using QuEChERS for extraction

ANALYTES	RECOVERY 1	RECOVERY 2	RECOVERY 3	AVG RECOVERIES	STD DEV
AFLATOXIN B1	105.09	105.06	105.03	105.06	0.03

AFLATOXIN B2	104.9	104.04	104.16	104.3666667	0.465761026
AFLATOXIN G1	76.9	77.5	77.6	77.33333333	0.37859389
AFLATOXIN G2	63.01	63.02	63.01	63.01333333	0.005773503
PATULIN	99.18	99.12	99.13	99.14333333	0.032145503

Table AI-5: recoveries obtained when using HLB Catridge

ANALYTES	**RECOVERY 1**	**RECOVERY 2**	**RECOVERY 3**	**AVG RECOVERIES**	**STD DEV**
AFLATOXIN B1	35.19	35.51	35.35	35.35	0.16
AFLATOXIN B2	47.89	47.71	46.99	47.53	0.476235236
AFLATOXIN G1	50.79	50.82	50.81	50.80666667	0.015275252
AFLATOXIN G2	43.69	43.55	43.99	43.74333333	0.224796204
PATULIN	12.19	12.31	12.55	12.35	0.183303028

Appendix II: Raw data for the cytotoxicity of patulin standard on human cancer cell lines

Table II-2: Fluorescence reading and correlated absorbance following treatment after 24 hours

Drug concentration (uM)	**Florescence**	**Asample - Ablank**	**StdD**
Media only	608.1106	503.3413	13.23535

0.05	560.3736	455.6043	28.92084
0.1	554.1770	449.4077	5.91584
0.25	551.3180	446.5487	2.101924
0.5	524.3493	419.5800	8.681945
1	492.9886	388.2193	6.287432
5	488.1586	383.3893	8.566765

Table AII-2: viability of cells at different concentrations of patulin

Average	**Patulin concentration (uM)**	**% cell viability**
A	Negative Control (0.0 uM)	100
B	0.05	92.15
C	0.1	86.23
D	0.25	77.01
E	0.5	65.27
F	1	59.01
G	5	58.69