

Doctoral Dissertation (Shinshu University)

**Development, Validation, and Application of  
Methods for Analysis of Fungal Contamination  
and Presence of Mycotoxins in Grains**

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## Abbreviations

AFB <sub>1</sub>	aflatoxin B <sub>1</sub>
AFB <sub>2</sub>	aflatoxin B <sub>2</sub>
AFG <sub>1</sub>	aflatoxin G <sub>1</sub>
AFG <sub>2</sub>	aflatoxin G <sub>2</sub>
AFs	aflatoxins
APCI	atmospheric pressure chemical ionization
CAST	Council for Agricultural Science and Technology
EU-RL	European Union Reference Laboratory
EC	European Commission
EFSA	European Food Safety Authority
ERG	ergosterol
EU	European Union
FAO	Food and Agricultural Organization
FL	fluorescence
FUMs	fumonisin
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
IAC	immunoaffinity column
IARC	International Agency for Research on Cancer
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
OCI	on-column injector
ODS	octadecylsilyl
OTA	ochratoxin A
PB	phosphate buffer
PBS	phosphate buffer saline

PTV	programmable temperature vaporizer
RSD	relative standard deviation
RSDr	relative standard deviation of repeatability
RSDRi	relative standard deviation (intermediate precision)
S/N	signal/noise
STC	sterigmatocystin
TRs	trichothecenes
UV	ultraviolet
ZEA	zearalenone

## Chapter 1: General introduction

### 1.1. Mycotoxigenic fungi and their products

Cereals are the staple food for most humans, and they are an important feed ingredient for livestock. Food and Agricultural Organization (FAO, 2013) estimated that approximately 2.3 billion tons of cereals are produced worldwide annually. Within that, roughly 1 billion tons of cereals are used for human food, 750 million tons are used for animal feed, and the remaining 500 million tons are processed for industrial use. Maize is the most widely produced cereals in the world, followed by rice and wheat. In terms of dietary intake, maize ranks third after rice and wheat, owing to its extensive use as animal feed (FAO, 2013).

Grains can be invaded by mycotoxigenic fungi before harvest, the time between harvest and drying, and during storage (CAST, 2003). Major mycotoxigenic genera such as *Aspergillus*, *Penicillium*, and *Fusarium* often contaminate agricultural commodities. These fungal species produces the primary metabolite ‘ergosterol’ and toxic secondary metabolites, which are collectively called ‘mycotoxins.’ Approximately 25 % of the world’s agricultural crops are contaminated with mycotoxins (Charmley *et al.*, 1995). However, the growth of fungi solely depends on climatic conditions, and the ability of fungi to produce toxin is greatly influenced by temperature, relative humidity, insect attack, and stress caused to the plants (Miraglia *et al.*, 2009). Among more than 300 known mycotoxins, the agriculturally important ones are aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FUMs), trichothecenes (TRs), and zearalenone (ZEA). Other important mycotoxins are citrinin, cyclopiazonic acid, sterigmatocystin (STC), and patulin. Chemical structures of common mycotoxins and ergosterol are shown in Fig. 1-1. The major mycotoxins produced by different fungi on agricultural commodities, with their toxic effects, are also shown in Table 1-1.

#### 1.1.1 Aflatoxins

AFs are among the most studied mycotoxin groups since their discovery in the 1960s. They were first identified as a probable toxin that killed more than 100,000 turkeys

(Turkey 'X' disease) in UK (Blount, 1961). Owing to their hazardous nature, AFs are still dominate in mycotoxin research. AFs are produced by *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis*, and *A. pseudotamarii* (Kurtzman *et al.*, 1987; Payne, 1998; Ito *et al.*, 2001; Paterson *et al.*, 2001). AFs are difuranocoumarin derivatives, and more than 20 derivatives are known (Hussein and Brasel, 2001; Papp *et al.*, 2002). Among them, there are four major naturally occurring AFs: AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub>. AFB<sub>1</sub> is the most abundant, and toxicity decreases in the following order: AFB<sub>1</sub>, AFG<sub>1</sub>, AFB<sub>2</sub>, and AFG<sub>2</sub>. The B designation of AFs is due to the exhibition of bluish fluorescence, whereas the G designation refers to a greenish fluorescence under UV light on thin layer chromatography (Sweeney and Dobson, 1998; Bennett and Klich, 2003). Another two metabolites, Aflatoxin M<sub>1</sub> and M<sub>2</sub>, are significant due to their direct contamination of food and feeds. The M designation was given because of the first isolation of these metabolites was from milk of lactating animals fed with AFs-contaminated feed. AFs are heat stable, and are usually not affected by cooking or pasteurization (Reddy and Waliyar, 2000).

#### 1.1.1.1. Occurrence

AFs contamination have been observed with different types of agricultural commodities such as maize, wheat, rice, cottonseed, peanut, figs, tree nuts, copra, milk, eggs, and cheese. Grains stored under high moisture or humidity at warm temperatures have a great chance of AFs production.

#### 1.1.1.2. Effects on human and animal health

AFs are mycotoxins of great importance in food and feed owing to their extremely high potency (Sweeney and Dobson, 1998). AFs have been known to have hepatocarcinogenic, mutagenic, teratogenic, and immunosuppressive effects in humans and animals (Moreno and Kang, 1999; Peraica *et al.*, 1999; Pitt, 2000; Hussein and Brasel, 2001; Papp *et al.*, 2002). AFB<sub>1</sub> (most potent mycotoxin) is classified as a group 1 carcinogen (carcinogen to humans) by the International Agency for Research on Cancer (IARC) (1993). Studies have linked dietary AFs and primary liver cancer in humans in several countries such as Kenya, Mozambique and China (Peers and Linsell, 1973; Van

Rensburg *et al.*, 1985; Li *et al.*, 2001; Casado *et al.*, 2001). In cattle, acute and chronic aflatoxicosis have been described with symptoms such as feed inefficiency, weight loss, reduced milk production, immunosuppression, and liver damage (Bodine and Mertens, 1983). In the poultry industry, aflatoxicosis causes severe economic losses. Clinical signs are anorexia, decreased weight gain and egg production, hemorrhage, and embryotoxicity (Edds and Bortel, 1983; CAST, 2003).

### 1.1.2. Ochratoxin A

OTA is a nephrotoxin primarily produced by *P. verrucosum*, *A. ochraceus* and *A. carbonarius*. OTA was first isolated from an *A. ochraceus* culture by South African scientists (van der Merwe *et al.*, 1965a, and b). There are three types of naturally occurring ochratoxins: OTA, OTB, and OTC. Among them, OTA was considered most toxic and more widely detected than OTB and OTC (van der Merwe *et al.*, 1965a).

#### 1.1.2.1. Occurrence

OTA is considered as a post-harvest mycotoxin rather than an in-field contaminant of grains. The occurrence of OTA has been reported in maize, wheat, sorghum, oats, rice, wine, beer, and green coffee (CAST, 2003). Barley, oats, wheat, and corn grown in Scandinavian countries, the Balkans and India have the highest incidence and level of OTA contamination. Animal feeds may be contaminated with high levels of OTA in Canada and European countries (Jelinek, 1987). Approximately 50 % of the dietary OTA intake in Europe comes from cereals and cereal-based products (Battaglia *et al.*, 1996).

#### 1.1.2.2. Effects on human and animal health

OTA has been associated with Balkan endemic nephropathy (Krogh *et al.*, 1977), and classified as a group 2B carcinogen (possible human carcinogen) by the IARC (1993). The major toxic effects of OTA are protein synthesis inhibition (Creppy *et al.*, 1984) and DNA single-strand breakages with a later stage of genotoxicity and carcinogenicity (Pfohl-Leszkowicz and Manderville, 2007). Immunotoxic effects of OTA have also been demonstrated by Pfohl-Leszkowicz and Manderville (2007). OTA contamination has



been implicated in nephrotoxicity and histological evidence of renal damage has been observed in pigs (Riley and Petska, 2005; Stoev *et al.*, 1998). Symptoms of ochratoxicosis in swine are anorexia, faintness, uncoordinated movements, increased water intake, and frequent urination (Glavitis and Vanyi, 1995). In poultry, OTA exposure reduces gluconeogenesis and leads to glycogen accumulation in the liver (JECFA, 2001). At lower levels of OTA exposure, pigs and poultry show decreased feed consumption and slower weight gain, and OTA-induced immunosuppression that leads to infections (Stoev *et al.*, 2000a and b).

### 1.1.3. Zearalenone

ZEA is non-steroidal estrogenic mycotoxin produced mainly by *F. graminearum* (*Gibberella zeae*) and *F. culmorum*; and it is a common contaminant of cereal crops worldwide (Bennett and Klich, 2003). ZEA is a resorcyclic acid lactone (6-[10-hydroxy-6-oxy-trans-1-undecenyl]- $\beta$ -resorcyclic acid lactone). ZEA derivatives such as  $\alpha$ -zearalenol,  $\beta$ -zearalenol,  $\alpha$ -zearalanol, and zearalanone can be found in corn stems contaminated with *Fusarium* in the field (Bottalico *et al.*, 1985).

#### 1.1.3.1. Occurrence

ZEA-producing fungi primarily contaminate corn, and to a lesser extent, barley, oats, wheat, sorghum, millet and rice (Zenedine *et al.*, 2007). Cereal products such as flour, malt, and beer may also be contaminated with ZEA. Moisture content (22-25 %) or delayed harvest may influence ZEA production in maize (Abbas *et al.*, 1988). *Fusarium* infected cereals are not only contaminated with ZEA in the field but also during improper storage (Kuiper-Goodman *et al.*, 1987). The co-occurrence of ZEA with other *Fusarium* toxins such as TRs and FUMs is commonly found in cereal grains and animal feed.

#### 1.1.3.2. Effects on human and animal health

US National Institute of Health (1982) was reported firstly about the capability of ZEA to induce liver lesions in rats and mice, with the development of hepatocarcinomas. Due to its carcinogenic nature, ZEA has been evaluated by the IARC (1999) and classified as a

group 3 carcinogen (not classifiable as carcinogenicity to humans). Studies have demonstrated that ZEA stimulates the growth of human breast cancer cells (MCF-7) through estrogen receptors (Ahamed *et al.*, 2001) and increases the occurrence of human breast cancer (Yu *et al.*, 2005). ZEA also shows genotoxic effects inducing DNA-adduct formation in *in vitro* cultures of bovine lymphocytes (Lioi *et al.*, 2004). ZEA has adverse effects on reproductive systems due to its ability to bind estrogenic receptors. In animals, swine are more sensitive to ZEA than cattle, poultry and rodents (Etienne and Jemmali, 1982). Calves show earlier sexual maturity, and cows suffer from vaginitis, prolonged estrus and/or infertility (Zenedine *et al.*, 2007).

#### 1.1.4. Stergimatozystin

STC is a polyketide mycotoxin, produced mainly by *A. versicolor* and *A. nidulans* (EFSA: European Food Safety Authority, 2013). Among these fungal species, *A. versicolor* is the most common source of STC production. STC is structurally similar to carcinogenic AFs and they share the same biosynthetic pathway (Sweeney and Dobson, 1998). Owing to structural and toxic effects that are similar to effects of AFs, STC is becoming a matter of concern in mycotoxin research.

##### 1.1.4.1. Occurrence

STC is known to contaminate grain and grain-based products during post-harvest conditions (EFSA, 2013). STC has also been reported on green coffee beans, pistachio nuts, spices, beer, and cheese (Versilovskis and De Saeger, 2010).

##### 1.1.4.2. Effects on human and animal health

Acute toxicity, immunotoxicity, genotoxicity, and carcinogenicity of STC have been recently reviewed by EFSA (2013). Like AFs, STC is considered as a hepatocarcinogen, and IARC (1987) classified STC as a group 2B carcinogen. Various studies from Asia, China in particular, have been conducted to correlate STC and the prevalence of gastric and liver cancer. A recent article from Hutanasu *et al.* (2011), who studied 166 human patients with liver cancer reported a correlation between a tumor marker (alfa-fetoprotein)

and STC in the liver cancer group. Feed contaminated with STC is associated with acute symptoms of bloody diarrhea, loss of milk production and death in dairy cattle (Versonder and Horn, 1985). Symptoms in pigs, with STC containing feed show reduce feed intake, diarrhea, and alteration in blood biochemical parameters (Kovalenko *et al.*, 2011). In poultry, STC is hepatotoxic and nephrotoxic. Although STC is genotoxic and carcinogenic like AFs, its presence in foodstuffs and the data for risk of exposure remain insufficient to characterize the risk presented by STC for human and animal health.

#### *1.1.5. Ergosterol (ERG)*

ERG is a sterol component specifically found in fungal cell membranes, but absent or present only in minor amounts in higher plants (Weete, 1980) (Fig. 1-1). Griffiths *et al.* (2003) found that ERG is the major sterol found in fungi, representing 95 % of their total sterols. ERG is better correlated with fungal biomass than traditional fungal colony forming unit count (Schnurer, 1993) which measures only viable fungi. Moreover, ERG is more sensitive than another fungal biomarker 'chitin' for the detection of early stage fungal growth (Matcham *et al.*, 1985).

ERG can be considered as a potential tracer of fungal invasion (Seitz *et al.*, 1977) and possible mycotoxin contamination (Seitz *et al.*, 1979; Saxena *et al.*, 2001) in grains. The correlation between ERG and mycotoxin contamination is not always absolute, as environmental factors such as temperature and humidity play a vital role for fungal proliferation and mycotoxin production. However, Pietri *et al.* (2004) reported that the quality of maize is acceptable if the level of ERG content is less than 3 mg/kg. There is a high possibility of fungal invasion and mycotoxin contamination if the level of ERG content exceeds more than 3 mg/kg.

## **1.2. Mycotoxin regulations**

Mycotoxins exert many adverse effects on human and animal health, including acute toxicity, mutagenicity, carcinogenicity, teratogenicity, immuno-toxicity, and estrogenic effects (CAST, 2003). Many countries have regulations for mycotoxins to protect consumers from their harmful effects. However, a variety of factors are involved in

setting regulatory limits for mycotoxins. Regulatory limits are chosen based on several important factors such as: a) availability of toxicological data, b) availability of mycotoxin contamination data, c) homogeneity of the concentration in a lot, d) availability of analytical methods, e) regulation in other countries where trade contracts exist, and f) the need for sufficient food supply (CAST, 2003). Approximately, 100 countries (covering 85 % of world inhabitants) had regulations/guidelines for mycotoxins by the end of 2003 (van Egmond, 2007), up from 33 countries in 1981 (Schuller *et al.*, 1983).

Regulations for mycotoxins in food and feed have been reviewed and published (Schuller *et al.*, 1983; van Egmond, 1989; FAO, 1997; FAO, 2004). FAO Food and Nutrition Paper 81 reviewed worldwide regulatory limits for various commodities, legal and responsible bodies, sampling and analysis methods and regulatory situations. Of all the mycotoxins, AF is the most regulated, and more than 100 countries have enacted regulation for AFs. Other mycotoxins that are regulated worldwide are TRs (dioxynivanelol, diacetoxyscirpenol, T-2 and HT-2 toxin), ergot alkaloids, FUMs (FUM B<sub>1</sub> and B<sub>2</sub>), phimopsins, OTA, patulin, STC and ZEA (van Egmond, 2007).

As mycotoxin contamination is a global concern, several economic communities and countries have harmonized mycotoxin regulations. In the EU, mycotoxin regulation not only set regulatory limits for foodstuffs but also evaluated risk based on exposure data of certain mycotoxins. EFSA is a regulatory body of the European commission, and give scientific opinions after gathering data and performing detailed risk assessment. Another EU activity is SCOOP (Scientific Co-operation on Questions relating to Food). SCOOP provides a scientific basis for evaluating dietary exposure to mycotoxins and for managing risk of exposure, after taking into account occurrence and consumption. In addition, EU assigned Joint Research Center's Institute for Reference Materials and Measurements (JRC-IRMM, Geel, Belgium) as a European Union Reference Laboratory (EU-RL) for mycotoxins. EU-RL has specified its tasks, duties and requirements of EU-RL in relation to food, feed, and animal health. The trend for mycotoxin regulation and limits have increased for many countries in the last decade. Owing to potential human and animal health hazards, many countries from the developing world have instituted

regulations for certain mycotoxins. Table 1-2 shows the maximum levels of AFs, OTA and ZEA in cereals set by European Commission (EC, 2006).

### **1.3. Analysis methods for fungal metabolites**

Analytical methods for fungal metabolites/mycotoxins usually involve three major steps: extraction, clean-up, and detection/determination of the toxins. During extraction, the toxins are separated from the solid sample and rendered into a liquid phase. The purpose of clean-up is to remove interfering compounds from the extract and to concentrate the toxins. Among clean-up techniques, immunoaffinity column (IAC) is selected in order to determine a single or a limited number of toxins in complex food or feed extracts containing potential interferences (Senyva and Gilbert, 2010). It produces 'clean' extracts than does solid phase or multifunctional column clean-up (Bradburn *et al.*, 1995; Sugita-Konishi *et al.*, 2006). Recently, commercial IACs are available for few mycotoxins such as AFs, OTA, deoxynivalenol, ZEA, T-2 and HT-2, FUMs and citrinin (Senyuva and Gilbert, 2010).

Different chromatographic methods are used for the determination of fungal metabolites/mycotoxins using gas chromatography (GC) with electron capture, flame ionization or mass spectrometry (MS) detector, and high performance liquid chromatography (HPLC) with ultraviolet (UV), fluorescence (FL) or MS detector. GC has been used for determining the presence of ergosterol in grains (Lamper *et al.*, 2000; Dong *et al.*, 2006). After the introduction of GC in the 1970s, GC was initially used for mycotoxin analysis, especially for the type A and B trichothecenes. In case of GC analysis, samples need to be sufficiently volatile at the column temperature or they need to be converted into volatile derivatives by a derivatization step. GC-MS methods have been reported for several mycotoxin determinations (Onji *et al.*, 2002; Tanaka *et al.*, 2000; Olsson *et al.*, 2002; Cunha *et al.*, 2010; Kharandi *et al.*, 2013).

HPLC is one of the most widely used analytical method for fungal metabolite or mycotoxin analysis. As most of the mycotoxins are small and polar compounds, analysis methods are based on reverse phase HPLC separation. A number of mycotoxins have fluorescence (e.g. AFs, OTA, citrinin) that can be detected by fluorescence detector.

Those that do not have chromophores (e.g. FUMs) require derivatization. Several mycotoxins have been analysed for single or multi-mycotoxins using the HPLC method in grains (Pietri *et al.*, 2004; Neuhof *et al.*, 2008; Ibanez-Vea *et al.*, 2011 and 2012; Iqbal *et al.*, 2014). In addition, ERG has also been widely reported by HPLC analysis in grains (Pietri *et al.*, 2004; Jedlickova *et al.*, 2008; Miyagawa *et al.*, 2009; Sasaki *et al.*, 2011).

In the last decade, liquid chromatography coupled with MS detector has become more popular than other traditional detection methods for the analysis of mycotoxin because of its higher sensitivity and selectivity. It eliminates the need for sample derivatization before injection, and provides confirmation of mycotoxins of interest according to their structural and molecular mass (Songsermsakul and Razzazi-Fazeli, 2008). However, accuracy, precision and sensitivity may also vary depending on mycotoxins, matrices and instruments. However, purification of a sample by multifunctional clean-up columns or IAC is usually needed before LC-MS injection to avoid matrix effects and ion suppression (Lattanzio *et al.*, 2007). Several LC-MS methods have been developed for the determination of different mycotoxins and ERG in grains (Scudamore *et al.*, 1996; Rosenberg *et al.*, 1998; Razzazi-Fazeli *et al.*, 1999; Palloroni and von Holst, 2003; Takino *et al.*, 2004; Varga *et al.*, 2006).

## Objectives

This thesis was focused on the development and validation of analytical methods for fungal and mycotoxin contamination in grains. The main purpose of this research was to develop sensitive and reliable analytical methods to determine four carcinogenic mycotoxins (AFs, OTA, ZEA and STC) and a fungal specific biomarker 'ERG'.

The first objective was the development and validation of STC analysis methods on grains. Unlike carcinogenic AFs, STC has not been studied well owing to the lack of sensitive and reliable analysis method for agricultural commodities. Therefore, an analysis method using an immuno-affinity column (IAC) clean-up followed by LC-MS and GC-MS determination were studied.

The second objective was the development of a rapid analytical method for ERG determination in grains using GC-MS with on-column injection. The developed method is rapid, sensitive, reliable, and could be useful to the grain industry.

The third objective was the development of a simultaneous method for AFs, OTA and ZEA determination using HPLC-FL. The simultaneous analysis method is very useful to monitor these three agriculturally important mycotoxins. After development of this method, the relationship between ERG content and mycotoxin (AFs, OTA and ZEA) contamination in maize was also studied.

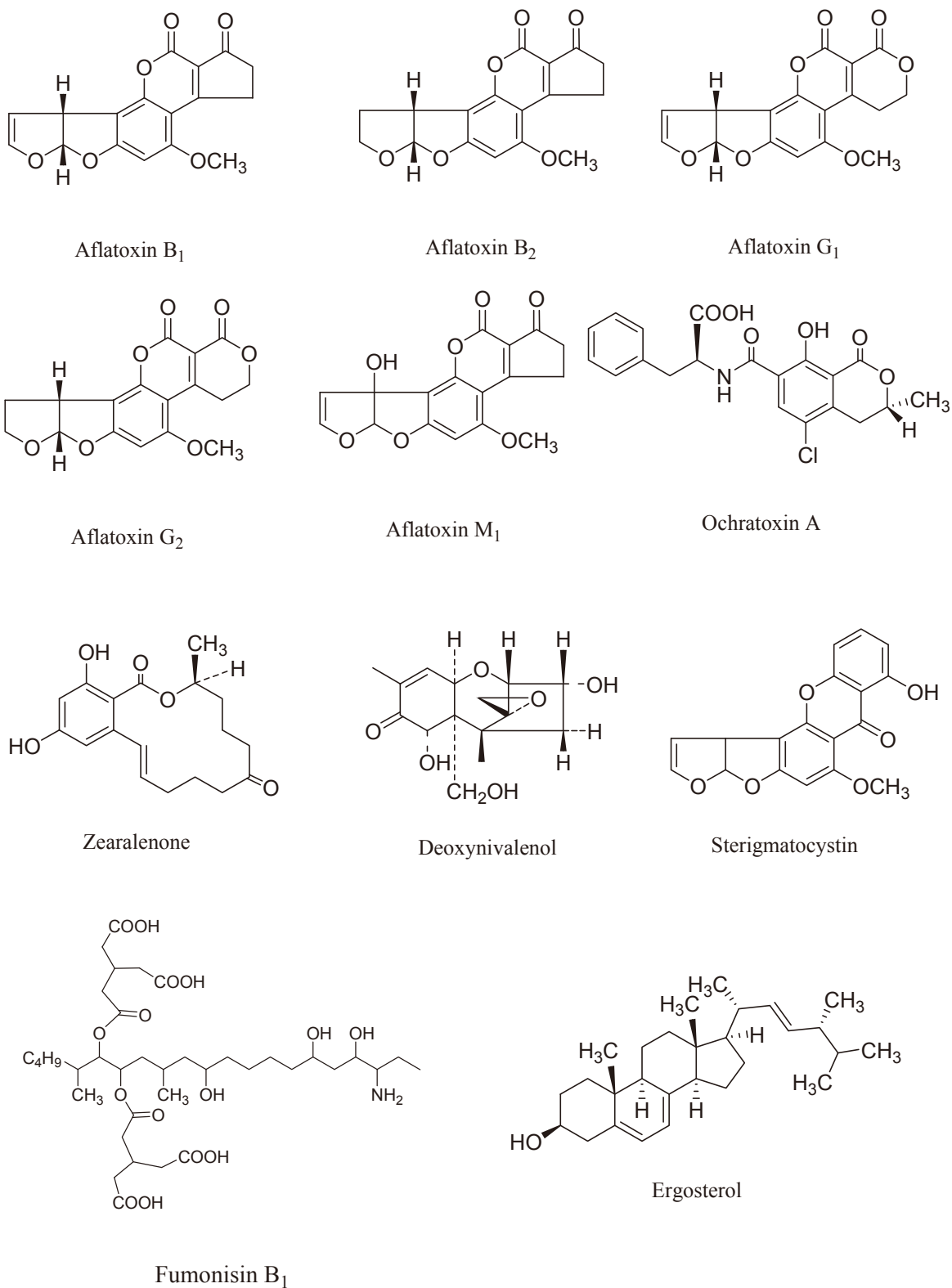


Fig. 1-1. Chemical structures of common mycotoxins and ergosterol.



Table 1-1 Mycotoxins contaminated commodities with different fungal species, and toxic effects on human and animal health (compiled from CAST, 2003)

Mycotoxins	Commodities	Fungi species*	Effects on human and animal health
Aflatoxins (AFs)	peanuts, maize, wheat, rice, nuts, cotton seed, milk, cheese	<i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. nomius</i>	Carcinogenesis Hepatotoxicity Bile duct hyperplasia Intestinal or kidney hemorrhage
Fumonisin (FUMs)	maize	<i>F. verticilloides</i> <i>F. proliferatum</i>	Pulmonary oedema Leukoencephalomalacia Hepatotoxicity Nephrotoxicity
Ochratoxin A (OTA)	wheat, barley, oats, maize, peanuts, bean, cheese	<i>A. ochraceus</i> <i>A. niger</i> <i>A. carbonarius</i> <i>P. verrucosum</i>	Carcinogenesis Nephrotoxicity Porcine nephropathy Teratogenesis Enteritis
Sterigmatocystin (STC)	maize, wheat, rice, barley, beer, cheese	<i>A. versicolor</i>	Carcinogenesis Hepatotoxicity
Trichothecenes (TRs)	maize, wheat, barley, oats	<i>F. graminearum</i> <i>F. culmorum</i> <i>F. poae</i> , <i>F. sporotrichioides</i>	Digestive disorders Reduced weight gain Hemorrhage Edema Dermatitis Immunosuppression
Zearalenone (ZEA)	maize, wheat, moldy hay, pelleted feed	<i>F. graminearum</i> <i>F. culmorum</i>	Estrogenic effects Atrophy of testicles Atrophy of ovaries Enlargement of breast Abortion

\*A: *Aspergillus*; F: *Fusarium*; P: *Penicillium*

Table 1-2 Regulations for mycotoxins on cereals set by European Commission (2006)

Mycotoxins	Cereals	Maximum levels ( $\mu\text{g}/\text{kg}$ )
Aflatoxins	All cereals and all products derived from cereals, including processed cereal products, with the exception of maize, processed food for infants and children, and dietary food for medical purpose	AFB <sub>1</sub> : 2 $\mu\text{g}/\text{kg}$ AFs (total): 4 $\mu\text{g}/\text{kg}$
	Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	AFB <sub>1</sub> : 5 $\mu\text{g}/\text{kg}$ AFs (total): 10 $\mu\text{g}/\text{kg}$
Ochratoxin A	Unprocessed cereals	5 $\mu\text{g}/\text{kg}$
	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	3 $\mu\text{g}/\text{kg}$
Zearalenone	Unprocessed cereals other than maize	100 $\mu\text{g}/\text{kg}$
	Unprocessed maize	200 $\mu\text{g}/\text{kg}$
Sterigmatocystin*	Foodstuffs	5-20 $\mu\text{g}/\text{kg}$

\* The Czech Republic and Slovakia had legislation for STC before entering EU.

## Chapter 2: Analytical methods development for sterigmatocystin determination in grains using LC-MS and GC-MS after immunoaffinity column purification

### 2.1. Introduction

Sterigmatocystin (STC; 3a, 12c-Dihydro-8-hydroxy-6-methoxy-7*H*-furo [2, 3-*c*] xanthen-7-one) is a toxic secondary metabolite produced by several fungal species of the genus *Aspergillus*, as well as those of the genera *Emericella*, *Chaetomium*, *Botryotrichum*, and *Humicola* (EFSA, 2013). Among these species, *A. versicolor* is the major producer of STC. *A. versicolor* is a xerophilic fungi, and can grow at low water activity (< 0.8). The optimum temperatures of STC producing fungi are between 23 to 29 °C, and the moisture content above 15 % (Versilovskis and De Saeger, 2010). Physically, STC can readily soluble in methanol, ethanol, acetonitrile, benzene and chloroform. The molecular mass and melting point of STC are 324.284 g/mol and 246 °C, respectively. Unlike AFs, STC has weak fluorescence (Maness *et al.*, 1976).

Grains and grain-based products are often contaminated with STC-producing fungi during the storage, transport, and processing stages (EFSA, 2013). In addition to its occurrence in grain and grain-based products (Sugimoto *et al.*, 1977; Scudamore and Hetmanski, 1995; Scudamore *et al.*, 1996; Versilovskis *et al.*, 2007; Versilovskis *et al.*, 2008a; Versilovskis and Bartkevics, 2012), STC is also found in rice (Sugimoto *et al.*, 1977), coffee beans (Bokhari and Aly, 2009), peanuts (Youssef *et al.*, 2008), spices (Saxena and Mehrotra, 1989; El-Kady *et al.*, 1995), beer (Versilovskis *et al.*, 2008b), and cheese (Abd Alla *et al.*, 1996; Scudamore *et al.*, 1996; Versilovskis *et al.*, 2009). In the UK, Scudamore and Hetmanski (1995) found 17 % of 46 samples contaminated with STC in poorly stored wheat, barley and oats. Versilovskis *et al.*, (2008a) reported that 26 % of the 215 analysed different Latvian grains (wheat, oats, ryes, barley and buckwheat) samples contaminated with STC, with concentration ranged from 0.7 to 83 µg/kg. STC was found in 7 % of 30 coffee beans from Saudi Arabia with concentration of 11 and 13 µg/kg (Bokhari and Aly, 2009). In Egypt, STC was detected in 15 % of roasted (12.2-16.8 µg/kg), and 5 % of roasted and salted nut (12.2 µg/kg) samples (Youssef *et al.*, 2008). In India, Sexana and Mehrotra (1989) reported the occurrence of STC on 15

different spices, where one fennel sample was detected positive (142 µg/kg) and two black pepper samples was detected positive (105 and 125 µg/kg). STC was found in 8 % of 26 beer samples (dark and light) from Latvia with concentration of 7.8 µg/L and 4.0 µg/L. STC is frequently found on hard cheeses that become infected with *A. versicolor* during production and ripening. Versilovskis *et al.*, (2009) analysed Latvian and Belgian cheeses, and detected 50 % of 8 Latvian cheese samples and 15 % of 13 Belgian cheese samples. Moreover, STC was also found in carpet dust from damp indoor environment (Engelhart *et al.*, 2002)

STC has carcinogenic properties (Purchase and van der Watt 1970) and shares its biosynthesis pathway with AFs (Sweeny and Dobson, 1998) as shown in Fig. 2-1. IARC (1976 and 1987) designated STC as a group 2B carcinogen. Furthermore, STC is genotoxic (Sekijima *et al.*, 1992; Wehner *et al.*, 1978). Currently, no countries have enacted official regulation governing the monitoring of STC contamination. The Czech Republic and Slovakia had legislated a level of 5–20 µg/kg for foodstuffs (FAO, 2004) before entering the European Union (EU). After recognising STC as a highly toxic compound, the California Department of Health Services issued a “no significant risk” STC intake level guideline for humans of 8 µg/kg body weight/day (EMAN, 2014). Owing to the limited information about the occurrence of STC and dietary risk exposure assessment, the EFSA (2013) panel recommended that more data should be collected on the occurrence of STC, and sensitive analytical methods should be developed to analyse STC content in food and feed across the EU.

STC detection in food and feed is performed by immunochemical or chromatographic analysis (Table 2-1). Li *et al.*, (1996) developed a sensitive monoclonal antibody for STC and applied it in an enzyme-linked immunosorbent assay (ELISA) for STC quantification in wheat. Among chromatographic methods, thin layer chromatography has been widely used as a technique for STC analysis (AOAC, 2005; Versilovskis and De Saeger, 2010). A few studies have been conducted using high performance liquid chromatography (HPLC) for STC determination (Schmidt *et al.*, 1981; Hurst *et al.*, 1987; Scudamore *et al.*, 1998; Tangni and Pussemier, 2007; Versilovskis *et al.*, 2008b). As STC has weak fluorescence, mass spectrometric detection (LC-MS/GC-MS) is preferable as a method to

monitor STC contamination at low levels. An STC determination method using liquid chromatography-mass spectrometry (LC-MS) with atmospheric pressure chemical ionization (APCI) was developed by Scudamore *et al.*, (1996) at a limit of detection (LOD) of 2 µg/kg in grains.

In a review, Versilovoskis and De Saeger (2010) reported that STC analysis is often extracted by acetonitrile, methanol, and 4 % potassium chloride solution, followed by clean-up with solid phase extraction. Although immunoaffinity column (IAC) is available for AFs, OTA, deoxynivalenol, but no commercial IAC has been developed for STC yet (EFSA, 2013). As STC is structurally similar to AFs, several IAC for AFs were tested for cross-reactivity. Among these IACs, a commercially available IAC, AFLAKING<sup>®</sup> manufactured by HORIBA (Japan) showed affinity for STC. So, at first an IAC clean-up method for STC was developed. According to EFSA (2013), LC-MS method showed the lowest limit of detection for STC, a single laboratory validation of STC determination was performed in grains using LC-MS after developing IAC clean-up.

However, compare to LC-MS, GC-MS operation is simpler and less expensive than LC-MS. In the history, STC was analysed by GC in the 1970s. However, published previous methods are few, not completely validated, and not sensitive enough for practical applications to monitor STC (Salhab *et al.*, 1976; Tanaka *et al.*, 2007). Owing to huge popularity of liquid chromatography in the last few decades, GC-MS based sensitive and reliable method for STC determination has not been developed and validated yet. Therefore, a GC-MS analytical method with on-column injection was developed and validated for STC in grains for the first time. Traditionally, GC-MS generally used split and splitless type injection, and requires sample derivatization prior to injection. As STC is not stable compound during heating, on-column injection was used to avoid STC decomposition and time consuming derivatization step. Therefore, the objectives of this study were to develop an IAC clean-up method for STC, and to determine STC in grains using mass spectrometric analysis method (LC-MS and GC-MS) separately.

## 2.2. Materials and methods

### 2.2.1. Chemicals

STC and AFs (AFB<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile, LC-MS-grade acetonitrile and methanol, spectroscopy-grade acetone, and formic acid were purchased from Wako Pure Chemical (Osaka, Japan). The IAC (AFLAKING<sup>®</sup>) was purchased from HORIBA (Kyoto, Japan). Ultrapure water was prepared using a water purification system (Autopure WT100 Yamato, Tokyo, Japan). All other chemicals were purchased from Kanto Chemical (Tokyo, Japan). Helium (99.9999 %) used as a carrier gas for GC-MS analysis, was obtained from Okaya Sanso (Nagano, Japan).

### 2.2.2. Preparation of standard solutions

STC stock solution (200 µg/mL) was prepared by dissolving 5 mg of STC powder in 25 mL of acetonitrile. For LC-MS analysis, STC working solutions of 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, and 10 µg/mL were prepared by diluting stock solution with acetonitrile. AFs stock solution (5.0 µg/mL for AFB<sub>1</sub> and AFG<sub>1</sub>, 1.5 µg/mL for AFB<sub>2</sub> and AFG<sub>2</sub>) was prepared by dissolving mixtures of 25 µg AFB<sub>1</sub> and AFG<sub>1</sub> powder and 7.5 µg AFB<sub>2</sub> and AFG<sub>2</sub> powder in 5.0 mL acetonitrile. AFs working solution (1.0 µg/mL for AFB<sub>1</sub> and AFG<sub>1</sub>, 0.3 µg/mL for AFB<sub>2</sub> and AFG<sub>2</sub>) was prepared by diluting stock solution with acetonitrile. To spike samples with STC, 250 µL of an appropriate concentration of STC working solution in acetonitrile (0.5, 1.0, 2.5, 5.0, and 10 µg/mL) was added to 25 g of ground samples. After spiking, samples were mixed and kept at 4 °C overnight in the dark.

For GC-MS analysis, working standard solutions of STC at concentrations of 0.01, 0.02, 0.03, 0.05, 0.075, 0.01, and 0.15 µg/mL were prepared by diluting the stock solution with acetone. All solutions were stored at -20 °C until they were used. For spiking purposes, 250 µL of STC working solution in acetonitrile at a concentration of 2.5 µg/mL was added to 25 g of ground grain samples. After STC spiking, the grain samples were mixed and kept at 4 °C overnight.

### 2.2.3. Samples

Maize, wheat, rice (polished and unpolished), barley and buckwheat samples used in this study were purchased from a retail market in Japan. Samples were ground by a Roter Mill (Pulverisette 14, Fritch, Germany) through a 0.5-mm mesh and stored at -20 °C until they were used. Artificially pre-treated grain samples were prepared by culturing *A. versicolor* on maize, wheat, and rice. *A. versicolor* NRRL 5219 was cultured on potato dextrose agar medium and inoculated onto maize, wheat, and rice. After inoculation, samples were incubated at 27 °C for 1-2 days, and then incubated at 20 °C for one week. After autoclaving, the samples were air-dried and ground. The concentration of STC in each grain sample was determined, after that the samples were diluted with uncontaminated grain samples for study, and designated as STC pre-treated grain samples.

### 2.2.4. Sample extraction and IAC clean-up

For LC-MS, 25 g of ground sample and 100 mL acetonitrile/water (84:16, v/v) were placed in a 300 mL Erlenmeyer flask. The flask was shaken for 1 h on a reciprocal shaker (SA-31 shaker, Yamato Scientific Co. Ltd., Tokyo, Japan) at 220 rpm. The extract was filtered through filter paper (No.113, Whatman, Maidstone, UK). Filtrate was diluted 10-fold with phosphate buffer (PB: pH 7.4, 0.01 mol/L), and 10 mL of the diluted extract was passed through the IAC. The IAC was then washed with 10 mL of PB, followed by 10 mL water, and the bound STC was eluted with 3 mL of acetonitrile. After drying of eluted sample by N<sub>2</sub>, the test sample was re-dissolved with 1 mL of acetonitrile for LC-MS analysis.

For GC-MS method, the above mentioned LC-MS method for STC was modified as to increase the instrumental sensitivity of GC-MS at two points. Firstly, the loading volume of diluted extract was increased from 10 mL to 25 mL for GC-MS. Secondly, after STC elution from IAC followed by N<sub>2</sub> evaporation, the residue was re-dissolved in 0.5 mL acetone instead of 1 mL acetonitrile.

### *2.2.5 Performance test of IAC purification*

For STC binding capacity of the IAC, STC working solution (0.01, 0.05, 0.1, 0.2 µg/mL) was diluted 10-fold with PB. Then 10 mL of the diluted solution (1.0, 5.0, 10, 20 ng/mL) was passed through the IAC, and the recovery was measured. As 10 and 20 ng/mL diluted solution were high in concentration, these solution, and PB and water washed solution was checked for leakage.

To know the binding capacity of IAC for STC in the presence of AFs, STC and AFs-spiked maize sample was prepared by adding 0.25 mL of 5.0 µg/mL STC, and AFs standard solutions to 25 g of maize sample (concentration of STC, AFB<sub>1</sub>, and AFG<sub>1</sub> was 50 µg/kg, and concentration of AFB<sub>2</sub> and AFG<sub>2</sub> was 15 µg/kg), and the recoveries were measured. Test sample was divided into half, one was used for STC analysis by LC-MS, and the other was used for AFs analysis by HPLC.

AFs were analysed by HPLC with a fluorescence detector (Shimadzu Co. Ltd., Kyoto, Japan). The HPLC system consisted of LC-10AD pump, a RF-10AxL fluorescence detector, a CTO-10ASvp column oven, a SIL-20AC auto sampler, and a CBM-20A system controller. Data were handled with LC solution (Shimadzu). AFs were separated with an ODS column (Develosil ODS-HG-5 4.6 mm i.d. x 150 mm with a guard cartridge, 4.0 mm i.d. x 10 mm, Nomura Chemical Co. Ltd., Seto, Japan). For each analysis, 10 µL of sample was injected, and the column and guard cartridge were kept at 40 °C. Mobile phase A was acetonitrile : methanol : water = 5 : 35 : 60 (v/v/v) and mobile phase B was acetonitrile : methanol : water = 70 : 10 : 20 (v/v/v). The flow rate of mobile phase was 1.0 mL/min in total. Fluorescence detection for AFs was carried out at an excitation wavelength of 365 nm and an emission wavelength of 435 nm. AFB<sub>1</sub> and AFG<sub>1</sub> were derivatized by photochemical reactor (PHRED, Aura Industries, Inc., New York, USA).

### *2.2.6. LC-MS and GC-MS analysis conditions*

The LC-MS system consisted of a LC-2010C HT and a LC-MS-2010 EV (Shimadzu). Data were handled with LC-MS solution software (Shimadzu). STC was separated with an ODS column (Develosil ODS-UG-5, 5 µm, 2.0 mm i.d. x 150 mm, with a guard cartridge, 1.5 mm i.d. x 10 mm; Nomura). For each analysis, 10 µL of sample was



injected, and the column was kept at 40 °C. Mobile phase A was water containing 0.1 % formic acid, and mobile B was acetonitrile containing 0.1 % formic acid. The flow rate of mobile phase was 0.3 mL/min in total. A gradient elution was performed as follows: the ratio of mobile phases A and B was set at 60:40. Then, the ratio of B was increased to 95 % over 10 min, and % B was held at 95 % for 8 min, then the ratio of B was returned to 40 % over 0.1 min, and held for 10 min before the next injection. For MS, STC was detected by APCI-positive mode. Temperature of the APCI, curved desolvation line and heat block were 450 °C, 250 °C and 300 °C, respectively. The flow rate of nebulizer gas (N<sub>2</sub>) was 2.5 L/min. STC was detected by selecting ion monitoring mode at m/z 325.

GC-MS analyses were performed using a GC-MS QP 2010 plus system with an OCI/PTV 2010 (on-column/programmable vaporizing temperature injection unit), and an AOC-20i auto-injector (Shimadzu). STC separation was carried out using an InertCap 5MS/NP (0.25 mm i.d. × 30 m, 0.25 µm, GL Science, Tokyo, Japan) low bleed capillary column, combined with a deactivated fused silica pre-column (0.53 mm i.d. × 0.5 m, GL Sciences). The pre-column and capillary column were connected by a fused silica connector (SUPELCO, Pennsylvania, USA).

Samples (2 µL) were injected in on-column injection (OCI) mode with the carrier gas at a constant column flow of 2.04 mL/min. The injection port temperature was maintained at 50 °C for 0.2 min, increased to 280 °C at 180 °C/min, and maintained at 280 °C for 25 min. The column oven temperature was maintained at 50 °C for 2 min, increased to 280 °C at 20 °C/min, and maintained at 280 °C for 15 min. The total run time was 28.5 min. Mass spectrometry parameters were set as follows: electron ionization (EI), 70 eV; ion source temperature, 290 °C; interface temperature, 300 °C.

#### *2.2.7. Single laboratory validation study*

For the within-day variation study, STC-spiked grain samples were prepared by adding 0.25 mL of 0.5 and 5.0 µg/mL STC working solutions to 25 g of six grain samples (5.0 and 50 µg/kg in the samples). The recovery and RSD<sub>r</sub> (relative standard deviation of repeatability) were determined by analysing each sample with six replicates. STC pre-treated grain samples of unpolished rice, wheat, and maize (50 µg/kg in the product) were

prepared by diluting the high concentration STC pre-treated samples with untreated grain samples.

For comparison of recovery and RSDr at different STC concentrations, STC-spiked and STC pre-treated grain samples of concentrations over the range of 5.0 to 100 µg/kg in grains were prepared and analysed in duplicate. For the intermediate precision study, STC-spiked wheat samples at 5.0 µg/kg and STC pre-treated maize samples at 20 µg/kg were analysed for 3 days, by three analysts with six replicates.

## **2.3. Results and discussion for LC-MS**

### *2.3.1. Performance of IAC purification*

The AFLAKING<sup>®</sup>, IAC used in this study contains an antibody be generated against AFB<sub>2</sub>, and this antibody reacts equally with AFB<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, and M<sub>1</sub>. Uchigashima *et al.*, (2009) reported that the binding capacity of the IAC (0.2 mL gel/ column) was at least 400 ng in terms of total AFs (AFB<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>: each 100 ng). As a result of the STC binding capacity of the IAC study, each amounts of STC (10, 50, 100, and 200 ng) were applied to the IAC, and STC recoveries were 101.1, 95.1, 95.4 and 98.6 %, respectively. So, this study confirmed that the binding capacity of the IAC for STC was at least 200 ng per column, and the recovery was more than 90 %. Table 2-2 shows the average recovery of STC and AFs from spiked maize samples. The average recovery of STC and AFs ranged from 87.4 % to 108.6 %, and these data suggested that the IAC could bind both STC and AFs at the same time.

### *2.3.2. Method performance*

#### *2.3.2.1. Selectivity, sensitivity and linearity*

Fig. 2-2 shows the chromatograms of the STC standard (A), the MS spectrum of STC (B), STC-spiked maize (C), and STC pre-treated unpolished rice sample (D) The LOD for STC was 2.5 pg (Signal/Noise ratio, S/N = 3), and the limit of quantification (LOQ, S/N = 10) for STC was 7.5 pg, and the calibration curve for STC standards was linear from 7.5 to 375 pg. As shown in Fig. 2-2, the entire STC peak was detected without the presence of any interfering peaks.

#### 2.3.2.2. Recovery and relative standard deviations

Table 2-3 shows the average recovery and RSDr of STC for the within-day variation study. In Table 2-3, at 5.0  $\mu\text{g}/\text{kg}$  STC, the average recovery and RSDr for the spiked samples ranged from 87.3 % to 102.5 %, and from 1.9 % to 6.5 %, respectively. The average recovery and RSDr for all spiked samples were 95.8 % and 3.3 %, respectively. At the 50  $\mu\text{g}/\text{kg}$  level, the average recovery and RSDr for all three types of STC-spiked samples (polished rice, barley, and buckwheat) ranged from 83.2 % to 93.2 %, and from 2.1 % to 3.3 %, respectively. Additionally, RSDr for the three types of STC pre-treated grain samples ranged from 3.1 % to 14.0 %. The RSDr of the STC pre-treated grain samples were greater than that of the STC-spiked samples; for example, the RSDr of the STC pre-treated maize sample was 14.0 %, while the RSDr of the maize samples spiked with STC at a concentration of 5.0  $\mu\text{g}/\text{kg}$  was 2.0 %; thus, the homogeneity of the pre-treated sample may affect the RSDr of these samples. Table 2-4 shows the average recovery and RSDr of STC from different STC-spiked and STC pre-treated grain samples. For spiking levels of STC in the concentration range of 5.0 to 100  $\mu\text{g}/\text{kg}$ , the recoveries were ranged from 83.2 % to 102.5 %.

#### 2.3.2.3. Intermediate Precision

Table 2-5 shows the average recovery and RSDRi (intermediate precision) of STC. The average recovery of STC from spiked wheat samples by the three analysts were 95.2 %, 107.5 %, and 95.2 %, and the RSDRi were 4.0 %, 4.2 %, and 7.1 %, respectively. The RSDRi obtained by the three analysts for the recovery of STC from STC pre-treated maize samples were 5.4 %, 4.8 %, and 10.4 %. For STC-spiked wheat samples, the recovery of STC by analyst 2 was slightly higher than those of the others, and in STC pre-treated maize samples, the RSDRi determined by analyst 3 was slightly higher than those of the other analysts.

## 2.4. Results and discussion for GC-MS

### 2.4.1. Optimization of GC-MS conditions

The column temperature during injection was set at 50 °C to prevent sample loss due to rapid evaporation and back-flow (Grob Jr. and Neukom, 1980). The rate at which the column oven temperature increased was evaluated from 15 °C/min to 25 °C/min, and set at 20 °C/min (Fig. 2-3). The injection temperature during injection was set at 50 °C. The optimum ion source temperature was evaluated by testing temperatures between 230 °C and 290 °C, and maximum sensitivity was showed at 290 °C (Fig. 2-3). The interface temperature was set at 300 °C (Fig. 2-3).

### 2.4.2. Selection of GC injection solvent

Acetone was selected as the injection solvent for the GC-MS system after comparing it with acetonitrile. Acetonitrile containing STC in grain matrices showed peak distortion during GC analysis. The STC chromatograms with acetone and acetonitrile solvent were shown in Fig. 2-4. In contrast to the results using acetonitrile, acetone containing STC in grain matrices did not show any peak distortion during the analysis.

### 2.4.3. Method performance

#### 2.4.3.1. Selectivity

The method was determined to be selective for STC in the presence of interfering or co-eluting compounds, because no overlapping of matrix compounds were observed. STC was determined in selecting ion monitoring mode using a target ion ( $m/z = 324$ ) and two reference ions ( $m/z = 295$  and  $m/z = 306$ ), as shown in Fig. 2-5. The retention time for STC was consistent in subsequent analyses.

#### 2.4.3.2. Matrix effects

IAC clean-up is used prior to chromatographic separation in mycotoxin analysis owing to its selective purification of target compounds. The matrix effect was investigated by adding STC standard solution to blank matrix extracts of maize, wheat, and rice. The matrix effect for STC in maize, wheat, and rice was insignificant (less than 15 %), as

shown in Table 2-6. After observing a slight matrix effect after IAC clean-up, STC containing acetone solutions were used to construct the calibration curve for STC quantification.

#### 2.4.3.3. Linearity of the detection

The linearity of the detection was evaluated at seven concentration levels of the STC standard solution between 10 ng/mL and 150 ng/mL (10, 20, 30, 50, 75, 100, and 150 ng/mL) with two replicates injection. The calibration curve was plotted by peak height against the concentration of STC. The results demonstrated good linearity with a coefficient of determination of 0.998, as shown in Fig. 2-6.

#### 2.4.3.4. Recovery and relative standard deviations

In this study, an IAC clean-up procedure identical to that of Sasaki *et al.*, (2014) was conducted, with the exception of the sample loading volume (25 mL). Due to the adequate binding capacity of the IAC for STC, the increased sample volume was not expected to affect the recovery of STC. However, this expectation was confirmed by spiking maize with 25 µg/kg STC and recovering STC at a rate of 93.2 %, with RSDr of less than 10 %.

#### 2.4.3.5. Sensitivity of the method

The LOD of the instrument was 6 pg (equivalent to 2.4 µg/kg in grain), as determined by decreasing the level of STC standard solution in acetone until the S/N ratio reached 3. The LOQ was determined to be 20 pg (equivalent to 8 µg/kg in grain). The LOD indicated that this method was more sensitive than previously reported gas chromatographic methods (Salhab *et al.*, 1976; Tanaka *et al.*, 2007), and compatible with LC-APCI-MS studies (Scudamore *et al.*, 1996) in grain. The sensitivity of the method is sufficient to monitor low levels of STC contamination in grains. Fig. 2-7 shows the chromatograms of STC contamination from pre-treated maize (43 µg/kg), wheat (47 µg/kg), and rice (101 µg/kg) samples.

## 2.5. Conclusion

At first, a new analytical method was developed for detecting STC in grains using a commercially available IAC purification followed by LC-MS analysis, and performed a single laboratory validation. The chromatograms of LC-MS analysed grains showed no interference for STC detection, and the LOD for STC was 2.5 pg. After successfully determined STC using LC-MS, another analysis method was developed for STC determination in grains using GC-MS method after on-column injection. For GC-MS, acetone was chosen as an injection solvent because it produced better separation than acetonitrile, and did not produce peak distortion during GC separation. The matrix effect was investigated in grain matrices, and was found to be insignificant effect (<15 %). The LOD of the GC-MS method for STC in grains was 2.4 µg/kg, and the LOQ was 8 µg/kg. In conclusion, the results revealed that both newly developed LC-MS and GC-MS methods reported herein could be useful analytical tools with which to monitor STC contamination in grains.

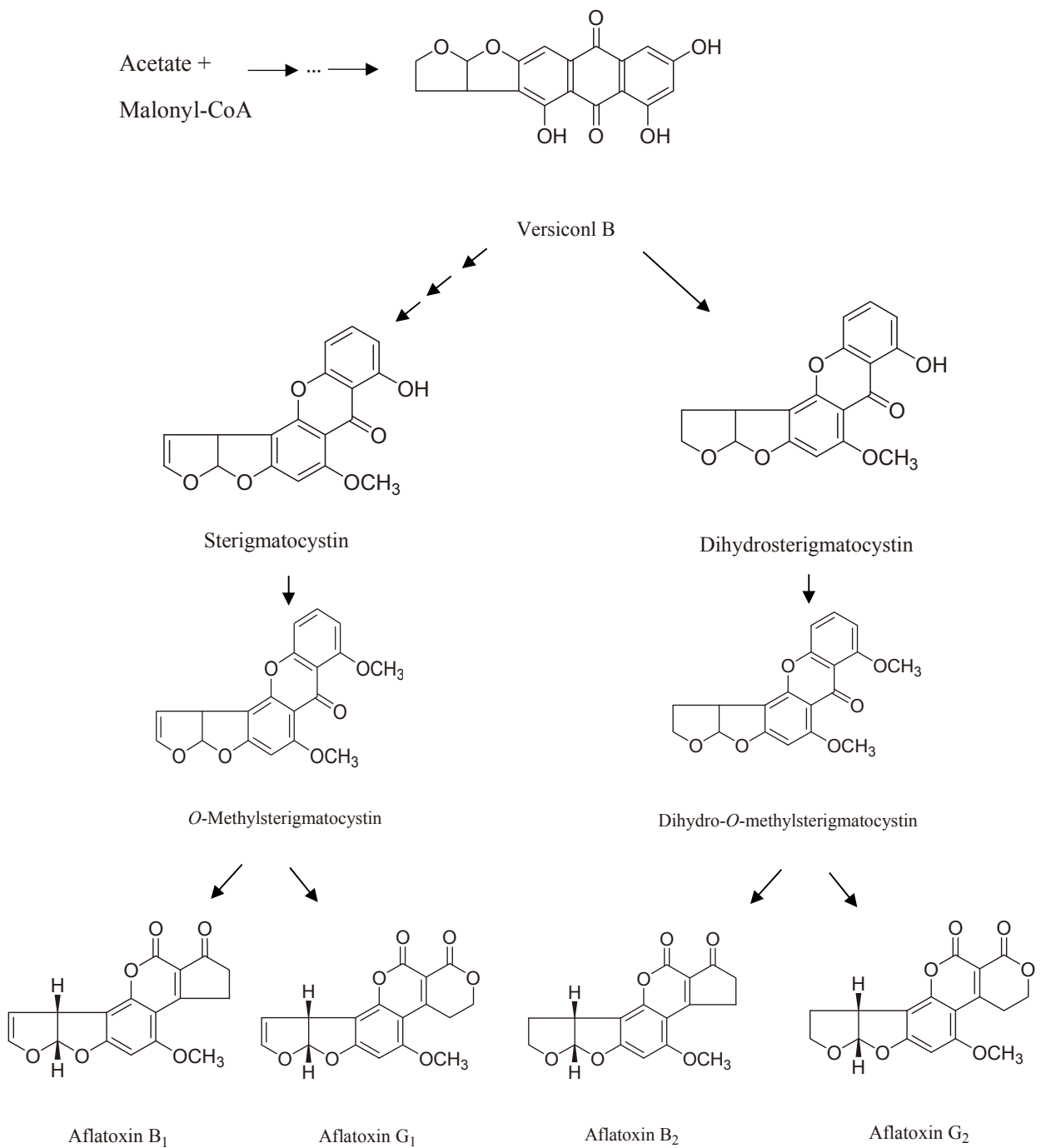


Fig. 2-1. Biosynthesis pathway of aflatoxins with sterigmatocystin.

Source: Sweeney and Dobson (1998)

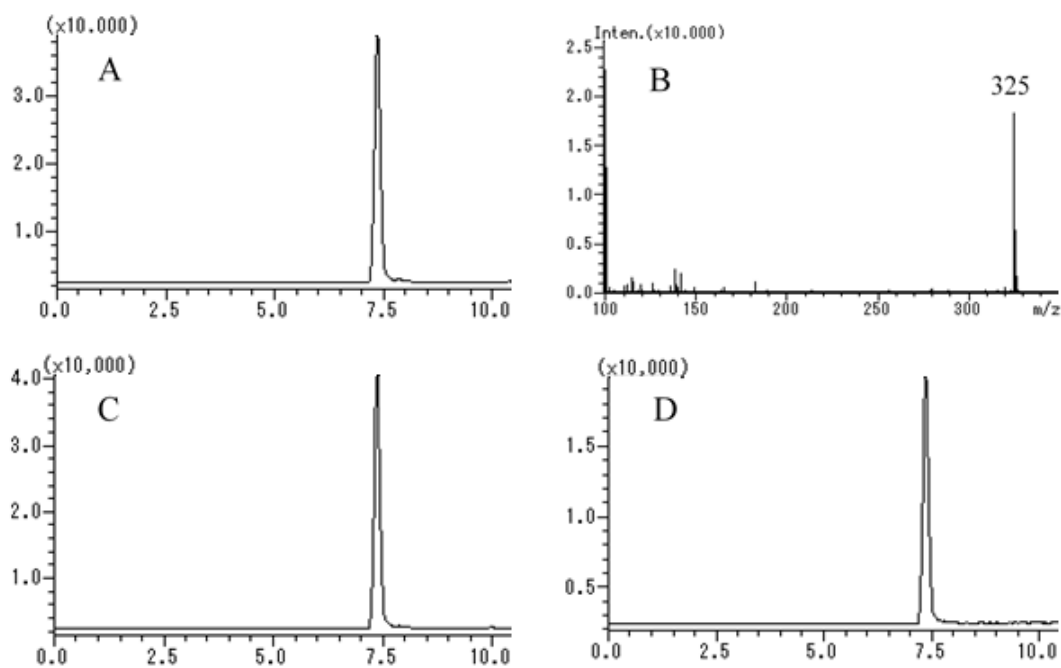


Fig. 2-2. LC-MS chromatograms and MS spectrum of STC.

A: STC standard at 25 ng/mL

B: MS spectrum of STC standard at 25 ng/mL

C: Spiked maize sample at 100 µg/kg

D: STC-pretreated unpolished rice sample at 50 µg/kg

(STC-pretreated grains were prepared by culturing *Aspergillus versicolor* on maize, wheat and unpolished rice. Then the STC levels in these samples were determined, and these stock 'contaminated' samples were then diluted with uncontaminated grain samples in subsequent experiments; such samples are termed the STC-pretreated grain samples)



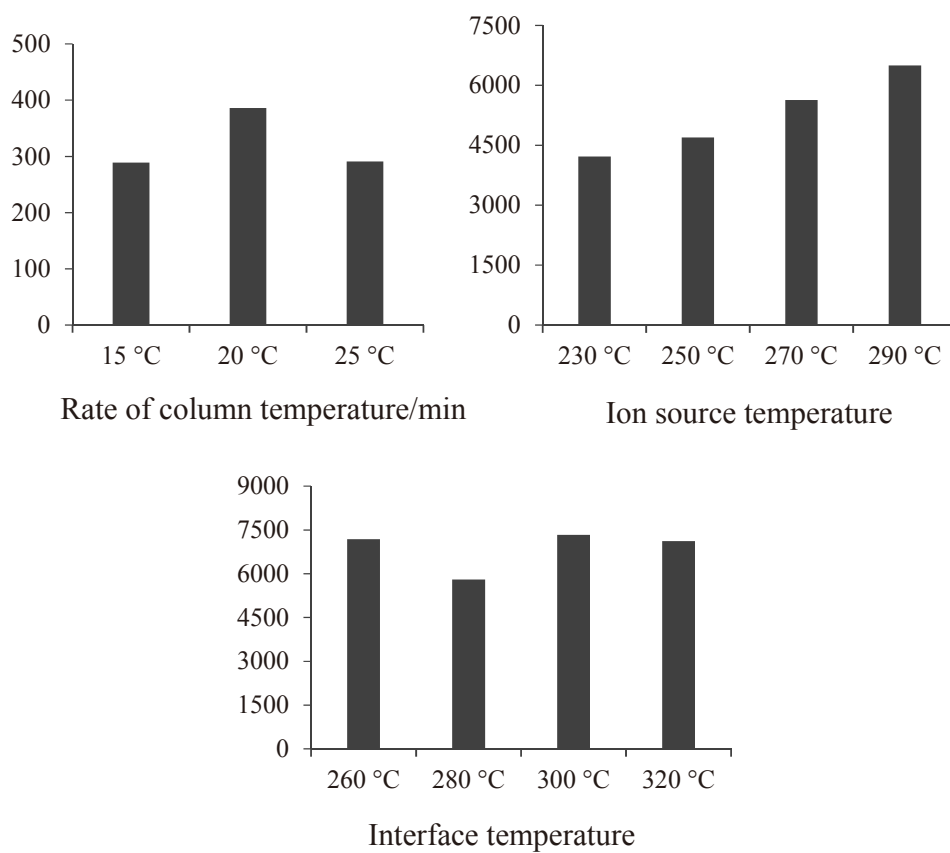


Fig. 2-3. Optimisation of GC-MS conditions for STC.

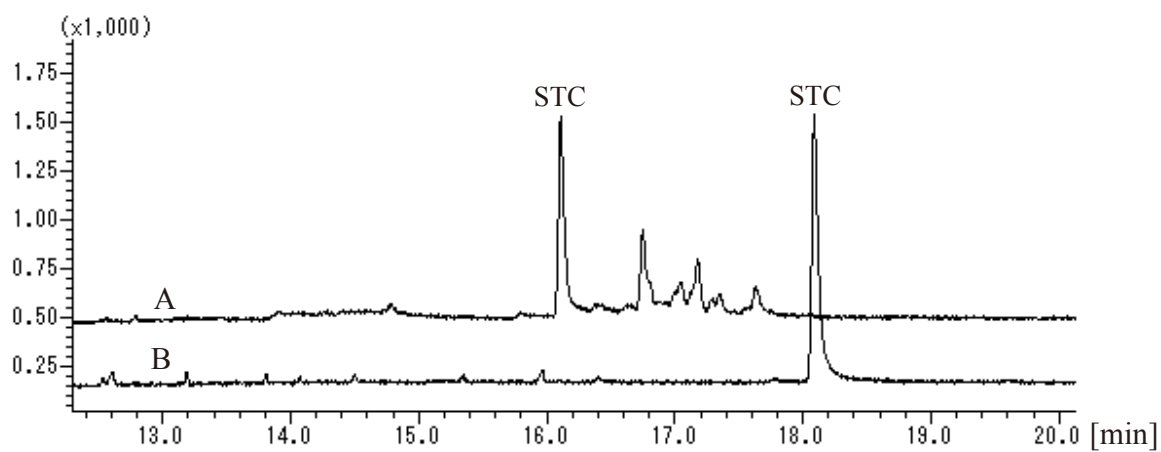


Fig. 2-4. Solvent effects for STC ( $m/z = 324$ ) separation in grain matrix.

A: Acetonitrile solution (initial oven temperature 65 °C)

B: Acetone solution (initial oven temperature 50 °C)

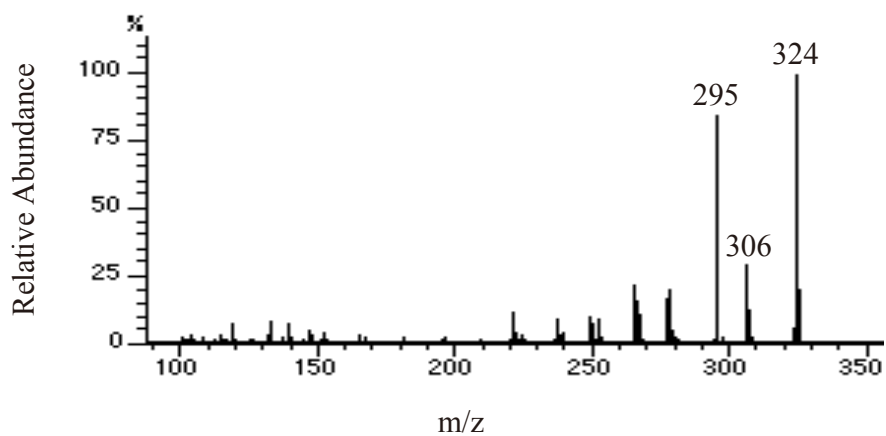


Fig. 2-5. GC-MS spectrum of STC

[ $m/z = 324 (M^+)$ ,  $306 (M-18)^+$  and  $295 (M-29)^+$ ]

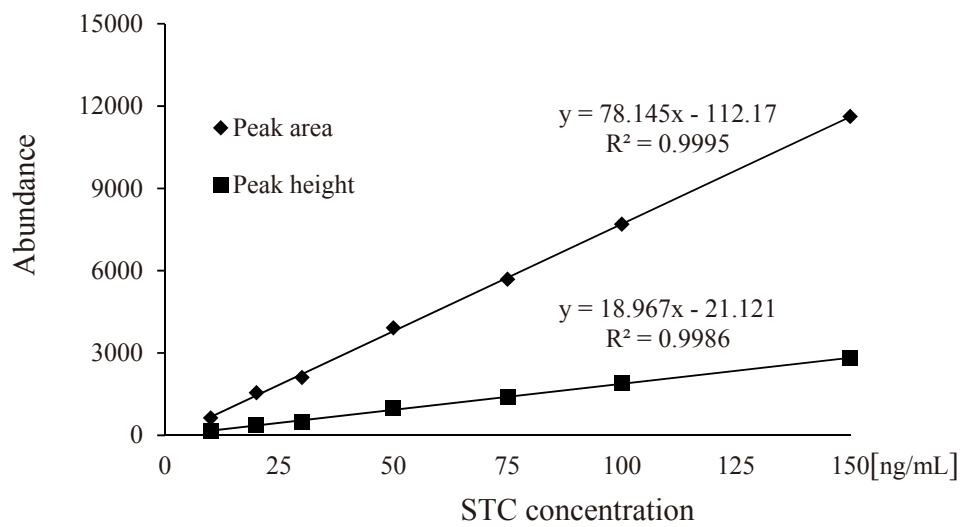


Fig. 2-6. Calibration curve for STC determination by GC-MS at seven concentration levels (10, 20, 30, 50, 75, 100, and 150 ng/mL)

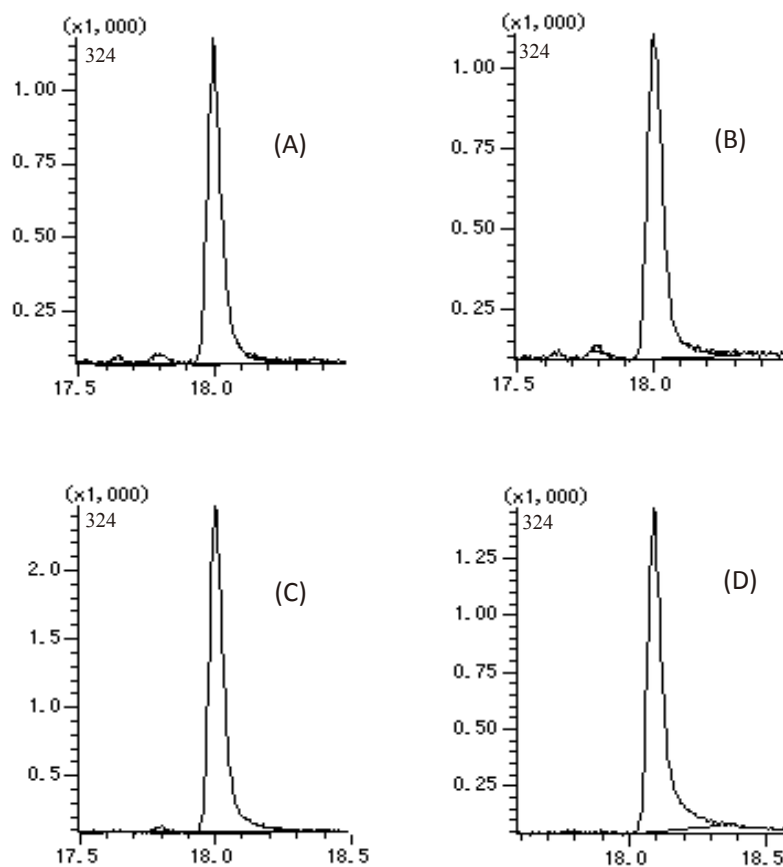


Fig. 2-7. GC-MS chromatograms ( $m/z = 324$ ) of STC in pre-treated grain samples.

A: Wheat sample at  $47 \mu\text{g/kg}$

B: Maize sample at  $43 \mu\text{g/kg}$

C: Rice sample at  $101 \mu\text{g/kg}$

D: Standard STC concentration at  $75 \text{ ng/mL}$

Table 2-1 Overview of analytical methods used for STC detection in food and feed.

(Adapted from EFSA, 2013)

Analytical methods	Method characteristics	LOD ( $\mu\text{g}/\text{kg}$ )
ELISA	Screening (qualitative and semi quantitative)	not reported <sup>a</sup>
TLC	Screening (qualitative and semi quantitative)	2.0 -140
HPLC (UV/FL)	Confirmation (semi-quantitative and quantitative)	0.3 -100
GC	Confirmation (semi-quantitative and quantitative)	5.0 - 50
LC-MS	Confirmation (semi-quantitative and quantitative)	0.4 - 10 <sup>b</sup>

<sup>a</sup> LOD of 31  $\mu\text{g}/\text{kg}$  was reported by Li et al. (1996)

<sup>b</sup> LOD of 2  $\mu\text{g}/\text{kg}$  in grains was reported by Scudamore et al. (1996)

ELISA: Enzyme linked immunosorbent assay

TLC: Thin layer chromatography

UV: Ultraviolet; FL: Fluorescence

LOD: Limit of detection

Table 2-2 Average recovery of STC and AFs from spiked maize samples (n=2)

Maize	STC	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>
Spiking levels (µg/kg)	50	50	15	50	15
Recovery	108.6	88.7	87.4	94.6	93.5

Table 2-3 Average recovery and RSD of STC for within-day variation study (n=6)

Matrix	5.0 µg/kg		50 µg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Unpolished rice	97.7	3.4	<sup>a</sup>	3.1
Polished rice	96.7	2.6	89.7	3.3
Wheat	95.3	6.5	<sup>a</sup>	9.0
Barley	95.2	3.2	83.2	2.5
Buckwheat	87.3	1.9	93.2	2.1
Maize	102.5	2.0	<sup>a</sup>	14.0
Average	95.8	3.3	88.7	5.7

<sup>a</sup> STC pre-treated grains were prepared by culturing *Aspergillus versicolor* on maize, wheat and unpolished rice. Then the STC levels in these samples were determined, and these stock 'contaminated' samples were then diluted with uncontaminated grain samples in subsequent experiments; such samples are termed the STC pre-treated grain samples.

Table 2-4 Average recovery and RSD of STC from different spiked and STC pre-treated samples

Matrix	Spiking levels			
	5.0 µg/kg	10 µg/kg	25 µg/kg	50 µg/kg
				100 µg/kg
			Recovery (%) (RSD (%))	
Unpolished rice	97.7 (3.4)		(2.4) <sup>a</sup>	95.1 (2.2)
Polished rice	96.7 (2.6)	93.1 (2.7)		89.7 (3.3)
Wheat	95.3 (6.5)		(2.8) <sup>a</sup>	98.5 (0.58)
Barley	95.2 (3.2)	97.7 (0.24)		83.2 (2.5)
Buckwheat	87.3 (1.9)		94.6 (0.42)	93.2 (2.1)
Maize	102.5 (2.0)	(11.0) <sup>a</sup>		(14.0) <sup>a</sup>
Average	95.8 (3.3)	95.4 (4.6)	94.6 (1.9)	88.7 (5.7)

<sup>a</sup> STC pre-treated grains were prepared by culturing *Aspergillus versicolor* on maize, wheat and unpolished rice. Then the STC levels in these samples were determined, and these stock 'contaminated' samples were then diluted with uncontaminated grain samples in subsequent experiments; such samples are termed the STC pre-treated grain samples.



Table 2-5 Average recovery and RSD of STC for intermediate precision study (n = 6)

Time	analyst 1			analyst 2			analyst 3		
	Recovery (%)		RSD (%)	Recovery (%)		RSD (%)	Recovery (%)		RSD (%)
	Spiked wheat (5.0 µg/kg)	STC pre-treated maize	maize (6.7) <sup>a</sup>	Spiked wheat (5.0 µg/kg)	STCpre-treated maize	maize (5.4) <sup>a</sup>	Spiked wheat (5.0 µg/kg)	STC pre-treated maize	maize (18.5) <sup>a</sup>
day 1	95.3 (6.5)		(6.7) <sup>a</sup>	113.9 (3.0)		(5.4) <sup>a</sup>	95.9 (6.8)		(18.5) <sup>a</sup>
day 2	94.6 (3.5)		(3.5) <sup>a</sup>	97.4 (5.1)		(6.7) <sup>a</sup>	95.7 (10.8)		(6.6) <sup>a</sup>
day 3	95.8 (2.1)		(6.1) <sup>a</sup>	111.2 (4.5)		(2.4) <sup>a</sup>	94.1 (3.8)		(6.0) <sup>a</sup>
Average	95.2 (4.0)		(5.4) <sup>a</sup>	107.5 (4.2)		(4.8) <sup>a</sup>	95.2 (7.1)		(10.4) <sup>a</sup>

<sup>a</sup> STC pre-treated maize (about 20 µg/kg) were prepared by culturing *Aspergillus versicolor* to maize. Then the STC levels in these samples were determined, and these stock 'contaminated' samples were then diluted with uncontaminated maize samples in subsequent experiments; such samples are termed the STC pre-treated maize samples.

Table 2-6 Matrix effects of STC on different matrices spiked at 40 µg/kg

Matrix	Recovery (%)	RSD (%)
Maize (n=3)	108	13.8
Wheat (n=3)	106	1.9
Rice (n=3)	92	10.8
Average	102	8.8

## Chapter 3: A rapid analytical method for the determination of ergosterol in grains using GC-MS without derivatization

### 3.1. Introduction

Ergosterol (ERG: ergosta-5,7,22-trien-3 $\beta$ -ol) is a major sterol found in fungal cell walls, however absent or present at a negligible amounts in higher plants (Weete, 1980). The degree of fungal contamination caused by mycotoxigenic fungi in grains can be estimated by knowing the level of one fungal specific compound 'ERG'. ERG has been used as an indicator of fungal invasion in grains (Seitz *et al.*, 1977), and may correlates with mycotoxin contamination (Saxena *et al.*, 2001). The relationships between the level of ERG and possible mycotoxin synthesis have been investigated in several studies such as AFs (Gourama and Bullerman, 1995; Castro *et al.*, 2002), OTA (Olsson *et al.*, 2002; Varga *et al.*, 2002), FUMs (Pietri *et al.*, 2004), ZEA (Zill *et al.*, 1988; Pietri *et al.*, 2004), deoxynivalenol (Lamper *et al.*, 2000; Pietri *et al.*, 2004) and patulin (Kadalkal *et al.*, 2005).

Owing to huge economic losses and barrier in international trade of mycotoxin contaminated grains, and the potential health consequences associated with the toxicity of the mycotoxins, a rapid and sensitive analytical method is needed to determine fungal contamination of grains. ERG analysis might be useful to the grain industry as a primary indicator to know the level of potential fungal contamination, prior to individual or multi-mycotoxin analysis. Many chromatographic methods have been established to determine ERG in different matrices using thin layer chromatography (Naewbanij *et al.*, 1984), HPLC (Neuhof *et al.*, 2008; Jeldičková *et al.*, 2008; Miyagawa *et al.*, 2009; Sasaki *et al.*, 2011), GC-MS (Lamper *et al.*, 2000, Nielson and Madsen, 2000; Axelsson *et al.*, 1995; Volker *et al.*, 2000; Ravelet *et al.*, 2001) and LC-MS (Varga *et al.*, 2006). Other than HPLC, GC-MS and LC-MS have also been investigated due to their higher selectivity and sensitivity (Varga *et al.*, 2006). Lamper *et al.*, (2000) reported a GC-MS method to determine ERG in *Fusarium* infected wheat grains to know deoxynivalenol contamination. However, the method lacks of validation related data. After that, Dong *et al.*, (2006) developed a GC-MS method with chemical derivatization for analysing ERG in single kernel, and ground barley and wheat. After saponification, the sample was extracted by

hexane and subsequently derivatized with trimethylsilylimidazole/trimethylchlorosilane at room temperature. Derivatization has been used to improve ERG peak shape by reducing decomposition (Nielson and Madsen, 2000). But derivatization steps are often interfere with the analysis, cause unintended chemical reactions, and may result in loss of analytes and increased analysis time. In this new method, an on-column injection technique was used to introduce samples directly into the GC-MS system. The advantages of on-column injection are the complete elimination of sample discrimination (Sandra, 1989) and reduced ERG decomposition.

Therefore, the purpose of this study was to develop and validate a rapid method using on-column GC-MS technique for determination of ERG in grains. The efficiency of the developed GC-MS method with an HPLC method on all naturally contaminated samples was also compared.

## **3.2. Materials and methods**

### *3.2.1. Chemicals*

ERG standard (98 %) was purchased from Acros (Geel, Belgium). Chemicals including methanol (reagent grade), ethanol (HPLC grade), hexane (reagent and spectroscopy grade) and potassium chloride were purchased from Kanto Chemical while methanol (HPLC grade) was purchased from Wako Pure Chemical. Water was purified by an ultrapure water system (Autopure WT 100, Yamato). High purity (99.9999 %) helium used as a carrier gas for GC-MS, was obtained from Okaya Sanso.

### *3.2.2. Standard solutions*

ERG stock solution (2000 mg/L) was prepared by dissolving 10 mg of ERG in 5 mL hexane. Standard working solutions containing ERG at concentrations ranged from 0.05 mg/L to 5 mg/L (0.05, 0.1, 0.5, 1, 2, 3.5 and 5 mg/L) were prepared in hexane to construct the calibration curve. ERG free blank matrices of maize and wheat were used to construct matrix matched calibration curves for ERG quantification. All the solutions were stored at -20 °C in dark amber bottles until use.

For recovery studies, an appropriate amount of 100 µL of ERG standard solutions in hexane (100 mg/L, 300 mg/L and 800 mg/L) were spiked to 10 g of ground grain samples. Samples were mixed and kept at 4 °C overnight in the dark before the day of extraction.

### 3.2.3. Samples

Maize (n=24) and wheat (n=13) samples were collected from Asian countries, including Japan. Before analysis, these samples were ground through a 1 mm mesh screen by milling (Rotor Mill, Fritsch, Germany). All samples were stored at -20 °C in the dark until the time of analysis.

### 3.2.4. Sample preparation and analysis conditions for GC-MS

Extraction procedure for ERG was performed according to the method of Sasaki *et al.*, (2011) with few modifications. Briefly, 10 g of sample and 40 mL of methanol were placed in a 100 mL Erlenmeyer flask, and then shaken by a horizontal shaker (SA-31, Yamato Scientific) at 320 rpm for 60 min. After filtration (Whatman No. 2, Maidstone, UK), 10 mL of filtrate was transferred into a 200 mL separating funnel, and 10 mL of 3 % potassium chloride aqueous solution was added and mixed. After adding 10 mL of hexane, the mixture was shaken vigorously by hand for 3 min. After separating the two layers, the upper 1 mL of the hexane layer was collected into a GC vial, and 1 µL was injected by on-column GC-MS injection system.

### 3.2.5. GC-MS analysis conditions

GC-MS analyses were performed using a GC-MS QP 2010 plus system (Shimadzu) and an AOC-20i auto-injector equipped with an OCI/PTV sample introduction system. ERG separation was carried out using an InertCap 5MS/NP (30 m x 0.25 mm i.d., 0.25 µm, GL Science) capillary column, combined with a deactivated fused silica pre-column (0.5 m x 0.53 mm i.d., GL Science). Pre-column and analytical column were connected by a fused silica connector (SUPELCO).

Samples (1 µL) were injected in on-column injection mode (OCI) with a carrier gas at a constant flow of 1.92 mL/min. The injection port temperature started from 70 °C for 0.2

min, increased to 280 °C at 180 °C/min, and maintained for 25 min. The column oven temperature was started from 90 °C for 2 min, ramped to 280 °C at 20 °C/min, and maintained for 15 min. Total run time was 26.5 min. Mass spectrometry parameters were set as follows: EI at 70 eV; ion source temperature at 250 °C; interface temperature at 300 °C. GC-MS solution software (ver. 2.5, Shimadzu) was used for data handling and GC-MS control.

### 3.2.6. Sample preparation and analysis conditions for HPLC

Sample extraction, analysis conditions and quantification were followed according to the HPLC method of Sasaki *et al.*, (2011). ERG was analysed by HPLC with UV detection system (Shimadzu LC-10 series, Shimadzu) consisting of double LC-10AD pumps, a SIL-10A auto sampler, a SCL-10A system controller, a CTO-10ASvp column oven and a SPD-M10Avp photodiode array detector. ERG was separated with an ODS column (ODS-SP, 4 mm i.d. x 150 mm, GL Sciences) and a guard cartridge (Inertsil ODS-SP, 4 mm i.d. x 10 mm, GL Sciences). The column oven temperature was maintained at 40 °C. Mobile phase A was a mixture of methanol and water (80:20, v/v), and mobile phase B was a mixture of methanol and ethanol (70:30, v/v). The total flow rate was 1 mL/min, and 10 µL of sample was injected into the HPLC system. The UV absorption was set at 282 nm.

## 3.3. Results and discussion

### 3.3.1. Optimization of GC-MS conditions

Several parameters were investigated to obtain optimum sensitivity based on peak height. The optimum temperature for column oven and rate, initial injection and ion source was evaluated (Fig. 3-1). At first, initial oven temperature was studied by changing the temperature from 50 °C to 110 °C. Higher sensitivity and sharper peaks were observed at 90 °C. Initial injection temperature for the injection port was also checked from 50 °C to 90 °C, and set at 70 °C. The rates of column oven temperature were evaluated using a temperature ranged from 15 °C/min to 25 °C/min, and maximum signal intensity was observed at 20 °C/min. Optimum ion source temperature was investigated

between the range 230 °C and 270 °C. The response of the signal reached a maximum at 250 °C.

### 3.3.2. Selectivity

Selectivity of the method was checked to quantify ERG accurately in the presence of interfering compounds. ERG was determined based on peak height using one target ion ( $m/z = 363$ ) and two reference ions ( $m/z = 337$  and  $396$ ) in selected ion monitoring mode as shown Fig. 3-2. This new method showed enough fragmentation to allow accurate identification of ERG in samples. Retention time of ERG was also appeared at the exact retention time in subsequent analysis.

### 3.3.3. Linearity

Initially, the calibration curve was constructed with standard ERG solutions in hexane ranging from 0.05 to 5 mg/L at seven points. The curve was constructed by plotting the peak height versus concentration and showed an excellent linearity. The coefficient of determination was higher than 0.999 with triplicate injections (Fig. 3-3), with RSDr from 1.88 to 5.7 %.

### 3.3.4. Matrix effects

To be clear on the matrix effects of maize and wheat, we evaluated solvent (hexane) and matrix (maize and wheat) calibration by comparing the slopes of the solvent matched standards with those obtained in the matrix matched standards. The results obtained showed an enhancement of chromatographic response for ERG. According to Erney *et al.* (1993), the matrix-induced enhancement was due to the blockage of active sites by the matrix compounds which reduces adsorption of analyte molecules in the GC system. However, several factors are associated with matrix effects such as GC injection (split/splitless/PTV/on-column), matrix (type and amount), sample pre-treatment and analytes (type and amount) (Erney *et al.*, 1995; Zrostilicova *et al.*, 2001). To reduce/compensate the matrix effect, these studies suggested to use hot on-column injection or matrix matched calibration curves. However, Zrostilicova *et al.* (2001)

studied PTV, pulsed splitless and on-column injection for GC, and observed that on-column injection has also a matrix induced enhancement effect. As on-column GC-MS injection was not completely eliminated the matrix enhancement effects, matrix matched calibration is one good alternative to obtain accurate results (Poole, 2007). After considering matrix enhancement effect, matrix matched calibration curves were decided to use for ERG quantification. The calibration curves were constructed at 7 concentration levels (0.05 to 5 mg/L) in blank matrices of maize and wheat with ERG standard solutions. The results showed a good linearity for ERG, with the coefficients of determination were in excess of 0.99 in both matrices. In Fig. 3-3 shows the matrix matched calibration curves at seven points in maize and wheat, along with solvent calibration at the same concentration levels.

#### *3.3.5. Recovery*

The recovery study was conducted in blank samples of maize and wheat at 1, 3 and 8 mg/kg (100  $\mu$ L of standard solutions at 100, 300, 800 mg/L of ERG, respectively) spiking levels. Each level was performed six times, and the value shown is the average of six measurements (Table 3-1). The mean recovery values were ranged from 98 % to 110 % in maize, and from 96 % to 110 % in wheat.

#### *3.3.6. Repeatability*

RSD<sub>r</sub> was calculated from six replicates of maize and wheat samples at three concentration levels (1, 3 and 8 mg/kg). Their values were less than 8 % in maize and 7 % in wheat (Table 3-1).

#### *3.3.7. Limit of detection and quantification*

The LOD of the method was determined with decreasing spiking levels of the ERG standard in the blank matrix until S/N of 3 was reached. The LOD of the method was determined to be 10  $\mu$ g/L, which is equivalent to 40  $\mu$ g/kg in grains. The LOQ (S/N=10) of the method was determined to be 50  $\mu$ g/L, which is equivalent to 200  $\mu$ g/kg in grains. This simple method was sensitive enough to monitor fungal invasion, and may useful to



indicate possible mycotoxin contamination in grains. The sensitivity of this method was higher than reported for HPLC (Neuhof *et al.*, 2008; Jeldičková *et al.*, 2008; Miyagawa *et al.*, 2009) and LC-MS (Varga *et al.*, 2006) detection.

#### 3.3.8. Analysis of naturally contaminated maize and wheat samples

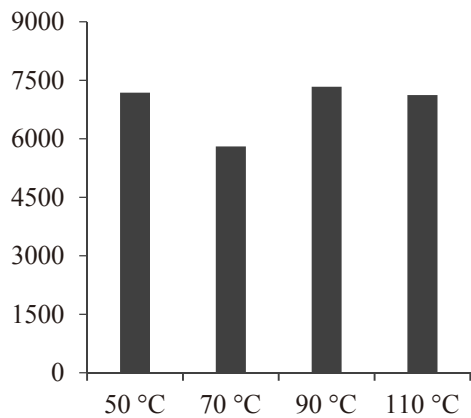
After optimization and validation, ERG levels in maize and wheat samples were determined. ERG content was found in all studied samples (n=37) with contamination levels ranged from 1.3 to 13.7 mg/kg in maize, and 1.0 to 14.4 mg/kg in wheat (Table 3-2). According to Pietri *et al.*, (2004), the quality of maize is acceptable if ERG in maize is less than 3 mg/kg. If ERG is more than 8 mg/kg, the potential of fungal invasion in grains is high. Pietri *et al.*, (2004) also studied the relationship between the amount of ERG and possible mycotoxin contamination, and concluded that the acceptable levels of mycotoxin contamination in maize is an ERG level of less than 3 mg/kg. Among the studied samples as observed in Table 3-2, 10 samples were determined to be less than 3 mg/kg, 22 samples were between 3 to 8 mg/kg, and 5 samples were more than 8 mg/kg of ERG level. Wheat samples showed higher ERG content than maize samples. In Fig. 3-4, typical chromatograms of ERG obtained from naturally contaminated maize (6.49 mg/kg) and wheat (3 mg/kg) were shown.

#### 3.3.9. Comparison of GC-MS and HPLC method

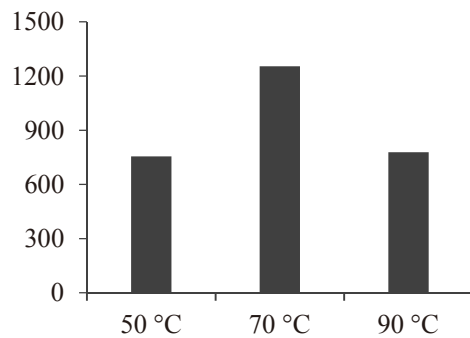
All samples were determined for ERG by the HPLC method, and the results were compared to the GC-MS determined ERG levels. The values found in analysing samples by both chromatographic methods were similar, as shown by the correlation in Fig. 3-5. The ERG content determined by the GC-MS method correlated with the HPLC method in maize ( $r^2 > 0.96$ ) and wheat samples ( $r^2 > 0.97$ ). However, a few advantages for using the GC-MS method versus HPLC-UV method includes less solvent consumption and no additional solvent evaporation by N<sub>2</sub> gas, which reduces total the analysis time and expenses.

### **3.4. Conclusion**

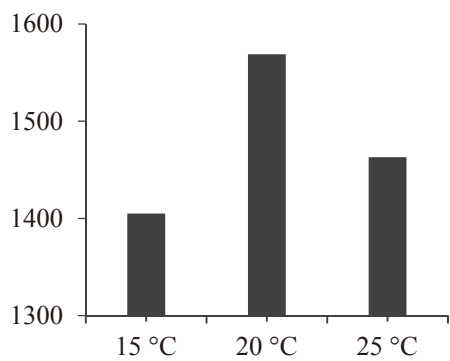
A simple and rapid GC-MS method was developed and validated for ERG determination in grains without a derivatization step. Matrix matched calibration curves were used to compensate matrix effects. Method validations such as linearity, recovery, repeatability and sensitivity were evaluated for accurate ERG determination. The average recoveries of ERG in maize and wheat with six replicates were ranged from 96 to 110 % with low RSDr (< 8 %). The method was applied to 37 naturally contaminated grains and successfully determined ERG levels. Between the two methods, GC-MS results were in agreement with results from the HPLC method for ERG presence in grains. This new GC-MS method is useful for routine analysis of ERG to monitor fungal contamination, and may help to predict possible mycotoxin contamination in grains.



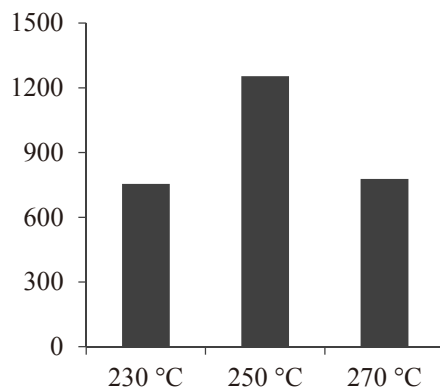
Column oven temperature



Initial injection temperature



Rate of column temperature/min



Ion source temperature

Fig. 3-1. Optimisation of GC-MS conditions for ERG analysis

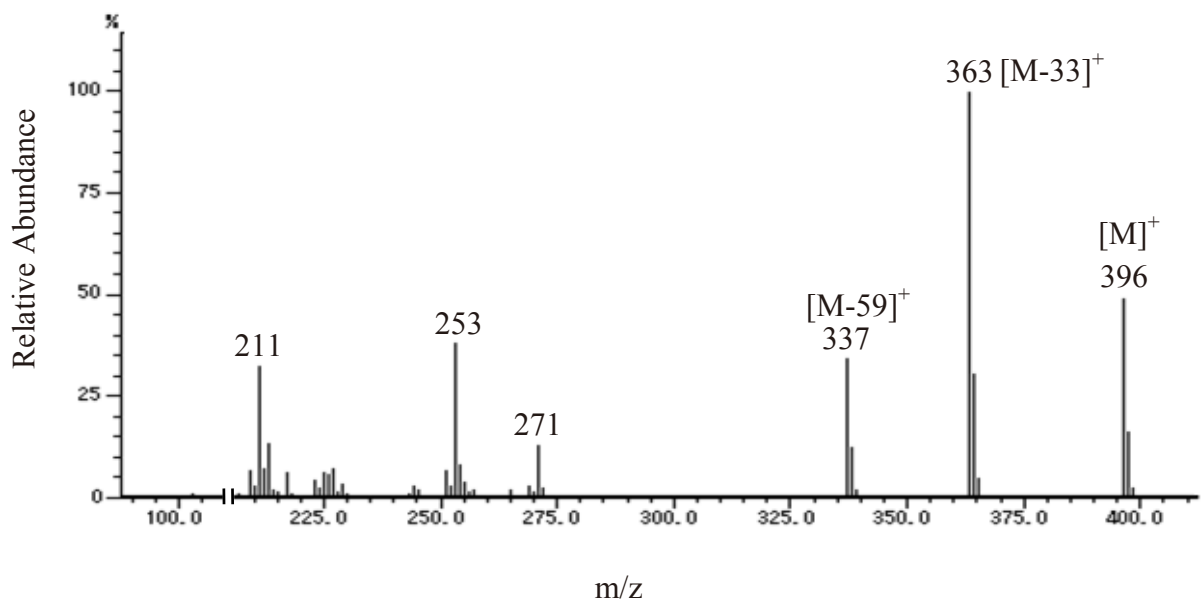


Fig. 3-2. GC-MS spectrum of ERG

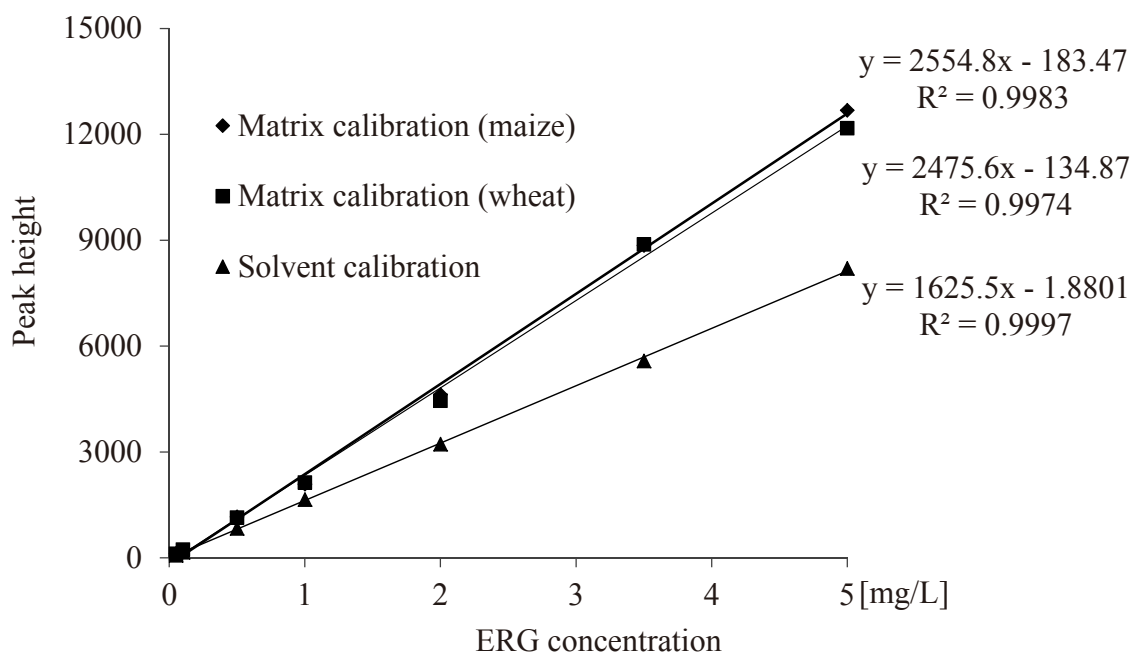


Fig. 3-3. Plot of matrix matched calibration curves in maize and wheat, along with solvent calibration

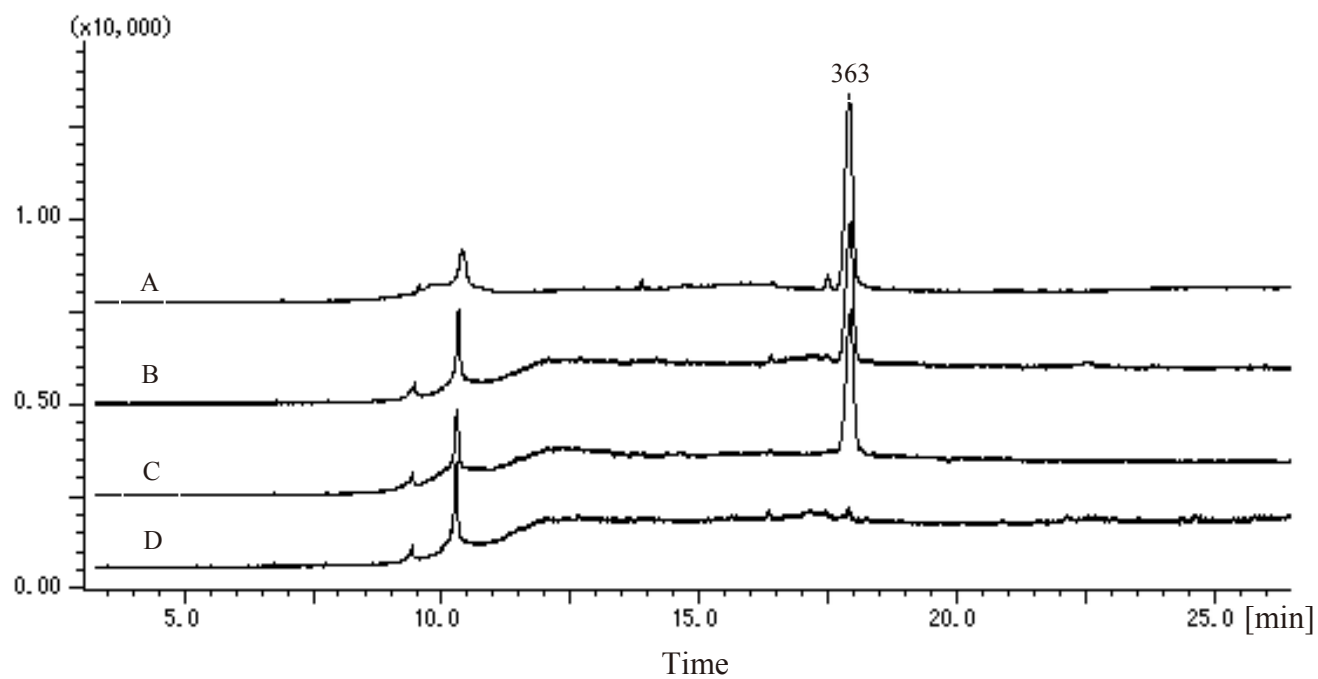


Fig. 3-4. Chromatograms for ERG ( $m/z=363$ ) in selected ion monitoring mode.

- A: Natural contaminated maize sample at 6.49 mg/kg
- B: Natural contaminated wheat sample at 3 mg/kg
- C: ERG standard at 1 mg/L (equivalent to 4 mg/kg)
- D: Blank matrix (maize)

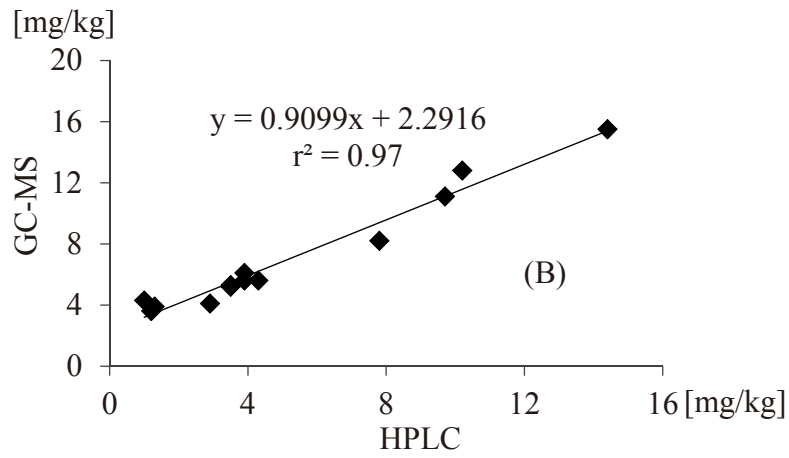
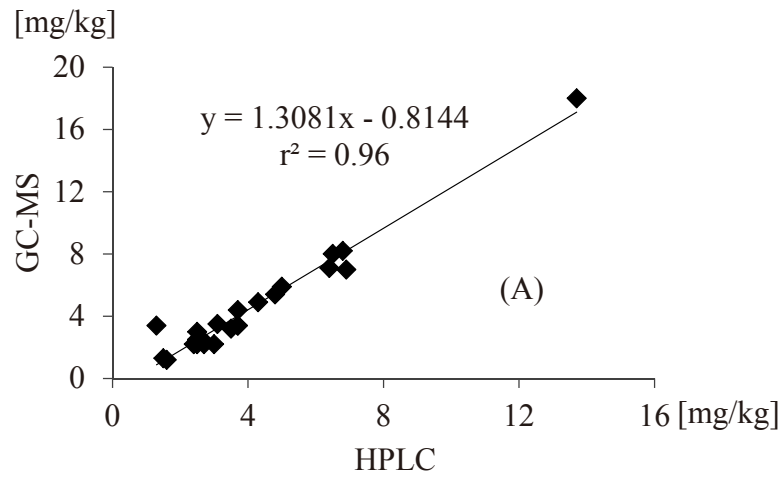


Fig. 3-5. Regression of ERG was compared with GC-MS and HPLC method.

A: Maize (n=24)

B: Wheat (n=13)

Table 3-1 Mean (%) recoveries and RSD (%) of developed GC-MS method (n=6)

ERG spiked (mg/kg)	Maize		Wheat	
	Recovery (%)	(RSDr %)	Recovery (%)	(RSDr %)
1	98	8	101	5
3	110	5	96	7
8	98	5	110	6

Table 3-2 ERG content ranges, medians and distributions determined by GC-MS

Matrix	ERG content (mg/kg)	Median	ERG distribution (mg/kg)		
			< 3	3 - 8	> 8
Maize (n=24)	1.3-13.7	3.5	10	13	1
Wheat (n=13)	1.0-14.4	3.9	0	9	4



## **Chapter 4: Development of an analytical method for simultaneous mycotoxins determination, and the relationship between ergosterol and mycotoxin contamination in maize**

### **4.1. Introduction**

Maize is one of the most important cereal crop that grows in many regions of the world. Mycotoxigenic fungal species of *Aspergillus*, *Penicillium*, and *Fusarium* infect to maize, and produce mycotoxins. Among more than 300 mycotoxins, AFs, OTA and ZEA are of major concern owing to their toxicity and occurrence (Pitt, 2006).

Mycotoxins represent a wide range of acute and chronic toxicological effects on human and animal health. AFs have demonstrated carcinogenic, mutagenic, and teratogenic properties (CAST, 2003). Liver is a primary target organ of AFs. OTA is a potent nephrotoxin that also shows carcinogenic, hepatotoxic, nephrotoxic, teratogenic, and immune-toxic effects. Besides, ZEA is an estrogenic mycotoxin that causes reproductive disorders in female swine and hyper-oestrogenic syndromes in human (Zinedine *et al.*, 2007). Owing to their toxic consequences on human health, IARC (1993) considered AFs and OTA as a group 1 and group 2B carcinogen, respectively. Later, IARC (1999) has been classified ZEA as a group 3 carcinogen.

A recent worldwide survey of over 19,000 samples of feed and feed ingredients revealed that the contamination frequencies of 26 % for AFs, 25 % for OTA, and 37 % for ZEA (Schatzmayr and Streit, 2013). The co-occurrence of several mycotoxins in the same sample may produce antagonistic, additive, or synergistic effects. Due to the chemical diversity of mycotoxins and their varying concentrations between samples, simultaneous analysis of multiple mycotoxins is a great challenge for analytical chemists (Ibáñez-Vea *et al.*, 2011). A few reports have described the simultaneous analysis of AFs, OTA, and ZEA by HPLC-FL after immunoaffinity column (IAC) purification in cereals (maize, wheat, and rice) from Malaysia (Rahmani *et al.*, 2010; Soleimany *et al.*, 2011); breakfast cereals, and barley from Spain (Ibáñez-Vea *et al.*, 2011; Ibáñez-Vea *et al.*, 2012); and breakfast cereals from Pakistan (Iqbal *et al.*, 2014).

An application note from R-Biopharm Rhône Ltd. (Glasgow, UK) has described AFs, OTA and ZEA determination using HPLC-FL after an AO-ZON PREP<sup>®</sup> IAC column clean-up. Owing to their chemical diversity, these three mycotoxins (AFs with OTA, and ZEA) were determined separately. As simultaneous mycotoxins determination can reduce analysis time and labour, an analytical method was developed for the determination of these mycotoxins in a single run using HPLC.

Most analysis methods for mycotoxins have been developed and validated for specific mycotoxin in a specific matrix. Even with advanced LC-MS/MS (tandem MS) instrument, it is impossible to analyse all mycotoxins in a single run (Tang *et al.*, 2013). It would be preferable if the level of fungal and mycotoxin contamination in grains can be estimated by the contents of ERG, a major sterol found in fungal cell wall (Seitz *et al.*, 1977). The relationship between ERG levels and mycotoxin contamination have also been investigated, particularly with AFs (Gourama and Bullerman 1995; Castro *et al.*, 2002; Pietri *et al.*, 2004, Karaca and Nas 2006; Ekinici *et al.*, 2014), OTA (Olsson *et al.*, 2004; Varga *et al.*, 2002), ZEA (Zill *et al.*, 1988; Pietri *et al.*, 2004), deoxynivalenol (Lamper *et al.*, 2000), and patulin (Kadikal *et al.*, 2005; Ekinici *et al.*, 2014).

Therefore, the major objectives of this study were to develop and validate a simultaneous determination method for these three agriculturally important mycotoxins (AFs, OTA and ZEA) using AO-ZON PREP<sup>®</sup> column purification, and to investigate the relationship between ERG content and mycotoxin contamination. Another objective was to identify regional variations in ERG content and mycotoxin contamination in maize samples collected from different geographical regions.

## **4.2. Materials and methods**

### *4.2.1. Samples*

One hundred and thirty nine maize samples were obtained from North America (n=76), South America (n=24), Asia (n=32), and Europe (n=7) in the year of 2011 and 2013. These samples were at first submitted to the two analytical laboratories (Singapore and the US) for analytical purpose. Samples from Asian countries included Thailand, Taiwan, Cambodia, Indonesia, Philippines, Australia, India, and Pakistan. American

countries included the US, Brazil, and Argentina. European countries included Ukraine, Russia, and Azerbaijan. The minimum size of each sample was 500 g. All samples were ground by milling through a 1-mm mesh (Rotter mill, Fritsch), and stored at -20 °C until analysis.

#### 4.2.2. Materials and reagents

ERG standard (98 %) was obtained from Acros Chemical. Analytical standards of AFs (AFB<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) and OTA were purchased from Biopure (Tulln, Austria), and ZEA was purchased from Sigma-Aldrich. AO-ZON PREP<sup>®</sup> IAC was purchased from R-Biopharm Rhône Ltd. HPLC-grade methanol was purchased from Wako Pure Chemical. All other chemicals and reagents were purchased from Kanto Chemical. The water was purified by a water purification system (Autopure WT100, Yamato).

PB (0.1 mol/L) was prepared by dissolving sodium phosphate dibasic and potassium dibasic in ultrapure water, adjusted to pH 7.4. After autoclaving at 121 °C for 15 min, the solution was stored at 4 °C. To make phosphate buffer saline (PBS, 0.01 mol/L), the solution was diluted 10-fold and sodium chloride was added.

#### 4.2.3. Standard solutions

ERG stock solution (2000 µg/mL) was prepared by dissolving in methanol. Standard calibration solutions (0.2, 0.5, 1, 5, 10, 20, 40 µg/mL) were made by diluting the stock solution with methanol. Standard stock solutions of AFB<sub>1</sub> and AFG<sub>1</sub> (9 ng/mL), AFB<sub>2</sub> and AFG<sub>2</sub> (2.25 ng/mL), OTA (135 ng/mL), and ZEA (4.5 µg/mL) were prepared daily by diluting in acetonitrile. Standard calibration curves were made daily by diluting the stock solution as follows: AFB<sub>1</sub> and AFG<sub>1</sub> (0.1-4.5 ng/mL), AFB<sub>2</sub> and AFG<sub>2</sub> (0.025-1.125 ng/mL), OTA (1.5-67.5 ng/mL), and ZEA (0.05-2.25 µg/mL). All solutions were stored at -20 °C in the dark.

#### 4.2.4. ERG analysis

ERG extraction, clean-up and analysis was performed as described by Miyagawa *et al.*, (2009). Briefly, 10 g sample, 5 g sodium hydroxide, and 40 mL methanol were placed in

a 300-mL flat-bottom flask. The samples were refluxed with a mantle-type heater for 1 h. After cooling at room temperature, methanol was added to compensate for evaporation during refluxing. The samples were filtered through Whatman No. 2 filter paper. Then, 10 mL of the filtrate was transferred to a 200-mL separating funnel, 10 mL 3 % aqueous potassium chloride was added and mixed. After adding 10 mL hexane, the mixture was shaken vigorously by hand for 3 min. A 5-mL sample of the hexane layer was collected and passed through a Sep-Pak<sup>®</sup> Plus silica cartridge containing 690 mg sorbent/cartridge (Waters, Milford, USA) after pre-conditioning with 5 mL hexane. Elution was performed with 5 mL methanol in an 8-mL amber vial and stored at -20 °C until HPLC-UV analysis.

ERG was analysed by HPLC with UV detection (Shimadzu LC-10 series, Shimadzu) on an ODS column (ODS-SP, 4 mm i.d. × 150 mm, 5 µm, GL Sciences) with a guard cartridge (Inertsil ODS-SP, 4 mm i.d. × 10 mm, 5 µm, GL Sciences). The column oven temperature was maintained at 40 °C. Mobile phase A was a mixture of methanol and water (80:20, v/v) and mobile phase B was a mixture of methanol and ethanol (70:30, v/v). The ratio of mobile phase A and B was 1:1 for the first 5 min. After that, the ratio of B was increased to 70 % for 5 min, and 90 % for 3 min. This last ratio was maintained for another 5 min. The mobile phase flow rate was 1 mL/min, and 10 µL of the sample was subjected to HPLC analysis. The UV absorption was measured at 282 nm.

#### 4.2.5. *Mycotoxins analysis*

Twenty-five grams of ground sample and 100 mL of a methanol and water mixture (80:20, v/v) were placed in a 300 mL of an Erlenmeyer flask. This flask was shaken for 30 min at 220 rpm by a reciprocal shaker (SA-31, Yamato Scientific). The sample was filtered through Whatman No. 113 filter paper (Maidstone, UK) and 10 mL of the filtrate was diluted with 40 mL PBS (0.01 mol/L). The IAC was conditioned with 3 mL PBS before loading the filtrate. A 20-mL sample of the diluted filtrate was passed through the AO-ZON PREP<sup>®</sup> IAC. After passing the sample, the IAC was washed with 20 mL PBS followed by 10 mL of water. Air was passed through the column to remove residual liquid. Elution was performed with 2 mL acetonitrile followed by 1 mL methanol. After

N<sub>2</sub> gas evaporation of the eluted samples, the residue was re-dissolved in 1 mL of 50 % acetonitrile for HPLC analysis.

AFs, OTA, and ZEA were analysed by HPLC with FL detection (Shimadzu LC-10 series, Shimadzu). Mycotoxins were separated on an ODS column (Develosil ODS-UG-5, 5 µm, 4.6 mm i.d. × 100 mm, Nomura Chemical) with a guard cartage (4 mm i.d. × 10 mm, Nomura Chemical). The column oven temperature was set at 45 °C. A 20-µL sample was injected with the mobile phase flow rate at 1 mL/min. Mobile phase A was a mixture of acetonitrile: methanol: 0.1 % acetic acid in water (5:35:60, v/v/v) and mobile phase B was a mixture of acetonitrile: methanol: 0.1 % acetic acid in water (70:10:20, v/v/v). The initial ratio of mobile phase A and B was set at 88:12 for the first 3.5 min. The ratio of B increased to 20 % for 4.5 min, and then 30 % for 21 min. The ratio of B increased to 60 % for the next 6 min and then returned to 12 %. To enhance detection of aflatoxin B<sub>1</sub> and G<sub>1</sub>, post-column derivatization was performed with a photochemical reactor (PHRED, Aura Industries). Excitation and emission wavelengths were set at 365 and 435 nm for AFs, 274 and 440 nm for ZEA, and 336 and 464 nm for OTA.

#### 4.2.6. Statistics

Concentration, means and medians were calculated in Microsoft Excel (ver. 2010). Regression analysis was used to assess the correlation between ERG and each mycotoxin group. The Kruskal-Wallis or Median test was used to evaluate regional differences (SPSS ver. 15, IBM, USA). A probability value of 0.05 indicated statistical significance.

### 4.3. Results and discussion

#### 4.3.1. Method optimisations for mycotoxins analysis

##### 4.3.1.1. IAC elution solvents

For selection of elution solvents from IAC, three different combination of solvent conditions (acetonitrile 2 mL and methanol 1mL; acetonitrile 1 mL and methanol 2 mL; mixture (3 mL) of acetonitrile and methanol 1:1, v/v) were studied as shown in Table 4-1. As shown in Table 4-1, acetonitrile (2 mL) followed by methanol (1 mL) gave better performance for AFs and OTA. So, acetonitrile followed by methanol was decided to use

as an elution solvents. After observing low recovery (%) of ZEA at 2000 µg/kg level in Table 4-1, lower concentration of ZEA (1000 µg/kg) was spiked and 70.7 % of ZEA was recovered. Low recovery of ZEA was observed owing to the overloading problem of IAC.

#### 4.3.1.2. Changing the HPLC column

Initially, Develosil ODS-HG-5 column was used for simultaneous mycotoxin separation. After observing good separation and shortened retention time (Fig. 4-1) for AFs, the column was changed to Develosil ODS-UG-5 for subsequent mycotoxins analysis.

#### 4.3.1.3. Mobile phase compositions and gradient

After experimenting different mobile phase compositions, mobile phases were set as follows: mobile phase A consists of acetonitrile: methanol: 0.1 % acetic acid in water (5:35:60, v/v/v) and mobile phase B consists of acetonitrile: methanol: 0.1 % acetic acid in water (70:10:20, v/v/v), and a better separation of AFs, OTA and ZEA was shown in Fig. 4-2. For HPLC analysis, gradient conditions were optimised for mycotoxins analysis (Fig. 4-3). After AFs (Ex: 365 nm/Em: 435nm) detection by fluorescence (0-10 min), the wavelengths of fluorescence detector were changed for ZEA (Ex: 274 nm/Em: 440 nm) from 10-20.8 min, and OTA (Ex: 335 nm/Em: 464 nm) from 20.8-35 min.

#### 4.3.2. Method performance for ERG and mycotoxins

The performance characteristics of the analytical method were assessed in terms of selectivity, linearity, sensitivity, and recovery. Both ERG and mycotoxin method were selective after using Silica cartridge and IAC clean-up, and determined by HPLC with UV and FL detection, respectively. Fig. 4-4 shows the chromatograms of a standard ERG solution with a naturally contaminated maize sample at 13.1 mg/kg. Naturally contaminated maize samples with different levels of AFs, OTA, and ZEA are shown in Fig. 4-5. Retention times for ERG and each mycotoxin peak corresponded with the standard solutions with a tolerance of  $\pm 2.5$  %. Calibration curves for ERG and mycotoxins (AFB<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, OTA, and ZEA) showed linearity within the tested ranges

with a co-efficient of determination in excess of 0.999. The LOD was determined to be 0.24 mg/kg for ERG, 0.025 µg/kg for AFB<sub>1</sub>, 0.0125 µg/kg for AFB<sub>2</sub>, 0.05 µg/kg for AFG<sub>1</sub>, 0.025 µg/kg for AFG<sub>2</sub>, 0.5 µg/kg for OTA, and 15 µg/kg for ZEA in maize. The LOQ was determined to be 0.1 µg/kg for AFB<sub>1</sub>, 0.05 µg/kg for AFB<sub>2</sub>, 0.2 µg/kg for AFG<sub>1</sub>, 0.08 µg/kg for AFG<sub>2</sub>, 1.5 µg/kg for OTA, and 50 µg/kg for ZEA in maize.

Recovery of ERG was checked at two concentration levels (3 and 8 mg/kg) with six replicates on ERG-free maize samples, which yielded mean recoveries of 71 %-81 % with RSDr of 5.7-12 %. For mycotoxins, blank maize samples were spiked simultaneously at 5 µg/kg AFB<sub>1</sub> and AFG<sub>1</sub>, 1.5 µg/kg AFB<sub>2</sub> and AFG<sub>2</sub>, 2.5 µg/kg OTA, and 0.5 mg/kg of ZEA with triplicates. The mean recoveries were AFB<sub>1</sub> (76 %), AFB<sub>2</sub> (83 %), AFG<sub>1</sub> (80 %), AFG<sub>2</sub> (85 %), OTA (90 %), and ZEA (89 %), RSDr of 0.6-4.9 %

#### 4.3.3. ERG levels in maize

ERG was detected in all 139 maize samples, with an average content of 9.5 mg/kg and a maximum of 119 mg/kg (Table 4-2). According to Pietri *et al.*, (2004), the quality of maize is acceptable if the ERG content is less than 3 mg/kg. If the ERG content is more than 8 mg/kg, the potential of fungal invasion/mycotoxin contamination is high. Low mycotoxin contamination was observed in samples with less than 3 mg/kg ERG. As shown in Fig. 4-6, 20 % of the maize samples contained less than 3 mg/kg ERG; 48 % of the samples contained 3-8 mg/kg; 13 % of samples contained between 8 and 12 mg/kg ERG; 19 % of samples contained more than 12 mg/kg.

#### 4.3.4. AFs, OTA and ZEA levels in maize

After determination of ERG content, all 139 maize samples were analysed for mycotoxins. The frequency and contamination of AFs, OTA, and ZEA are presented in Table 4-2. Mycotoxin occurrence was considered positive if contamination were higher than the LOD. Results showed that 74 % of the samples were positive for AFB<sub>1</sub>, with mean and maximum levels of 11.8 and 327 µg/kg, respectively. The contamination frequencies of AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub> were 43 %, 4 %, and 0.7 % respectively; AFB<sub>2</sub> contamination ranged from 0.01 to 33.8 µg/kg. Surprisingly, only one sample contained a



high level of OTA (19.8 µg/kg) that exceeds the EU commission permissible limits for unprocessed cereals (5 µg/kg). In addition, 24 % of samples were contaminated with ZEA, with mean and maximum levels of 118 and 626 µg/kg, respectively (Table 4-2).

In this study, 25 % of samples contained more than the maximum permissible limit of AFB<sub>1</sub> (5 µg/kg; Fig. 4-6), and 73 % of the samples contained AFB<sub>1</sub> levels greater than the LOQ. About 10 % of the samples contained above-limit levels of total AFs (10 µg/kg). Besides, 22 % of samples contained more than 100 µg/kg ZEA (Fig. 4-6) whereas only 4 % of samples were above the EU maximum permissible limits in unprocessed maize (200 µg/kg).

#### 4.3.5. Co-occurrence of AFs, OTA, and ZEA

Thirty-four percent of samples contaminated with one mycotoxin, 42 % contaminated with two mycotoxins, 7 % contaminated with three, and only 1 % of the samples contaminated with four mycotoxins. No mycotoxins were detected in 16 % of samples. Grains are often contaminated with multiple mycotoxins that potentiate their toxic effects (Prelusky *et al.*, 1994). After reviewing 100 studies on mycotoxin interactions with adverse effects on animal health, Grenier and Oswald (2011) concluded that most co-occurring mycotoxins produce additive or synergistic effects. Thus, determining single mycotoxins in grains cannot assure the toxicity of fungal metabolites. Two or more mycotoxins were found in 50 % of samples, higher than the findings of Schatzmayr and Streit (2013), who reported that 39 % of samples contained two or more mycotoxins. This study also revealed the co-occurrence of AFs and ZEA in 47 % of samples. This confirms previous findings ZEA-contaminated corn samples are frequently contaminated with AFs (EFSA, 2004a). AFB<sub>1</sub> and AFB<sub>2</sub> were detected on 58 % of the maize samples. Therefore, the co-occurrence of mycotoxins should be taken into account when making exposure risk assessments.

#### 4.4. Relationships between ERG content and mycotoxin contamination

ERG presence in grain matrices indicates fungal growth, and may give a signal for possible mycotoxin production (Seitz *et al.*, 1977 and 1979; Zill *et al.*, 1988; Saxena *et al.*,



2001). The relationship between carcinogenic AFB<sub>1</sub> and ERG content has been investigated in previous studies (Pietri *et al.*, 2004; Castro *et al.*, 2002; Gourama and Bullerman, 1995). No significant correlation was observed between ERG and the presence of AFB<sub>1</sub>/total AFs in maize, agreement with the results of Pietri *et al.*, (2004). When ERG content in 25 samples with >5 µg/kg AFB<sub>1</sub> was compared, a very weak correlation ( $r^2=0.253$ ) was observed. All 25 samples contained more than 3 mg/kg ERG. After analysing 139 samples, of which 26 samples (19 %) contained less than 3 mg/kg ERG and AFB<sub>1</sub> levels of 0.03-3.1 µg/kg. With the exception of two samples that contained 1.5 and 3 µg/kg AFB<sub>1</sub>, the samples contained less than 1 µg/kg AFB<sub>1</sub>). Castro *et al.*, (2002), and Gourama and Bullerman (1995) reported that AFB<sub>1</sub> positively correlates with ERG content in grains, but AFB<sub>2</sub> and AFG<sub>1</sub> do not follow similar trends, as also observed in this study. ERG content is not always consistent with AFs production in grains because of the differences in fungal species and environment.

No significant correlation was also observed between ERG and OTA production in maize as only one sample contained OTA at 19.3 µg/kg with an ERG level of 4.79 mg/kg at this time. However, a positive correlation between ERG content and the presence of OTA has been observed in grains (Saxena *et al.*, 2001; Olsson *et al.*, 2002; Abramson *et al.*, 2005; Tangni and Pussemier, 2006). To understand the correlation of occurrence between ERG and OTA, more samples with an OTA contamination in maize need to be examined.

ZEA production is usually favoured in humid and low-temperature regions, and found mainly in high moisture maize (CAST, 2003). A significant correlation ( $r^2=0.82$ ) was observed between ERG content and ZEA production in maize (Fig. 4-7). This relationship between ERG and ZEA levels has also been reported in a separate studies (Pietri *et al.*, 2004; Neuhof *et al.*, 2008; Zill *et al.*, 1988). A positive correlation was reported between ERG and combined DON with ZEA contamination by Pietri *et al.*, (2004). Another study by Neuhof *et al.*, (2008) showed a relationship between ERG and ZEA with a correlation coefficient of 0.999 on fractioned wheat kernels. In this study, samples contaminated with higher ZEA levels (>100 µg/kg) also contained more than 3 mg/kg ERG. No samples

with ZEA <50 µg/kg showed ERG levels above 3 mg/kg. Among 26 samples with less than 3 mg/kg ERG, only one sample contained 32 µg/kg ZEA.

#### 4.4.1. ERG and production of mycotoxins based on origins of samples

Mycotoxin contamination on grains was caused by both plant pathogenic and storage fungi. Therefore, mycotoxin contamination was effected by various factors such as climate, plant, storage, insect damage, pest attack and so on (Paterson and Lima, 2010). ERG and levels of mycotoxins by different geographic region are summarised according to geographic origin in Table 4-3. Higher mean and maximum values (> 8 mg/kg) of ERG were found in North American and Asian samples. North American and Asian samples also showed higher levels of contamination with AFB<sub>1</sub>, AFB<sub>2</sub> and ZEA. Nevertheless, no significant difference was observed for ERG between regions (Kruskal-Wallis or median test). More than 3 mg/kg ERG was observed in 83 % of North American samples, 79 % of South American samples, 72 % of Asian samples, and 79 % of European samples (Fig. 4-8). ERG content >8 mg/kg was observed in 32 % of North American samples, 29 % of South American samples, 37 % of Asian samples, and 7 % of European samples.

Aflatoxigenic fungi are native to arid, semi-arid, warm, tropical climate. Therefore, changes in climate may generate large fluctuations in the quantity of aflatoxin producers (Bock *et al.*, 2004; Shearer *et al.*, 1992). In this study, the mean values of AFB<sub>1</sub> from North American and Asian samples were 8.2 and 23.3 µg/kg, both of which exceed the EU maximum permissible limits (5 µg/kg) for AFB<sub>1</sub> (Table 4-3). As expected, significant differences ( $p < 0.05$ ) were observed for AFB<sub>1</sub> and AFB<sub>2</sub> between regions. According to Wu *et al.*, (2011), high temperature and drought stress directly impact maize and *A. flavus* growth. High temperature and low rainfall favour the infection of maize with *A. flavus*, which produce high levels of AFs (Jones *et al.*, 1980; Payne *et al.*, 1985; Shearer *et al.*, 1992). This study observed that North American and Asian maize samples have a high level of fungal invasion and AFs contamination, especially AFB<sub>1</sub> and AFB<sub>2</sub>. Grains generally grown in warm climates have a greater chance of aflatoxigenic fungal infection and in some regions, infection only observed when temperatures rise in association with drought (Sanders *et al.*, 1984; Schmitt and Harburgh, 1989). In contrast, AFG<sub>1</sub> and AFG<sub>2</sub>

contamination were detected on five samples from North America. No European maize samples were contaminated with AFs as these toxins are mainly favoured by tropical and sub-tropical climate (EFSA, 2004b).

OTA producing fungi were found across a wide range of climatic conditions and include the species of *Aspergillus* and *Penicillium*. The production of OTA was considered a storage-related rather than pre-harvest problem (Petzinger and Weidenbach, 2002). In the four geographical regions covered in this study, only one North American sample contained 19.3 µg/kg OTA (Table 4-3). This findings differ from those of Schatzmayer and Streit (2013), whose study on feed and raw materials showed OTA in 25 % of samples.

ZEA-producing fungi may be found in the field and in improperly stored animal feeds (Kuiper-Goodman *et al.*, 1987). In this study, ZEA did not significantly differ by region. North American samples showed the highest maximum level of ZEA contamination (626 µg/kg), with a mean value of 186 µg/kg (Table 4-3). The higher maximum (331 µg/kg) and mean values (83.2 µg/kg) were also observed in Asian maize with a contamination frequency of 22 %. South American (25 %) and European (57 %) samples showed low levels (mean values <50 µg/kg) of ZEA contamination.

The natural co-occurrence of mycotoxin in individual maize samples varies by geographic region (Table 4-4). Two or more mycotoxins co-occurred in North American (52 %) and South American (22 %) maize samples. Asian countries showed the highest number of samples (79 %) with more than two mycotoxins. No mycotoxin combinations were found in European samples.

#### **4.5. Conclusion**

Fungal and mycotoxin contamination are unavoidable problem for agricultural commodities. As an economically important crop worldwide, mycotoxin-contaminated maize is a global trade concern. For ensuring frequently encounter mycotoxins contamination, a simultaneous mycotoxins method is useful and highly desirable. Therefore, a simultaneous determination of three agricultural important mycotoxins (AFs, OTA and ZEA) was developed and validated using HPLC-FL detection. Method

performance such as selectivity, recovery, LOD and LOQ were evaluated. The developed simultaneous method was successfully validated on 139 naturally contaminated maize samples. As ERG analysis is a good indicator for initial screening of bulk samples to know fungal and mycotoxin contamination, all maize samples were analysed to determine ERG using HPLC-UV detection. After determining ERG content, the relationship between ERG and AFs, OTA, and ZEA contamination were investigated in maize samples. Although the correlation between ERG and mycotoxin is not always absolute as observed in previously reported studies, here a good correlation ( $r^2=0.82$ ) was observed between ERG and ZEA contamination. However, no significant correlation was observed between ERG and total AFs or OTA. Results also indicate that North American and Asian samples showed the highest frequency and the levels of mycotoxin contamination. However, maize samples containing less than 3 mg/kg of ERG were less likely to exceed the maximum permissible limits of AFs, OTA, and ZEA set by the EU. This indication of ERG might be useful in the grain industry to monitor fungal invasion and, on a merely qualitative basis, mycotoxin contamination on maize.

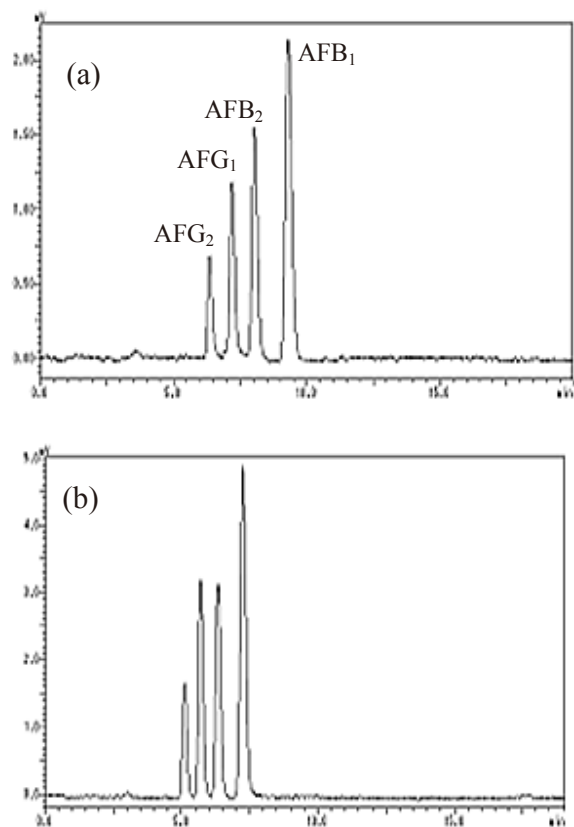


Fig. 4-1. Chromatograms of AFs with Develosil ODS-HG-5 (a) and ODS-UG-5 (b) column.

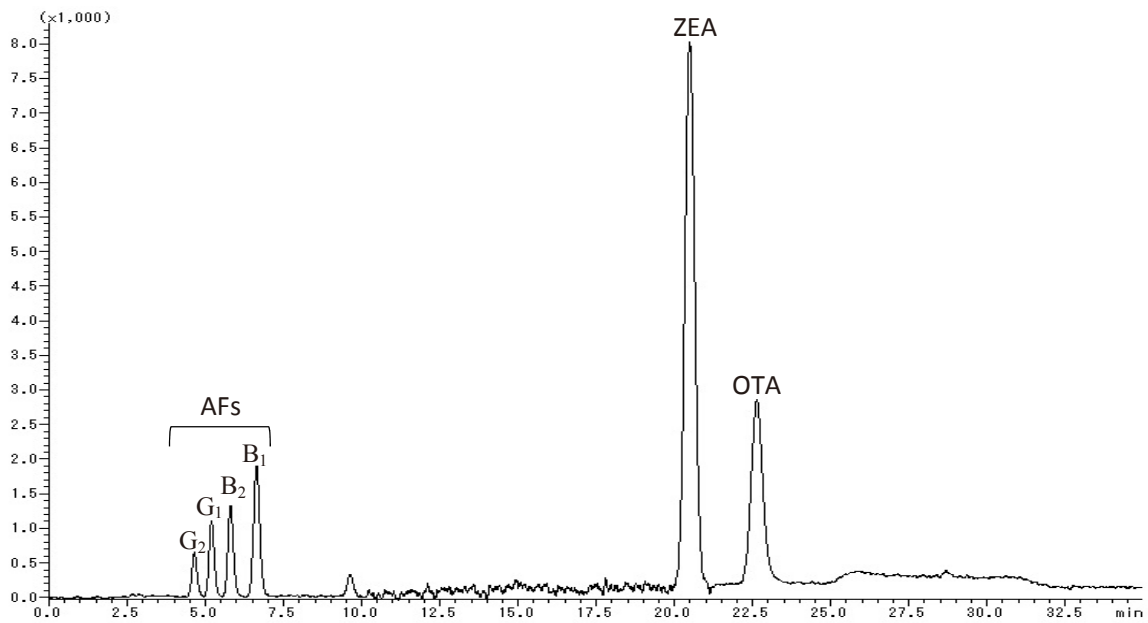


Fig. 4-2. Chromatograms of AFs (0.01 ng of AFB<sub>1</sub> and AFG<sub>1</sub>, 0.0025 ng of AFB<sub>2</sub> and AFG<sub>2</sub>), OTA (0.15 ng), and ZEA (5 ng) standards.

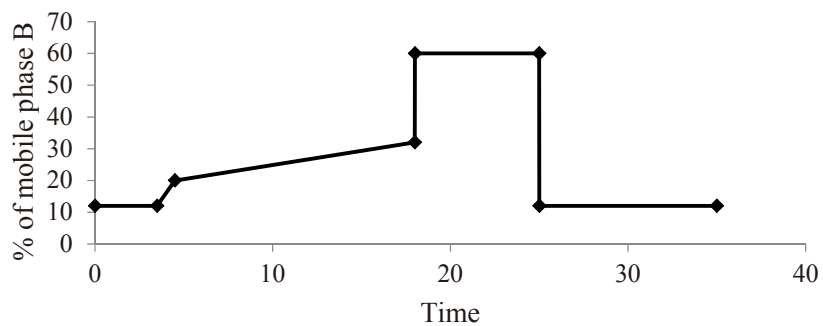


Fig. 4-3. Gradient conditions for simultaneous AFs, OTA and ZEA analysis.

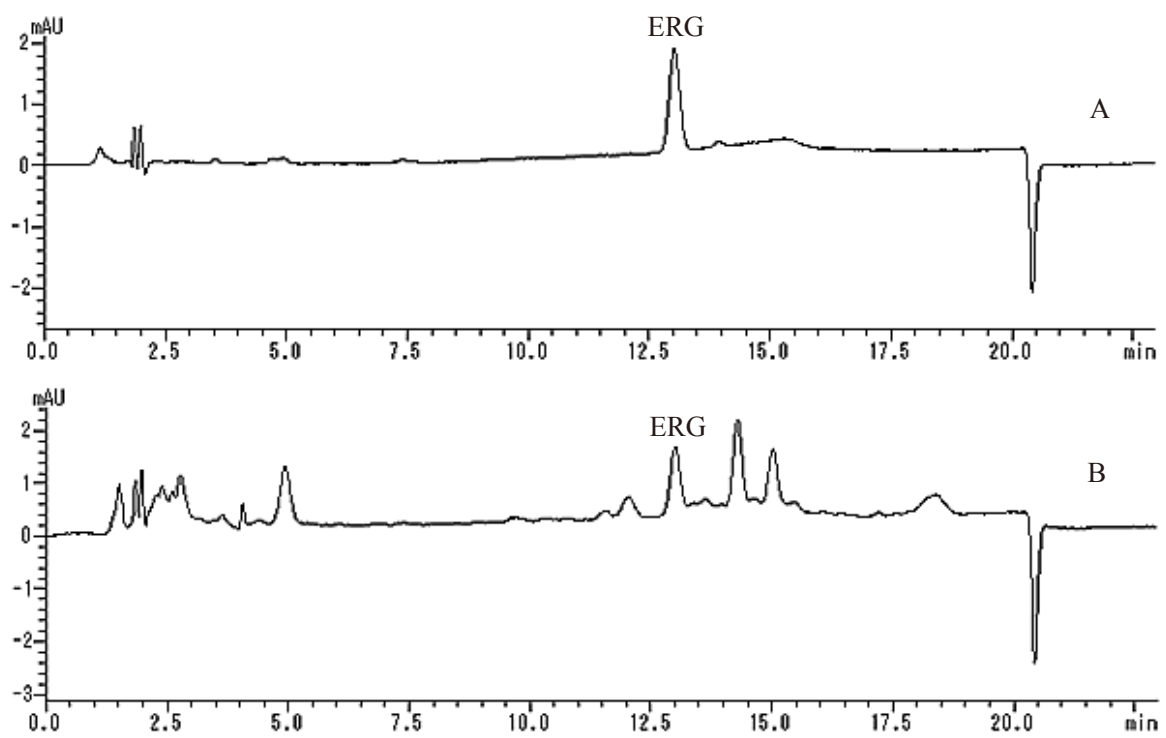


Fig. 4-4. HPLC chromatograms of ERG in maize samples.

A) Standard solution of ERG at 5 mg/L

B) ERG in a naturally contaminated maize at 13.1 mg/kg.

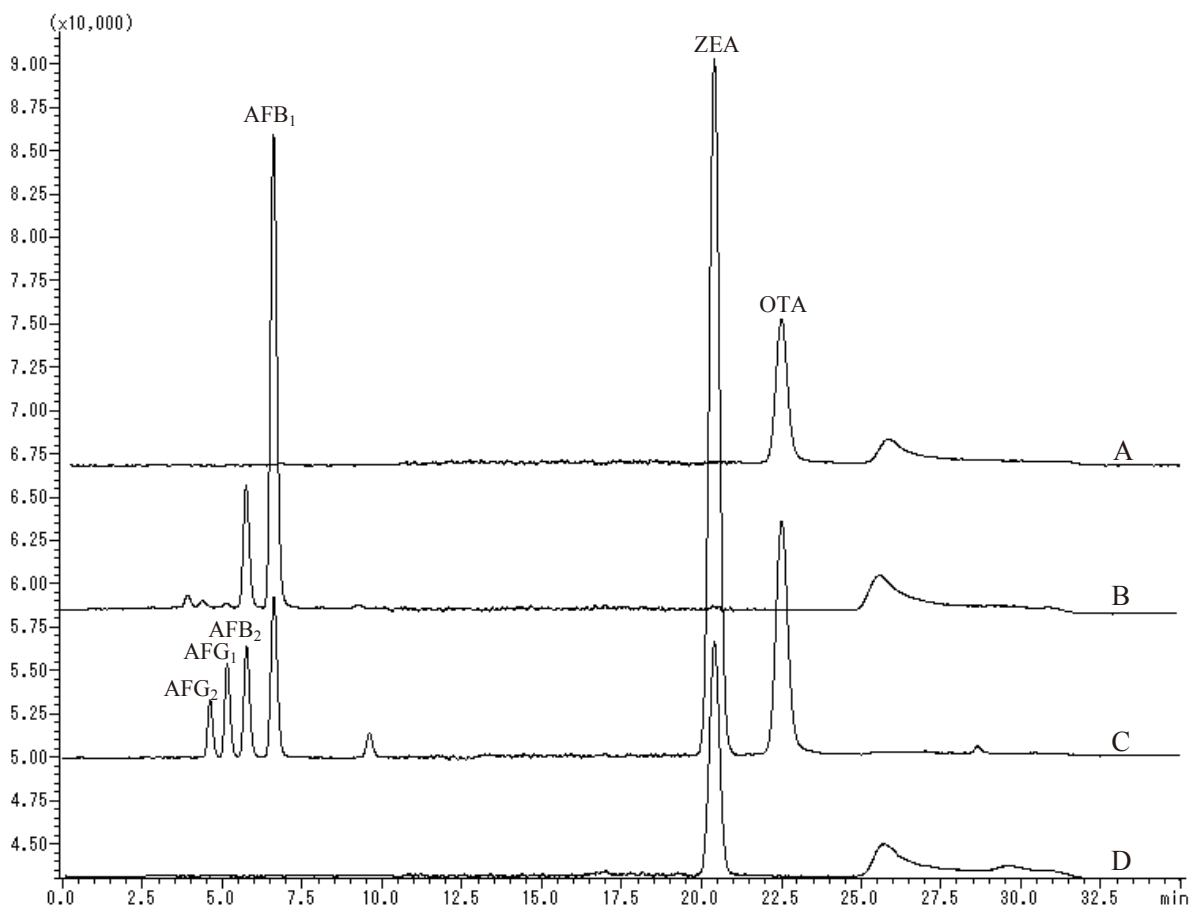


Fig. 4-5. HPLC chromatograms of AFBs, OTA and ZEA in maize samples.

A) OTA in a naturally contaminated maize sample at 19.3  $\mu\text{g}/\text{kg}$

B) Naturally contaminated AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> at 6.2, 0.6, 0.1  $\mu\text{g}/\text{kg}$ , in maize respectively

C) Standard solutions of AFBs (AFB<sub>1</sub> and AFG<sub>1</sub> at 2 ng/mL, AFB<sub>2</sub> and AFG<sub>2</sub> at 0.5 ng/mL), OTA at 45 ng/mL and ZEA at 1  $\mu\text{g}/\text{mL}$

D) Naturally contaminated ZEA at 331  $\mu\text{g}/\text{kg}$  in maize sample



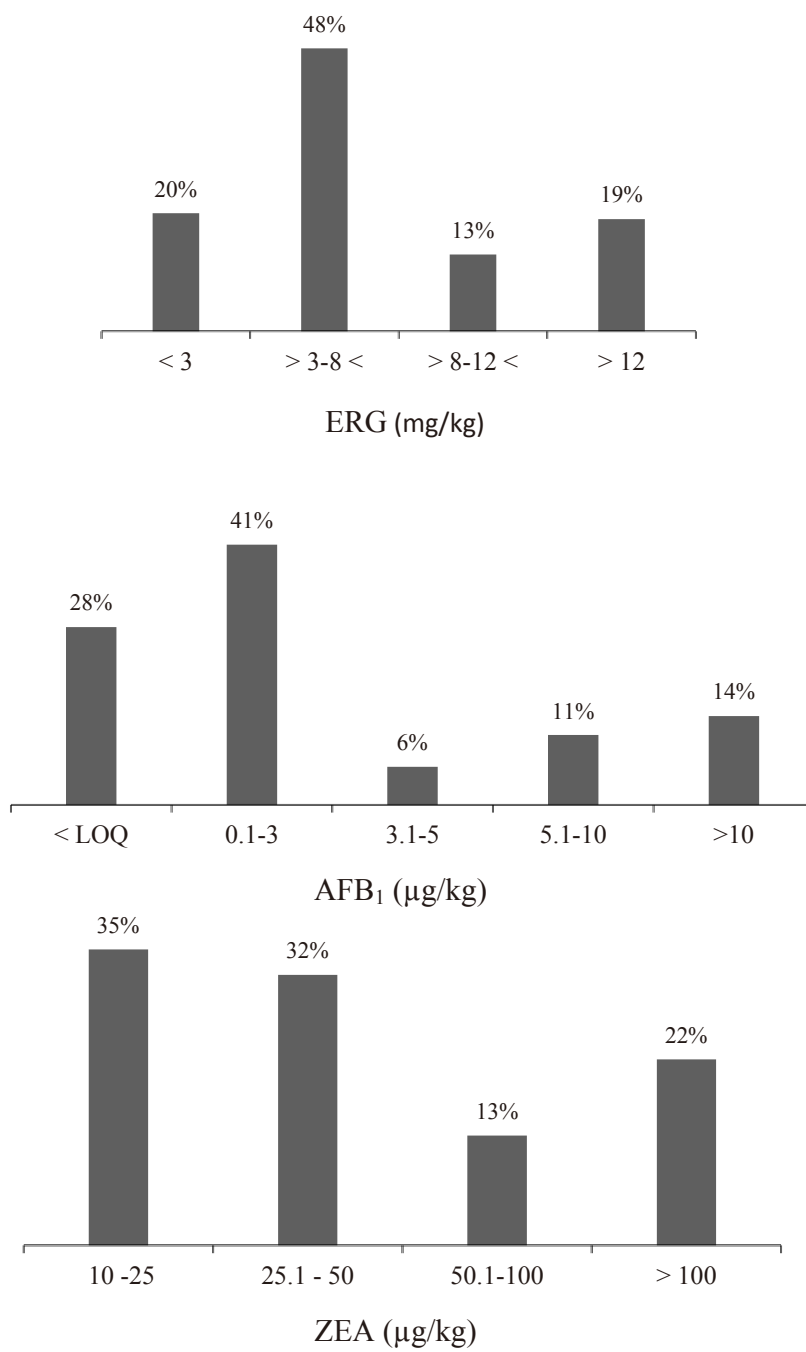


Fig. 4-6. Distributions of ERG, AFB<sub>1</sub> and ZEA in maize samples.

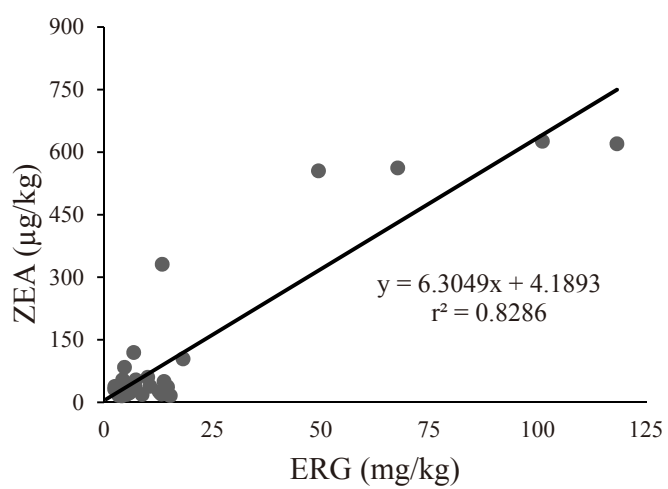


Fig. 4-7. Regression of ERG content and ZEA concentration in maize

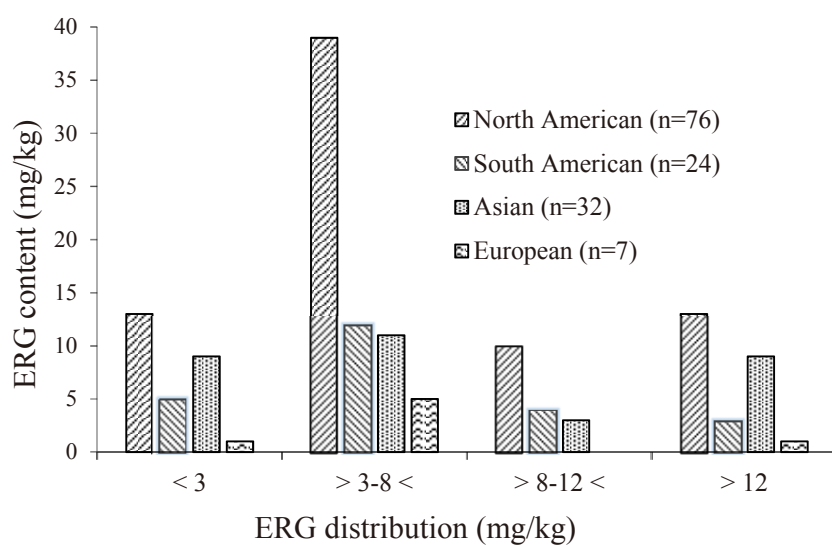


Fig. 4-8. Distributions of ERG content (mg/kg) from different geographical regions

Table 4-1 Different solvent combinations for IAC elution of AFs, OTA and ZEA

Mycotoxins	Spiking levels (µg/kg)	Recovery (%)		
		Eluent (1) <sup>a</sup>	Eluent (2) <sup>b</sup>	Eluent (3) <sup>c</sup>
Aflatoxin B <sub>1</sub>	20	86.5	83.8	75.0
Aflatoxin B <sub>2</sub>	6	90.1	86.8	79.7
Aflatoxin G <sub>1</sub>	20	74.3	63.2	44.1
Aflatoxin G <sub>2</sub>	6	76.6	63.5	43.9
Zearalenone <sup>d</sup>	2000	37.9	38.0	38.5
Ochratoxin A	10	98.1	101.4	102.4

<sup>a</sup> Eluent 1 (acetonitrile 2 mL + methanol 1 mL)

<sup>b</sup> Eluent 2 (acetonitrile 1 mL + methanol 2 mL)

<sup>c</sup> Eluent 3 (mixture of acetonitrile: methanol, 3 mL)

<sup>d</sup> Low recovery owing to overloaded of IAC by ZEA

Table 4-2 Ergosterol level with mycotoxin contamination found in maize samples

Mycotoxins	Maize samples (n = 139)			
	Contamination frequency (%)	Ranges in positive samples (µg/kg)	Mean contamination (µg/kg)	Median (µg/kg)
Aflatoxin B <sub>1</sub>	74	0.03 - 327	11.8	0.65
Aflatoxin B <sub>2</sub>	43	0.01 - 33.8	1.3	0.2
Aflatoxin G <sub>1</sub>	4	0.1 - 0.5	0.25	0.2
Aflatoxin G <sub>2</sub>	0.7	0.05	0.05	0.05
Ochratoxin A	0.7	19.3	19.3	19.3
Zearalenone	24	15.1 - 626	118.0	37.35
Ergosterol	100	0.54 - 119 <sup>a</sup>	9.5 <sup>a</sup>	6.18 <sup>a</sup>

<sup>a</sup> mg/kg

Table 4-3 ERG and mycotoxin contamination found in maize samples from different regions

Regions	Different parameters	ERG (mg/kg)	AFB <sub>1</sub> (µg/kg)	AFB <sub>2</sub> (µg/kg)	AFG <sub>1</sub> (µg/kg)	AFG <sub>2</sub> (µg/kg)	OTA (µg/kg)	ZEA (µg/kg)
	% of positive	100	83	38	15	-	1	20
North	Mean value	11.1	8.2	1.6	0.2	-	19.3	186
American (n=76)	Median value	6.1	0.2	0.1	0.2	-	19.3	25.3
	Maximum level	119	327	33.8	0.2	-	19.3	626
	% of positive	100	42	21	-	-	-	25
South	Mean value	7.2	1.4	0.2	-	-	-	44.5
American (n=24)	Median value	6.8	0.25	0.2	-	-	-	42.4
	Maximum level	14.7	5.4	0.4	-	-	-	60
	% of positive	100	91	78	9	3	-	22
Asian (n=32)	Mean value	8.1	23.3	1.7	0.3	0.05	-	83.2
	Median value	5.53	3.9	0.4	0.2	0.05	-	38
	Maximum level	22.5	173	6.5	0.5	0.05	-	331
	% of positive	100	-	-	-	-	-	57
European (n=7)	Mean value	5.5	-	-	-	-	-	31.5
	Median value	4.6	-	-	-	-	-	26.8
	Maximum level	12.6	-	-	-	-	-	53.4
K-W test <sup>a</sup>	Statistics <sup>b</sup>	6.18	0.65	0.2	0.2	-	-	37.35
	Significance	0.72	0.00*	0.29*	1.0	-	-	0.76

- Not detected; <sup>a</sup>Kruskal-Wallis test; <sup>b</sup>Median test; \*Significant at p <0.05 level

Table 4-4 Co-occurrences of mycotoxins in maize samples from different geographical regions

Regions	Co-occurrence /sample <sup>a</sup> (%)				
	One mycotoxin	Two mycotoxin	Three mycotoxin	Four mycotoxin	No mycotoxin
North American (n=76)	40	49	3	0	8
South American (n=24)	38	13	9	0	40
Asian (n=32)	13	60	19	4	4
European (n=7)	58	0	0	0	42

<sup>a</sup> Each maize sample containing number of mycotoxins.

## Chapter 5. General conclusion

Grains are susceptible to fungal attack at different stages of production from pre-harvest to post-harvest. Mycotoxigenic genera such as *Aspergillus*, *Penicillium*, and *Fusarium* produce ‘mycotoxins’ in grains (Ibáñez-Vea *et al.*, 2012). Mycotoxins have toxic effects on humans and animals, including carcinogenic, teratogenic, mutagenic, nephrotoxic and immunotoxic effects (Steyn, 1995; Murphy *et al.*, 2006). Apart from this, mycotoxin-contaminated grains are becoming a worldwide concern in a recent years. To protect human and animal health, sensitive and reliable analytical methods are essential to monitor fungal and mycotoxin contamination in grains at low levels (Rahmani *et al.*, 2009). Therefore, this thesis describes the development, validation and application of analytical methods for four agriculturally important mycotoxins (AFs, OTA, ZEA and STC), and a fungal biomarker ‘ERG’ in grains using available HPLC-UV/FL, GC-MS and LC-MS analytical instruments.

First, a new clean-up method for STC in grains was developed for the first time using a commercially available IAC (AFLAKING<sup>®</sup>) that was originally developed for AFs clean-up. This newly developed IAC clean-up method shows good recovery for STC. After successful IAC purification, STC was determined using LC-MS and a single laboratory validation was performed on different types of grains. The method was selective for STC without showing any interference peaks in the entire chromatogram. The average recoveries of STC on grains at five spiking levels ranged from 83.2 to 102.5 % with RSDr (0.24-6.5 %). The method was reliable and sensitive (1 µg/kg) enough to monitor STC contamination in grains at low levels.

After determination of STC using LC-MS, GC-MS based STC determination method was developed in grains. To develop this method, IAC clean-up method that was previously developed for LC-MS analysis was slightly modified. Matrix effect was investigated in three different grains (maize, wheat and rice), and showed insignificant effects (<15%). The developed GC-MS method was selective, more sensitive (2.4 µg/kg) than previously published GC methods, and comparable with the LC-MS method.

To date, more than 300 mycotoxins have been reported worldwide (Milićević *et al.*, 2010). Even using modern instruments like LC-MS/MS, analysing all of these hundreds of mycotoxins within a short time is practically impossible. A good analysis method for fungal



biomarker to recognize fungal invasion in agricultural commodities is highly desirable. So a simple and rapid GC-MS method was developed and validated for the first time to determine ERG content in grains without any chemical derivatization step. The recovery of ERG content in maize and wheat at three levels ranged from 96 to 110 % with RSDr (<8%). The method was rapid, and more sensitive (40 µg/kg) than previously published HPLC and LC-MS methods for ERG determination. The method was also successfully applied to 37 naturally contaminated market grains to determine ERG levels. As this method is quick and sensitive, it will be helpful to detect fungal and possible mycotoxin contamination in the grain industry.

As AFs, OTA and ZEA are important mycotoxins that frequently contaminate grains, a simultaneous determination of AFs, OTA and ZEA using HPLC-FLD was developed and validated on 139 maize samples. Previously, AFs, OTA and ZEA were analysed separately which requires more time and expense. This new, simultaneous method was selective, sensitive, and useful for monitoring three important agricultural mycotoxins in a single run.

To correlate these determined AFs, OTA and ZEA with ERG, same maize samples were analysed to determine ERG levels using a simple existing HPLC-UV analysis method. Although the correlation between ERG and mycotoxin is not always absolute, a good correlation ( $r^2 = 0.82$ ) was found between ERG and ZEA contamination. However, no significant correlation was observed between ERG and AFs or OTA. More than two mycotoxins were found in 50 % of the analysed samples. The levels of mycotoxin contamination and ERG vary according to the origins of the sample. North American and Asian samples showed higher level of mycotoxin and ERG contamination. To conclude that samples containing less than 3 mg/kg ERG in most cases do not exceed the EU maximum limits for AFs, OTA, and ZEA. This ERG level might be a useful indicator to check fungal invasion and, also mycotoxin contamination in grains on a qualitative basis.

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