Development, evaluation and application of methods for mycotoxin analysis

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“พ่อไม่มีเงินทองมากองให้ จงตั้งใจพากเพียรเรียนหนังสือ

หาวิชาความรู้เป็นคู่มือ เพื่อยึดถือเอาไว้ใช้เลี้ยงกาย

พ่อกับแม่มีแต่จะแก่เฒ่า จำเลี้ยงเจ้าเรื่อยไปนั้นอย่าหมาย

ใช้วิชาช่วยตนไปจนตาย เจ้าสบายแม่กับพ่อก็ชื่นใจ”

~พระราชนิพนธ์ในรัชกาลที่ ๕~

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Chapter 1: General Introduction

Mycotoxins are natural contaminants produced by fungal species, and commonly occur in food and feed. The name mycotoxin is derived from 'mykes', a Greek word for fungus, and the Latin word 'toxicum' meaning poison. Mycotoxin contamination may occur at any stage in the food chain and more than a hundred mycotoxins have been discovered so far. Due to their adverse effects on humans and animals, mycotoxins remain challenging to classify. *Aspergillus, Penicillium, Fusarium,* and *Alternaria* are the major fungi that produce mycotoxins (Moss 1992; Placinta et al., 1999). The most important mycotoxins are aflatoxins, ochratoxins, fumonisins, trichothecene and zearalenone. Table 1 shows the different species of fungi and their corresponding mycotoxin production.

To address the adverse affects of mycotoxin contaminants in food and feed, national and international institutions and organisations, such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) of the United Nations have evaluated mycotoxin toxicological data based on the no observed adverse effect levels (NOAEL; μg/kg of body weight/day). The EC has set maximum levels for some mycotoxins in foods and feeds (EC 2006a). To protect consumers and apply these regulatory limits, development and validation of mycotoxin analytical methods are urgently required. Mycotoxin analysis methods include sampling, sample preparation (extraction and clean-up) and determination.

Sampling

The objective of the sampling step is to obtain a good representative sample from a large quantity. Since mycotoxin contamination is heterogeneous in food and feed samples, the traditional method of sampling is not suitable for mycotoxin analysis. The distribution of mycotoxins in the sample matrix is an important factor to be considered in establishing regulatory sampling criteria. Consequently, the European Commission (2006b) defined the method for sampling for mycotoxins in agricultural commodities. After a sample is taken, a milling step should follow. The choice of sample method is based on the size of the test sample and the type of commodity. The goal is to obtain small particles for accurate mycotoxin analysis.

Table 1. Major mycotoxins, their production fungi, health effects and commodities

Mycotoxin	Fungi species*	Health effects	Commodities
Aflatoxins	*A. flavus, A. parasiticus, A. nomius*	Liver diseases, carcinogenic and teratogenic effects	Nuts and groundnuts, cereals, milk, species
Fumonisins	*F. verticillioides, F. proliferatum*	Pulmonary oedema, nephro- and hepatotoxic, immune suppression	Maize
Oochratoxins	*A. ochraceus P. nordicum*	Nephrotoxic, carcinogenic, immune suppression	Cereals, wine, grape juice
Patulin	*A. clavatus, A. terreus, P. expansum, P. carviforme*	Mutagenic, genotoxic, carcinogenic	Fruit
Trichothecenes	*F. graminearum, F. culmorum, F. poae, F. sambucinum, F. sporotrichioides*	Digestive disorders, reduced weight gain, heamorrhages, oral lesions, dermatitis, infertility, degeneration of bone marrow, slow growth, immune suppression	Cereals
Zearalenone	*F. graminearum, F. culmorum, F. sporotrichioides*	Oestrogenic effects, prolapse of vagina, enlargement of uterus, atrophy of ovaries, infertility, abortion	Maize, wheat

**A. = Aspergillus; F. = Fusarium; P. = Penicillium*

Extraction

In the mycotoxin analytical process, sample extraction and preparation are the most time-consuming steps. The purpose of the extraction step is to remove as much of the mycotoxin from the solid sample as possible and render it into a liquid phase. Extraction can be performed by liquid-liquid extraction (LLE) by using two immiscible phase solvents, or solid phase extraction (SPE) by using a solid and a liquid phase.

Liquid-liquid extraction (LLE)

This extraction method is simple and is also a useful technique for the analysis of liquid samples. The typical extraction organic solvents are polar solvents (Saez et al., 2004; Hayashi and Yoshizawa 2005; Juan et al., 2005; Hinojo et al., 2006; Sulyok et al., 2006). Acid aqueous solvents also help in the extraction process (Dunne et al.,

1993; Sulyok et al., 2006; Zitomer et al., 2008). The polarity of the analytes, the polarity of the solvent, and pH are the most important factors and play a key role during the extraction step.

In general, this LLE method is easy to perform. However, it is labour intensive because multiple extractions are necessary and large volumes of organic solvents are required. In some case, the different toxins and different commodities need variable extraction solvent mixtures to ensure a high recovery rate. Consequently, the development of extraction solvents is still under urgent study.

Solid-phase extraction (SPE)

This technique is based on the partition of analytes and interfering compounds between a liquid phase and a stationary phase. The stationary phase is contained in the SPE-cartridge and is composed of a solid adsorbent and immobilized phase such as ethyl (C2), octyl (C8), octadecyl (C18), cyanopropyl (CN), diol (2OH), aminopropyl (NH2), and an ion exchange phase. After the crude samples are placed into the cartridge, the analyte and matrix are adsorbed to the solid-phase. The matrix is washed, and then the analyte compound is eluted with organic solvents.

The most common solid adsorbents are silica, alumina, diatomaceous earth, Florisil®, modified silica, porous polymers or carbon. The immune affinity column (IAC) is one of the most popular SPE methods, and is prepared by binding antibodies specific to mycotoxins to the solid-phase. IACs are being increasingly used because of their specificity (Danicke et al., 2004; Saez et al., 2004; Zinedine et al., 2006; Hussain et al., 2010). To analyse multiple mycotoxins, the antibodies against more than one mycotoxin have been immobilized (Lattanzio et al., 20079; Villa and Markaki, 2009).

The ion-exchange column is another kind of SPE that is employed for some mycotoxin extraction such as fumonisins and moniliformin (Parich et al., 2003; Kushiro et al., 2008;). These kinds of mycotoxins can be changed to ionic form and interact with the sorbent by electrostatic force. Then, this force is disrupted and the analyte is eluted from the cartridge.

The advantages of SPE methods are that they are simple to use, have high specificity, require small amounts of solvents, and can perform with automated. However, these cartridges have limited use because they are expensive and the antibodies are not provided for all kinds of mycotoxins and commodities.

Mycotoxin determination and analytical methods

Mycotoxin determination methods should be rapid, simple, accurate and selective for multiple analyses in several foods and feed matrices. Most mycotoxins have low molecular mass and are soluble in a range of organic and aqueous organic solvents. For this reason, they can be separated and detected by chromatographic methods such as thin-layer chromatography (TLC), liquid chromatography (LC) and gas chromatography (GC). However, these chromatographic methods are time consuming for sample preparation and need hyphenating instruments which cost effective for routine analysis. Therefore, screening methods based on immuno assays, such as the enzyme-linked immunosorbent assay (ELISA), and biosensors have become popular techniques for mycotoxin determination nowadays.

Thin-layer chromatographic (TLC) method

TLC was the first chromatographic method for mycotoxin determination since it was first reported in 1964 for aflatoxin analysis. After extraction and clean-up, a sample is applied to a silica plate and separated using organic solvent. Visual inspection under UV light is carried out and the sample is compared with a standard. This technique can yield semi-quantitative results. The AOAC International office approved TLC methods for determination of several mycotoxins including deoxynivalenol (DON), patulin (PUT), ochratoxin (OTA) and zearalenone (ZEA) (Trucksess, 2000). These techniques are still in routine use in many laboratories, especially in developing countries. However, TLC has limited use for mycotoxin detection. Many mycotoxins cannot be detected under UV or fluorescence, and sometimes a derivatization step must be performed, which uses expensive reagents and is harmful for health.

Gas chromatographic (GC) method

GC is used to detect mycotoxins if they are sufficiently volatile at the column temperature or can be converted into volatile derivatives. Trichothecenes are extensively determined by GC. They are characterized by the 12, 13-epoxy-trichothec-9-ene ring system. Commonly, GC is coupled with electron capture detection (Cirillo et al., 2003; Tanaka et al., 2007); FID (Schothorst and Jekel 2001;

Wu and Smith 2007) or MS detection (Tanaka et al., 2000; Jostoi et al., 2004; Neuhof et al., 2009) are applied for trichothecenes and *Fusarium* toxin detection. However, since most mycotoxins are small, non-volatile compounds and have to be derivatized prior to GC analysis, this technique is not suitable for commercial purposes. Thus, the use of high performance liquid chromatography (HPLC) coupled with UV, fluorescence or a mass spectrometer is much more advantageous and such methods are in widespread use for mycotoxin analysis.

High-performance liquid chromatography (HPLC) coupled to classical detectors

Most mycotoxins are relatively small polar compounds and can be separated by reverse-phase HPLC using a mobile phase made from the composition of water, acetonitrile or methanol. The stationary phase or column of this technique contains silica particles of small size (5 μm or less) which modified this particle with a hydrophobic layer, mostly is C18. Due to selectivity and sensitivity, a fluorescence detector is preferred for mycotoxin analysis, whereas UV is used for patulin and moniliformin because of its strong UV absorption.

Trichothecene and fumonisin also lack UV absorption and require derivatization for measurement by HPLC-FLD. T-2 and HT-2 have been determined to be ester derivatives after reacting with fluorogenic agent (Lippolis et al., 2008). Fluorescamine has been used for derivatization with fumonisin (Ross et al., 1991) before HPLC analysis. Shephard et al., 1996 reported that o-phthaldialdehyde (OPA) is the most useful pre-column fluorogenic derivatizing agent.

The evaporative light scattering detector (ELSD) is a conventional detector, which has limited use in mycotoxin analysis. This detector has been used to detect fumonisins in fungal cultures (Plattner, 1995; Wilkes et al, 1995). These researchers reported that the calibration curve was non-linear and had low detection sensitivity.

Liquid chromatography with mass spectrometric detection (LC-MS)

Within the last few decades, LC-MS has become a popular technique for mycotoxin analysis. This technique can be used to detect simultaneous mycotoxins in food and feed commodities without dramatization. LC-MS has more sensitivity and selectivity when compared with conventional detection techniques. After separating the sample

into chemical compounds by HPLC, a mass spectrometer will ionize, sort and identify these compounds based on the mass-to-charge ratio (m/z). Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are ionization techniques that are widely used for different molecular weights and polarity of compounds. Modern LC-MS instruments enable ionization in both negative and positive mode; likewise switching between them in the same chromatographic run is possible, and this is useful for simultaneous mycotoxin determination. Furthermore, the most important mass analyzers in mycotoxin analysis are triple quadrupole, ion-trap and time of flight. After selecting an appropriate mass analyzer, all MS parameters such as cone voltage, capillary voltage, nebulizer gas, cone gas flow, desolvation temperature, mass resolution and collision energies have to be set for target mycotoxins.

Several LC-MS methods for multiple mycotoxin analysis have been reported, which include extraction, sample pre-treatment and reverse-phase LC-MS quantification (Razzazi et al., 2002; Klotzel et al., 2005). Sulyok et al. (2006) improved the LC-MS/MS for determination of 39 mycotoxins in cereal. In another study, multiple mycotoxin determination including aflatoxins, trichothecene-A and B, OTA, zearalenone, fumonisins and patulin have been analyzed in cornflakes with no clean-up in a single run by LC-MS/MS (Rudrabhatla et al., 2007). However, injecting a crude extract has an effect on the ion source and disturbs the ionization process of the compound or the so-called matrix-effect. This effect will enhance or suppress signals in target mycotoxin analysis. Consequently, the use of an internal standard is recommended for quantification. The ideal internal standard behaviour should be the same as the interested compound and cannot be found in a natural contaminated sample. The proper internal standard is isotope-labelled isomers. Nowadays, not all isotopic standards (ISs) are commercially available for all mycotoxins, but they will be in the near future.

Bretz et al. (2005) synthesized 3-d_3-ADON and developed methods for DON and 3-ADON analysis using LC-MS/MS. Häubl et al., (2006) used $^{13}C_{15}$ DON for mycotoxin analysis in maize and wheat extracts without any clean-up. Cramer et al. (2007) produced 3,5-d_2-zearalenone and analyzed corn flakes. The use of isotopic labelled standard can overcome the problem of the related matrix effect by being spiked to the sample before extraction to correct the overall process of analysis. Nevertheless, these ISs are discussed due to the high cost of analysis.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA is a rapid screening method that involves a reaction between an antigen and an antibody in micro-plate wells. The direct competitive ELISA is commonly used in mycotoxin analysis. Briefly, mycotoxin is extracted from a ground sample with solvent, a portion of the sample extract and a conjugate of an enzyme coupled mycotoxin are mixed and then added to the antibody-coated microtiter wells. After washing, an enzyme substrate is added and color develops. The intensity of the color is inversely proportional to the concentration of mycotoxin in the sample.

Due to low sample volume requirements and the fact that a clean-up step is not needed, ELISA test kits are used more than TLC or HPLC. Although the antibodies have high specificity and sensitivity, overestimation or underestimation can occur due to the interaction of antibodies with similar chemical groups of antigens. This is known as a cross reaction. Therefore, an evaluation study of the accuracy and precision of the ELISA method is needed, and full validation of the ELISA method is essential.

Thesis outline

Mycotoxins contaminate various foods, and agriculture products regularly contain multiple mycotoxins. One of the important steps for mycotoxin analysis is extraction. Therefore, it was necessary to develop extraction solvents for multiple mycotoxin analysis in multiple matrices such as wheat, maize and rice. Chapter 2 is about the results obtained with the new developed extraction solvent when compared with conventional solvents. The method of analysing mycotoxins is also important; therefore chapter 3 gives a comparison between the enzyme-linked immunoabsorbent assay (ELISA) and liquid-chromatography mass spectrometry in inoculated maize samples for deoxynivalenol, fumonisins and zearalenone analysis.

Rice is a staple food in Asia and has become a popular food in European countries. *Fusarium* and mycotoxin contamination in rice have been reported. However, these contaminations depend on the type of rice and the geographical region. Therefore, chapter 3 deals with the analysis of fumonisins in unpolished rice from Thailand (Thai red cargo rice), which has high mineral content and is popular nowadays.

Objectives

- To develop new extraction solvents for simultaneous mycotoxin analysis, for three kinds of matrices, namely wheat, maize, and rice (Chapter 2)
- To evaluate the performance of the ELISA method when compared with the hyphenated method, LC-MS/MS. Moreover, in order to investigate the fluctuation of ELISA, inter-laboratory results were also compared (Chapter 3)
- To monitor the occurrence of fumonisins, particularly the major toxicant (FB1), in Thai red cargo rice.

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Chapter 2: Acetone-based extraction solvents for the determination of mycotoxins in wheat, maize, and rice grain by LC-MS/MS*

*This manuscript is prepared for publication.

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Abstract

Current HPLC methods for mycotoxin determination in grain rely on the extraction of samples with acetonitrile-based solvents, the most commonly used being acetonitrile/water 84:16 (v/v). The recovery of fumonisins in this solvent is poor. Methanol-based extraction solvents are suitable for fumonisins but inefficient for other mycotoxins such as trichothecenes, aflatoxins, and zearalenone. In this work, we investigated the suitability of acidified acetone/water mixtures as a substitute for acetonitrile/water. We compared 13 solvents based on acetone, acetonitrile, and methanol regarding the extraction of ground wheat, maize, and rice grain flour spiked with deoxynivalenol, zearalenone, fumonisins B1 and B2, and beauvericin. The extracts were analyzed by HPLC-MS/MS without dilution in order to make matrix effects visible and apparent recoveries were determined. The best performing methanol-based and acetone-based solvents were selected for further analysis, together with two standard acetonitrile-based solvents. Maize, wheat, and rice flour were spiked with 27 mycotoxins encompassing aflatoxins, beauvericin, citrinin, enniatins, fumonisins, gliotoxin, ochratoxin A, patulin, sterigmatocystin, trichothecenes type A and B, verrucarol, and zearalenone. Extraction efficiency determined by comparing HPLC-MS/MS signals with the signals of spiked matrix

extracts revealed that acetone/water/acetic acid (80:19:1, v/v/v) was the best extraction solvent. We propose this solvent as a replacement for acetonitrile-based solvents for mycotoxin extraction for multitoxin methods.

Keywords: Mycotoxins; Extraction solvents; Matrix effects; LC-MS/MS

Abbreviations

ACN	Acetonitrile
2-PrOH	Iso-propanol
HAc	Acetic acid
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-ESI-MS/MS	Liquid chromatography electrospray tandem mass spectrometry
MeOH	Methanol
R_A	Apparent recovery
R_E	Extraction recovery
SPE	Solid phase extraction
SSE	signal suppression/enhancement
SAX	Strong anion exchange chromatography

Introduction

Mycotoxins are fungal secondary metabolites commonly present in agricultural commodities worldwide. The most important mycotoxins are trichothecenes, aflatoxins, ochratoxin A, fumonisins, and zearalenone, produced by the fungal genera *Aspergillus*, *Penicillium*, and *Fusarium*. Because of their frequency in food and feed and their toxic effects on human and animal health (Hussein and Brasel, 2001), legal limits were set for maximum levels of mycotoxins in grain and food/feed products (Directive 2002; Commission Regulation 2006 and 2007, Commission Recommendation 2006) and monitoring schemes have been established in most countries. Liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) is the most popular method for the determination of multiple mycotoxins because it allows analysis of a variety of mycotoxins of different polarities without derivatization and offers a high specificity (Biselli and Hummert, 2007; Tanaka et al., 2010). The need to determine the levels of multiple mycotoxins in a single analysis

sparked the development of so-called multitoxin methods (Sulyok et al., 2006). Because mycotoxins comprise secondary metabolites of enormous structural diversity, extraction protocols used in multitoxin methods are inferior to protocols optimized for individual mycotoxins. Clean-up protocols aiming at the removal of interfering matrix constituents have had to be abandoned completely in most multitoxin methods owing to differences in hydrophobicity, acidity, and other properties of target analytes. However, the savings of time and cost offered by multitoxin methods outweigh their inherent drawbacks. Multitoxin methods based on LC-MS/MS have been widely adopted in the last decade and have become a quasi-standard in analytical practice. Among factors limiting the performance of multitoxin methods, the recovery of the analytes in extraction solvent and inadequate removal of matrix components interfering with the ionization are the most serious. The signals of analytes were suppressed or enhanced during the ionization step of LC-ESI-MS/MS technique or so-called matrix effects. These effects can be compensated for using isotope-labelled internal standards (Trebstein et al., 2009), but these are not applicable for all mycotoxins and they are expensive. Therefore, reduction of the level of matrix components before MS is a possible strategy to diminish matrix effects. This can be achieved by improving the sample preparation step.

Consequently, the sample preparation procedure is critical for mycotoxin determination. Previously, various extraction techniques were developed, including liquid-liquid extraction and solid-phase extraction (SPE). Many researchers have studied methods and devices for the clean up of samples by SPE, such as immuno-affinity column (Songsermsakul et al., 2006; Sáez et al., 2004), strong-anion exchange column (SAX; Humpf et al., 2004), and molecular imprinted solid phase extraction (MISPE; Urraca et al., 2008, Baggiani et al., 2008). However, these techniques are not available for all mycotoxins and not suitable for routine analysis because of the cost and time involved. Therefore, liquid-liquid extraction is frequently used for multiple mycotoxin analysis. The traditional solvent, acetonitrile-water, has been used in the past several years (Klötzel et al., 2005; Cavaliere et al., 2005; Neuhof et al., 2009). Recently, Sulyok et al. (2006) reported that acidified acetonitrile (acetonitrile-water-79:20:1 v/v/v) was the best compromise for the extraction of 39 mycotoxins from wheat and maize. However, the extraction recovery of FB was low.

Acetone is established as an extraction solvent for aflatoxin analysis (e.g. Nawaz et al., 1992; Möller and Nyberg, 2004), but it has rarely been used for other mycotoxins.

Recently, Mol et al. (2010) compared acetone/water with other solvents in a generic method for the determination of 172 pesticides, mycotoxins, plant toxins, and veterinary drugs. The results were promising but only recoveries for feed matrix were reported. In biochemical approaches and on one occasion in mycotoxin analysis (Sørensen et al., 2010), acetone was used to precipitate proteins. These findings inspired us to investigate whether acetone-based solvent can improve the recovery of mycotoxins that can be extracted efficiently into methanol- or acetonitrile-based solvents but not into both.

Owing to the harmful effects and cost of acetonitrile, we developed two new solvents: methanol/isopropanol/water/acetic acid (79:5:15:1 v/v/v/v) and acetone/water/acetic acid (80:19:1 v/v/v), to extract multiple mycotoxins in this experiment by comparing the extraction efficiencies with those of acetonitrile with and without 1% acetic acid. Mol et al. (2008) found the best extraction recoveries of acetone for simultaneous use for mycotoxins, plant toxins, and veterinary drugs in maize when compared with acetonitrile solvents.

Rice has become an interesting source of samples to study mycotoxin production since it was reported to suffer from mycotoxigenic fungal contamination (Abbas et al., 1999; Reddy et al., 2007; Makun et al., 2007). Therefore, the objective of this work is to develop new solvents for simultaneous mycotoxin analysis, which are suitable for three kinds of matrices, those of wheat, maize, and rice.

Materials and methods

Chemicals and reagents

Mycotoxin standards were purchased from Sigma Aldrich (Steinheim, Germany), except for enniatins, which were purchased from Axxora Europe (Germany). Stock solutions of each analyte were prepared in acetonitrile or methanol. Twenty-seven combined standard mycotoxins were prepared in acetonitrile/water 1:1 (v/v) for spiking experiments. The high-performance liquid chromatography (HPLC) grade solvents (methanol, acetonitrile, 2-propanol, acetone, and acetic acid) for extraction experiments were purchased from Roth Company (Germany), whereas LC-MS grade solvents (methanol, acetonitrile, and acetic acid) for mobile phase were purchased from Fisher Scientific (Germany).

Instrumentation

HPLC

The HPLC system consisted of two Prostar 240 pumps and Prostar 410 autosampler from Varian Inc. (Canada). The mycotoxin separation was performed at 40°C on a Kinetex® C_{18}-column, 50 × 4.6 mm i.d., 2.6 µm, equipped with a C_{18} security guard cartridge, 4 mm × 2 mm i.d., from Phenomenex (CA, USA). The mobile phase consisted of solvent A, 5 mM in water containing 5% acetonitrile and solvent B, and 5 mM in methanol. The gradient program was set up for 0-0.5 min 5% B, then went to 98% B from 0.5 to 1.5 min, and was then held at this condition for 4.5 min before being returned to 5% B for 4.0 min. The flow rate was 0.20 mL/min. To protect the MS interface, mobile phase was directed to the MS instrument only from 0.5 to 6.0 min using a switching valve.

Mass spectrometry

MS/MS was performed on a 1200MS triple quadrupole mass spectrometer equipped with an electro-spray ionization (ESI) interface (Varian Inc., Canada). The setting of the ESI source was heating at 270°C in the negative and positive ionization modes. The ion spray voltages were set at -4,000 V and +4,000 V, respectively. The nebulizing gas, the drying gas, and the curtain gas pressures were 50 psi, 18 psi, and 20 psi, respectively. ESI-MS/MS was performed in the multiple reaction monitoring (MRM) mode with both positive and negative modes. To optimize MS tuning parameters, standard solutions (10 µg ml^{-1} methanol) for each compound were infused (2 µl min^{-1}) using a syringe pump.

Sample preparation

Comparison of extraction solvents

Preliminary experiment

Ground wheat, maize, and rice (1 g) were spiked with deoxynivalenol, zearalenone, fumonisin B1, fumonisin B2, and beauvericin at 1.0 mg kg^{-1}. To select the optimized extraction solvent for the next experiment, 10 ml of each of the 13 solvents listed below was added to the matrices (n=3).

1) Acetonitrile/water 84:16 (v/v)
2) Methanol/Iso-propanol/water 80:5:15 (v/v/v)

3) Methanol/water 75:25 (v/v)

4) Acetonitrile/water/acetic acid 79:20:1 (v/v/v)

5) Methanol/Iso-propanol/water/acetic acid 79:5:15:1 (v/v/v/v)

6) Acetone/water 20:80 (v/v)

7) Acetone/water 40:60 (v/v)

8) Acetone/water 60:40 (v/v)

9) Acetone/water 80:20 (v/v)

10) Acetone/water/acetic acid 20:79:1 (v/v/v)

11) Acetone/aater/acetic acid 40:59:1 (v/v/v)

12) Acetone/water/acetic acid 60:39:1 (v/v/v)

13) Acetone/water/acetic acid 80:19:1 (v/v/v)

After shaking at 200 rpm overnight, the crude extracts were centrifuged at 4500 rpm for 10 min. A sample of 0.5 ml of supernatant was evaporated to dryness using speedVac (Christ, Osteredo, Germany) and the residue was redissolved with 0.5 ml of mobile phase. Finally, 0.5 ml of hexane was added to the defatted sample, then 10 µl of supernatant was injected into LC-ESI-MS/MS without further clean up. To evaluate the solvent efficiency, apparent recovery (R_A) was calculated using the following formula:

$$RA\ (\%) = 100 \times \text{peak area}_{\text{spiked samples}} / \text{peak area}_{\text{liquid standards.}}$$

Undiluted extracts with a large injection volume (as compared to flow rate/column) were used to make the matrix effects visible. Five mycotoxins representing different structure classes were spiked at a single concentration to maize, wheat, and rice flour and relatively large volumes of undiluted extracts were injected to the ion source to make the matrix effects visible.

Extraction solvent optimization for 27 mycotoxins

Ground wheat, maize, and rice (1 g) were spiked with 27 mycotoxin standards (Table 1) at a concentration of 1.0 mg kg^{-1}. Then, the best four extraction solvents from the preliminary experiment, namely;

(1) acetonitrile/water 84:16 (v/v)

(2) acetonitrile/water/acetic acid 79:20:1 (v/v/v)

(3) methanol/iso-propanol/water/acetic acid 79:5:15:1 (v/v/v/v)

(4) acetone/water/acetic acid 79:20:1 (v/v/v)

were added to the samples (n=3) and extracted as described above. To study the recovery of extraction, 1 g of samples was extracted with the above four different solvents, then standard mycotoxins were spiked to the dry residue at the same concentration levels (matrix-matched standard) before injection and the peak area was compared with that of spiked samples. Extraction recovery was calculated using the following formula:

Extraction recovery (%)=100×peak area$_{\text{spiked samples}}$/peak area$_{\text{matrix-matched standards.}}$

To reduce the matrix effect, the supernatant sample of dry residue was diluted 10 and 20 times and the apparent recoveries were compared.

Method validation

For validation of the extraction method, ground wheat, maize, and rice were spiked at seven concentration levels (n=3) from 0.02-0.64 mg kg^{-1} or 0.2-6.4 mg kg^{-1} (depending on the sensitivity of the mycotoxin). Blank extracts were fortified for matrix-matched calibration. For external calibration, the 27 mycotoxins were prepared in the mobile phase at the same seven concentration levels. To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the apparent recovery (R_A), the signal suppression/enhancement (SSE), and the recovery of the extraction step ($R_{E)}$ as defined by Sulyok et al. (2006) were used:

R_A (%) = 100 × slope$_{\text{spiked samples}}$ / slope$_{\text{pure standards}}$

SSE (%) = 100 × slope$_{\text{matrix-matched standards}}$ / slope$_{\text{pure standards}}$

R_E (%) = 100 × slope$_{\text{spiked samples}}$ / slope$_{\text{matrix-matched standards.}}$

Results and discussion

Acetone as extraction solvent for mycotoxins

All solvents used in the established protocols for mycotoxin extraction for HPLC analysis are acetonitrile/water and methanol/water mixtures, either without additives or acidified with acetic acid. Acetone is solvent miscible with water at any ratio, which is known to possess high elution power (it is used to "clean" TLC plates before a run) and to efficiently precipitate proteins and other macromolecules.

HPLC-MS/MS detection and column efficiency

To evaluate the analytical column, two Phenomenex columns with different lengths and particle sizes, Synergi fusion (100 mm × 4.6 mm, 4 μm) and Kinetex (5 mm × 4.6 mm, 2.6 μm), were tested. The Phenomenex Kinetex column performed with low-noise, good separation efficiency. Furthermore, the separation speed was reduced when using a short and fine-particle-size column. Therefore, Kinetex was chosen for this study.

Because of the variability of mycotoxin polarity, the gradient program of mobile phase was set to change from 5% to 98% acetonitrile. To improve the sensitivity of the detection, 15 mycotoxins were separated by two chromatographic runs with negative detection mode (Figure 1, a and b), whereas 12 mycotoxins were separated by another chromatographic run with positive detection mode (Figure 1c). All 27 mycotoxins were detected in only 30 minutes, including the equilibration time of the column. Nivalenol was the first mycotoxin eluted at 1:52 min.

The MS/MS parameters of each mycotoxin were optimized by direct infusion of the standard. The precursor ion and fragment ions are shown in Table 1. Trichothecenes-B, aflatoxins, citrinin, gilotoxin, patulin, sterigmatocystin, verrucarol, and zearalenone gave fragment ion intensities in the negative mode detection higher than those in the positive mode, which suit for trichothecenes-A, enniatins, beauvericin, fumonisins, ochratoxin, diacetoxyscripenol, and neosolaniol analysis

Extraction solvent optimization

Screening of solvents for mycotoxin extraction

The objective of this work was to develop a new solvent optimized for simultaneous mycotoxin extraction from wheat, maize, and rice without an SPE column. To optimize the composition of organic solvents for mycotoxin extraction, acetone was varied from 20 to 80% with and without 1% acetic acid. These solvents were compared with conventional solvent (acetonitrile-water mixture, 84:16 v/v) and methanol-water with and without acetic acid. The acidified mixture of acetonitrile and water was also used in this experiment since they were found to be the best extraction solvents for 39 mycotoxins in wheat and maize (Sulyok et al., 2006). The results of preliminary experiments are shown in Figure 2.

The apparent recoveries of DON, ZEN, and BEAU were high when using conventional solvent, acidified acetonitrile, and 80% acetone with or without acetic

acid in all three matrices. In the case of FB, the apparent recoveries were high when using acidified organic solvents. According to these results, we choose the conventional solvent, acetonitrile-water mixture (84:16 v/v), and compared the extraction efficiency with that of acetonitrile/water/acetic acid (79:20:1 v/v/v), and also those of two new acidified solvents (methanol/isopropanol/water/acetic acid, 79:5:15:1, v/v/v/v, and acetone-water-acetic acid, 80:19:1, v/v/v) in the next step.

Solvent optimization for 27 mycotoxins

To evaluate the efficiency of each solvent, the extraction recovery (%) was established for three matrices as summarized in Tables 2, 3, and 4. Acetone/water/acetic acid generated comparable recoveries for trichothecenes, aflatoxins, enniatins, and zearalenone in wheat and maize as acetonitrile-water-acetic acid. Interestingly, fumonisin recovery was below 60% when using conventional extraction solvent. This result correlated with the findings of Sulyok et al. (2006), who reported that acetonitrile is not appropriate for FB1 and FB2. Among acidic solvents from our experiment, acidified acetone gave the highest extraction recovery in both wheat and maize matrices.

In the case of rice matrix, in which many researchers have become interested in terms of mycotoxigenic fungal contamination and mycotoxin production in the last few years, no significant differences of recoveries of DON, DAS, STER, enniatins, and aflatoxins were found among four solvents. The results also showed that a conventional solvent is not suitable to extract fumonisins. Because of the solvent cost and user-friendliness of acetone extraction in routine analysis compared with acetonitrile extraction, and the short time required for the evaporation step, we decided to use acidified acetone for the method validation procedure. However, crude extracts should be diluted to reduce the matrix effect before analysis. Therefore, we compared the ratio of dilution between 1:10 and 1:20, as indicated in Table 5. Unfortunately, 15-ADON, T-2, and HT-2 were not evaluated in terms of the apparent recovery owing to spikes of these standards at low concentration levels. For other toxins, the apparent recoveries decreased after 20-fold matrix dilution. Consequently, 10-fold matrix dilution was chosen for sample preparation prior to analysis by LC-ESI-MS/MS.

Method validation

The mycotoxin analysis method was validated for wheat, maize, and rice by spiking 27 standards at multiple concentrations into blank (n=3) and blank extracted samples and compared with liquid standards. The validation was evaluated in terms of extraction recovery, apparent recovery, and signal suppression/enhancement as shown in Table 6. High signal suppression was found for enniatins, BEAU, and STER analysis. There were no differences between extraction recovery and apparent recovery when SSE (%) approached 100% (no matrix effect). Moreover, most mycotoxins had satisfactory extraction recovery when using acetone-water-acetic acid (80:19:1 v/v/v) as extraction solvent.

Conclusions

The developed extraction solvent for use for mycotoxins simultaneously in wheat, maize, and rice was validated in this work. Acetone-water-acetic acid (80:19:1 v/v/v) is an interesting solvent with high extraction recovery and low matrix effects when the crude extracted samples are diluted 10 times. Moreover, this method can be performed at low cost and in a short time because SPE avoids the need to clean up the samples.

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Tables and Figures

Table 1. Precursor and product ions and MS/MS condition of the analytes

Analyte	Abbreviation	Retention time (min)	Precursor ion (m/z)	Primary product ion (m/z)	Secondary product ion (m/z)	Collision energy (V)
3-Acetyldeoxynivalenol	3-ADON	3.05	337.3	307.0	170.0	-11/-9
15-Acetyldeoxynivalenol	15-ADON	3.05	337.3	150.0	277.0	-10/-7
Aflatoxin B1	AF B1	3.29	313.4	285.0	241.0	22/41
Aflatoxin B2	AF B2	3.21	315.4	287.0	259.0	25/28
Aflatoxin G1	AF G1	3.14	329.4	243.0	200.0	26/42
Aflatoxin G2	AF G2	3.09	331.4	245.1	189.0	33/49
Beauvericin	BEAU	5.09	806.5	384.1	266.0	45/45
Citrinin	CIT	4.07	251.5	233.1	205.0	15/26
Deoxynivalenol	DON	2.48	295.0	265.0	138.0	-10/-14
Diacetoxyscirpenol	DAS	3.34	384.5	307.1	229.2	11/14
Enniatin A	ENN A	5.19	682.5	210.0	228.3	27/27
Enniatin A1	ENN A1	5.31	668.5	210.0	228.3	27/27
Enniatin B	ENN B	4.87	640.5	196.0	214.0	25/25
Enniatin B1	ENN B1	4.99	654.5	196.0	214.0	30/30
Fuminisin B1	FB1	3.27	722.6	334.3	352.3	32/28
Fuminisin B2	FB2	3.57	706.6	336.0	318.5	33/27
Fusarenon-X	FUSX	2.84	353.3	262.9	204.8	-10/-11
Gliotoxin	GLIO	3.29	327.4	263.2	245.1	8/16
HT-2 Toxin	HT-2	3.54	447.5	285.0	345.0	18/17
Neosolaniol	NEO	2.86	400.5	185.2	215.3	19/16
Nivalenol	NIV	1.52	371.0	281.0	311.0	-15/-10
Ochratoxin A	OTA	3.89	404.4	239.0	221.0	24/38
Patulin	PUT	1.53	153.4	109.0	81.0	-2/-12
Sterigmatocystin	STER	3.99	325.0	310.0	281.0	21/49
T-2 Toxin	T-2	3.66	484.0	215.0	185.0	18/11
Verrucarol	VER	2.93	267.5	249.0	231.2	6/7
Zearalenone	ZEN	3.93	317.3	174.8	131.1	23/31

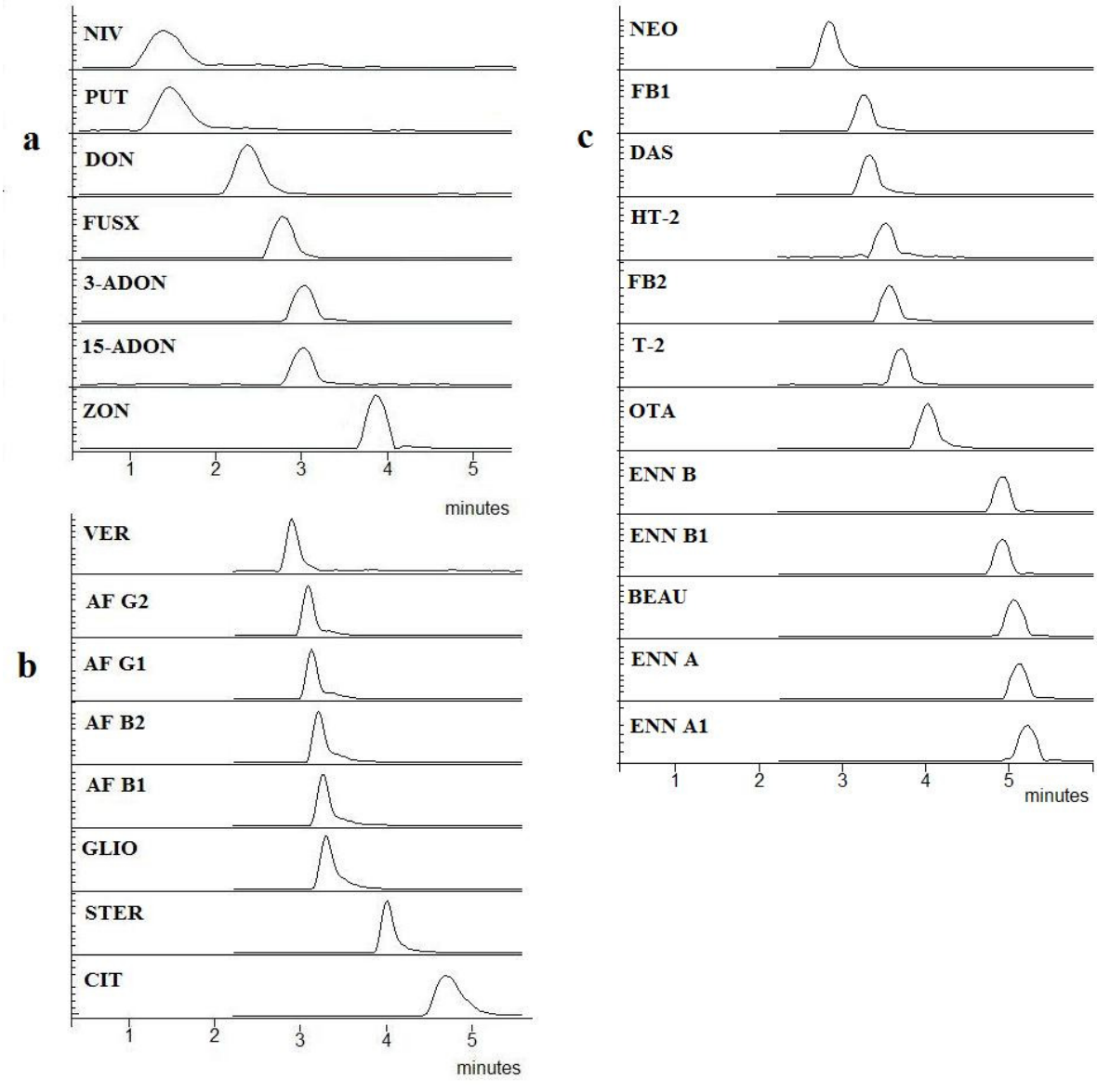

Figure 1. Mass chromatograms of a mycotoxin standards (0.2 µg ml^{-1}). Data were acquired in the multiple reaction monitoring mode by negative mode for 1st LC running (a) and positive mode for 2nd and 3rd LC running (b,c).

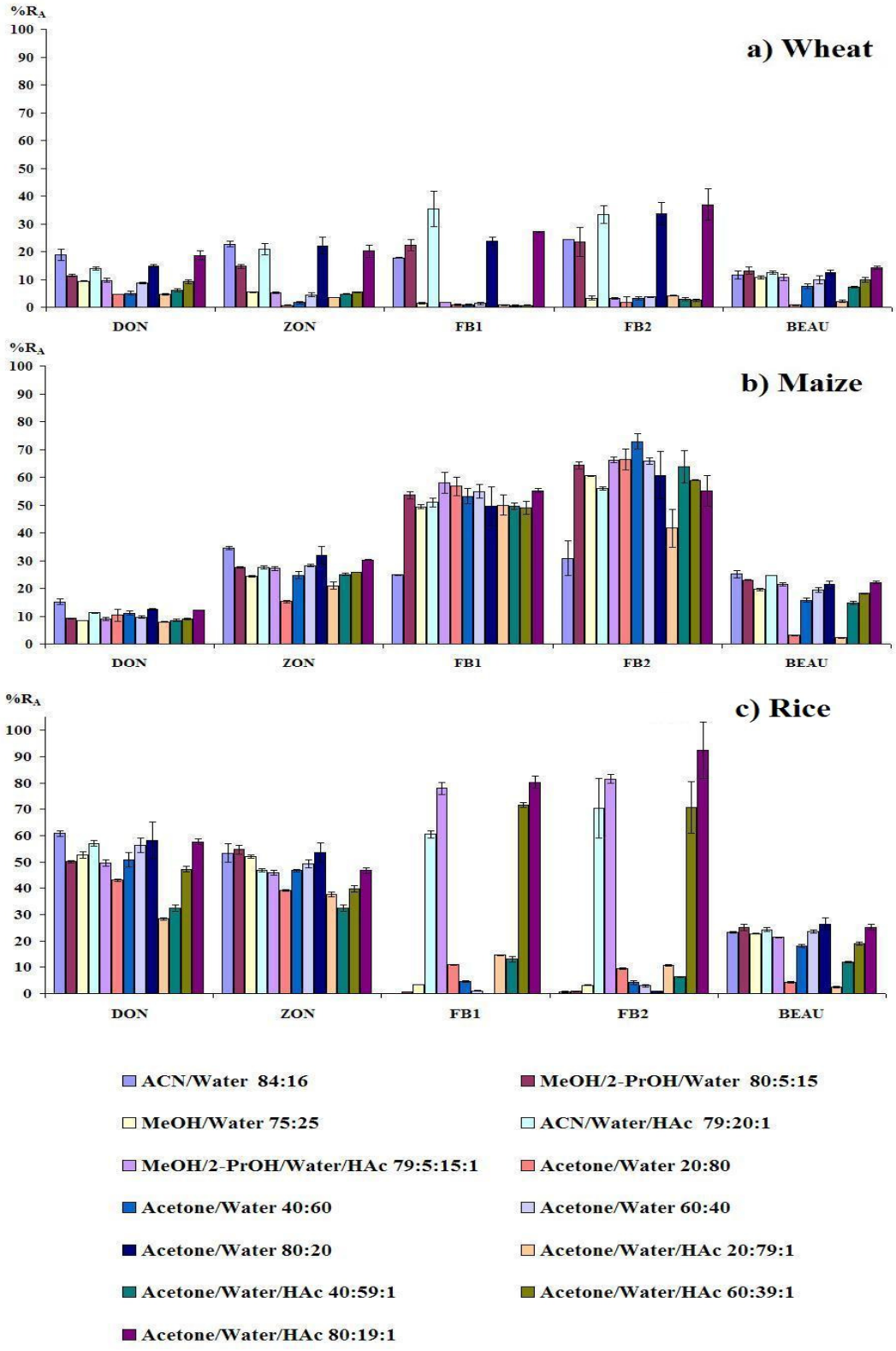

Figure 2. Apparent recovery (R_A) of five mycotoxins from spiked wheat (a), maize (b) and rice (c) samples (n=3) after extraction with 13 solvents. Apparent recovery was determined by comparing the signal obtained for undiluted extracts of spiked matrix with the signals of pure standards. HAc; Acetic acid

Table 2. Mean extraction recoveries and relative standard deviations (RSD) of mycotoxins when using different extraction solvents for wheat samples (n=3).

Analyte	Extraction Recovery* (%) ± RSD			
	ACN/H_2O (84:16, v/v)	ACN/H_2O/HAc (79:20:1,v/v/v)	MeOH/2-PrOH/H_2O/HAc (79:5:15:1, v/v/v/v)	Acetone/H_2O/HAc (80:19:1, v/v/v)
3-Acetyldeoxynivalenol	78 ± 6	105 ± 11	50 ± 12	102 ± 11
15-Acetyldeoxynivalenol	66 ± 4	89 ± 6	65 ± 18	58 ± 17
Aflatoxin B1	102 ± 4	102 ± 6	55 ± 4	97 ± 5
Aflatoxin B2	98 ± 10	97 ± 3	61 ± 1	87 ± 9
Aflatoxin G1	109 ± 3	94 ± 7	74 ± 3	94 ± 3
Aflatoxin G2	96 ± 5	94 ± 4	71 ± 8	98 ± 3
Beauvericin	102 ± 5	93 ± 4	69 ± 2	98 ± 3
Citrinin	55 ± 5	122 ± 2	63 ± 1	53 ± 2
Deoxynivalenol	60 ± 4	72 ± 4	68 ± 0	73 ± 2
Diacetoxyscirpenol	94 ± 6	105 ± 1	0 ± 0	81 ± 4
Enniatin A	82 ± 3	82 ± 4	72 ± 1	82 ± 2
Enniatin A1	78 ± 3	86 ± 7	69 ± 4	78 ± 4
Enniatin B	82 ± 2	82 ± 4	73 ± 2	80 ± 2
Enniatin B1	79 ± 3	81 ± 6	73 ± 1	77 ± 2
Fuminisin B1	35 ± 1	64 ± 2	65 ± 3	83 ± 3
Fuminisin B2	51 ± 1	64 ± 3	95 ± 10	89 ± 1
Fusarenon-X	69 ± 3	90 ± 2	78 ± 2	93 ± 5
Gliotoxin	97 ± 2	108 ± 6	0 ± 0	88 ± 8
HT-2 Toxin	84 ± 11	130 ± 13	0 ± 0	98 ± 0
Neosolaniol	80 ± 3	85 ± 2	41 ± 6	89 ± 3
Nivalenol	110 ± 15	116 ± 10	136 ± 3	137 ± 5
Ochratoxin A	97 ± 2	126 ± 2	123 ± 2	91 ± 2
Patulin	46 ± 3	76 ± 2	49 ± 3	62 ± 2
Sterigmatocystin	136 ± 4	130 ± 7	156 ± 9	93 ± 3
T-2 Toxin	66 ± 7	80 ± 11	0 ± 0	90 ± 14
Verrucarol	101 ± 0	114 ± 1	34 ± 6	86 ± 5
Zearalenone	96 ± 3	142 ± 7	89 ± 5	100 ± 5

*Extraction recovery for spiked wheat samples (n=3) was determined by comparing the signal obtained for undiluted extracts of spiked matrix with the signals of spiked extracts of mycotoxin-free matrix (matrix-matched standards).

Table 3. Mean extraction recoveries and relative standard deviations (RSD) of mycotoxins when using different extraction solvents for maize samples (n=3).

Analyte	Extraction Recovery (%) ± RSD			
	ACN/H_2O (84:16, v/v)	ACN/H_2O/HAc (79:20:1, v/v/v)	MeOH/2-PrOH/H_2O/HAc (79:5:15:1, v/v/v/v)	Acetone/H_2O/HAc (80:19:1, v/v/v)
3-Acetyldeoxynivalenol	77 ± 1	71 ± 4	78 ± 7	85 ± 7
15-Acetyldeoxynivalenol	90 ± 5	88 ± 2	94 ± 5	71 ± 1
Aflatoxin B1	191 ± 28	185 ± 27	172 ± 24	211 ± 16
Aflatoxin B2	115 ± 20	81 ± 10	96 ± 4	85 ± 5
Aflatoxin G1	87 ± 11	110 ± 26	94 ± 0	101 ± 11
Aflatoxin G2	93 ± 3	105 ± 3	100 ± 4	99 ± 14
Beauvericin	85 ± 3	76 ± 1	80 ± 1	74 ± 0
Citrinin	21 ± 1	24 ± 2	27 ± 0	25 ± 1
Deoxynivalenol	69 ± 4	78 ± 1	72 ± 1	81 ± 3
Diacetoxyscirpenol	91 ± 7	82 ± 3	82 ± 4	92 ± 6
Enniatin A	75 ± 1	69 ± 2	74 ± 1	73 ± 2
Enniatin A1	73 ± 2	67 ± 7	70 ± 4	70 ± 1
Enniatin B	75 ± 1	72 ± 2	73 ± 1	73 ± 1
Enniatin B1	74 ± 3	69 ± 2	72 ± 2	71 ± 2
Fuminisin B1	56 ± 0	85 ± 4	84 ± 1	94 ± 3
Fuminisin B2	63 ± 1	90 ± 2	86 ± 3	103 ± 2
Fusarenon-X	99 ± 5	83 ± 6	73 ± 17	90 ± 7
Gliotoxin	61 ± 11	75 ± 2	76 ± 12	91 ± 7
HT-2 Toxin	97 ± 2	72 ± 7	103 ± 16	58 ± 7
Neosolaniol	93 ± 6	101 ± 6	97 ± 1	96 ± 8
Nivalenol	70 ± 8	55 ± 5	41 ± 3	68 ± 6
Ochratoxin A	103 ± 4	87 ± 2	82 ± 1	100 ± 2
Patulin	24 ± 2	48 ± 2	41 ± 2	62 ± 1
Sterigmatocystin	145 ± 6	99 ± 5	94 ± 1	96 ± 2
T-2 Toxin	91 ± 10	82 ± 8	93 ± 7	107 ± 1
Verrucarol	95 ± 6	88 ± 6	120 ± 0	92 ± 9
Zearalenone	96 ± 12	142 ± 5	89 ± 4	100 ± 2

Table 4. Mean extraction recoveries and relative standard deviations (RSD) of mycotoxins when using different extraction solvents for rice samples (n=3).

Analyte	Extraction Recovery (%) ± RSD			
	ACN/H_2O (84:16, v/v)	ACN/H_2O/HAc (79:20:1, v/v/v)	MeOH/2-PrOH/H_2O/HAc (79:5:15:1, v/v/v/v)	Acetone/H_2O/HAc (80:19:1, v/v/v)
3-Acetyldeoxynivalenol	16 ± 0	41 ± 1	39 ± 2	34 ± 1
15-Acetyldeoxynivalenol	17 ± 0	39 ± 1	33 ± 1	33 ± 0
Aflatoxin B1	151 ± 4	134 ± 2	121 ± 2	130 ± 7
Aflatoxin B2	96 ± 1	103 ± 1	104 ± 3	79 ± 6
Aflatoxin G1	86 ± 2	62 ± 4	62 ± 1	69 ± 7
Aflatoxin G2	75 ± 5	79 ± 5	69 ± 4	73 ± 2
Beauvericin	86 ± 2	94 ± 4	77 ± 2	87 ± 5
Citrinin	6 ± 0	3 ± 0	5 ± 1	5 ± 0
Deoxynivalenol	128 ± 3	157 ± 3	153 ± 5	133 ± 3
Diacetoxyscirpenol	105 ± 3	114 ± 5	104 ± 2	117 ± 6
Enniatin A	103 ± 2	111 ± 10	84 ± 2	97 ± 7
Enniatin A1	110 ± 8	93 ± 21	63 ± 2	95 ± 7
Enniatin B	102 ± 1	109 ± 9	85 ± 2	95 ± 6
Enniatin B1	101 ± 4	105 ± 15	74 ± 1	89 ± 8
Fuminisin B1	0 ± 0	84 ± 1	97 ± 4	74 ± 4
Fuminisin B2	0 ± 0	83 ± 3	93 ± 5	74 ± 4
Fusarenon-X	13 ± 0	32 ± 0	31 ± 1	23 ± 1
Gliotoxin	38 ± 2	41 ± 1	36 ± 0	44 ± 0
HT-2 Toxin	24 ± 2	112 ± 5	0 ± 0	53 ± 3
Neosolaniol	94 ± 2	83 ± 0	64 ± 1	80 ± 2
Nivalenol	17 ± 0	19 ± 1	19 ± 1	16 ± 0
Ochratoxin A	72 ± 1	131 ± 3	129 ± 2	98 ± 3
Patulin	42 ± 1	70 ± 4	45 ± 2	62 ± 2
Sterigmatocystin	115 ± 3	141 ± 5	145 ± 4	107 ± 4
T-2 Toxin	17 ± 2	53 ± 2	73 ± 3	62 ± 7
Verrucarol	96 ± 4	154 ± 1	211 ± 11	126 ± 2
Zearalenone	23 ± 1	55 ± 1	57 ± 0	47 ± 0

Table 5. Effect of dilution of the apparent recovery of mycotoxins extracted with acetone-based solvent extraction (Acetone/H_2O/Acetic acid 80:19:1, v/v/v) from wheat, maize and rice (n=3).

Analyte	Apparent recovery					
	Wheat		Maize		Rice	
	1:10 dilution	1:20 dilution	1:10 dilution	1:20 dilution	1:10 dilution	1:20 dilution
3-Acetyldeoxynivalenol	55 ± 7	72 ± 17	41 ± 3	59 ± 2	68 ± 5	61 ± 7
Aflatoxin B1	76 ± 6	122 ± 11	67 ± 14	ND	97 ± 10	103 ± 24
Aflatoxin B2	63 ± 11	104 ± 5	57 ± 4	84 ± 5	85 ± 8	81 ± 16
Aflatoxin G1	67 ± 9	100 ± 8	56 ± 10	84 ± 17	63 ± 4	57 ± 25
Aflatoxin G2	69 ± 7	83 ± 12	57 ± 4	93 ± 6	68 ± 8	57 ± 22
Beauvericin	69 ± 12	41 ± 13	57 ± 11	77 ± 11	79 ± 9	45 ± 17
Citrinin	26 ± 4	34 ± 4	14 ± 2	19 ± 1	24 ± 2	21 ± 3
Deoxynivalenol	59 ± 6	89 ± 27	51 ± 5	68 ± 13	94 ± 7	84 ± 15
Diacetoxyscirpenol	83 ± 10	49 ± 2	67 ± 5	54 ± 5	109 ± 7	53 ± 9
Enniatin A	17 ± 2	ND	19 ± 4	19 ± 2	25 ± 4	16 ± 3
Enniatin A1	28 ± 2	15 ± 1	29 ± 6	31 ± 11	35 ± 4	24 ± 5
Enniatin B	44 ± 4	24 ± 5	46 ± 8	36 ± 1	55 ± 3	28 ± 8
Enniatin B1	40 ± 5	16 ± 3	36 ± 8	29 ± 1	45 ± 4	25 ± 5
Fuminisin B1	126 ± 22	59 ± 9	104 ± 7	76 ± 10	112 ± 11	44 ± 7
Fuminisin B2	108 ± 14	65 ± 6	96 ± 12	83 ± 4	102 ± 17	50 ± 14
Fusarenon-X	54 ± 5	62 ± 13	43 ± 3	43 ± 4	83 ± 2	68 ± 21
Gliotoxin	51 ± 7	78 ± 2	35 ± 2	57 ± 5	72 ± 6	76 ± 18
HT-2 Toxin	ND	ND	ND	ND	151 ± 27	ND
Neosolaniol	87 ± 6	62 ± 6	58 ± 5	64 ± 5	110 ± 8	53 ± 6
Nivalenol	27 ± 1	ND	42 ± 5	ND	84 ± 6	ND
Ochratoxin A	106 ± 7	68 ± 6	77 ± 8	67 ± 0	112 ± 12	60 ± 13
Patulin	66 ± 4	84 ± 11	60 ± 3	39 ± 5	ND	ND
Sterigmatocystin	25 ± 3	37 ± 3	28 ± 5	35 ± 1	33 ± 2	32 ± 10
T-2 Toxin	97 ± 10	ND	ND	ND	90 ± 16	ND
Verrucarol	55 ± 13	ND	48 ± 6	ND	70 ± 2	ND
Zearalenone	62 ± 11	67 ± 21	63 ± 8	49 ± 15	75 ± 8	64 ± 9

Table 6 Apperent recovery (%) of spiked wheat, maize and rice samples (n=3) after extracted with Acetone/H_2O/Acetic acid (80:19:1, v/v/v)

	Wheat			Maize			Rice		
	RE	RA	SSE	RE	RA	SSE	RE	RA	SSE
3-Acetyldeoxynivalenol	71 ± 11	61 ± 10	86 ± 12	68 ± 7	51 ± 5	75 ± 1	113 ± 4	79 ± 3	69 ± 8
15-Acetyldeoxynivalenol	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aflatoxin B1	177 ± 6	106 ± 4	60 ± 5	86 ± 19	57 ± 12	67 ± 17	132 ± 16	86 ± 11	65 ± 5
Aflatoxin B2	109 ± 23	78 ± 16	71 ± 16	102 ± 6	78 ± 5	77 ± 4	93 ± 11	79 ± 9	86 ± 2
Aflatoxin G1	116 ± 17	89 ± 13	77 ± 11	123 ± 3	74 ± 2	60 ± 6	88 ± 3	54 ± 2	61 ± 7
Aflatoxin G2	90 ± 7	94 ± 8	104 ± 0	114 ± 26	88 ± 20	77 ± 23	ND	ND	ND
Beauvericin	184 ± 27	51 ± 8	27 ± 10	104 ± 15	51 ± 7	50 ± 0	192 ± 38	62 ± 12	33 ± 2
Citrinin	37 ± 5	29 ± 4	79 ± 5	30 ± 2	23 ± 1	78 ± 4	21 ± 3	18 ± 3	87 ± 0
Deoxynivalenol	84 ± 21	80 ± 19	95 ± 14	75 ± 3	87 ± 4	116 ± 25	91 ± 8	120 ± 10	132 ± 6
Diacetoxyscirpenol	101 ± 1	71 ± 0	70 ± 6	147 ± 1	73 ± 0	50 ± 0	156 ± 10	112 ± 7	71 ± 10
Enniatin A	ND	ND	ND	ND	ND	ND	ND	ND	ND
Enniatin A1	ND	ND	ND	ND	ND	ND	ND	ND	ND
Enniatin B	104 ± 34	26 ± 8	25 ± 0	100 ± 11	29 ± 3	29 ± 4	143 ± 30	34 ± 7	24 ± 1
Enniatin B1	92 ± 4	28 ± 1	30 ± 5	71 ± 14	25 ± 5	34 ± 5	175 ± 6	33 ± 1	19 ± 1
Fuminisin B1	82 ± 5	78 ± 4	94 ± 14	91 ± 2	92 ± 2	101 ± 13	64 ± 4	73 ± 4	114 ± 15
Fuminisin B2	80 ± 4	85 ± 5	106 ± 7	92 ± 7	83 ± 7	90 ± 1	74 ± 7	71 ± 7	96 ± 16
Fusarenon-X	67 ± 12	75 ± 13	111 ± 29	57 ± 3	55 ± 3	97 ± 10	61 ± 6	86 ± 8	142 ± 9
Gliotoxin	80 ± 12	68 ± 10	85 ± 8	82 ± 10	53 ± 7	65 ± 19	92 ± 10	91 ± 10	99 ± 4
HT-2 Toxin	ND	ND	ND	ND	ND	ND	ND	ND	ND
Neosolaniol	117 ± 2	91 ± 1	78 ± 15	123 ± 20	63 ± 10	51 ± 4	115 ± 19	84 ± 14	73 ± 5
Nivalenol	ND	ND	ND	ND	ND	ND	35 ± 3	71 ± 7	202 ± 4
Ochratoxin A	101 ± 7	88 ± 6	88 ± 5	96 ± 4	74 ± 3	77 ± 4	122 ± 9	94 ± 7	78 ± 9
Patulin	48 ± 13	74 ± 20	156 ± 25	0 ± 0	0 ± 0	84 ± 15	ND	ND	ND
Sterigmatocystin	121 ± 9	34 ± 2	28 ± 3	110 ± 5	32 ± 1	29 ± 0	124 ± 10	30 ± 2	24 ± 2
T-2 Toxin	ND	ND	ND	ND	ND	ND	ND	ND	ND
Verrucarol	ND	ND	ND	ND	ND	ND	ND	ND	ND
Zearalenone	79 ± 5	81 ± 5	103 ± 5	79 ± 2	86 ± 2	108 ± 23	95 ± 12	88 ± 11	92 ± 13

Chapter 3: Comparison of LC-MS/MS and ELISA for *Fusarium* toxin detection in maize*

*This manuscript is prepared for publication.

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Abstract

In this study, the uses of ELISA and LC-MS/MS techniques for measurement of DON, FB, and ZEN in unprocessed maize were compared. Maize plants were inoculated with *F. verticillioides* and *F. graminearum* in experimental fields in Germany from 2006 to 2009 and mycotoxins were detected by ELISA at three laboratories and by LC-MS/MS at one laboratory. Good correlations and good agreement between methods were found upon analysis by linear regression and Bland-Altman plot. However, the performance of ELISA depended on the skill of the technician and the cross-reactivity of the ELISA test kits with similar compounds. ELISA is valuable to use as a screening method for samples with a high level of mycotoxin contamination; it is rapid and easy-to-use. In cases of a low level of mycotoxin contamination, the sample results should be confirmed by LC-MS/MS.

Keywords: Mycotoxins; LC-MS/MS; ELISA

Abbreviations

3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
DON	Deoxynivalenol
ELISA	Enzyme-linked immunosorbent assay
FB	Fumonisin
F. verticilliodes	*Fusarium verticilliodes*
F. graminerum	*Fusarium graminerum*

GC	Gas chromatography
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
TLC	Thin layer chromatography
ZEN	Zearalenone

Introduction

Mycotoxins are secondary metabolites produced by fungi, which have toxic effects for humans and animals (Smith et al., 1995; Rezar 2007). *Fusarium* fungi are often found in contaminated cereals. In Europe, *F. verticillioides* and *F. graminearum* are the major fungi in maize (Dimitrov et al., 1983; Miadenov 1974). They produce several mycotoxins such as trichothecene, fumonisin, and zearalenone.

Because of their toxic effects, the European Union has set maximum levels of 1.75 mg kg-1, 4.0 mg kg-1, and 0.35 mg kg-1 for DON, FB, and ZEN in unprocessed maize, respectively (EC 2006). Commonly used methods for the identification of mycotoxins are GC, HPLC, TLC, and immunochemical methods such as ELISA. ELISA test kits are rapid, easy-to-use, and have become the most widely used method. However, the cross-reactivity of the antibody with compounds similar to the target mycotoxin is a disadvantage of ELISA and results in underestimation or overestimation of the level detected. LC-MS/MS is a specific method for mycotoxin measurement that has high sensitivity and accuracy. Comparison of LC-MS/MS and ELISA has been reported for samples both naturally and artificially contaminated with mycotoxins (Sydenham et al., 1996; Ono et al., 2000; Bolduan et al., 2009). They found good correlation between these methods using linear regression analysis.

However, Bland and Altman (1986, 1999) suggested that a high correlation is no guarantee of good agreement between methods, and not only how far the two methods correlation but is also how closely they agree. They suggested a plot of the difference against the average of the two methods of measurement. In such a plot, the x-axis shows the mean of the results of the two methods whereas the y-axis represents the absolute difference between the two methods. Therefore, the objective of this study was to evaluate the performance of the ELISA method when compared with the hyphenated method, LC-MS/MS. Moreover, to investigate the fluctuation of ELISA, inter-laboratory results were also compared.

Materials and methods

Reagents and instruments

DON, 3-ADON, 15-ADON, FB1, FB2, and ZEN were purchased from Biopure (Tulln, Austria). LC-MS grade methanol, acetonitrile, and acetic acid were from Fisher Scientific (Schwerte, Germany). HPLC grade methanol, acetonitrile, and hexane were supplied by Carl-Roth Company (Karlsruhe, Germany). The ELISA test kits (Ridascreen®) for DON, FB, and ZEN were purchased from R-Biopharm Company (Darmstadt, Germany).

The LC-MS/MS module consisted of the Varian HPLC system (Varian Inc., Canada) including Prostar 240 pumps connected to Prostar 410 autosampler and 1200MS triple quadrupole mass spectrometer equipped with an electro-spray ionization (ESI) interface (Varian Inc., Canada). This system was operated by MS-Work Station software from Varian (Canada).

Chromatographic separation was achieved using a Kinetex® C18-column (50 × 4.6 mm i.d., 2.6 µm) with a C18 security guard cartridge (4 mm × 2 mm i.d.), both supplied by Phenomenex (Torrance, USA) and kept at 40°C. The mobile phase consisted of solvent A, 5 mM acetic acid in water containing 5% acetonitrile, and solvent B, 5 mM acetic acid in methanol. The gradient program was set up with 0-0.5 min at 5% B, then went to 98% B from 0.5 to 1.5 min and was held at this condition for 4.5 min before being returned to 5% B for 4.0 min. The flow rate was 0.20 mL/min. To protect the MS interface, mobile phase was directed to the MS instrument only from 0.5 to 6.0 min using a switching valve. The injection volume was 20 µl.

LC-ESI-MS/MS determination was performed by operating the MS system in the negative mode for DON, 3-ADON, 15-ADON, and ZEN, whereas a positive mode was used for FB1 and FB2. Quantitative analysis was carried out using the multiple reaction monitoring (MRM) modes. The setting of the ESI source was heating at 270°C in the negative and positive ionization modes. The ion spray voltages were set at -4,000 V and +4,000 V, respectively. The nebulising gas, the drying gas, and the curtain gas pressures were 50 psi, 18 psi, and 20 psi, respectively. Table 1 show the precursor and fragment ions of each target toxin in this experiment.

Samples

Four hundred and twenty-eight maize samples were collected in the field from 2006 to 2009. Maize plants were inoculated with *F. verticillioides* and *F. graminearum* in experimental fields across Germany in the remit of different research projects carried out by Hohenheim University (Stuttgart, Germany), Goettingen University (Göttingen, Germany), and KWS Saat AG (Einbeck, Germany). The number of samples for each toxin is given in Table 2. ELISA was performed by three laboratories: samples from the year 2006 were analyzed by laboratory A, samples from the year 2007 by laboratory B, and samples from the years 2008 and 2009 by laboratories A and C in parallel. LC-ESI MS/MS was used to determine the amounts of DON, FB1, FB2, and ZEN in the same samples as used for ELISA. This analysis was performed by Göttingen University. In addition, samples for DON analysis from the years 2008 and 2009 were also analyzed for 3-ADON and 15-ADON content by HPLC-MS.

Sample preparation

ELISA method

Maize samples were extracted and analyzed for mycotoxins with the ELISA test kit ″Ridascreen″ (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 5 g of samples was supplemented with 25 ml of distilled water for DON extraction, whereas 70% methanol-water was used for FB and ZEN extraction. After 3 min of sample shaking, the crude extracts were filtered. Then, the supernatant was diluted and pipetted into an ELISA well plate.

LC-MS/MS method

Five grams of maize sample was extracted with 40 ml of acetonitrile:water (84:16 v/v) for DON and ZEN determination and extracted with 40 ml of methanol:water (75:25 v/v) for FB1 and FB2 determination. After shaking at 200 rpm overnight, the crude extract was centrifuged at 4500 rpm for 10 min. A 0.5 ml sample of supernatant was evaporated to dryness using speedVac (Christ, Osteredo, Germany) and the residue was re-dissolved with 0.5 ml of mobile phase. Finally, 0.5 ml of hexane was added to the defatted sample, then 10 µl of supernatant was injected to LC-ESI-MS/MS without further clean up. The calibration curve was prepared by spiking of different amounts of DON, 3-ADON, 15-ADON, FB1, FB2, and ZEN into blanks of maize and extract as described above (matrix-matched standard calibration curve).

Correlation analysis of ELISA against LC-ESI-MS/MS

ELISA and LC-ESI-MS/MS detected DON, FB, and ZEN in inoculated maize samples. For comparison between the two methods, linear regressions were plotted and the correlation coefficient was calculated. Moreover, Bland-Altman graph was also plotted to study the limit of agreement between the two methods. The x-axis shows the mean of the results of the two methods whereas the y-axis represents the absolute difference between the two methods.

Results and discussion

ELISA methods have been widely used for the quantification of *Fusarium* toxins in recent years. To evaluate the correlation between this method and an instrumental method such as LC-MS/MS, inoculated maize samples were analyzed for DON (n=157), FB (n=126), and ZEN (n=145).

DON determination

The comparisons of method between ELISA and LC-MS/MS in each year and each laboratory for DON determination are shown in Figure 1. There was no correlation between these two methods in 2006 as employed by Lab-A and 2007 by Lab-B. However, a correlation of the results obtained by Lab-A increased in 2008 (r^2 = 0.8274) and 2009 (r^2 = 0.9594). These results can be explained in the terms of technical skill: Lab-A improved its skill in using the ELISA method to detect DON. For inter-laboratory study, Lab-C detected the same samples as Lab-A, and a good correlation between their results was found in both 2008 (r^2 = 0.8632) and 2009 (r^2 = 0.9787).

Bland-Altman plot analysis was applied to determine the agreement between ELISA and LC-MS/MS methods. High variability between methods was found at a low concentration of DON. From these plots, we also found overestimation by ELISA at a high concentration (absolute difference between two methods > 0), especially in 2008 and 2009. These results can be explained in terms of cross-reactivity of the ELISA kit with other compounds. Twenty maize samples were evaluated for DON, 3-ADON, and 15-ADON by LC-MS/MS and DON by ELISA. The results are shown in Table 3. 3-ADON and 15-ADON were found at high concentrations when samples had a high DON level. These results confirmed the ELISA overestimation and its relationship to

the cross-reactivity in the manufacturer's information of the ELISA-kit, which stated 100% and 19% cross-reactivity of 3-ADON and 15-ADON, respectively.

Many researchers (Krska et al., 2007; Zachariasova et al., 2008; Goryacheva et al., 2009) have reported that 3-ADON and 15-ADON can cross-react in DON determination by ELISA. Moreover, Zachariasova et al. (2008) pointed out that aqueous extraction by the ELISA method might be the cause of overestimation when compared with HPLC using acetonitrile-water as an extraction solvent. However, from these experiments, most sample results are within the limit of agreement between the two methods.

FB determination

A good correlation was found between the ELISA and chromatographic method to detect FB in maize samples in 2006 from Lab-A and 2007 from Lab-B, whereas there was a low correlation in 2008 and 2009 in both Lab-A and Lab-C. Moreover, the inter-laboratory study from 2009 showed that Lab-C detected more FB than Lab-A when analyzing the same samples. The Bland-Altman plots were related to these results. In 2009, overestimation by ELISA was found for Lab-C but underestimation for Lab-A. This inter-laboratory result suggested that the ELISA analysis performance depended on the technician's skill.

Nilfer and Boyacolu (2002) suggested that low regression between the results of ELISA method and HPLC method may occur owing to the absence of a clean-up step in the ELISA method. In addition, Ono et al. (2000) found that extract dilution of samples decreased the ELISA/HPLC ratio and reduced the matrix effect. Pestka et al. (1994) explained the differences of FB levels determined between ELISA and a chromatographic approach. They suggested that not only a matrix effect but also additional compounds might cross-react with monoclonal antibody of ELISA; then, false positive or false negative results can occur. Moreover, some of the samples were heavily contaminated and had to be diluted. Technicians who perform ELISA seldom use matrix for sample dilution; they usually use just water. Toxin diluted in water may give a different ELISA response than toxin in the matrix.

ZEN determination

Figure 3 shows the correlation coefficient between the two methods for ZEN determination. All samples had a good correlation, except for the absence of a

correlation for the results obtained by Lab-C in 2009. These results were related to the DON and FB results in that the skill of the technician had an effect on the use of ELISA. Regarding the Bland-Altman plots, underestimation by ELISA was found for Lab-B in 2007 when a high level of ZEN contamination was present in the maize sample. This underestimation might have originated from the use of an unsuitable extraction method (type of organic solvents and solvent volume). Bennet and Nielsen (1994) performed collaborative study between laboratories to analyze ZEN in corn, wheat, and feed. They pointed out that a high level of technical skill for ELISA analysis was helpful for improving the results. The important feature is the washing step to remove unbound antigen and other materials, which should be performed carefully. In terms of the overall data, most of them are within the limit of agreement between these methods, which means that ELISA can be used to screen for ZEN in samples.

The comparison between methods at maximum residue limit (MRL)

As described in European Union legislation (EC 2006), the MRLs of these toxins in unprocessed maize samples are 1.75, 4.0, and 0.35 mg kg-1 for DON, FB, and ZEN, respectively. These levels were chosen to be criteria for comparison of the methods of mycotoxin analysis between ELISA and LC-MS/MS. Table 4 shows the frequency of samples with levels above and below the MRL level. Different numbers of samples with levels higher than the MRLs were found when using different methods to detect DON, FB, and ZEN. LC-MS/MS detected 135 and 100 samples for DON and FB, whereas only 131 and 96 samples were found by the ELISA method. Regarding food safety control, there are errors in the rejection of samples when using ELISA for mycotoxin determination. These results show that LC-MS/MS should be used to confirm the mycotoxin levels if they are close to the MRL after screening by ELISA.

Conclusions

In conclusion, the research presented herein found that ELISA method produced overestimation or underestimation when compared with LC-MS/MS as a reference method. Moreover, the skill of the technician in applying ELISA should be focused on. ELISA can be used easily and at low cost when analyzing many samples for routine work at high levels of contamination. In cases of low levels of mycotoxin

contamination, LC-MS/MS should be used as a confirmatory method to decide whether to reject samples.

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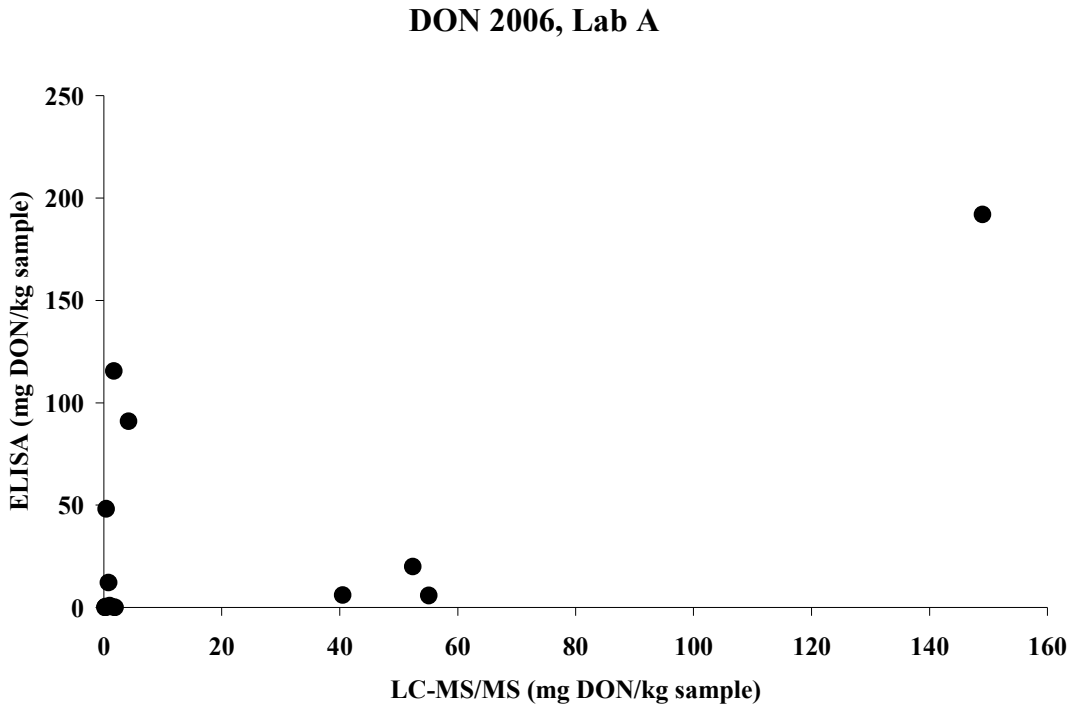
Tables and Figures

Table 1. Precursor and product ions of target *Fusarium* toxins.

Analyte	Precursor ion (m/z)	Primary product ion (m/z)	Secondary product ion (m/z)
3-Acetyldeoxynivalenol	337.3	307.0	170.0
15-Acetyldeoxynivalenol	337.3	150.0	277.0
Deoxynivalenol	295.0	265.0	138.0
Fuminisin B1	722.6	334.3	352.3
Fuminisin B2	706.6	336.0	318.5
Zearalenone	317.3	174.8	131.1

Table 2. Number of analyzed maize samples according to type of mycotoxin and harvest year.

Year of harvest	DON	FB	ZEN
2006	14	10	8
2007	103	76	97
2008	20	20	20
2009	20	20	20
Total	157	126	145



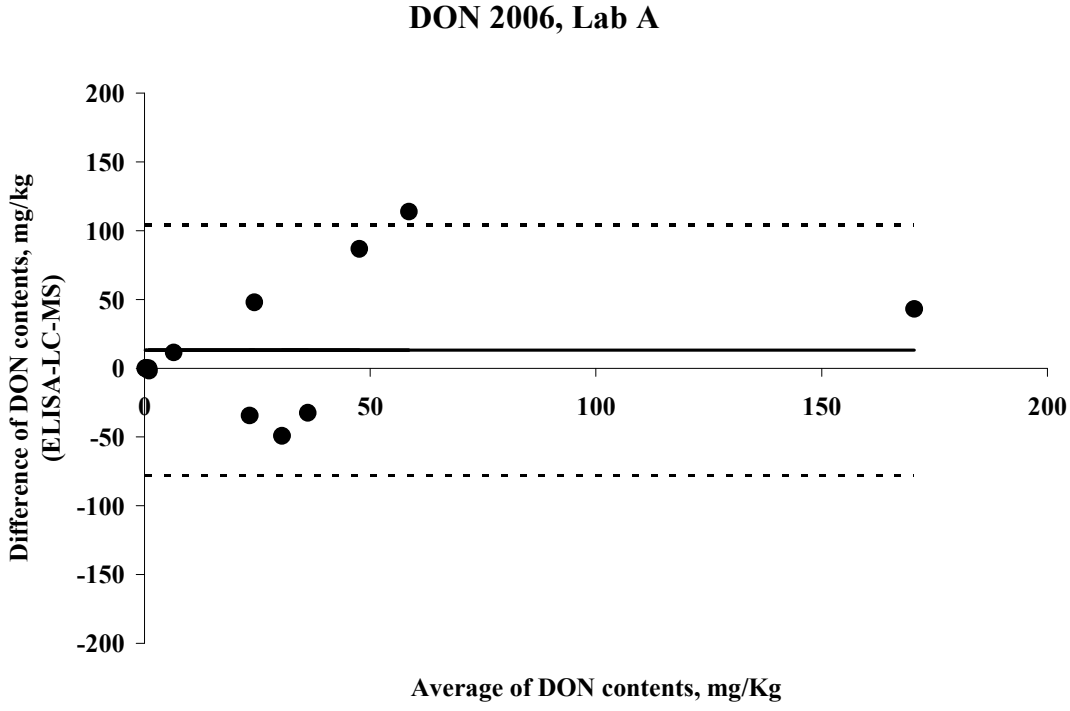


Figure 1a. The linear regression and Bland-Altman plots for DON determination by ELISA and LC-MS/MS in each laboratory from 2006.

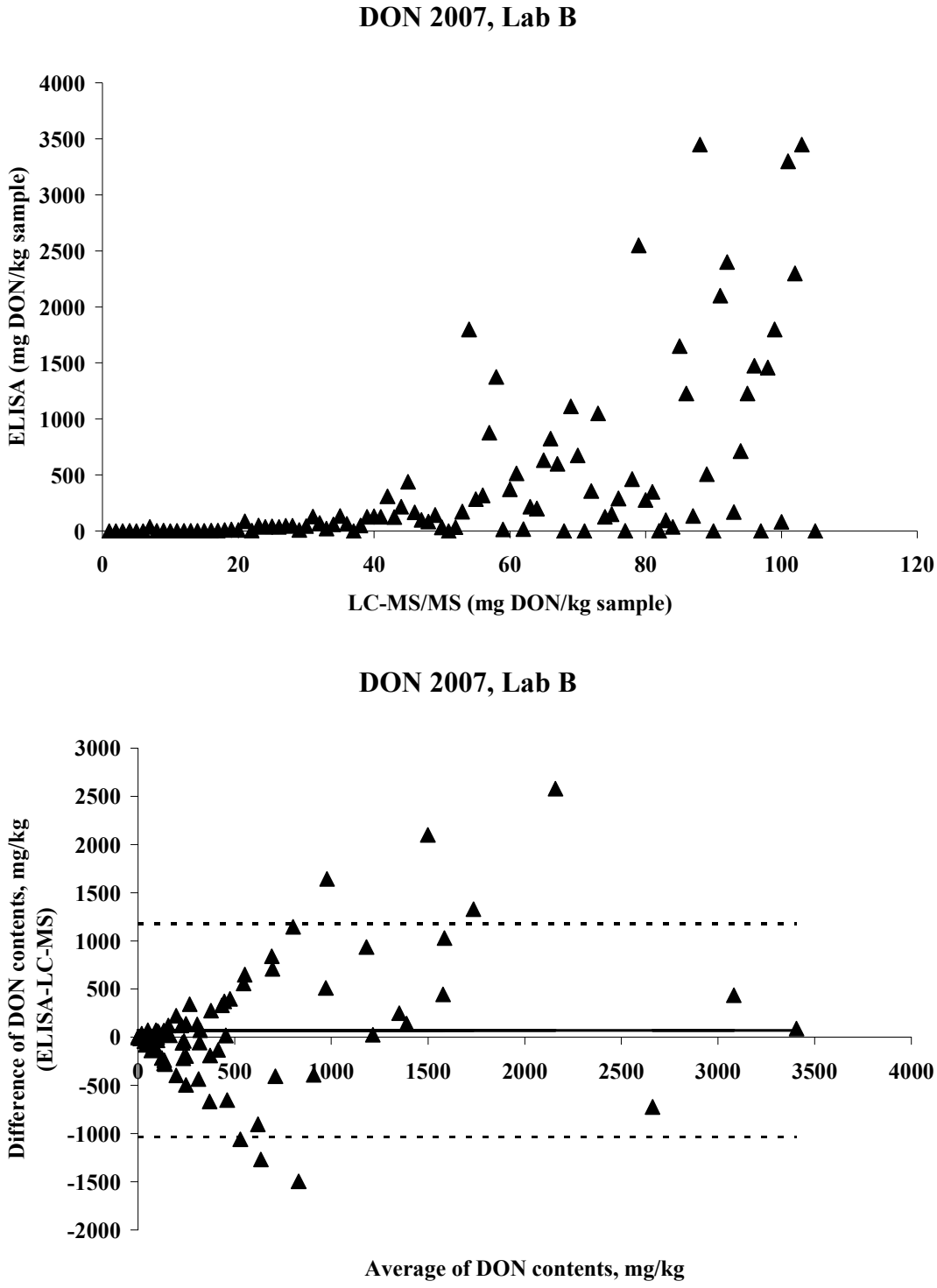
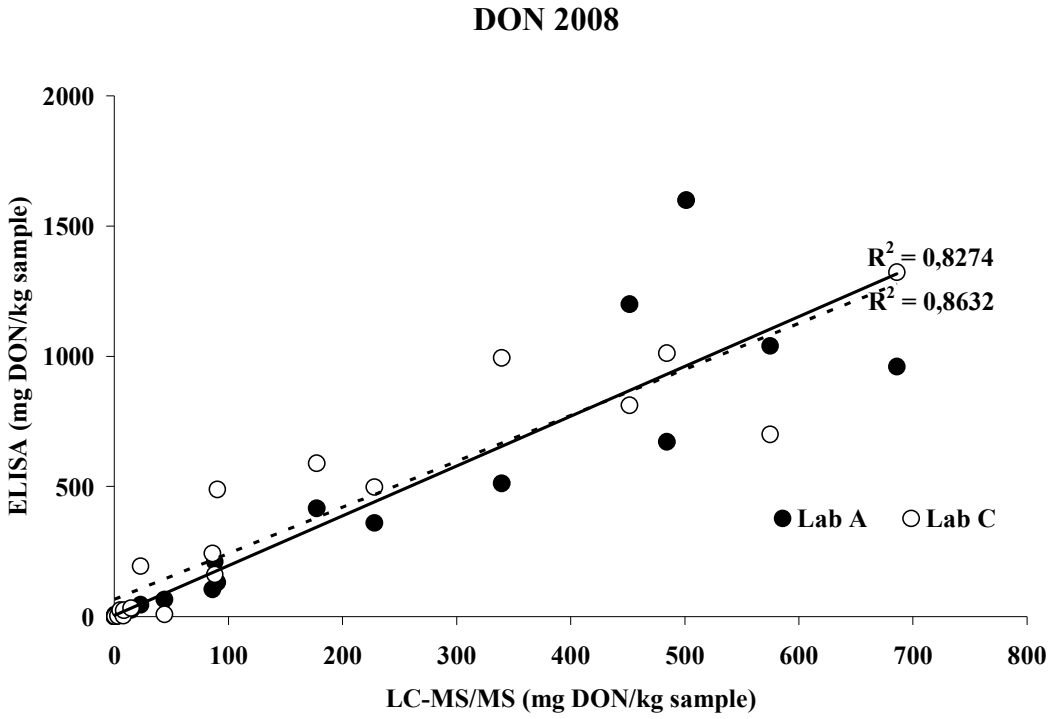


Figure 1b. The linear regression and Bland-Altman plots for DON determination by ELISA and LC-MS/MS in each laboratory from 2007.



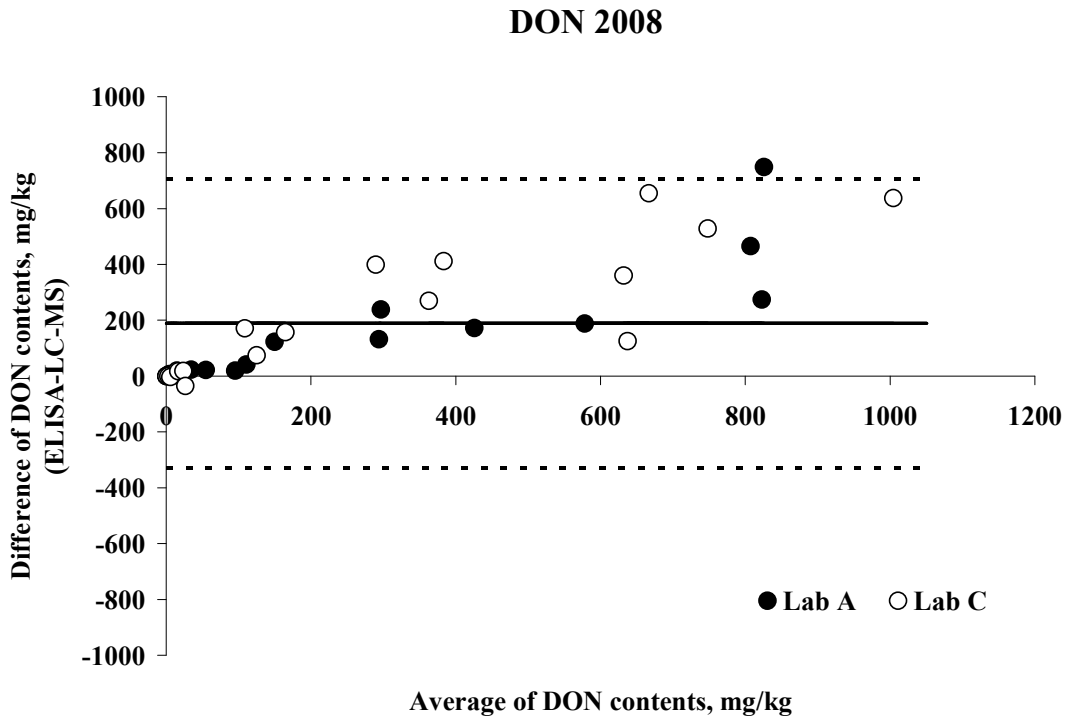


Figure 1c. The linear regression and Bland-Altman plots for DON determination by ELISA and LC-MS/MS in each laboratory from 2008.

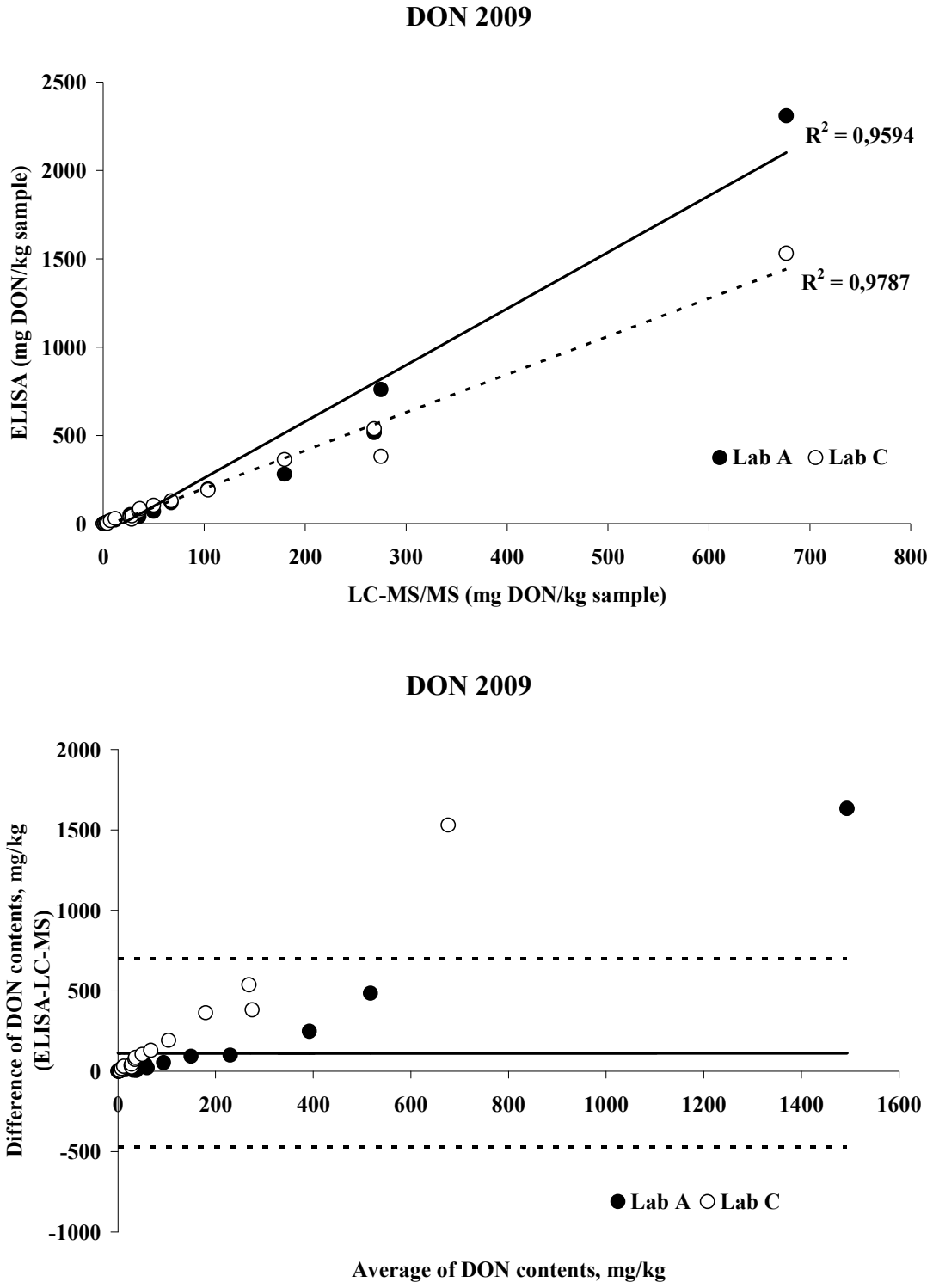
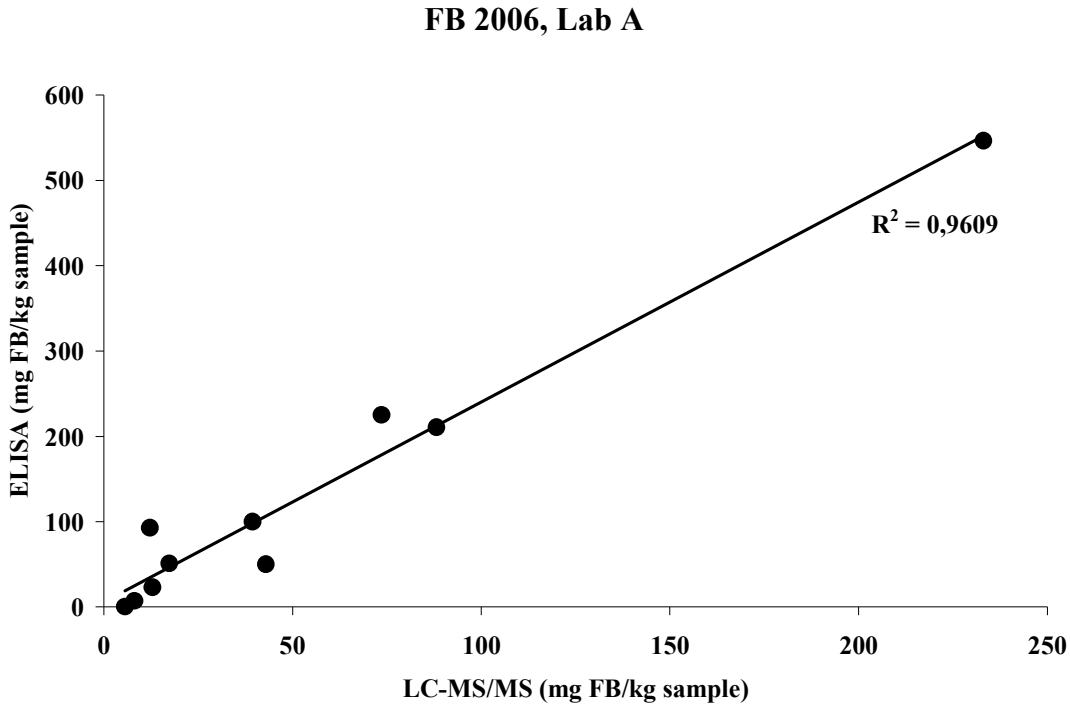


Figure 1d. The linear regression and Bland-Altman plots for DON determination by ELISA and LC-MS/MS in each laboratory from 2009.



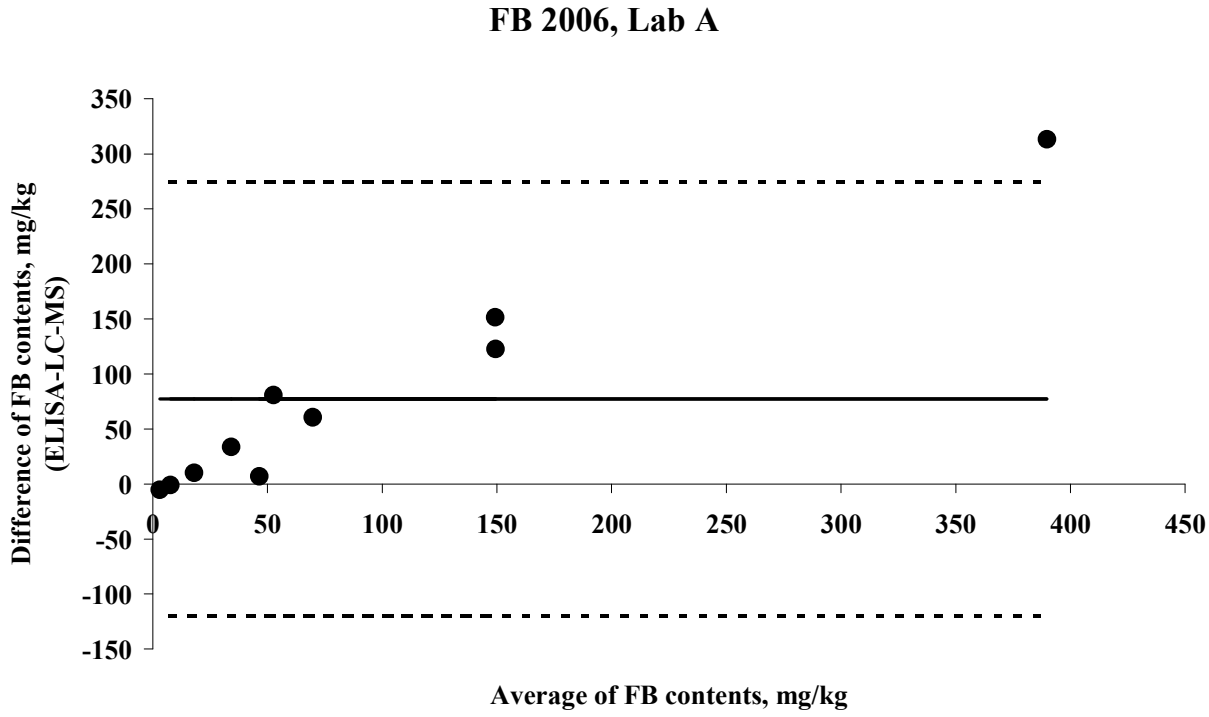


Figure 2a. The linear regression and Bland-Altman plots for FB determination by ELISA and LC-MS/MS in each laboratory from 2006.

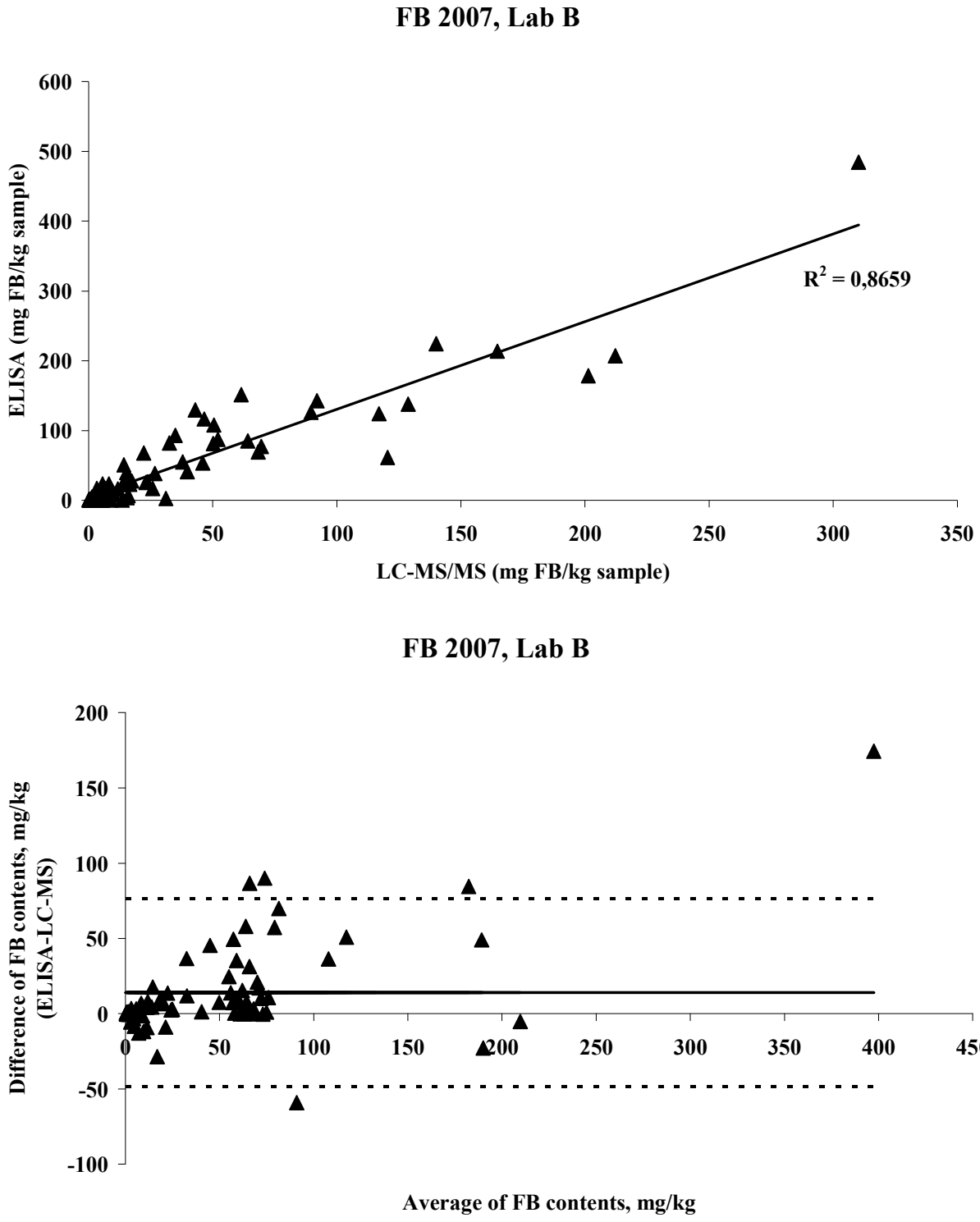


Figure 2b. The linear regression and Bland-Altman plots for FB determination by ELISA and LC-MS/MS in each laboratory from 2007.

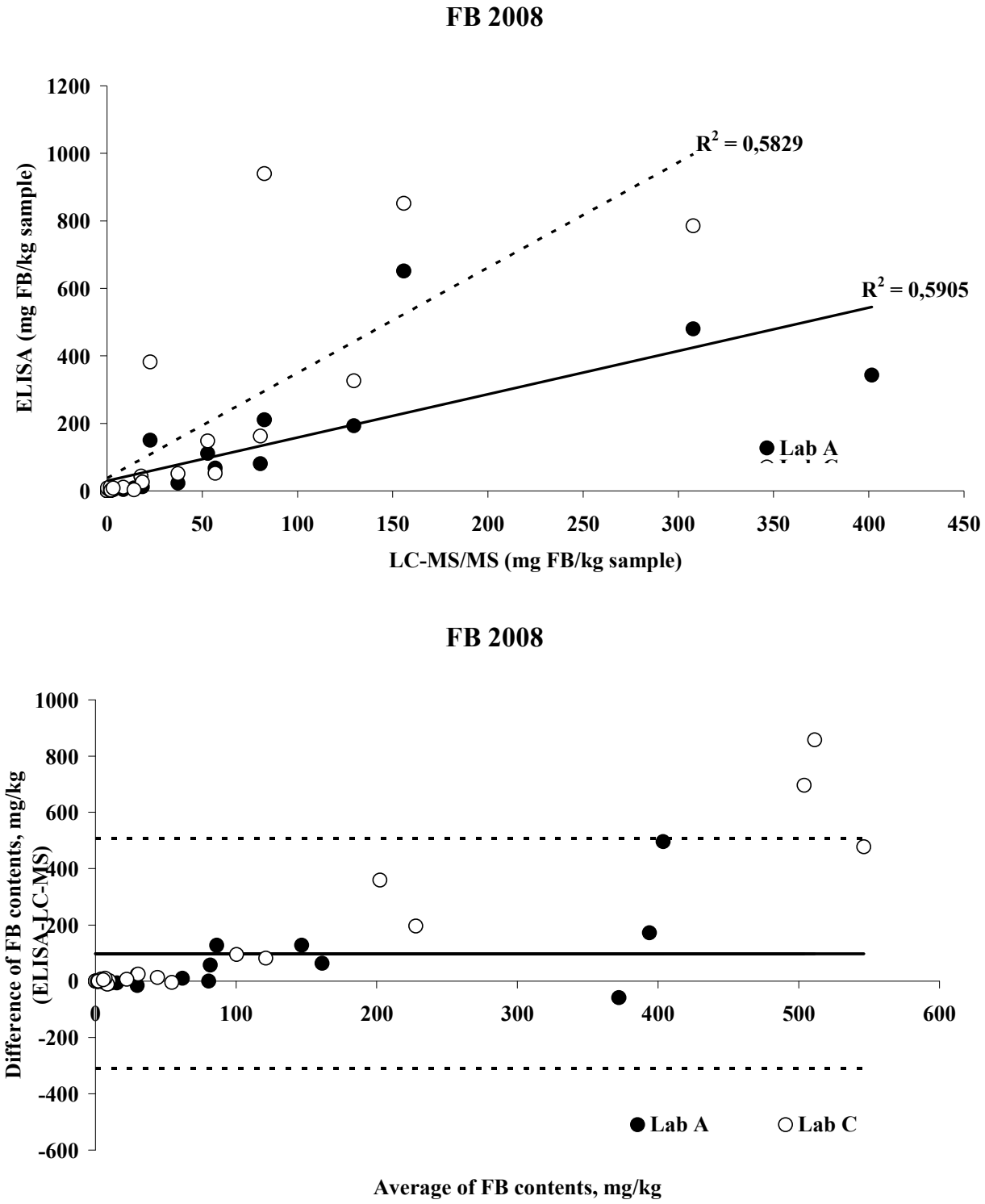


Figure 2c. The linear regression and Bland-Altman plots for FB determination by ELISA and LC-MS/MS in each laboratory from 2008.

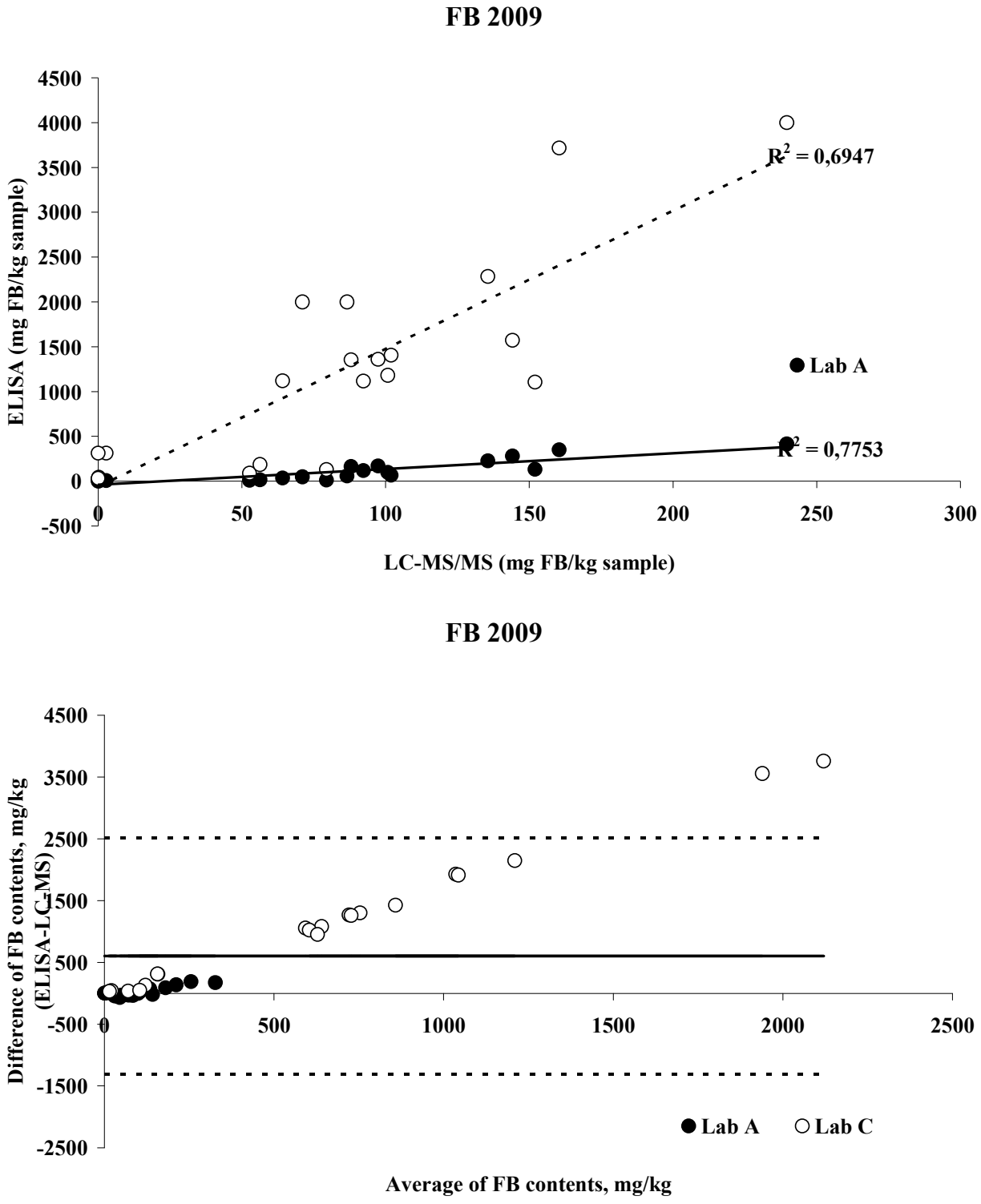
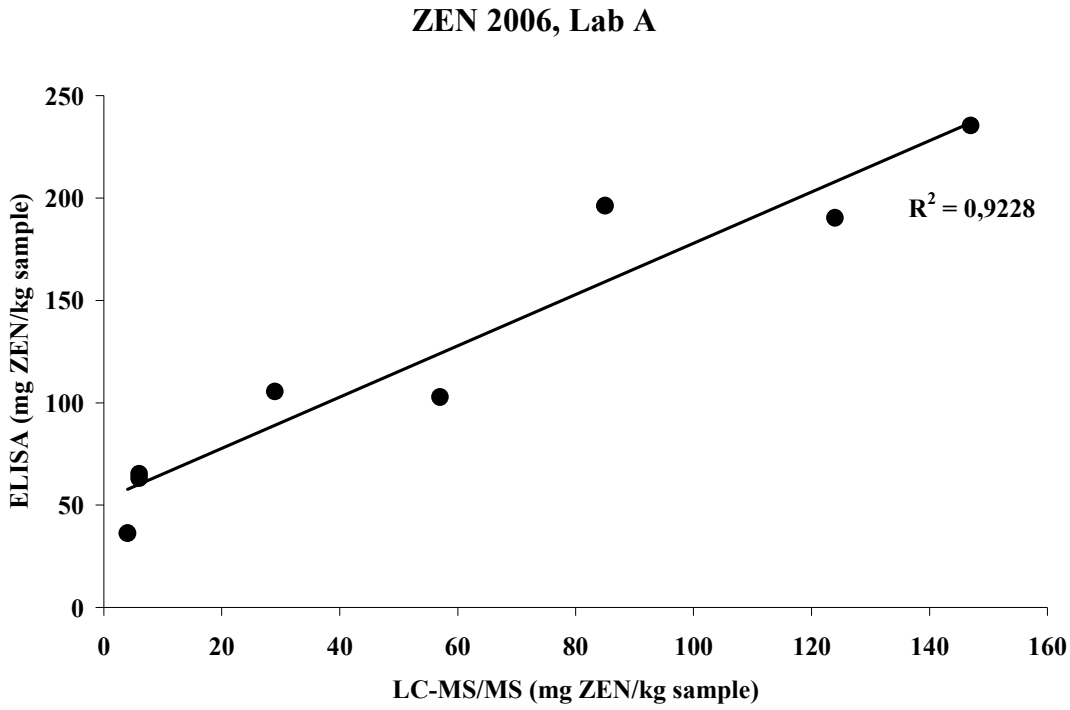


Figure 2d. The linear regression and Bland-Altman plots for FB determination by ELISA and LC-MS/MS in each laboratory from 2009.



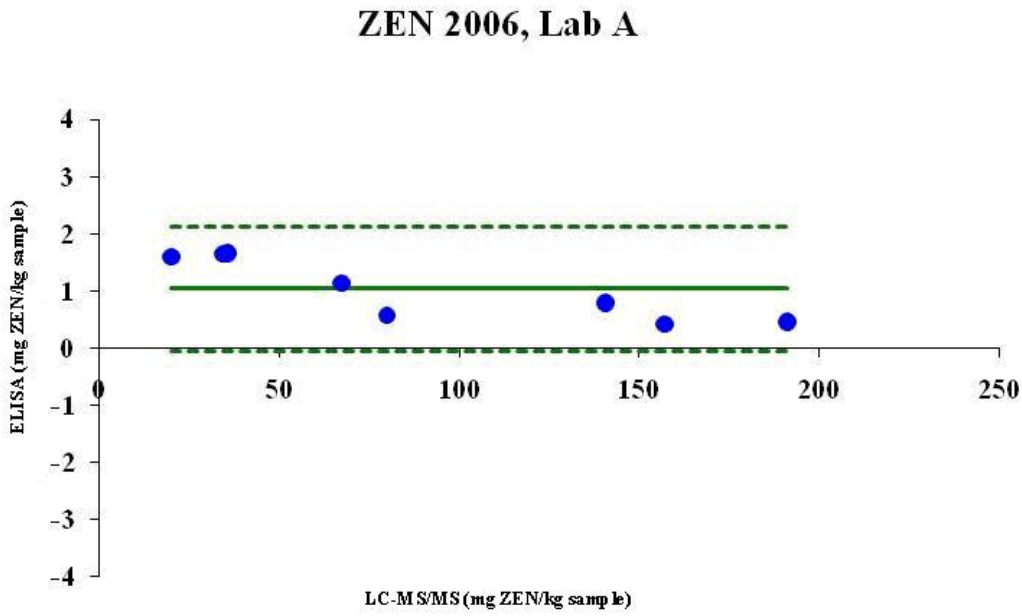
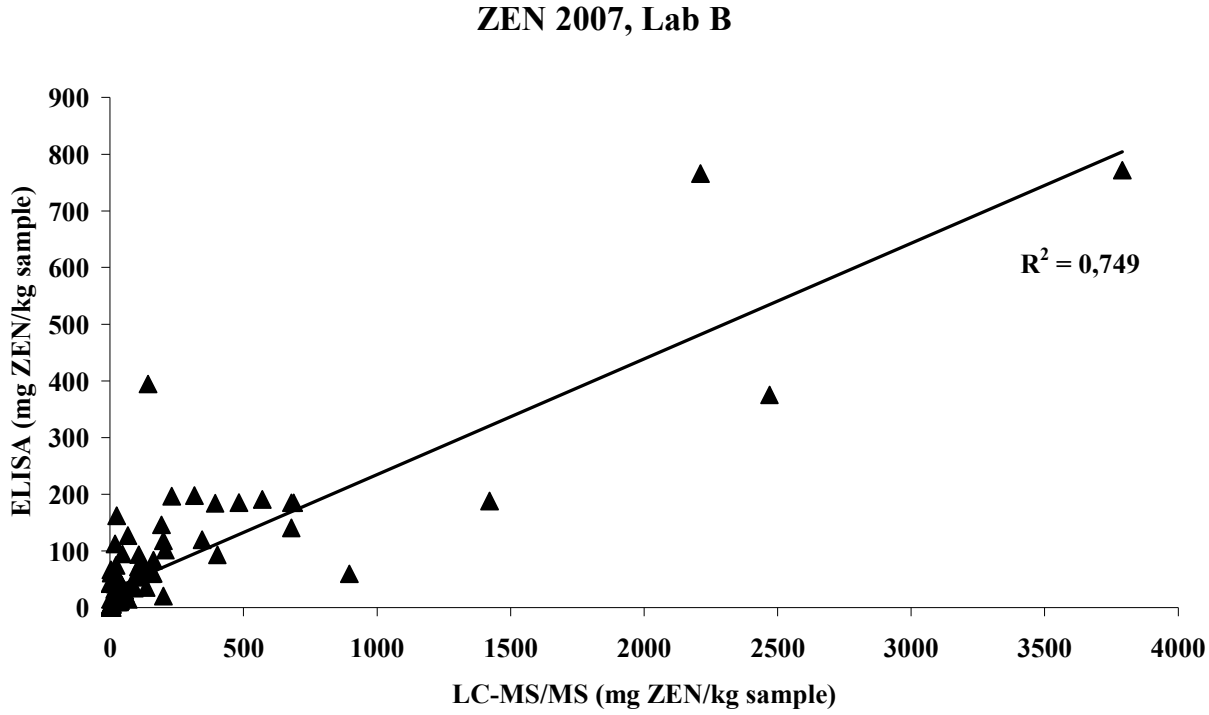


Figure 3a. The linear regression and Bland-Altman plots for ZEN determination by ELISA and LC-MS/MS in each laboratory from 2006.



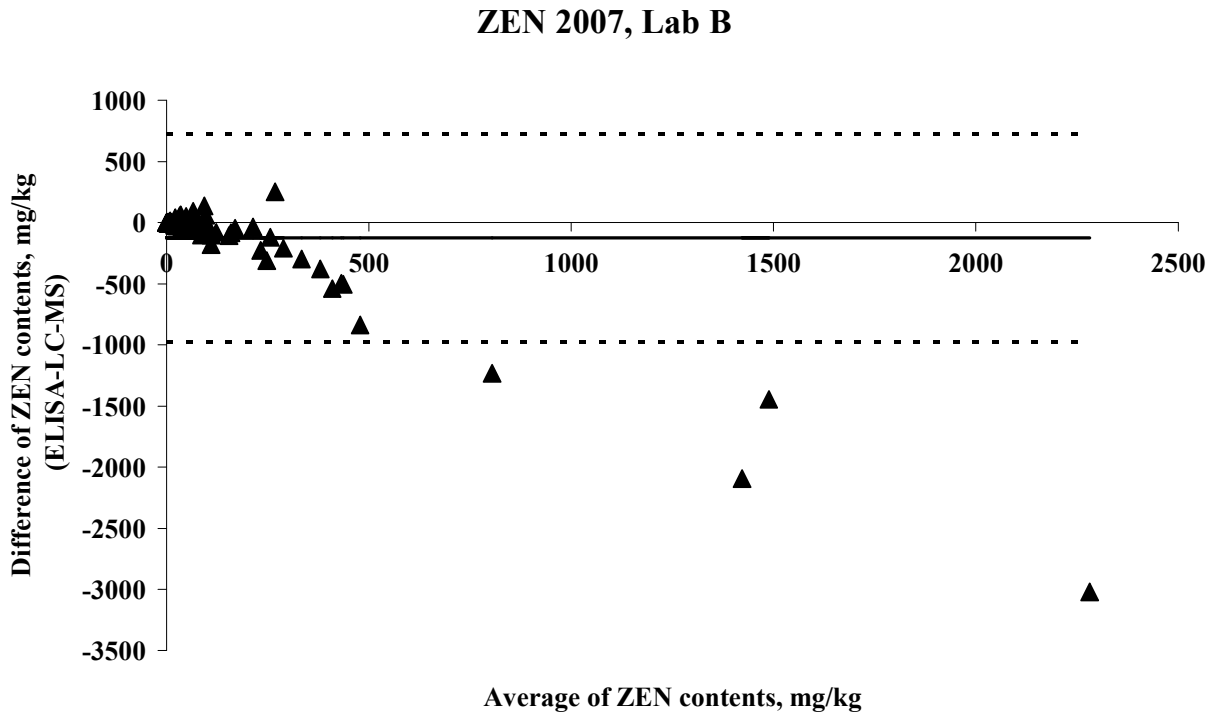
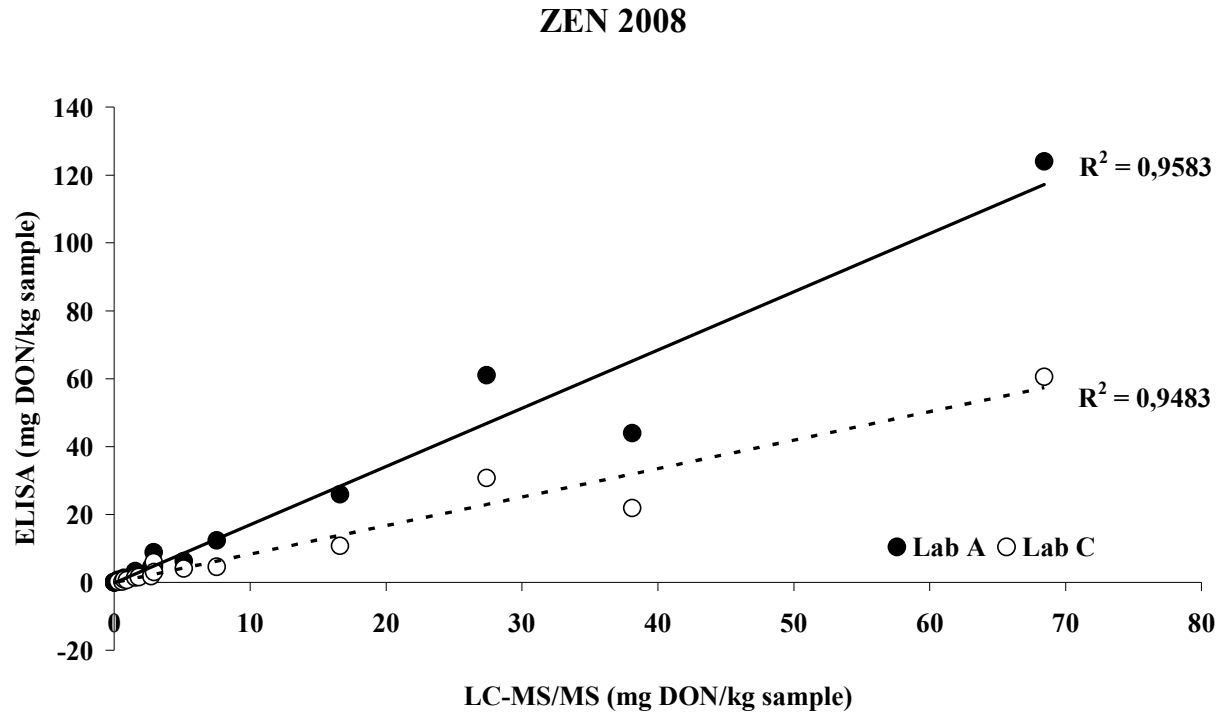


Figure 3b. The linear regression and Bland-Altman plots for ZEN determination by ELISA and LC-MS/MS in each laboratory from 2007.



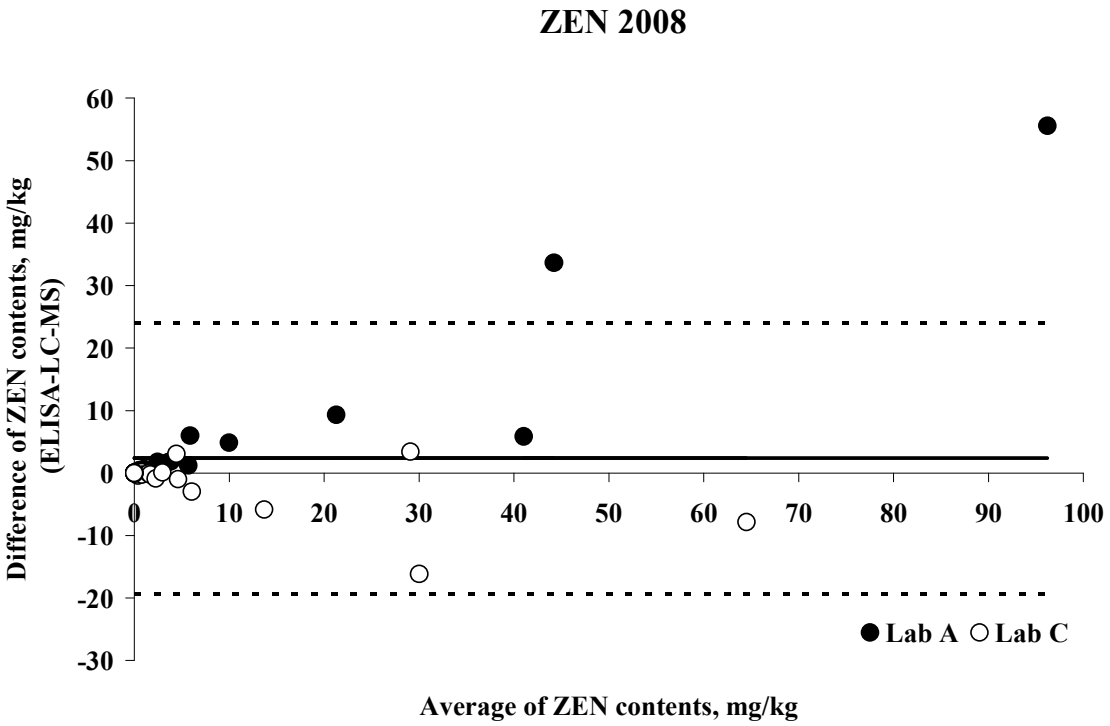


Figure 3c. The linear regression and Bland-Altman plots for ZEN determination by ELISA and LC-MS/MS in each laboratory from 2008.

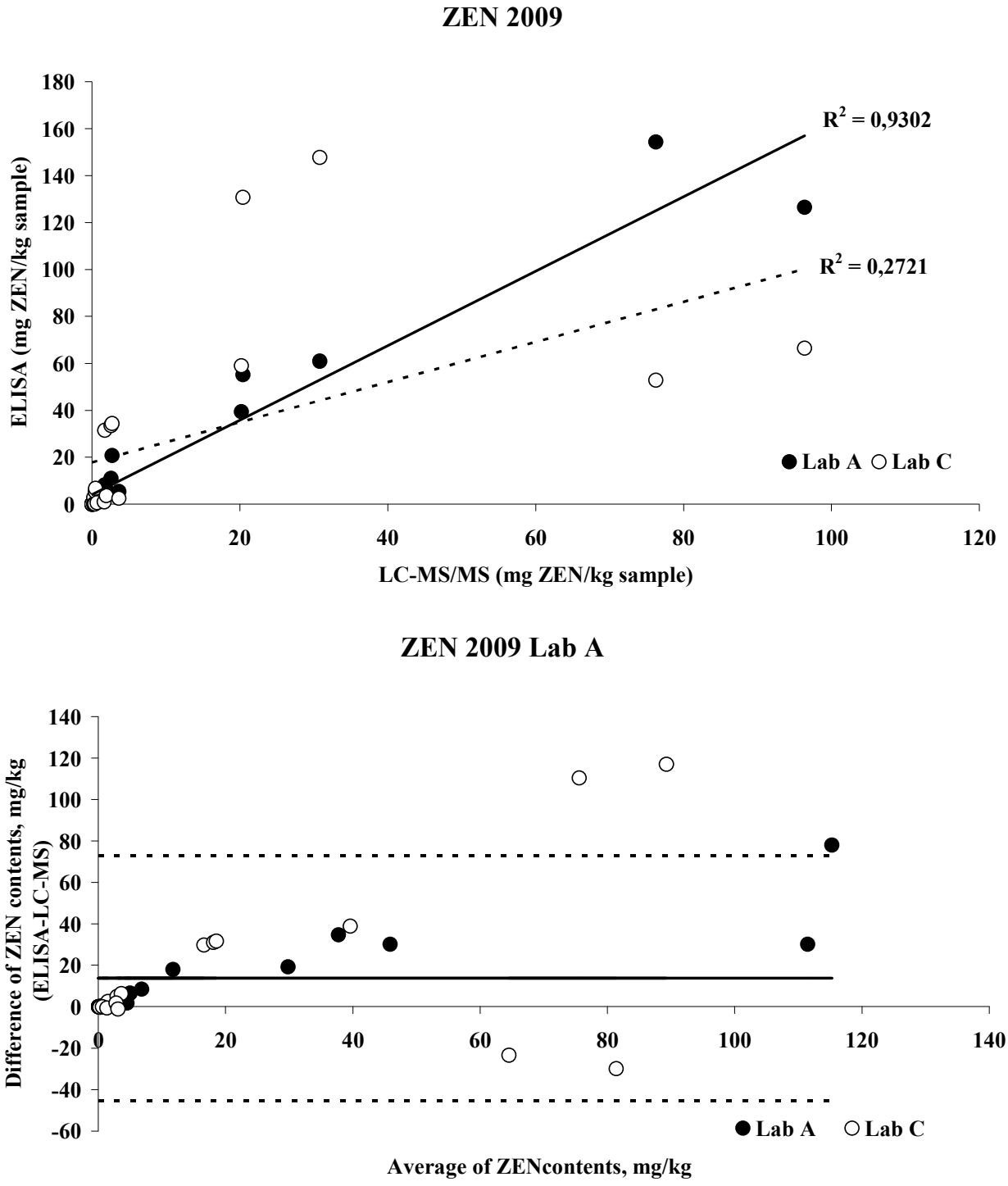


Figure 3d. The linear regression and Bland-Altman plots for ZEN determination by ELISA and LC-MS/MS in each laboratory from 2009.

Table 3. Concentration levels of DON, 3-ADON and 15-ADON obtained by LC-MS/MS and DON obtained by ELISA in 20 maize samples

	LC-MS/MS			ELISA
Sample no.	DON (mg/kg)	15-ADON (mg/kg)	3-ADON (mg/kg)	DON (mg/kg)
1	3.1	1.6	<0.1	3.9
2	451.7	76.3	8.5	1200.0
3	86.0	7.4	0.8	104.8
4	7.9	2.0	0.4	11.8
5	177.4	21.9	4.4	416.0
6	0.2	<0.1	<0.1	0.2
7	5.0	0.8	0.1	9.7
8	484.4	30.1	5.0	672.0
9	23.1	3.4	0.5	45.7
10	90.3	15.3	3.9	131.8
11	0.7	<0.1	<0.1	8.1
12	686.1	86.7	9.2	960.0
13	574.8	77.0	8.5	1040.0
14	339.5	25.5	4.7	512.0
15	8.4	1.6	<0.1	12.7
16	228.1	27.5	4.8	360.0
17	88.2	10.1	3.6	211.4
18	14.7	2.8	0.4	25.7
19	44.0	2.8	0.5	65.8
20	501.3	7.1	5.6	1600.0

Table 4. Frequent of samples by using LC-MS/MS and ELISA of each mycotoxin

Analyte	MRL ($mg\ kg^{-1}$)	Number of sample			
		LC-MS-MS		ELISA	
		< MRL	≥ MRL	< MRL	≥ MRL
DON	1.75	22	135	26	131
FB	4.00	26	100	30	96
ZEN	0.35	22	123	21	124

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Fumonisin monitoring in Thai red cargo rice by reversed-phase high-performance liquid chromatography with electrospray ionization ion trap mass spectrometry

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Keywords

Fumonisin B1, Fumonisin B2, red cargo rice, mycotoxins, LC-MS/MS

Abstract

The occurrence of fumonisins in red cargo rice from Thailand was studied by high-performance liquid chromatography with electrospray ionization ion trap mass spectrometry (LC-ESI-MS/MS). A quantification method for fumonisin B1 (FB1) was developed and the chromatogram of fumonisin B2 (FB2) was observed. The present method provides a sensitive detection limit at 1.0 ng g^{-1}. The limit of quantification was 5.0 ng g^{-1}. The recovery rate showed high yield of accuracy at 110.1±13.3, 89.3±11.1 and 91.9±4.6 % after fortification (n=5) at 50, 100 and 500 ng g^{-1}, respectively. Of the fifty eight samples from the retail markets, two samples were found to be naturally contaminated with FB1 at a trace level (lower than 5.0 ng g^{-1}). None of FB2 was found in any of the samples. This is the first report about the natural occurrence of FB in red cargo rice from Thai market.

Introduction

Foods and feeds contaminated with mycotoxins is a global serious problem that has high potential risk on human and animal health. Mycotoxins are toxics secondary metabolites produced by molds growing in foodstuffs and animal feeds. As known, the major mycotoxin-producing fungi are species of *Aspergillus*, *Fusarium*, and *Penicillium*. At present, the monitoring of mycotoxins contamination in staple food such as rice is highlighted. Currently, the occurrence of mycotoxins in rice, mostly known major toxin, has been reviewed (Tanaka *et al.*, 2007).

Rice is one of the staple foods which is needed and consumed by human and animals all over the world. World rice production was recorded at 434.3 million tons (milled basis) in 2008/09 (Child, 2009). However, food safety is of interest to the consumer. Despite Thailand is the largest rice exporter in the world but data available on diversity of fungal flora and mycotoxin-producing fungi in Thai rice grains is scarce. At present, health organizations are focusing on natural substance contamination e.g. mycotoxins in grains. In Thailand, Pitt *et al.* (1994) suggested that Thai rice is susceptible to fungi following *Gibberella fujikuroi*, *Fusarium semitectum* and *Alternaria padwickii*. Fumonisins in rice were first detected in 1998 with Fusarium sheath rot disease (Abbas *et al.*, 1998). Japanese scientists have developed a method to measure the level of fumonisins in rice, infected by *Gibberella fujikuroi*, by HPLC-Fluorescence (HPLC-FL) and LC-MS/MS and then detected the natural contaminant at 70.0-100.0 ng g^{-1} (in total) in 2 out of 6 samples (Kushiro *et al.*, 2008). As known, the LC-MS/MS is a powerful instrument as a feasible analytical technique in the analysis of food contaminants including mycotoxins. The applicability of the LC-MS/MS method includes simple sample preparation, no derivatization is needed and a very small quantity of compounds can be detected. However, the disadvantage of LC-MS/MS is the matrix effect which obstructs the ionization of mycotoxins during analysis.

More recently, contamination of fumonisin in rice infected with *Fusarium* spp. was confirmed (Maheshwar *et al.*, 2009). Commonly, the occurrence of mycotoxins in rice is at a minimal level and is not found as frequently as other cereals such as maize, wheat and barley (Tanaka *et al.*, 2007). However, contaminations of certain mycotoxins in rice have been reported (Weidenboerner, 2000; Hussaini *et al.*, 2007; Reddy *et al.*, 2009). Red Cargo rice is a kind of unpolished rice similar to brown rice, only color of bran is red, purple or maroon. Unpolished rice is a healthy food which is richer in essential nutrients than milled rice, for instance vitamins (vitamin B1, B2, B3, B6 and E) and minerals (potassium, calcium, sodium, magnesium, iron, zinc, copper and manganese) (Anonymous1, 2001; Jiang *et al.*, 2008). It has become a popular dish as a nourishing food

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in Asia, particularly in Thailand, and other parts of the world by cooking it either with or without normal polished rice. Red cargo rice retains its bran layer, and hence may be contaminated by mycotoxins such as fumonisins. Fumonisins are produced by *Fusarium* spp., known as pre-harvest or soil fungi. They reportedly have 28 structural analogs but FB1 is the major detected toxicant (Šegvić *et al.*, 2001).

Fumonisins reportedly have a potential to affect sphingolipid biosynthesis (Wang *et al.*, 1991). Moreover, they reportedly have hepatotoxicity effects and are classified in group 2B as a possibly carcinogenic substance to humans (IARC, 2002; Murphy, 2006). As well as in animals, fumonisins increasingly have become a problem in farm production. It causes softening of the white matter in the brain (leukoencephalomalacia) and is known as Moldy Corn poisoning in horses and Porcine Pulmonary Edema (PPE) in swine (Bucci and Howard, 1996; Haschek *et al.*, 2001). To minimize the problem, the Scientific Committee on Food of the European Commission (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) allocated a provisional maximum tolerable daily intake of 2.0 ng g^{-1} of body weight to fumonisins B1, B2, and B3, alone or in combination(European Commission, 2003; WHO, 2002). Moreover, the maximum levels of total FB1 and FB2 range from0.2 to 2.0 μg g^{-1} in maize-based products and unprocessed maize and have been regulated by the European Union (European Commission, 2006). In addition, The Food and Drug Administration (FDA) has also released guidance levels of total fumonisins in corn and corn-based products at 2.0–4.0 μg g^{-1} for foods and 5.0–100 μg g^{-1} for animal feeds (FDA, 2001).

Despite unavoidable and long-term exposure even at low-doses, natural contamination of fumonisins is harmful for health but little information on the determination and detection of fumonisins in rice has been reported. Therefore, the aim of this study was to optimized detection method for fumonisins and limited survey of the occurrence of fumonisins, particularly the major toxicant (FB1), in Thai red cargo rice.

Materials and Methods

Chemical and reagents

A standard of Fumonisin B1 and B2 were purchased from Sigma Co. (St. Louis, MO., USA). Methanol, acetonitrile and acetic acid were purchased from Fisher Scientific (UK). For solid phase extraction, we used strong-anion-exchange cartridges (SAX, 500 mg, 3 ml, Varian, Inc. CA, USA) as a clean-up and collection step.

Red cargo rice sample and sample preparation

The occurrence of fumonisins contamination in Thai red cargo rice were investigated using 58 samples collected from retail markets in the central region of Thailand in 2009. Rice samples of 2 kg were collected from market and 10 sub-sampling (20 g each) were thoroughly mixed. Then, 10 g of mixed sampling was soaked under 5 mL of water for 30 min. Then, 15 mL of methanol was added and shaken at 150 rpm in an orbital shaker for 1 hr. The matrix was then centrifuged at 4500 rpm for 10 min and 5 mL of supernatants were collected. The extraction method was modified following Kushiro *et al.*, (2008).To clean up the sample, a strong anion exchange (SAX) cartridges (500 mg sorbent, Varian, CA, USA) column was first prepared by washing with 10 mL of methanol and then precondition column was used with 10 mL of 75% methanol. The collected samples were loaded by passing into the column for 2 mL followed by 2 steps washing with 8 mL of 75% methanol and 4 mL of methanol, respectively. The elution was achieved by slowly passing 10 mL of 1% acetic acid in methanol. Then the eluate from SAX was transferred to evaporate under a gentle stream of nitrogen gas. The remainder was redissolved with 500 μL of methanol/water (50:50, v/v) and analysed with the LC-ESI-MS/MS.

Apparatus and method of quantification

Fumonisins were analysed by high-performance liquid chromatography coupled to electrospray ionization ion trap mass spectrometry. The HPLC system was carried out with an Agilent 1100 Series (Palo, Alto, CA, USA) equipped with a binary pump, a vacuum degasser and a autosampler. Gradient HPLC separation was performed on a Phenomenex Gemini C-18, 150 mm × 4.6 mm, 3 μm (Phenomenex, Macclesfield, UK) attached with a guard column, Phenomenex Gemini C-18, 3 mm, 3μm. The columns were controlled at 40°C. Solvent A was 0.05% acetic acid in water (v/v), and solvent B was 0.05% acetic acid in methanol. The gradient program was: 0-1 min, 30% B; 1-6 min linear gradient to 95% B; hold 95% B for 3 min; then equilibrate column with 30% B for 5 min between each injection at a flow rate of 0.2 mL min^{-1}. The column effluent was directly coupled to an Agilent 1100 MSD Trap SL mass spectrometer equipped with an atmospheric-pressure ESI source and an IT mass analyzer. Standard FB1 and FB2 were infused by syringe pump at a flow rate 20 μl min^{-1} for optimizing the mass spectrometer parameter. The MS^2 measurements of the protonated molecules were

made in full-scan mode. The spray needle voltage was +3.5 kV, the optimal capillary temperature was 350°C, sheath gas pressure 80 psi, and auxiliary gas setting 40 units. To identify and confirm, at least three fragment specific ions were monitored.

No matrix effect of the rice sample appeared after using SAX clean up compared with chromatograms with fortified samples and standard samples. Thus, we obtained a standard calibration curve without a matrix. The method was linear (R^2>0.998) using triplicate standard solution at six different concentrations range from 5-1000 ng g^{-1} for the calibration curve. Accuracy, intra-day (n=5) and inter-day (n=3) precision were determined using quality control samples at concentration of 50, 100, and 500 ng g^{-1}. The recovery rate was reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Briefly, 10 g of blank red cargo rice, thus assumed not to contain fumonisins, were spiked with standard fumonisin B1 to obtain total levels of 50, 100, and 500 ng g^{-1}, followed by the extraction and clean-up described above. The limit of detection (LOD) was based on signal-to-noise ratio (S/N) that provided signals at three times above the background noise level. The limit of quantification (LOQ) was defined as the lowest concentration of standard calibration curve which was validated.

Table 1. Extraction recoveries of the method used for determination of FB1 in Thai red cargo rice

FB1 spiking level (ng g^{-1})	Recovery rate ± RSD[a]
50.0	110.1 ± 13.3
100.0	89.3 ± 11.1
500.0	91.9 ± 4.6
Mean of means	97.1 ± 11.3

[a]: relative standard deviation (n= 5 replicates)

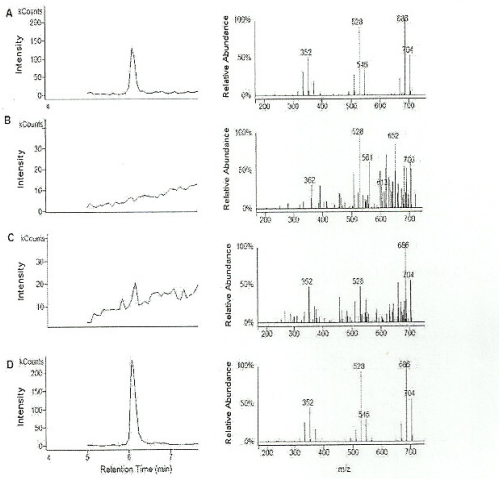

Figure 1. LC-ESI-MS/MS chromatograms of fumonisin B1 (m/z 722) and MS/MS spectrum; (A) standard of FB1 at 5.0ng g^{-1}, (B) Blank red cargo rice sample, (C) positive sample No.35, and (D) fortified sample at 50.0ng $g^{-1}$of FB1

Result and Discussion

Method of quantification

HPLC consisting of ESI/MS/MS permitted a highly sensitive and selective detection of fumonisin B1 in red cargo rice. FB1 and FB2 have highest intensive signal at m/z 722 and m/z 706 respectively. A typical chromatogram, showed in figure 1, was obtained from a rice blank sample (B) and FB1 spiked at 50 ng g^{-1} (D). Figure 1 (A) and 2 (A) showed the spectra of the product ions of FB1 and FB2 standards. The main fragment ions of FB1 were 686, 528 and 352. The fragmentation of FB2 showed the product ions at m/z 670, 512, and 336. These protonated ions were used to confirm FB1 and FB2 in red rice samples with retention time at 6.1 min for FB1 and 6.8 min for FB2. The mean recoveries of fortified samples were 89-110% (see table 1).

The biological-matrix effect in the LC-MS/MS analysis has been documented (Niessen, 2003; Spanjer *et al.*, 2008). However, in the current method has no matrix effect. We observed neither signal suppression nor enhancement after the clean-up step by SAX-solid phase extraction column. The SAX cartridge has been extensively used in fumonisin detection as an excellent purification procedure (Shephard, 1998). However, the cartridge needs to control range of pH in the extracted sample to yield good recovery. Therefore, the current method adjusts acidic condition by adding 10 mL of 1% acetic acid into methanol for the elution process.

The smallest signal of FB1 that could be detected based on a signal-to noise ratio greater than three (LOD) was 1.0 ng g^{-1} and the limit of quantification was 5.0 ng g^{-1}. The mean recoveries of fortified samples were 89-110 %. The method provided good intra-day repeatability and inter-day reproducibility with acceptable relative standard deviation (RSD) range 4.6-13.3% and an excellent linearity (r^2) of the calibration curve at 0.9989. The parent and daughter fragment ion of FB2 in standard and samples were also investigated (see figure 2). The LOD of FB2 was 1.0 ng g^{-1}. Unfortunately, FB2 did not validate as there was insufficient data of spiked samples. Kushiro *et al.*, (2009) who developed method for fumonisin detection in rice has limited the LOD level at 12 ng g^{-1}. The modified method reported here has better LOD (1 ng g^{-1}) might be due to using different solvent solution for preparing standard stock solution, mobile phase and even instrument sensitivity. Thus, the result

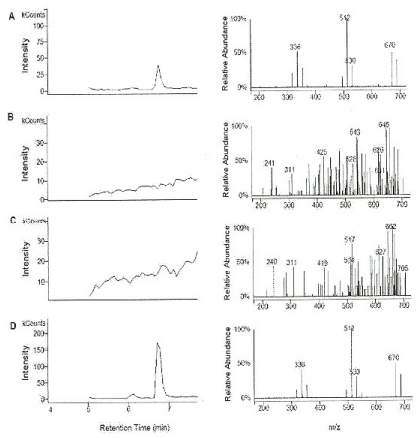

Figure 2. LC-ESI-MS/MS chromatograms of fumonisin B2 (m/z 706) and MS/MS spectrum; (A) standard of FB2 at 5.0ng g^{-1}, (B) Blank red cargo rice sample, (C) sample No.35, and (D) fortified sample at 50.0 ng g^{-1} of FB2

clearly showed that the fumonisin determination was well validated and appropriate to apply.

Occurrence of FB1 in Thai red cargo rice

It was estimated that mean of unpolished rice consumption by Thai was 25.60 g/person/day (Jankhaikhot, 2005). The current detection method was applied to quantify the presence of FB1 in red cargo rice by testing fifty eight samples collected from retail markets in the central region of Thailand. As results, rice has low risk of contamination with FB1 as it was found in 3.45% of the samples (2 of 58 samples) at a trace level (concentration between LOD and LOQ). In addition, none of the rice samples presented a signal of FB2 following product ions monitoring at m/z 670, 512 and 336 as shown in figure 2.

Although fumonisins contamination have been extensively studied in food commodities including maize, wheat, barley, cornflake, and wine (Castellá *et al.*, 1999; Spanjer *et al.*, 2008). As mentioned above, fumoinisins contamination in rice has also been documented (Abbas *et al.*, 1998). In USA, high level of fumonisins found in rice (600-4,300 ng g^{-1}) has been recorded (Weidenboerner, 2000). In Japan, fumonisins has been found at the level ranging from 0.061-0.101 mg/kg for FB1 and from 0.011-0.027 mg/kg for FB2 in rice seed by LC–MS/MS detection (Kushiro *et al.*, 2009). Nonetheless, data determination of fumonisins in rice, particularly in unpolished, is scarce.

Recently, the association of fumonisins producing a *F. verticillioides* isolate in paddy rice was confirmed (Maheshwar *et al.*, 2009). However, in this present study *Fusarium* spp. was not isolated. More recently, focusing on fumonisins contaminated Thai rice, the FB1 has been detected (14 ng g^{-1}) only 1 sample from 100 rice sample in Thai black sweet rice (Bansal *et al.*, 2011). In addition, as it's relatively low-level of fumonisin contamination, Thai rice has also been used as in-house reference material containing fumonisins (Awaludin *et al.*, 2009). However, due to *Fusarium* spp. occurs worldwide on cereal grains, therefore, its mycotoxicology and its residue, particularly in rice, warrants further studied.

Regarding mycotoxogenic fungi in rice, rice is generally immune to fungal infection as compared to corn and wheat (Kushiro *et al.*, 2009). However, a wide variety of fungi infected rice field and grain has been reported (Reddy *et al.*, 2009). Thailand geographically has wide diversity. Thus, rice growing in field is commonly contaminated with various fungi and may also remain certain mycotoxins (Jankhaikhot, 2005). Although little data of fumonisins contamination in rice are available, other mycotoxins in rice such as aflatoxin, ochratoxin, deoxynivalenol, citrinin and zealarenone have been found (Weidenboerner, 2000; Tanaka, 2007). Reddy *et al.*, (2009) found high incidence of aflatoxin contamination in rice in India. More recently, Fredlundet *et al.*, (2009) reported a survey of mycotoxins in rice in the Swedish market identified the presence of aflatoxin and ochratoxin A. Moreover, co-occurrence of aflatoxin, citrinin and ochratoxin A in Vietnamese rice has been documented (Nguyen *et al.*, 2007). Thus, a high specific quantification technique and monitoring program of mycotoxins in rice in order to protect human and animal health should be investigated.

Conclusion

The current method using LC-ESI-MS/MS is sufficient to detect fumonisin B in red cargo rice. As FB1 detected at very low level, fumonisins contamination in Thai red cargo rice may negligible. Although the maximum residue limit (MRLs) of mycotoxins in rice have not yet been established, to prevent risk of long-term and low-dose exposure of natural contamination, regular monitoring of mycotoxins in rice should be carried out. However, mould and mycotoxins contamination may variable occur in different condition. Data available on less-known mycotoxins produced by fungi-infected rice is scarce. Therefore, further study on the occurrence of mycotoxigenic fungi and related mycotoxins in rice should be investigated.

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Chapter 5: General Discussion

Develop a new extraction solvent for simultaneous mycotoxin analysis.

Our results suggest that acetone/water/acetic acid (80:19:1 v/v/v) is the best extraction solvent for 27 mycotoxins including aflatoxins, beauvericin, citrinin, enniatins, fumonisins, gliotoxin, ochratoxin A, patulin, sterigmatocystin, trichothecenes types A and B, verrucarol, and for zearalenone analysis in wheat, maize and rice when compared with conventional solvent (acetonitrile-water mixture, 84:16 v/v) and acidified acetonitrile (acetonitrile/water/acetic acid 79:20:1 v/v/v). These mycotoxins have diversity of polarity and chemical structure.

Many researchers have used acetonitrile-based solvents during the past few years (Klötzel et al. 2005; Cavaliere et al., 2005; Sulyok et al., 2006; Neuhof et al. 2009) to extract multiple toxins in cereal, but solvents have not yet been optimized for fumonisin, which is one of the most important contaminating mycotoxins and is found in food and agriculture commodities worldwide.

Although, Mol et al. (2010) compared acetone/water with other solvents for the determination of 172 pesticides, mycotoxins, plant toxins, and veterinary, the results have reported recoveries for only feed matrices, whereas our results show good recoveries in a variety of matrices, including wheat, maize and rice. Moreover, our results show that we can avoid the SPE clean-up by dilution 1:10 after extracting the sample with our newly developed solvent, acetone/water/acetic acid (80:19:1 v/v/v).

These results provide strong evidence that the newly developed solvent is an interesting one, with high extraction recovery and low matrix effects. Moreover, this method can be performed at low cost and in a short time for the evaporation step and clean-up step. We propose this solvent as a user-friendly replacement for acetonitrile-based solvents for mycotoxin extraction that use multiple toxin methods.

Currently, more than 300 mycotoxins have been identified. The choice of the type of extraction solvent will be based on the analysis propose, type of mycotoxin, and type of food matrix. To study the metabolites of fungal and known target mycotoxins, we can use solvents with high performance to extract those toxins. In the case of multiple

toxin analysis in various matrices, or routine work, the selected solvent should be compromized for most of target mycotoxins .

Evaluation of mycotoxin detection methods, comparing ELISA and LC-MS/MS

The present study was designed to evaluate the performance of the ELISA method when compared with LC-MS/MS for DON, FB and ZON in *Fusarium*-inoculated maize. Moreover, to investigate the fluctuation of ELISA, inter-laboratory results were also compared. The results of this study indicate that good correlations between the methods were found upon analysis. The findings of the current study are consistent with Sydenham et al. (1996), Ono et al. (2000) and Bolduan et al. (2009).

Moreover, our important finding was that there is good agreement between ELISA and LC-MS/MS. This finding corroborates the ideas of Emmanuel et al. (2010), who suggested that the agreement between methods of analysis should be studied by plotting a Bland-Altman graph (1999).

However, the results showed that the performance of ELISA depended on the skill of the technician and the cross-reactivity of the ELISA test kits with similar compounds. In previous findings, many researchers (Krska et al. 2007; Zachariasova et al. 2008; Goryacheva et al. 2009) reported that 3-ADON and 15-ADON can cross-react in DON determination using ELISA. Zachariasova et al. (2008) pointed out that aqueous extraction by the ELISA method might be the cause of overestimation when compared with HPLC using acetonitrile-water as an extraction solvent. Bennet and Nielsen (1994) performed a collaborative study between laboratories to analyze ZEN in corn, wheat, and feed. They pointed out that a high level of technical skill for ELISA analysis was helpful for improving the results. The important feature in ELISA analysis is the washing step to remove unbound antigen and other materials. This step should be performed carefully.

Errors of estimation are especially important with samples that have contaminant levels close to the permitted limit or MRL (maximum residue limit). The present results suggest that LC-MS/MS should be used to confirm the mycotoxin levels if they are close to the MRL after screening by ELISA. ELISA can be effectively used as a rapid screening method for samples with a high level of mycotoxin

contamination. In cases of a low level of mycotoxin contamination, the sample results should be confirmed by LC-MS/MS. However, more research on this topic should be carried out to investigate the correlation, method agreement and cross-reactivity of ELISA to detect different mycotoxins in various matrices of food and agriculture commodities.

The monitoring of fumonisin in red cargo rice from Thailand

Fumonisins are mycotoxins affected by hepatotoxicity (Wang et al. 1991). They are possibly carcinogenic to humans (IARC 2002; Murphy 2006). Fumonisins are produced by *Fusarium* spp., known as pre-harvest or soil fungi. They reportedly have 28 structural analogs but FB1 is the major detected toxicant (Šegvić et al. 2001). Fumonisins have been found in various food and agricultural commodities. Although fumonisin contamination has been extensively studied in food commodities including maize, wheat, barley, cornflake, and wine (Castellá et al. 1999; Weidenboerner 2000; Spanjer et al. 2008), data determination of fumonisins in rice is scarce.

In the present study, we validated a determination method for FB1 analysis and monitored this toxin in red cargo rice, which has become a favourite staple food due to its high nutrient content. The results of this study show that LC-MS/MS has high sensitivity, specificity and accuracy in detecting FB1 at ppb level. Moreover, we found that using an anion exchange cartridge, SAX, is preferable for clean-up and concentrate sample.

Based on our results, only 3.45% of samples were found to be contaminated with FB1 at trace level (lower than 5.0 ng g-1) and none of the samples contained FB2. Contamination by certain mycotoxins in rice has been also reported (Weidenboerner 2000; Hussaini et al. 2007; Reddy et al. 2009). Kushiro et al. (2008) have developed a method to measure the level of fumonisins in rice infected by Gibberella fujikuroi. This method uses HPLC-Fluorescence (HPLC-FL) and LC-MS/MS, and they detected the natural contaminant level at 70.0-100.0 ng g-1 (in total) in two out of six samples. Recently, it was confirmed that fumonisins produce an *F. verticillioides* isolate in paddy rice (Maheshwar et al. 2009). Although the MRLs of mycotoxins in rice have not yet been established, to prevent risk of long-term and low-dose exposure to natural contamination, regular monitoring of mycotoxins in rice should be carried out.

The type of mycotoxin and the contamination level in rice may differ from one location to another. These differences can be explained in part by various factors such as temperature, relative humidity and agricultural practice (Reddy et al., 2009). Moreover, mould contamination may occur to varying degrees in different conditions. Data available on less-known mycotoxins produced by fungi-infected rice is scarce. Therefore, further study on the occurrence of mycotoxigenic fungi and related mycotoxins in rice should be carried out. It is expected that the outcomes will provide useful data to establish the MRLs of mycotoxins in rice, which is a staple food for humans and thus consumed in large quantities.

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Summary

Mycotoxins are toxic secondary metabolites produced by fungi. Due to their high toxicity and the widespread presence of these compounds in various food commodities and agricultural products, mycotoxins are still a worldwide problem and it is important to establish maximum permissible levels in diverse types of food. Consequently, the development of a mycotoxin detection method that is rapid, sensitive, and specific is essential. Different analytical methods have a variety of performance factors and are thus suited for different purposes. Nowadays, over 300 mycotoxins are known and they show great diversity in their chemical and physicochemical properties. The different mycotoxins in different food matrices require specific methods of extraction, clean-up and determination, which affect the performance of analysis.

We investigated the suitability of acidified acetone/water mixtures as a substitute for acetonitrile/water for simultaneous mycotoxin analysis for three kinds of matrices: those of wheat, maize, and rice. Thirteen extraction solvents based on acetone, acetonitrile, and methanol were compared for deoxynivalenol, zearalenone, fumonisins B1 and B2, and beauvericin analysis by LC-MS/MS without sample dilution. In further analysis, methanol-based and acetone-based solvents were selected for 27 mycotoxins including aflatoxins, beauvericin, citrinin, enniatins, fumonisins, gliotoxin, ochratoxin A, patulin, sterigmatocystin, trichothecenes type A and B, verrucarol, and zearalenone. Extraction efficiency was determined by comparing LC-MS/MS signals with the signals of spiked matrix extracts, and this revealed that acetone/water/acetic acid (80:19:1, v/v/v) was the best extraction solvent. We propose this solvent as a replacement for acetonitrile-based solvents for mycotoxin extraction for multi-toxin methods.

ELISA is a rapid mycotoxin screening method and has become a favourite for routine analysis, but it is still important to validate this method. Therefore, we investigated the performance of the ELISA when compared with the hyphenated method, LC-MS/MS by measuring deoxynivalenol, fumonisin B and zearalenone in maize samples inoculated with *Fusarium verticillioides* and *Fusarium graminearum* in experimental fields in Germany. This was done from 2006 to 2009. To investigate the fluctuation of ELISA, three inter-laboratory results were also compared with one LC-MS/MS

laboratory. Good correlations and good agreement between methods were found upon analysis by linear regression and Bland-Altman plot. However, the performance of ELISA depended on the skill of the technician and the cross-reactivity of the ELISA test kits with similar compounds. Furthermore, we found that the ELISA is valuable to use as a screening method for samples with a high level of mycotoxin contamination, in which case it is rapid and easy to use. In cases of a low level of mycotoxin contamination, the sample results should be confirmed by LC-MS/MS.

Finally, we investigated the occurrence of fumonisin in unpolished rice or Red cargo rice, which is a staple human food. Red cargo rice retains its bran layer, and hence may be contaminated by mycotoxins such as fumonisins produced by *Fusarium* spp. However, little information on the determination and detection of fumonisins in rice has been reported. Therefore, we optimized a detection method for fumonisins and surveyed the occurrence of fumonisins, particularly the major toxicant (FB1), in Thai red cargo rice by LC-MS/MS. This method provides a sensitive detection limit at 1.0 ng g^{-1}. The limit of quantification was 5.0 ng g^{-1}. An accuracy showed high yield of mean recovery after fortification sample. Of the 58 Thai red cargo rice samples from the retail markets, two samples were found to be naturally contaminated with fumonisin B1 at a trace level (lower than 5.0 ng g^{-1}). No fumonisin B2 was found in any of the samples.

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Conference lecture

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Limsuwan, S., Tansakul, N., and Karlovsky P., "Evaluation of extraction solvents for the determination of mycotoxins in sugar beet by LC-ESI-MS/MS", The 31st Mycotoxin Workshop, 15th-17th June, 2009, Münster, Germany, Book of abstracts.

Eggert, K., **Limsuwan, S**., Weinert, J., Karlovsky, P., and Pawelzik, E., "Resistance of emmer and nacked barley against *Fusarium culmorum* and *Fusarium graminearum* after artificial infection", The 30th Mycotoxin Workshop, 28th-30th April, 2008, Utrecht, The Netherlands, Book of abstracts.

Döll, K., **Limsuwan, S**., and Karlovsky, P., "Control of fumonisin synthesis in *Fusarium verticillioides* and *F.proliferatum*", The 30th Mycotoxin Workshop, 28th-30th April, 2008,Utrecht, The Netherlands, Book of abstracts.

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Kreuzberger, M., **Limsuwan, S**., Karlovsky, P., and Pawelzik, E., "Correlation between *Fusarium graminearum* DNA and deoxynivalenol content in wheat flours and bran", The 32nd Mycotoxin Workshop, 14th-16th June, 2010, Lyngby, Denmark, Book of abstracts.

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