# Development of Simultaneous Determination Methods for Mycotoxins in Foods by LC-MS/MS and LC-Orbitrap MS

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Dissertation

# Development of Simultaneous Determination Methods for Mycotoxins in Foods by LC-MS/MS and LC-Orbitrap MS

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# List of Abbreviations

**Reagents**

| | |
|---|---|
| 3-ADON | 3-acetyl deoxynivalenol |
| 15-ADON | 15-acetyl deoxynivalenol |
| AFB1 | aflatoxin B1 |
| AFB2 | aflatoxin B2 |
| AFG1 | aflatoxin G1 |
| AFG2 | aflatoxin G2 |
| AFM1 | aflatoxin M1 |
| APA | 2-amino-1-propanol |
| DAS | diacetoxyscirpenol |
| DON | deoxynivalenol |
| FA1 | fumonisin A1 |
| FA2 | fumonisin A2 |
| FA3 | fumonisin A3 |

| | |
|---|---|
| FB1 | fumonisin B1 |
| FB2 | fumonisin B2 |
| FB3 | fumonisin B3 |
| Fe | iron |
| FUX | fusarenon-X |
| HT-2 | HT-2 toxin |
| IPA | isopropanol |
| MeCN | acetonitrile |
| MeOH | methanol |
| $MgSO_4$ | anhydrous magnesium sulfate |
| NaCl | sodium chloride |
| NEO | neosolaniol |
| Ni | nickel |
| NIV | nivalenol |
| OTA | ochratoxin A |
| PAT | patulin |
| PCB | polychlorinated biphenyl |
| PEEK | polyether ether ketone |

| | |
|---|---|
| Pt | platinum |
| SUS | stainless steel |
| T-2 | T-2 toxin |
| TAF | total aflatoxins: the sum of aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 |
| TCA | tricarballylic acid |
| α-ZAL | α-zearalanol |
| β-ZAL | β-zearalanol |
| α-ZEL | α-zearalenol |
| β-ZEL | β-zearalenol |
| ZEN | zearalenone |

**Methods and Instruments**

| | |
|---|---|
| C18 | octadecylsilyl silica gel |
| dSPE | dispersive solid phase extraction |
| ESI | electrospray ionization |

| | |
|---|---|
| GC | gas chromatography |
| GC-ECD | gas chromatography-electron capture detection |
| GC-FID | gas chromatography-flame ionization detection |
| GCB | graphite carbon black |
| HSQC | heteronuclear single-quantum coherence |
| IAC | immunoaffinity column |
| LC | liquid chromatography |
| LC-FL | liquid chromatography-fluorescence spectroscopy |
| LC-MS | liquid chromatography-mass spectrometry |
| LC-MS/MS | liquid chromatography-tandem mass spectrometry |
| LC-Orbitrap MS | liquid chromatography-Orbitrap mass spectrometry |
| AGC | auto gain control |
| dd-$MS^2$ | data-dependent $MS^2$ mode |
| HESI-II | heated electrospray ionization source |
| IT | injection time |
| NCE | normalized collision energy |
| LC-UV | liquid chromatography-ultraviolet spectroscopy |
| MFC | multi-functional cartridge |

| | |
|---|---|
| MRM | multiple reaction monitoring |
| NMR | nuclear magnetic resonance spectroscopy |
| PSA | primary-secondary amine |
| PFP | pentafluorophenyl |
| PTFE | polytetrafluoroethylene |
| QuEChERS | Quick, Easy, Cheap, Effective, Rugged, and Safe |
| SPE | solid phase extraction |
| TIC | total ion chromatogram |
| UHPLC | ultra high performance liquid chromatography |

**Organizations**

| | |
|---|---|
| AOAC | Association of Official Analytical Chemists |
| CODEX | Codex Alimentarius Commission |
| EC | European Committee |
| EU | European Union |
| FAO | Food and Agriculture Organization of the United Nations |

| | |
|---|---|
| FSCJ | Food Safety Commission of Japan |
| IARC | International Agency for Research on Cancer |
| JECFA | Joint FAO/WHO Export Committee on Food Additives |
| MAFF | Ministry of Agriculture, Forestry and Fisheries of Japan |
| MHLW | Ministry of Health, Labour and Welfare of Japan |
| NIHS | National Institute of Health Sciences of Japan |
| US | United States |
| WHO | World Health Organization |

**Other abbreviations**

| | |
|---|---|
| kg-bw | kilogram of body weight |
| LOQ | limit of quantification |
| *m/z* | mass-to-charge ratio |
| TDI | tolerable daily intake |
| PMTDI | provisional maximum tolerable daily intake |
| RSD | relative standard deviation |

# General Introduction

## An overview of mycotoxins

Food safety has become an important research topic owing to the many related incidents and accidents in the recent past. The various risk factors of food safety include natural substances, synthetic substances such as pesticide residues, byproducts of processing of foods, and contaminants consisting of foreign substances such as insects and manufactured materials. In particular, because natural substances appear during food growth and storage, it is difficult to remove them completely. There are many kinds of natural substances that act as risk factors. One group of such substances that contaminate crops and the related products is *mycotoxins*.

Mycotoxins are toxic secondary metabolites produced by fungi. These substances can cause severe health problems in humans and animals. Aflatoxin B1 (AFB1), which is known to be the strongest cancer-causing agent among natural substances, was discovered as a cause of the “turkey X disease” around 1960 in the United Kingdom. At the time, more than one hundred thousand turkeys died from the disease and

as a result, mycotoxins became known widely as a risk factor in foods. Even recently, some fatal accidents as a result of AFB1 ingestion have been reported: 125 people died after eating corn contaminated with AFB1; this corn was stored under conditions of high humidity in Kenya in 2004. More than three hundred mycotoxins have been discovered to date. Among them, some of the key mycotoxins that cause food-borne illnesses include aflatoxins, ochratoxin A (OTA), patulin (PAT), trichothecenes, fumonisins, and zearalenone (ZEN). Outline of key mycotoxins are shown in Table 1. These mycotoxins pose various health hazards such as carcinogenesis, hepatopathy, gastrointestinal hemorrhage, immunodeficiency, and estrogenic syndrome. Additionally, mycotoxins do not disintegrate after heat treatment during food processing because of their high heat stability. Therefore, there is also a risk of their staying in food products even after heating.

In order to reduce economic losses and adverse effects on the health of humans and animals as a result of mycotoxins, the CODEX Alimentarius Commission (CODEX) has been working on setting the maximum levels for each type of mycotoxin in food products and on establishing guidelines regarding food management [1, 2]. The regulatory levels of mycotoxins are set for country-specific among the developed countries. In Japan, the Ministry of Health, Labour and Welfare (MHLW) and the Ministry of Agriculture, Forestry and Fisheries (MAFF) have been working on defining regulatory levels and

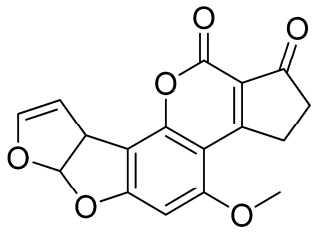
**Table 1** Outline of key mycotoxins that cause issues with food hygiene.

| Mycotoxin | Fungus | Contaminated food | Toxicity | Maximum level (CODEX) | Regulatory level (Japan) |
|---|---|---|---|---|---|
| Aflatoxins (AFB1,AFB2, AFG1, AFG2, and AFM1) | *A. flavus* *A. parasiticus* | Cereals, nuts Milk (AFM1) | Hepatocarcinoma, hepatitis | 10–15 µg/kg (TAF in nuts) 0.5 µg/kg (AFM1 in milk) | 10 µg/kg (TAF in all foods) 0.5 µg/kg (AFM1 in milk) |
| OTA | *A. niger* *A. ochraceus* *P. verrucosum* | Cereals, coffee, cocoa, wine | Kidney cancer, nephritis | 5 µg/kg (Wheat, barley, rye) | - |
| PAT | *P. patulum* | Apple juice | Hemorrhaging in the digestive | 50 µg/kg (Apple juice) | 50 µg/kg (Apple juice) |
| Trichothecenes (DON) | *F. culmorum* *F. graminearum* *F. sporotrichioides* | Cereals | Gastrointestinal dysfunction, immunodeficiency | 1–2 mg/kg (DON in wheat, barley, and corn) | 1.1 mg/kg (DON in wheat) |
| Fumonisins | *F. proliferatum* *F. verticillioides* | Corn | Esophageal cancer | 2–4 mg/kg (Fumonisin B1 (FB1) and fumonisin B2 (FB2) in corn) | - |
| ZEN | *F. culmorum* *F. graminearum* | Cereals | Estrogenic syndrome | - | - (1 mg/kg: feed) |

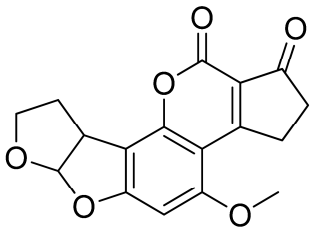
establishing guidelines for mycotoxin management [3–7]. At present in Japan, regulatory levels of some mycotoxins are set: total aflatoxins (TAF), which are the sum of AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), in all foods; PAT in apple juice; and deoxynivalenol (DON) in wheat. Additionally, because the definition of regulatory levels for other mycotoxins is under discussion on the basis of international trends, one can expect that the regulations will be further strengthened. Therefore, the development of accurate methods of determination is necessary in order to closely manage such mycotoxins in food. I describe below the various mycotoxins that occur globally as well as the relevant analytical methods.

## Aflatoxins

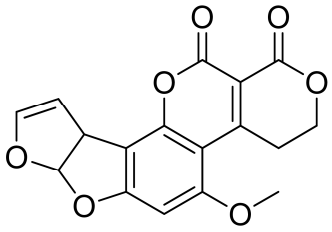
Aflatoxins are contaminants found in many types of food products such as cereals, nuts, and spices. They are produced by *Aspergillus flavus* (*A. flavus*) and *A. parasiticus*. The main aflatoxins are AFB1, AFB2, AFG1, AFG2, and aflatoxin M1 (AFM1). The structures of aflatoxins are shown in Figure 1. AFM1 is a metabolite of AFB1 in livestock that consume feed contaminated with AFB1, and it is detectable in milk. Aflatoxins are carcinogens and are classified as Group 1 substances (*carcinogenic to humans*) by the International Agency for Research on Cancer (IARC) [8]. The CODEX



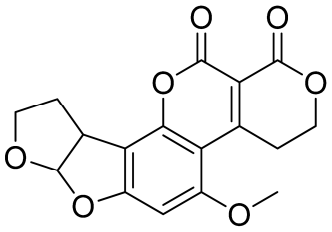
Aflatoxin B1
(AFB1)



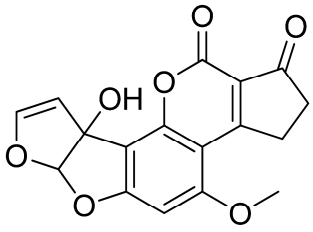
Aflatoxin B2
(AFB2)



Aflatoxin G1
(AFG1)



Aflatoxin G2
(AFG2)



Aflatoxin M1
(AFM1)

**Figure 1** Structures of aflatoxins.

has set the maximum level of TAF to 10 μg/kg in nuts [1], and many developed countries have also set regulatory levels [9, 10]. In Japan, the regulatory level of TAF was set to 10 μg/kg in all food products in 2011 [3]. In addition, the CODEX has set the maximum level of AFM1 in milk to 0.5 μg/kg [1]. In Japan, in line with the CODEX's levels, the regulatory level was set to 0.5 μg/kg in milk in 2015, and this level will be implemented starting in January 2016 [11].

The standard method for analysis of aflatoxins, which was announced by the Association of Official Analytical Chemists (AOAC) and adopted by CODEX, is performed as follows. Aflatoxins are extracted from samples by means of a multi-functional cartridge (MFC) or an immunoaffinity column (IAC) for aflatoxins. Following this, they are subjected to fluorescence derivatization by ultraviolet irradiation on a post-column. Subsequently, aflatoxins are measured by liquid chromatography-fluorescence spectroscopy (LC-FL) [12]. The method adopted in Japan involves extraction with an MFC or IAC followed by fluorescence derivatization with trifluoroacetic acid and measurement using LC-FL [13].

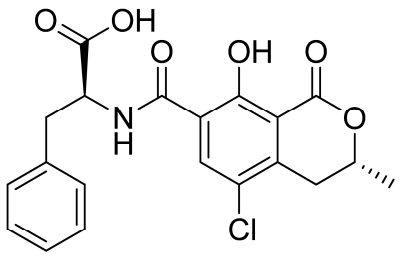
An MFC is an extraction cartridge optimized for each mycotoxin based on its chemical structure and physical property. An MFC contains several kinds of supports that bind to functional groups such as reverse-phase, normal-phase, and ion-exchange

supports. Most of MFCs can remove matrices from a food sample if a researcher passes an extraction solvent through them. Therefore, this extraction method represents easy sample preparation. On the other hand, IACs are an extraction cartridge that is based on antigen-antibody interactions. IACs are capable of providing strong purification.

Ochratoxin A (OTA)

OTA (Figure 2) is produced by fungi such as *A. niger*, *A. ochraceus*, and *Penicillium verrucosum* (*P. verrucosum*), and is found as a contaminant in such products as cereals, coffee, cocoa, and wine. OTA is strongly toxic toward the liver and kidneys. The IARC has classified OTA into Group 2B substances (*possibly carcinogenic to humans*) because it is suspected of contributing to kidney cancer and to nephritis in humans in the Balkan States (Balkan nephropathy) [14, 15]. Additionally, the CODEX has set the maximum level of OTA in wheat, barley, and rye to 5 μg/kg [1]. The regulatory levels of OTA in many food products have also been set in the Europe Union (EU), whereas in Japan, such levels are still under discussion.

The method for analysis of OTA involves extraction with an MFC or IAC followed by measurements by LC-FL. The analytical method in Japan is the same [12, 13].




Ochratoxin A
(OTA)

**Figure 2** Structure of ochratoxin A.

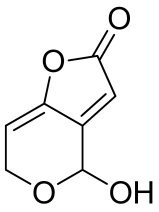
Patulin (PAT)

PAT (Figure 3) is produced by fungi such as *P. patulum* and is present as a contaminant in fruits, especially, apple and its products (e.g., apple juice). PAT is suspected of being carcinogenic according to studies on laboratory animals and is recognized as a contributor to hemorrhage in the digestive system [16]. The CODEX has set 50 μg/kg as the maximum level of PAT in apple juice [1]. The Japanese regulatory level is the same [4].

The analytical method for PAT, as adopted by the AOAC and in Japan, involves extraction with ethyl acetate followed by measurement using liquid chromatography-ultraviolet spectroscopy (LC-UV) [12, 13].

Trichothecenes

Trichothecenes are mycotoxins produced by *Fusarium* fungi such as *Fusarium culmorum*, *F. graminearum*, and *F. sporotrichioides*. Cereals infected with *Fusarium* fungi turn red at the time point of infection, and this sign is known as "*Fusarium* head blight." The toxicity of trichothecenes is lower than that of aflatoxins and OTA, but trichothecenes contaminate cereals including wheat, barley, and corn worldwide [17–20]. Trichothecenes are known to cause not only acute adverse effects such as vomiting,




Patulin
(PAT)

**Figure 3** Structure of patulin.

diarrhea, bleeding, skin inflammation, and decline in the functioning of marrow and hematopoietic systems but also chronic adverse effects such as gastrointestinal dysfunction and immunodeficiency [21–24]. Figure 4 shows the main trichothecenes that are relevant to food safety. DON, HT-2 toxin (HT-2), and T-2 toxin (T-2) levels in cereals are regulated in the EU and United States (US) [9, 10, 25], and the maximum levels of DON were set to 2 mg/kg in cereals (wheat, barley, and corn) and to 1 mg/kg in cereal products by the CODEX in 2015 [1]. The provisional regulatory level of DON in wheat was set to 1.1 mg/kg in Japan [5]. In contrast, nivalenol (NIV) levels are not regulated in the world and are reported to be detected in Asia [26]. Thus, the research on NIV is under way in Japan, and the tolerable daily intake (TDI) of NIV was set to 0.4 μg/[kg of body weight (kg-bw)]/day by the Food Safety Commission of Japan (FSCJ) in 2010 [27]. TDI is a level that does not appear to have harmful effects such as diseases even if a person consumes the substance in question every day throughout the lifespan. Because TDI of DON has been established at the level of 1 μg/kg-bw/day by the FSCJ in 2010 [27], this situation indicates that NIV may pose a higher risk to human health than DON does.

The method for analysis of DON consists of purification using a florisil support, silanization, and measurement using gas chromatography-electron capture detection (GC-ECD) or gas chromatography-mass spectrometry (GC-MS) [12]. On the other hand, the

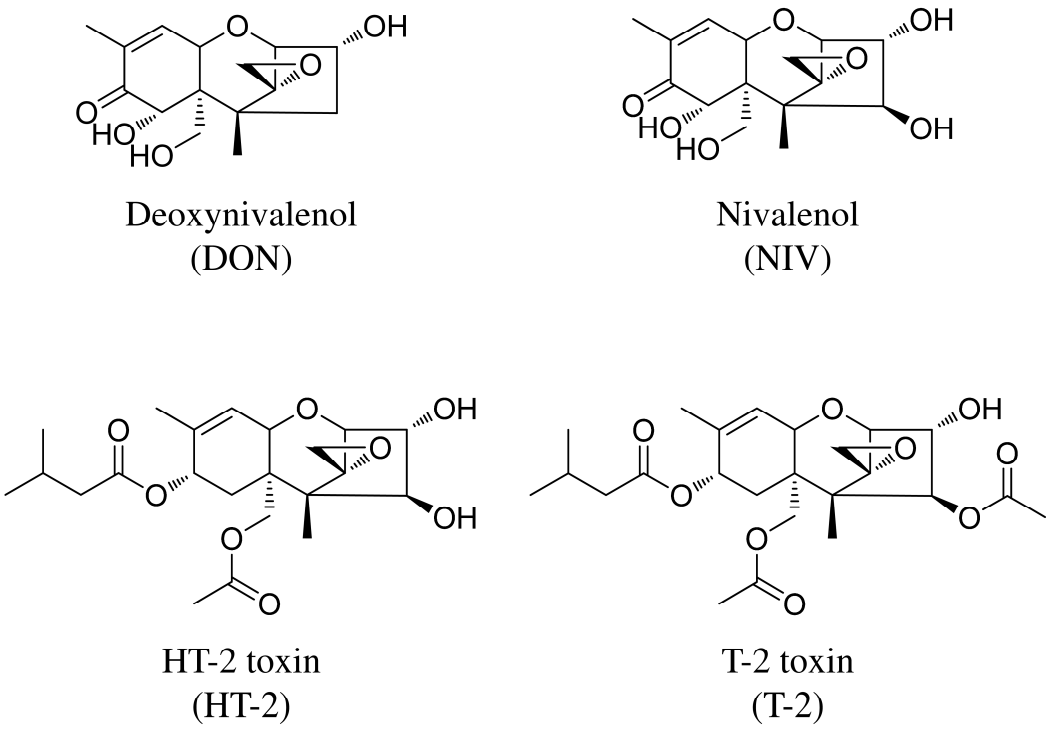



**Figure 4** Structures of trichothecenes.

Japanese methods for analysis of DON and NIV consist of purification using an MFC for trichothecenes, followed by measurements by LC-UV. The methods are reported by the National Institute of Health Sciences of Japan (NIHS) [13, 26].

Fumonisins

Fumonisins are produced by *Fusarium* fungi such as *F. proliferatum* and *F. verticillioides*. Although there are several fumonisins, the fumonisin B-series (Figure 5) is the most clinically important from the standpoint of food safety, and these fumonisins are found as contaminants in corn. They pose a major health risk because they may cause esophageal cancer in humans, equine leukoencephalomalacia in horse, and porcine pulmonary edema in pig [21–24]. Fumonisin B-series is classified into Group 2B substances (*possibly carcinogenic to humans)* by the IARC [8], and their levels in corn are subject to regulation in the EU and US [9, 10]. The CODEX has set the maximum levels for the sum of fumonisin B1 (FB1) and fumonisin (FB2) in raw corn grain to 4 mg/kg and in corn flour and corn meal to 2 mg/kg in 2014 [1]. There is currently no regulatory level in Japan. Because the FSCJ has started performing risk assessments on fumonisin B-series in 2015, it is expected that regulatory levels of fumonisin B-series will be set in the near future in Japan.

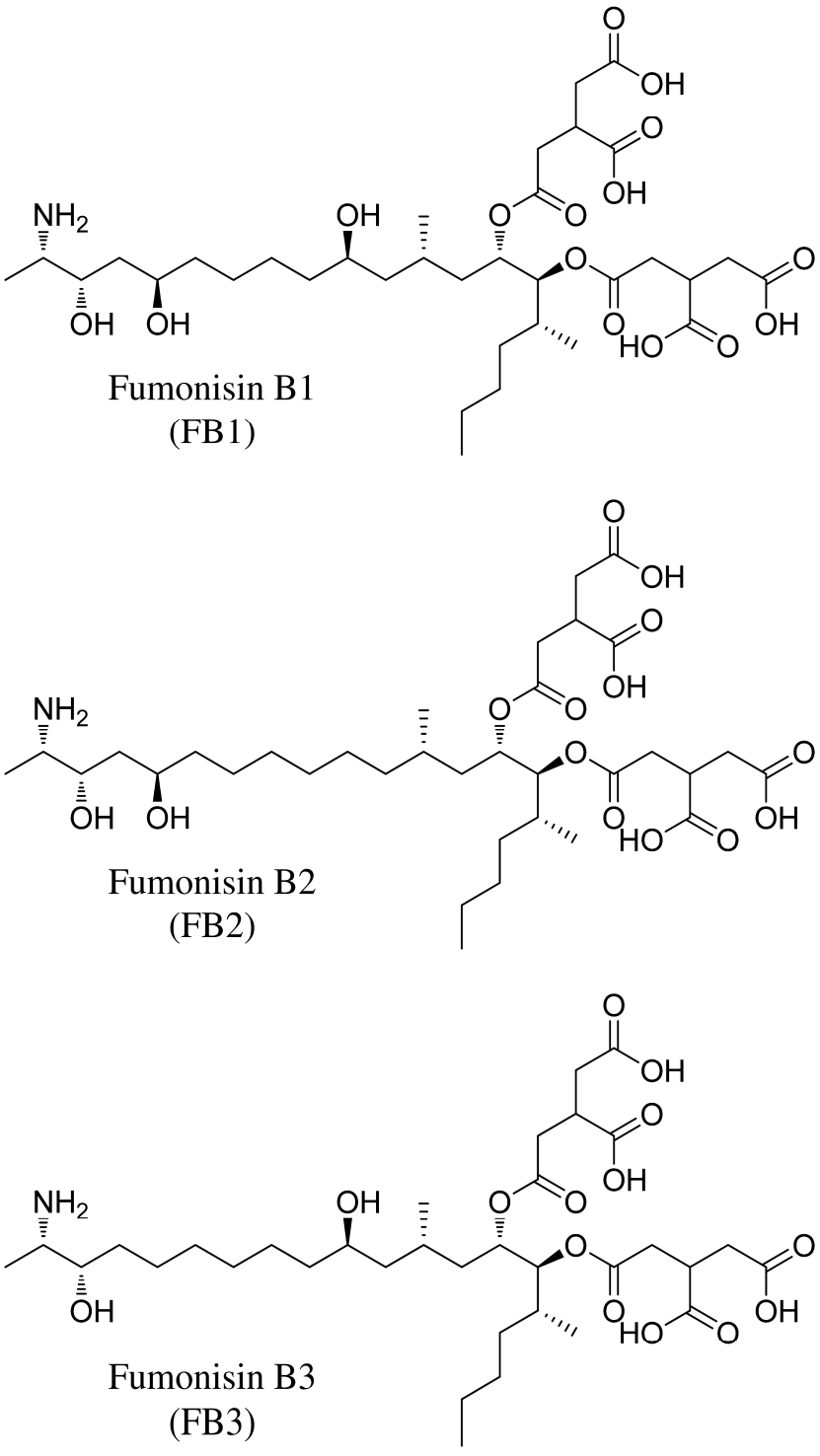


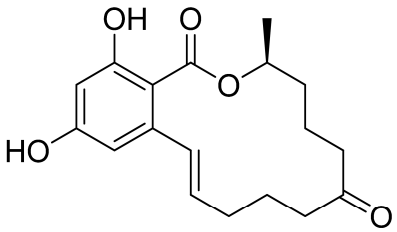

**Figure 5** Structures of fumonisin B-series.

The method for analysis of fumonisin B-series including FB1, FB2, and fumonisin B3 (FB3), as recommended by the AOAC, is as follows: purification by means of a solid phase extraction (SPE) cartridge of strong anion exchange (SAX) or an IAC for fumonisins, followed by measurement using LC-FL after fluorescent labeling with o-phthalaldehyde [12]. Although there is no official method of analysis in Japan, a method consisting of purification by SPE or IAC, followed by measurements using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was introduced by the NIHS [28].

Zearalenone (ZEN)

ZEN (Figure 6) is a *Fusarium* toxin produced by fungi such as *F. culmorum* and *F. graminearum*, and is known to be a contaminant of cereals. It exerts an estrogenic effect causing pseudopregnancy, swelling of breasts, uterus enlargement, ovarian changes, and infertility in livestock that consume feed contaminated with ZEN [21–24, 29]. Regulatory levels have been set for corn and cereals in the EU [9], whereas no maximum or regulatory levels have been set for food products by the CODEX and in Japan. Nonetheless, the regulatory level for animal feed was set to 1 mg/kg in Japan [30].

The AOAC method for analysis of ZEN involves liquid-liquid extraction




Zearalenone
(ZEN)

**Figure 6** Structure of zearalenone.

followed by measurement by LC-FL [12]. The Japanese method for analysis of ZEN in animal feed involves purification by means of an MFC followed by measurements using either LC-FL or LC-MS/MS [31].

**Simultaneous determination of mycotoxins and the associated issues**

In the future, multiple mycotoxins will need to be monitored simultaneously. The reasons are as follows. The regulatory levels will be set for more mycotoxins in Japan in response to international trends (e.g., those related to the CODEX). Additionally, various mycotoxins have different properties as contaminants in food products [17–20]. On the other hand, the official analytical methods adopted by the AOAC and Japanese government are geared toward individual mycotoxins, whereas methods for simultaneous determination of multiple mycotoxins have not yet been recommended. Therefore, the monitoring of multiple mycotoxins by individual methods is complicated and time-consuming, and simultaneous determination is required to monitor multiple mycotoxins. Under these circumstances, mass spectrometry has become an attractive analytical method for food safety studies. Next, I will describe LC-MS/MS, which has high sensitivity, and liquid chromatography-Orbitrap mass spectrometry (LC-Orbitrap MS), which has high resolution. These tools have received much attention worldwide.

LC-MS/MS

LC-MS/MS is an analytical method where the target compounds in the sample

are separated by liquid chromatography (LC) and measured by MS/MS. LC-MS/MS is capable of measuring compounds that are nonvolatile and thermally unstable without derivatization. Therefore, this method is versatile and has a wide range of practical applications. An MS/MS instrument is composed of an ion source, mass spectrometer, and detector (Figure 7). Additionally, the mass spectrometer contains the first quadrupole, collision cell, and second quadrupole. First, in the first quadrupole, the target compound, which is ionized in the ion source, is sorted according to the mass-to-charge ratio (*m/z*) specific to the compound. When the sorted compound is cleaved by collision with nitrogen or argon gas in the collision cell, specific product ions are obtained. The product ions are then sorted in the second quadrupole and detected in the detector. In other words, it is a highly sensitive instrument capable of detecting target compounds selectively, with the selection performed in two steps involving mass filters. LC-MS/MS is useful for simultaneous analysis of multiple mycotoxins with different properties.

LC-Orbitrap MS

An LC-Orbitrap MS was introduced in 2005 and represents high-resolution mass spectrometry. The Orbitrap functions as a mass spectrometer and enables measurement of exact masses up to four decimal places. Although an Orbitrap MS instrument also

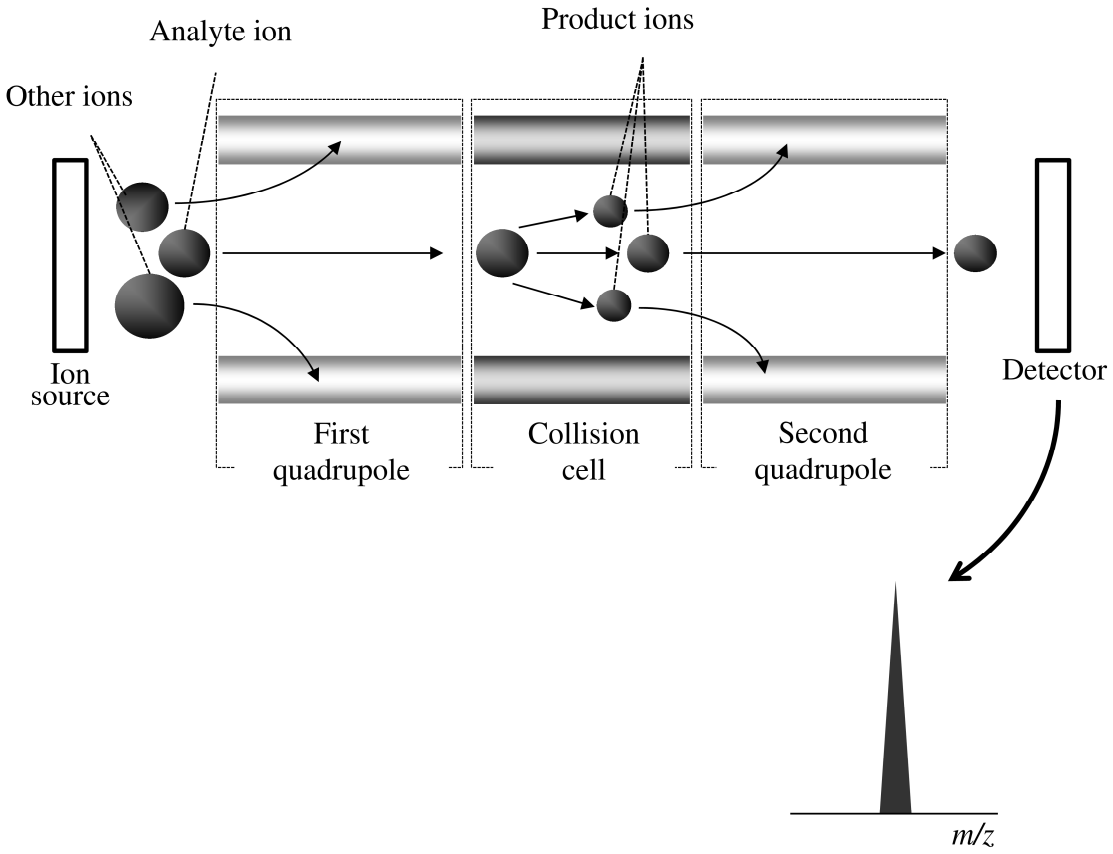



**Figure 7** Schematic illustration of MS/MS analysis.

contains an ion source, mass spectrometer, and detector, the components of the mass spectrometer and the detection principles are different from those of MS/MS (Figure 8). Each compound is ionized in the ion source (analyte ions A and B in Figure 8) and introduced into the mass spectrometer (Orbitrap), which is composed of outer electrodes and a central electrode. Static voltage is applied to the central electrode, and each analyte ion corresponds to a specific rotary amplitude around the electrode. By means of an amplitude campaign movement specific to each ion, the induced currents that are generated at the outer electrodes are detected as complex signals. A complex signal is decomposed to single signals by Fourier transformation, and the *m/z* of each ion is calculated from the angular frequency of each single signal obtained. Even minute differences in *m/z* can be detected by Orbitrap MS via lengthening of recording time. According to the above principle, Orbitrap MS is useful for not only estimation of the formula of unknown compounds on the basis of exact masses but also for accurate detection of known compounds with the known exact masses used as indices because exact masses can be measured at high resolution.

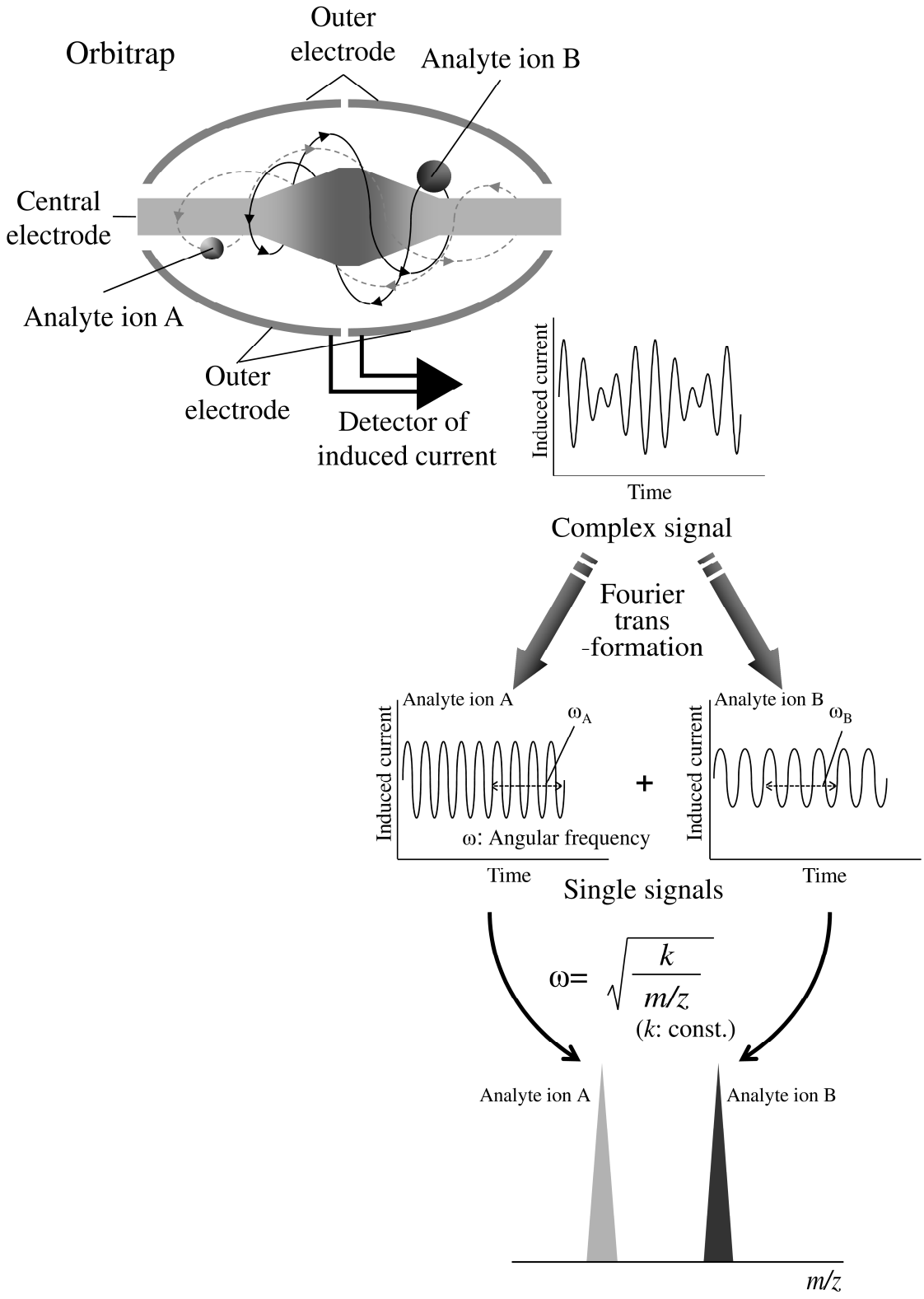



**Figure 8** Schematic illustration of Orbitrap MS analysis.

Measurement problems with mass spectrometry

Recently, the development of such technologies as mass spectrometry for simultaneous analysis of multiple mycotoxins was attempted [20, 32–36]. Because it is difficult to devise simultaneous purification processes for multiple mycotoxins with different properties, the sample preparation often involves only extraction of multiple mycotoxins from a sample. As a result of such preparation methods, matrix removal from food is insufficient, and therefore some mycotoxins show low peak intensity and repeatability. In other words, the methods are not quantitatively accurate. Because such a mass spectrometer has higher sensitivity, greater selectivity, and higher versatility than the previous detectors did, it is useful for analysis of trace amounts of compounds in food. Nonetheless, there are some specific problems associated with mass spectrometry that should be addressed.

The first problem is the influence of the matrix in food samples. Matrix components in a sample may change the ionization efficiency of the target compounds (ion enhancement or ion suppression) [37, 38] and may contaminate the instruments. As a result, the quantitative data are strongly affected, and quantitative accuracy is worsened. Therefore, it is important to remove the matrix during the sample preparation process. On the other hand, there are many complicated matrices in food, and quality and quantity of

matrices are different in various foods. Thus, it is necessary to develop simple and appropriate sample preparation procedures that are capable of removing the matrix from each food product and of recovering multiple mycotoxins with different properties simultaneously.

The second problem that is associated with mass spectrometry is carryover. This is a phenomenon where the target compound remains in an LC instrument and is detected during the next run. Although it is not a problem with low-sensitivity instruments, it often is for high-sensitivity instruments such as mass spectrometers. This phenomenon greatly influences the accuracy and results of quantification [39–41]. Therefore, it is important to reduce carryover when developing highly quantitative analytical methods.

The third problem has to do with the ability of a mass spectrometer to discriminate compounds that have different formulas. It cannot discriminate compounds with the same formula such as isomers. In order to quantify each of the compounds that have the same formula, separating them by LC is essential.

Therefore, optimization of sample preparation and LC conditions is necessary if a researcher wants to take full advantage of mass spectrometry and crucial for development of rapid and highly quantitative methods for the simultaneous determination of mycotoxins.

**The purpose of this study**

Simultaneous determination of mycotoxins using mass spectrometry has not been adopted yet as an official method, but this situation is expected to change: the methods for individual mycotoxins are expected to give way to simultaneous method for multiple mycotoxins. In order to develop a new official method for simultaneous determination, rapid and highly quantitative analysis of mycotoxins by LC-MS/MS and LC-Orbitrap MS is intended. In this study, simple and easy preparation procedures and optimization of LC conditions were examined for proper analysis of mycotoxins (that have different properties) in various food products.

In this doctoral thesis, Chapter 1 describes the development of methods for multiple mycotoxin determination in beers and wines by LC-MS/MS. Chapter 2 describes simultaneous determination of mycotoxins in corn grits by LC-MS/MS with the focus on minimizing carryover. Chapter 3 describes identification and quantification of some fumonisins by LC-Orbitrap MS in corn contaminated with mycotoxins. Chapter 4 describes a method for the simultaneous determination of *Fusarium* toxins, including trichothecenes, fumonisins, and zearalenone-group, in cereals by LC-Orbitrap MS.

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# Chapter 1

# Development of determination methods for multiple mycotoxins in beers and wines by LC-MS/MS

## 1.1 Introduction

In this study, beer and wine were selected as analytical samples for LC-MS/MS. This is because beer is prepared from cereals (e.g., corn, barley, wheat, or rice), which are at risk of contamination with aflatoxins, OTA, trichothecenes, fumonisins, and ZEN. Wine is prepared from grapes, which are at risk of contamination with OTA, and a recent study showed occurrence of fumonisins, in particular FB2, in red wine [1]. *A. niger,* which is an OTA producer, was found to be capable of producing fumonisins [2, 3]. Additionally, Tabata reported that PAT can be a contaminant not only in apples but also in grapes [4]. These observations indicate that contamination with OTA, fumonisins, or PAT is a substantial problem.

Preparation of mycotoxins was examined to apply the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) methodology, which was originally developed

for preparation of multiple pesticide residues [5–8]. QuEChERS is a simple and easy two-step preparation method and is performed follows: (1) Extraction into acetonitrile (MeCN) using hydrous MeCN; this task is accomplished by salting out and dehydration from MeCN using sodium chloride (NaCl) and anhydrous magnesium sulfate ($MgSO_4$); (2) purification by dispersive solid phase extraction (dSPE) from the MeCN extract; this procedure is performed to remove the matrix compounds by adsorption to the supports of the octadecylsilyl silica gel (C18), primary-secondary amine (PSA), and graphite carbon black (GCB) by mixing these supports and the complex by stirring. The first step with MeCN allows us to extract the target compounds and to remove hydrophilic matrices such as saccharides. At the next step, purification by dSPE by means of each support enables removal of ionic and hydrophobic matrices; therefore, the removal of matrices such as pigments and proteins in samples was expected. Thus, if the methodology is applicable to the mycotoxins under study, then the samples can be prepared simply and simultaneously, and the procedure's duration can be shortened significantly.

In this chapter, the following 15 mycotoxins (Figure 1.1) were selected for simultaneous determination by LC-MS/MS: AFB1, AFB2, AFG1, AFG2, AFM1, DON, and PAT, whose regulatory levels for foods have been set in Japan; and NIV, HT-2, T-2, FB1, FB2, FB3, ZEN, and OTA, which have attracted global attention.

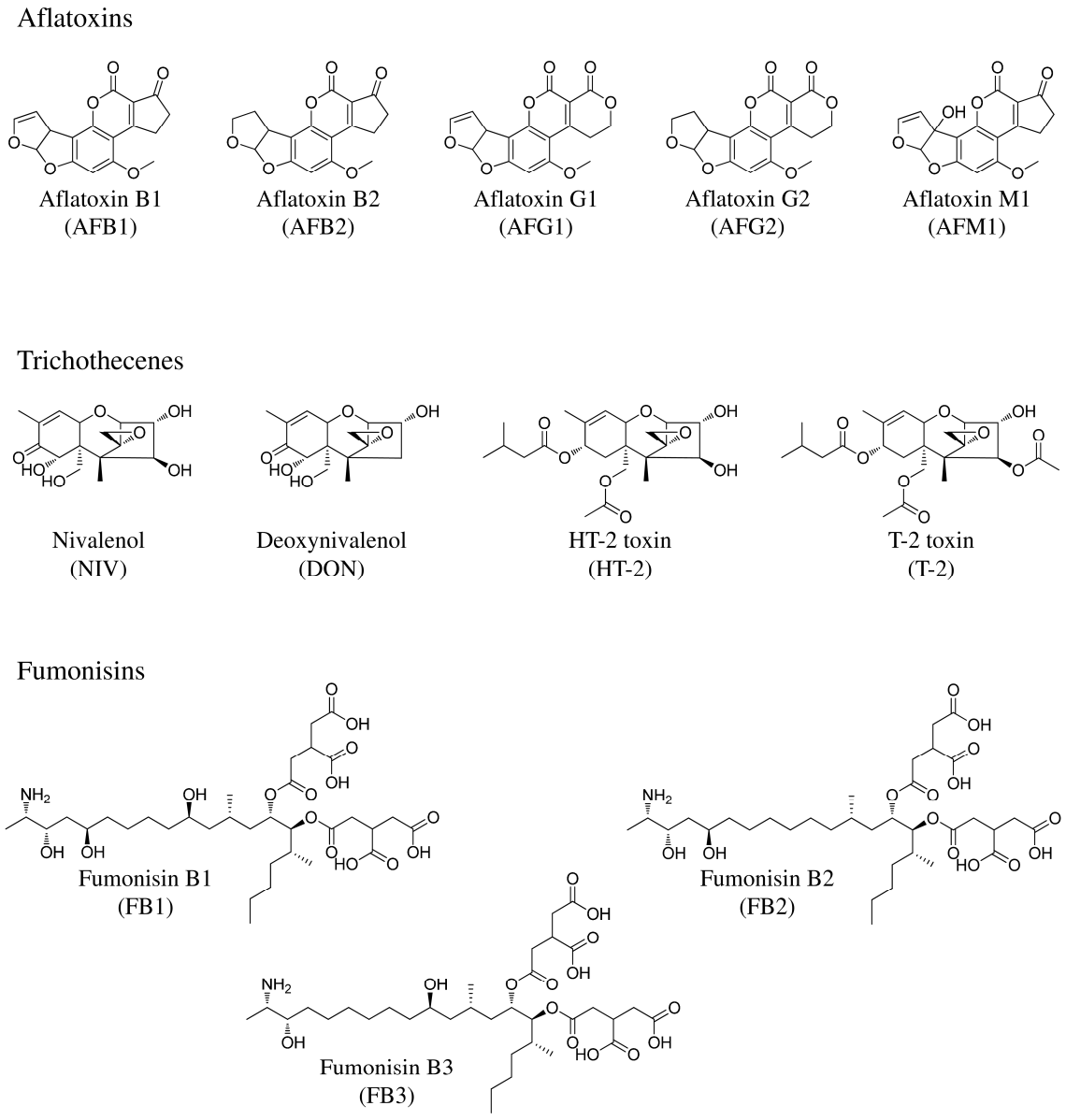


**Figure 1.1** Chemical structures of the mycotoxins under study.

## 1.2 Experimental section

### 1.2.1 Samples and reagents

Random samples of 24 beer-based drinks, including regular beer, low-malt-beer, new genre beer, and nonalcoholic beer, 14 red wines, and 13 white wines were acquired at local supermarkets in Japan between 2009 and 2010. All the samples were refrigerated until analysis.

Methanol (MeOH, for LC-MS), MeCN [for LC-MS and for pesticide residue and polychlorinated biphenyl (PCB)], ammonium acetate (guaranteed reagent grade), formic acid (guaranteed reagent grade), and acetic acid (guaranteed reagent grade) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). MeCN (for LC-MS) was used for preparation of working solutions and for LC-MS/MS analysis, and MeCN (for pesticide residue and PCB analysis) was used for sample preparation. Water was purified using a Milli-Q system from Millipore (Molsheim, France). A dSPE Citrate Extraction Tube, dSPE PSA/C18 SPE Clean Up Tube 1, and Supelclean ENVI-Carb cartridge (1 g/12 mL) were acquired from Supelco (Bellefonte, PA, USA). An InertSep C18 cartridge (1 g/6 mL) and InertSep PSA cartridge (1 g/6 mL) were purchased from GL Sciences

(Tokyo, Japan). An Oasis HLB cartridge (200 mg/6 mL) was purchased from Waters (Milford, MA, USA). A MultiSep 229 Ochra cartridge was purchased from Romer Labs Corp. (Bukit Merah, Singapore). Polytetrafluoroethylene (PTFE) filters (0.20-μm mesh pores) were acquired from Advantec Toyo Kaisha (Tokyo, Japan). Standard solutions of AFM1 (10 μg/mL), OTA (50 μg/mL), and Aflatoxin Mix containing AFB1, AFG1 (each 2 μg/mL), AFB2, and AFG2 (each 0.5 μg/mL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PAT, ZEN (each 100 μg/mL), FB1, FB2, and FB3 (each 50 μg/mL) standard solutions were purchased from Romer Labs Corp. NIV, DON, HT-2, and T-2 (each 100 μg/mL) standard solutions were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Working solutions were prepared as follows: a fumonisins solution containing FB1, FB2, and FB3 (each 5 μg/mL) was diluted with the mixture MeCN/water (50/50, v/v) and stored in a refrigerator; an aflatoxins solution containing AFB1, AFG1, AFM1 (each 1 μg/mL), AFB2, and AFG2 (each 0.25 μg/mL); an OTA solution (1 μg/mL); and a solution of other mycotoxins containing PAT, DON, NIV, and ZEN (each 50 μg/mL), HT-2, and T-2 (each 10 μg/mL) were diluted with MeCN and stored in a freezer.

### 1.2.2 LC-MS/MS analysis

LC-MS/MS analysis was performed on an ACQUITY UPLC system coupled with a Quattro Premier XE tandem quadrupole mass spectrometer (Waters). The MassLynx 4.1 software equipped with QuanLynx software (Waters) was used to control the instruments and to process the data. An ACQUITY UPLC system consisting of a binary pump, an autosampler, and a column heater was also used. Chromatographic separation was carried out on an ACQUITY UPLC BEH C18 (1.7 μm, 2.1 × 50 mm; Waters) for beer analysis and ACQUITY UPLC BEH C18 (1.7 μm, 2.1 × 100 mm; Waters) for wine analysis. Solvent A was water, and solvent B was 2% acetic acid with 0.1 mM ammonium acetate in MeOH. The two gradient profiles that were set up for beer analysis were as follows: 5% B (0 min), 80% B (4.5 min), and 5% B (4.51–6.0 min) for the mycotoxins except FB1, FB2, FB3, and OTA; and 55% B (0 min), 80% B (2 min), and 55% B (2.01–3.0 min) for FB1, FB2, FB3, and OTA. Similarly, the gradient profiles for wine analysis were as follows: 5% B (0–1.0 min), 80% B (8.0 min), and 5% B (8.01–10 min) for the mycotoxins except FB1, FB2, FB3, and OTA; and 55% B (0 min), 80% B (5.0 min), and 55% B (5.01–7.0 min) for FB1, FB2, FB3, and OTA. The flow rate was set at 0.5 mL/min for beer analysis and at 0.3 mL/min for wine analysis. The column

temperature was 40°C, and the autosampler was used to inject 5 μL of a sample to be analyzed.

The Quattro Premier XE tandem quadrupole mass spectrometer was operated both in positive and negative mode with an electrospray ionization (ESI) source. The operating parameters were optimized under the following conditions: capillary voltage, 3.0 kV (positive mode) or 2.8 kV (negative mode); ion source temperature, 120°C; desolvation temperature, 450°C; cone gas flow, 50 L/h; desolvation gas flow, 800 L/h (both gases were nitrogen); and collision gas flow, 0.3 mL/min (argon gas). The multiple reaction monitoring (MRM) transitions, the applied cone voltages, and the collision energies are summarized in Table 1.1.

### 1.2.3 Preparation of samples

#### 1.2.3.1 Beer

A 10-mL sample of beer was degassed by sonication for 15 min and added into a 50-mL polypropylene centrifuge tube. Then, 10 mL of MeCN was added, and the liquids were mixed thoroughly. The contents of a dSPE Citrate Extraction Tube were

**Table 1.1** MS/MS conditions for selected mycotoxins.

| Mycotoxin | Polarity | Cone voltage (V) | Precursor ion (*m/z*) | Quantification ion | | Certification ion | |
|---|---|---|---|---|---|---|---|
| | | | | Collision energy (eV) | Product ion (*m/z*) | Collision energy (eV) | Product ion (*m/z*) |
| AFB1 | ESI+ | 50 | 313 | 38 | 241 | 23 | 285 |
| AFB2 | ESI+ | 50 | 315 | 25 | 287 | 30 | 259 |
| AFG1 | ESI+ | 50 | 329 | 28 | 243 | 23 | 311 |
| AFG2 | ESI+ | 50 | 331 | 23 | 313 | 33 | 245 |
| AFM1 | ESI+ | 38 | 329 | 23 | 273 | 43 | 229 |
| PAT | ESI- | 18 | 153 | 7 | 135 | 10 | 109 |
| NIV | ESI- | 23 | 371 | 15 | 281 | 11 | 311 |
| DON | ESI+ | 23 | 297 | 12 | 249 | 13 | 231 |
| HT-2 | ESI+ | 15 | 442 | 13 | 263 | 13 | 215 |
| T-2 | ESI+ | 20 | 484 | 15 | 305 | 23 | 185 |
| ZEN | ESI- | 48 | 317 | 25 | 175 | 20 | 273 |
| FB1 | ESI+ | 50 | 722 | 40 | 334 | 35 | 352 |
| FB2 | ESI+ | 48 | 706 | 40 | 318 | 38 | 336 |
| FB3 | ESI+ | 48 | 706 | 40 | 318 | 38 | 336 |
| OTA | ESI+ | 25 | 404 | 25 | 239 | 15 | 358 |

added, mixed by vortexing for 20 s, and centrifuged at 1,580 × *g* for 5 min. Five milliliters of the MeCN phase was cleaned by passing it through an InertSep C18 cartridge conditioned beforehand with 5 mL of MeCN, followed by passing another 5 mL of MeCN through the cartridge, with collection in a test tube. The eluate was evaporated completely at 40°C under a nitrogen stream, and the residue was dissolved in 500 µL of 10 mM ammonium acetate/MeCN (85/15, v/v). Each sample was passed through a 0.20-µm PTFE filter immediately before the LC-MS/MS analysis.

**1.2.3.2 Wine**

A 5-mL sample of wine and 25 mL of 10 mM ammonium acetate were placed into a 50-mL polypropylene centrifuge tube and were mixed. The mixture was applied to an Oasis HLB cartridge conditioned beforehand with 5 mL of MeCN and 5 mL of 10 mM ammonium acetate. The cartridge was washed with 5 mL of 10 mM ammonium acetate. The mycotoxins that were retained in the cartridge were eluted with 5 mL of 10 mM ammonium acetate/MeCN (1/1, v/v) and then with 5 mL of MeCN. The eluates were mixed and evaporated completely at 40°C under a nitrogen stream. The dried sample was dissolved in 1 mL of water. After that, 60 µL of acetic acid and 5 mL of MeCN were added to the sample, and everything was mixed. The mixture was applied to a MultiSep 229

Ochra cartridge. Four milliliters of the purified eluate was evaporated completely at 40°C under a nitrogen stream, and the residue was dissolved in 400 μL of 10 mM ammonium acetate/MeCN (85/15, v/v). Each sample was passed through a 0.20-μm PTFE filter immediately before LC-MS/MS analysis.

### 1.2.4 Validation of methods

Because there were no official guidelines concerning the determination of multiple mycotoxins, I referred to the “Guideline for the in-house validation of analytical methods for agricultural chemicals in food” provided by the MHLW in 2007 [9] and “about the total aflatoxins analysis” provided by the MHLW in 2011 [10]. Additionally, prior to the evaluation, the samples were analyzed and confirmed to be free of any naturally present mycotoxins.

#### 1.2.4.1 Beer

Performance of the developed method was assessed using beer samples spiked with mycotoxins, and the coefficient of linearity was determined at the following

concentrations: 5, 10, 25, 50, and 100 μg/L for PAT, NIV, DON, ZEN, FB1, FB2, and FB3; 1, 2, 5, 10, and 20 μg/L for AFB1, AFG1, AFM1, HT-2, T-2, and OTA; and 0.25, 0.5, 1.25, 2.5, and 5 μg/L for AFB2 and AFG2. Recovery and repeatability as relative standard deviation (RSD) involved five replicate measurements that were carried out on the same day using beer samples spiked with each mycotoxin at the following concentrations: 50 μg/L for PAT, NIV, DON, ZEN, FB1, FB2, and FB3; 10 μg/L for AFB1, AFG1, AFM1, HT-2, T-2, and OTA; and 2.5 μg/L for AFB2 and AFG2.

#### 1.2.4.2 Wine

Performance of the developed method was evaluated on wine samples spiked with the mycotoxins under study. The coefficient of linearity was determined using samples spiked with each mycotoxin at the following concentrations: 5, 10, 20, 50, and 100 μg/L for PAT, NIV, DON, and ZEN; 0.2, 0.5, 1, 2, and 5 μg/L for AFB1, AFB2, AFG1, AFG2, AFM1, and OTA; and 1, 2, 4, 10, and 20 μg/L for HT-2, T-2, FB1, FB2, and FB3. Recovery and repeatability (as RSD) involved five replicate measurements that were carried out on the same day using samples spiked with each mycotoxin at the following concentrations: 20 μg/L for PAT, NIV, DON, and ZEN; 1 μg/L for AFB1, AFB2, AFG1, AFG2, AFM1, and OTA; 4 μg/L for HT-2 and T-2; and 5 μg/L for FB1, FB2, and FB3.

## 1.3 Results and Discussion

### 1.3.1 Optimization of LC-MS/MS conditions

First, MS/MS conditions for the 15 mycotoxins were optimized. The mycotoxins were detectable by ESI. DON, AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, FB1, FB2, FB3, and OTA were detected in positive mode, whereas PAT, NIV, and ZEN were detected in negative mode. All mycotoxins except NIV, HT-2, and T-2 were set as $[M+H]^+$ or $[M-H]^-$ precursor ions. The acetic acid adduct $[M+CH_3COO]^-$ of NIV and the ammonium adduct $[M+NH_4]^+$ of HT-2 and T-2 were set. Two product ions for a precursor ion in each mycotoxin were selected and set as quantification and certification ions, respectively. The selected parameters for each mycotoxin are shown in Table 1.1.

LC separation of each mycotoxin was performed to determine the optimal conditions, using a C18 column (ACQUITY UPLC BEH C18; 1.7 μm, 2.1 × 50 mm; Waters) as an analytical column and water/MeOH or water/MeCN as the mobile phase under the gradient conditions. Each mycotoxin was eluted as a single peak using water/MeOH, which yielded higher intensity of peaks than water/MeCN did, except for

OTA. Next, to improve the intensity of peaks, the additive agents in the mobile phase were examined under conditions of the gradient of water/MeOH as a mobile phase. Acetic acid (2%), ammonium acetate (10 mM), and formic acid (0.1%) were selected as the additives, and peak detection and intensity of peaks of mycotoxins with each additive agent were compared. When only acetic acid was used as the mobile phase, peaks of the mycotoxins in question were observed and their intensity was improved. When only ammonium acetate served as the mobile phase, the peaks of PAT, AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, and ZEN were sharper than those when only acetic acid was used as the mobile phase, whereas the peaks of FB1, FB2, and FB3 were not detected. Moreover, when only formic acid served as the mobile phase, the intensity of peaks of FB1, FB2, and FB3 was better than that when only acetic acid was used as the mobile phase although worse intensity was attained for AFB1, AFB2, AFG1, AFG2, and AFM1, whereas the peaks of PAT, NIV, and DON were not detected. According to the results, acetic acid and ammonium acetate were selected as additives in the mobile phase in order to detect all the mycotoxins analyzed and to obtain good intensity of peaks. According to the examination of LC conditions, additive concentrations and gradient profile were as follows: solvent A, water and solvent B, 2% acetic acid with 0.1 mM ammonium acetate in MeOH as the mobile phase, with a gradient of 5–80% of solvent B during a 5-min

period.

Carryover of FB1, FB2, FB3, and OTA was observed in the LC condition. Carryover is a phenomenon where a compound remains in an analytical instrument and is detected during the next run. To eliminate this phenomenon, the chromatographic conditions were optimized specifically for FB1, FB2, and FB3, whose carryover was noticeable. Solvents A and B that served as the mobile phases were identical to those used in the LC condition described above. The gradient starting points that I tested were 5%, 30%, 55%, and 80% of solvent B, increasing during 5 min to finish at 80% of solvent B. Injections of the standard solutions were followed by 10 injections of the blank solution. Figure 1.2 shows chromatograms of FB2 and FB3 standards, followed by three blank injections. Carryover was observed when starting with 5% or 30% of solvent B as shown in Figures 1.2 (A) and 1.2 (B). Carryover of FB2, in particular, was observed until the seventh blank injection when the gradient began at 5% of solvent B. No carryover was observed even for the first blank injection when the gradient began at 55% or 80% of solvent B as shown in Figures 1.2(C) and 1.2(D). FB1, FB2, and FB3 were not retained in the analytical column when 80% of solvent B was used [Figure 1.2(D)]. Judging by the results, two gradient conditions for beer sample analysis were selected: 5% B (0 min), 80% B (4.5 min), and 5% B (4.51–5.5 min) for all mycotoxins except FB1, FB2, FB3,

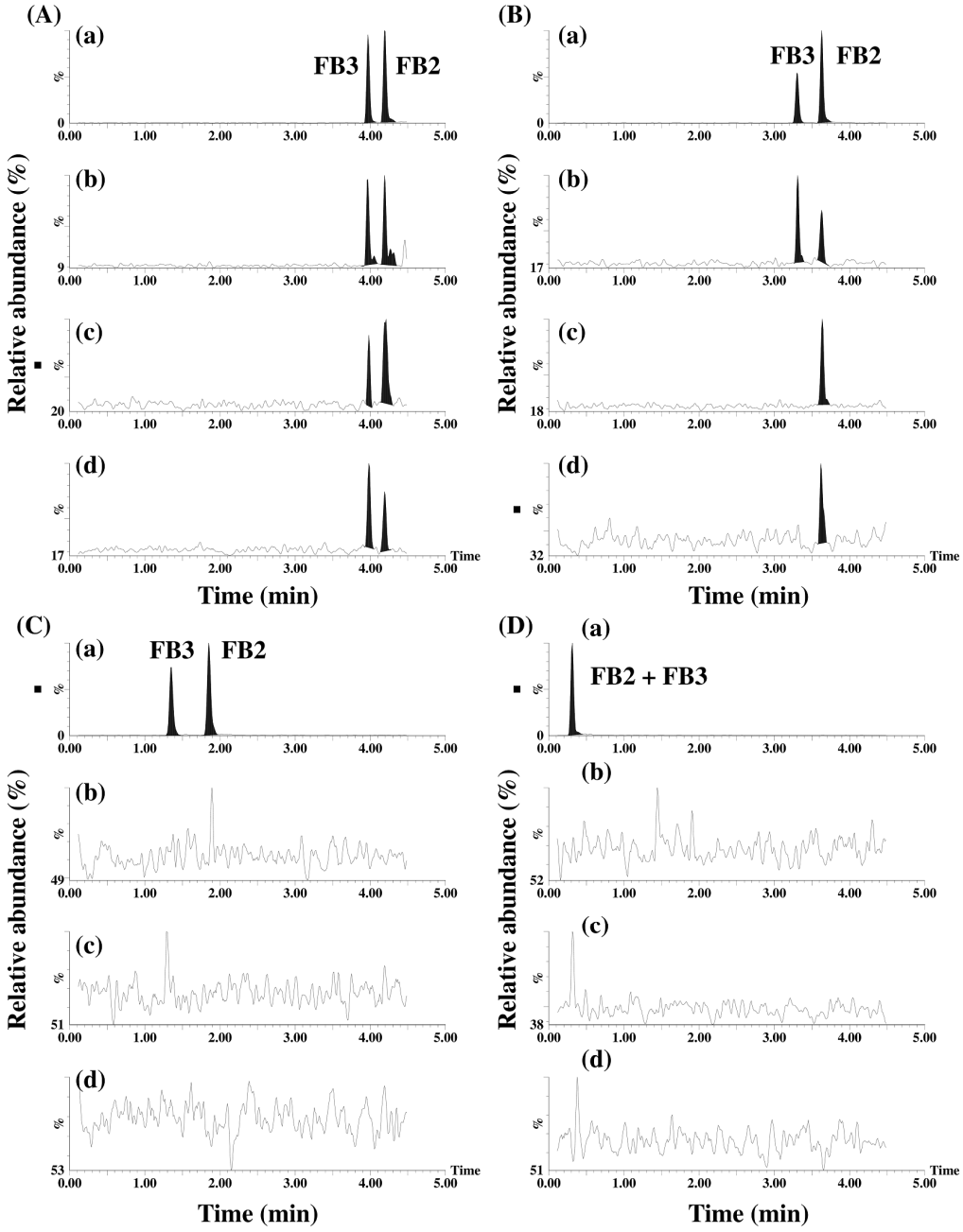


**Figure 1.2** Chromatograms showing carryover of FB2 and FB3. The mobile phase consisted of solvent A: water and solvent B: 2% acetic acid with 0.1 mM ammonium acetate in MeOH. Four linear gradients of changing proportions (v/v) of solvent B were applied at the flow rate of 0.5 mL/min, with these time-versus-concentration gradients expressed as [*t* (min), % B]: (A) (0, 5), (4.5, 80), (B) (0, 30), (4.5, 80), (C) (0, 55), (4.5, 80), and (D) (0, 80), (4.5, 80). Each chromatogram shows (a) the standards for FB2 and FB3 (each 5 µg/mL), (b) the first blank injection, (c) the second blank injection, and (d) the third blank injection for all 15 mycotoxins.

and OTA; and 55% B (0 min), 80% B (2.0 min), and 55% B (2.01–3.0 min) for FB1, FB2, FB3, and OTA. The total analysis duration was 8.5 min.

The LC conditions for wine sample analysis were different from those for the beer samples because it was necessary to eliminate the influence of matrices during LC separation as much as possible: the matrices in wine were assumed to be more varied and numerous than those in beer. The length of the analytical column was changed from 50 to 100 mm, and the flow rate was changed from 0.5 to 0.3 mL/min, taking into account pressure in the instrument. The two gradient profiles were as follows: 5% B (0–1.0 min), 80% B (8.0 min), and 5% B (8.01–10.0 min) for all the mycotoxins except FB1, FB2, FB3, and OTA; and 55% B (0 min), 80% B (5.0 min), and 55% B (5.01–7.0 min) for FB1, FB2, FB3, and OTA. The total analysis duration was 17 min for all 15 mycotoxins.

### 1.3.2 Optimization of sample preparation

#### 1.3.2.1 Beer

Recovery was confirmed using preparation by the QuEChERS method. The beer sample that was spiked with mycotoxins in question (at the following concentrations)

was extracted with MeCN using a dSPE Citrate Extraction Tube containing NaCl, $MgSO_4$, and citrate buffer: 50 μg/L for PAT, NIV, DON, ZEN, FB1, FB2, and FB3; 10 μg/L for AFB1, AFG1, AFM1, HT-2, T-2, and OTA; and 2.5 μg/L for AFB2 and AFG2. During the extraction, pigments in beer samples were found to be shifted to the water phase. Next, the MeCN phase was purified by means of a kit for purification involving $MgSO_4$ and supports of PSA and C18 (dSPE PSA/C18 SPE Clean Up Tube 1). Each mycotoxin was analyzed by optimized LC-MS/MS, and the recovery values were calculated from the intensity of peaks of each mycotoxin. The results are shown in Table 1.2 (A). More than 70% recovery was attained for most of the mycotoxins under study except FB1, FB2, FB3, and OTA, which could not be recovered. It was assumed that they were adsorbed to the PSA or C18 support. Thus, the recovery was confirmed using SPE cartridges: C18 (InertSep C18), PSA (InertSep PSA), and GCB (Supelclean ENVI-Carb). After extraction with the dSPE Citrate Extraction Tube, the extracts were subjected to purification by passing them through each SPE cartridge. The results are shown in Table 1.2 (B). Good recovery values (>70%) were obtained for the 15 mycotoxins with the C18 cartridge, but poor recovery was observed for FB1, FB2, FB3, and OTA with the PSA cartridge. It was assumed that FB1, FB2, FB3, and OTA were adsorbed by PSA because of the ionic affinity between the amines in the PSA support and the carboxyl groups in

**Table 1.2** Recovery for sample preparation by the QuEChERS method and SPE cartridges.

| Mycotoxin | (A) QuEChERS method (%) | (B) SPE cartridge (%) | | |
|---|---|---|---|---|
| | | C18 | PSA | GCB |
| AFB1 | 85 | 119 | 96 | 0 |
| AFB2 | 87 | 97 | 95 | 0 |
| AFG1 | 86 | 108 | 98 | 0 |
| AFG2 | 83 | 99 | 89 | 0 |
| AFM1 | 84 | 106 | 88 | 0 |
| PAT | 91 | 110 | 83 | 73 |
| NIV | 70 | 79 | 77 | 68 |
| DON | 79 | 88 | 85 | 79 |
| HT-2 | 87 | 102 | 94 | 85 |
| T-2 | 87 | 97 | 95 | 81 |
| ZEN | 84 | 103 | 91 | 0 |
| FB1 | 0 | 97 | 0 | 5 |
| FB2 | 0 | 92 | 0 | 0 |
| FB3 | 1 | 93 | 0 | 0 |
| OTA | 36 | 92 | 0 | 0 |

FB1, FB2, FB3, and OTA. Additionally, poor recovery was attained for AFB1, AFB2, AFG1, AFG2, AFM1, ZEN, FB1, FB2, FB3, and OTA with the GCB cartridge, due to π-π interactions between the $sp^2$ hybrid orbitals in the GCB six-membered rings and the planar aromatic rings in these mycotoxins. According to the results, PSA and GCB were not suitable for preparation of the mycotoxins, and this procedure was performed with purification by passing through a C18 SPE cartridge, an InertSep C18, after extraction of mycotoxins from beer samples using the dSPE Citrate Extraction Tube as a kit for QuEChERS extraction. Consequently, the proposed preparation procedure made possible the recovery of the 15 mycotoxins and removal of the matrices (such as pigments in beer). Figure 1.3 shows LC-MS/MS chromatograms of a prepared beer sample spiked with mycotoxins.

#### 1.3.2.2 Wine

The process of sample preparation for beer, which was extracted using a QuEChERS extraction kit followed by purification with a C18 cartridge, was examined to be applied to a red wine sample, whose pigments were removed insufficiently. The pigments seemed to worsen quantitative accuracy and pollute LC-MS/MS. Accordingly, MultiSep 229 Ochra cartridge, which is an MFC for OTA, was tested for adequate

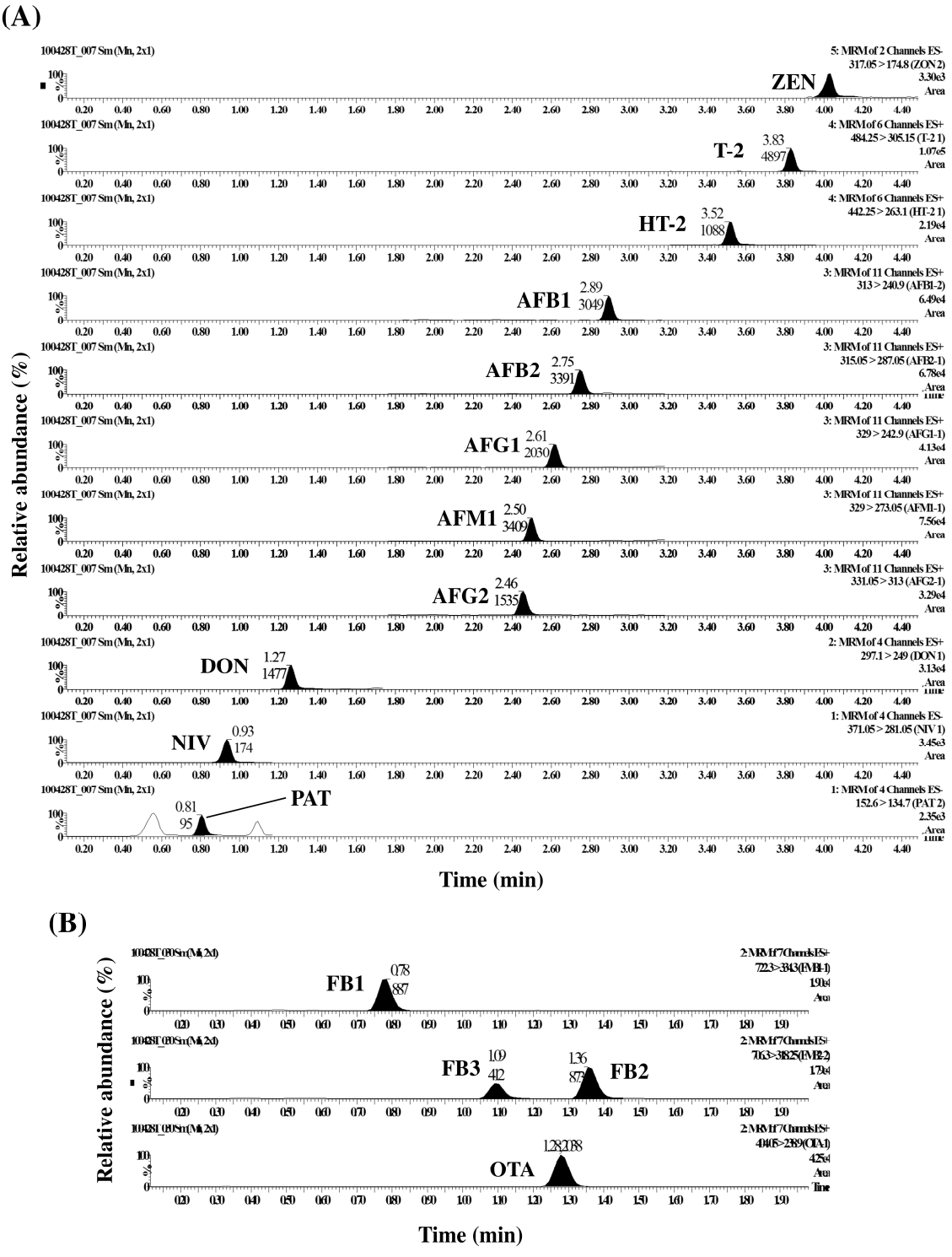


**Figure 1.3** Chromatograms of a beer sample spiked with the mycotoxins under study. (A) Chromatograms of 11 mycotoxins except FB1, FB2, FB3, OTA; (B) chromatograms of FB1, FB2, FB3, and OTA.

removal of the pigments in place of the C18 cartridge. This MFC cartridge, which is packed with supports of reverse phase, normal phase, and ion exchange conforming to the OTA property, enabling adsorption of the matrices and extraction of OTA from a sample after simple passage through the cartridge without conditioning steps. In the evaluation of beer sample preparation in subsection 1.3.2.1, it was obvious that some of the mycotoxins under study (including OTA) that have ionic functional groups or aromatic rings in their chemical structures were adsorbed to supports of PSA and GCB. Because MultiSep 229 Ochra cartridge is designed to not adsorb OTA, which has ionic functional groups and an aromatic rings, purification for other mycotoxins in question without adsorption in the MFC can be expected.

When a sample of red wine spiked with the mycotoxins under study was prepared by extraction wiht the QuEChERS extraction kit followed by purification with passage through MultiSep 229 Ochra cartridge, the pigments were removed from red wine. Nonetheless, in the chromatograms [Figure 1.4 (B)], the matrix peaks were observed near PAT, and the PAT peaks were not as sharp as those in the standard chromatograms [Figure 1.4 (A)]. Additionally, no peaks were identified for NIVs. In either case, PAT and NIV were affected by the presence of matrix compounds other than pigments. Considering the retention time, these matrices might have high polarity like that of organic and amino

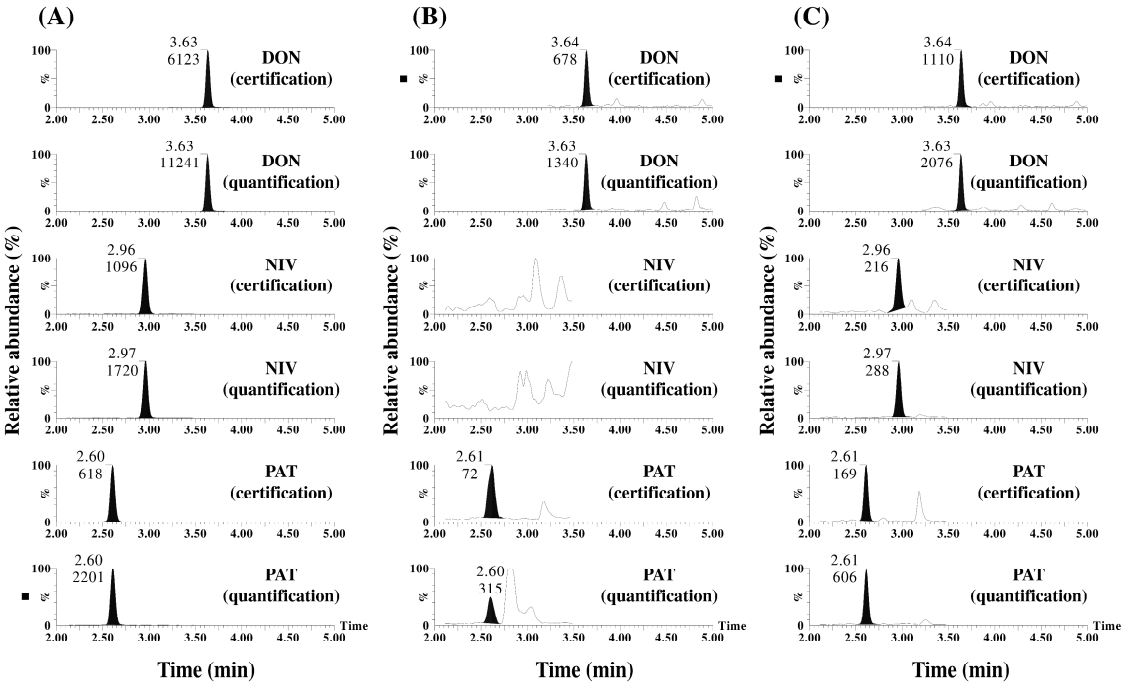


**Figure 1.4** Chromatograms of PAT, NIV, and DON after different pretreatment procedures. Each chromatogram was obtained for (A) the standards of PAT, NIV, and DON (each 20 μg/L); (B) red wine samples spiked with mycotoxins (each 20 μg/L) that were purified with a MultiSep 229 Ochra cartridge after QuEChERS extraction; (C) red wine samples spiked with mycotoxins (each 20 μg/L) that were purified with MultiSep 229 Ochra cartridge after being extracted and purified by Oasis HLB cartridge.

acids, which are abundant in wine. It was assumed that they were partitioned into the MeCN phase at the QuEChERS extraction step, and that they passed through MultiSep 229 Ochra cartridge without being adsorbed. It seemed difficult to remove highly polar matrices by this preparation procedure. Therefore, to remove such matrices, the sample was extracted and purified using Oasis HLB cartridge instead of the QuEChERS extraction.

Oasis HLB cartridge, which contains the divinylbenzene-*N*-vinylpyrrolidine co-polymer, is for SPE. It holds weakly to moderately polar substances and separates highly polar substances. Eventually, nearly all the pigments were removed from the wine samples that were purified by means of MultiSep 229 Ochra cartridge after being extracted and purified by means of Oasis HLB cartridge. No peaks of highly polar matrices were observed, and the peak shapes for PAT and NIV improved [Figure 1.4(C)]. Thus, with this preparation procedure, pigments and highly polar matrices were removed from the wine samples, and good chromatograms were obtained.

### 1.3.3 Validation of methods

Matrix effects are common problems during mass spectrometry and have adverse effects on the analytical results. In this phenomenon, a response of the target substance in a sample is either reduced or enhanced, compared to that in a solvent. While observing the matrix effects for a beer sample, I found FB1, FB2, and FB3 to be affected by ion enhancement, and the other mycotoxins were affected by ion suppression. This finding showed that the data from the 15 mycotoxins analyzed by these methods were influenced by matrices; therefore, to adjust the procedure for the influence of matrix effects and to quantify accurately, I used the standard addition method.

The standard addition method, which is a quantitative method, should be applied when the influence of matrices in samples is not negligible. The samples for analysis and the samples for calibration curves that were spiked with verified compounds at different concentrations were prepared and analyzed by the same method. It is possible to adjust the data for the influence of matrix effects because the matrices in samples for analysis and in samples for calibration curves were identical. Therefore, the standard addition method was used to conduct further quantitative analyses in this dissertation project.

The results obtained by this validation test for beer samples are presented in

Table 1.3. Linearity of the calibration curves for the beer samples (spiked with each mycotoxin) was >0.992. Recovery ranged from 70% to 111%, with repeatability ranging from 4.6% to 14.6 %. The limits of quantification (LOQs) were defined as the lowest concentration values of the mycotoxins in the calibration curves: 5 μg/L for PAT, NIV, DON, ZEN, FB1, FB2, and FB3; 1 μg/L for AFB1, AFG1, AFM1, HT-2, T-2, and OTA; and 0.25 μg/L for AFB2 and AFG2, as shown on the calibration curves. Thus, I successfully developed a rapid method for accurate determination of the 15 mycotoxins in beer samples, involving simple and easy preparation by a modified QuEChERS method.

The results of the evaluation of wine samples spiked with each mycotoxin are summarized in Table 1.4. Linearity of the calibration curves was >0.990. Recovery ranged from 76% to 105%, with repeatability ranging from 3.4% to 11.8 %, except for NIV. Recovery of NIV was 43%, which affected the quantification performance. It is assumed that the highly polar NIV was hardly retained by Oasis HLB cartridge and that some percentage of NIV was eluted with the matrices. LOQs for the mycotoxins were defined as the lowest concentration values visible on the calibration curves: 5 μg/L for PAT, DON, and ZEN; 0.2 μg/L for AFB1, AFB2, AFG1, AFG2, AFM1, and OTA; and 1 μg/L for HT-2, T-2, FB1, FB2, and FB3. Overall, I successfully developed a rapid method for accurate determination of 14 mycotoxins (with the exception of NIV) in wine samples.

**Table 1.3** Performance of the method used for determination of mycotoxins in beer.

| Mycotoxin | Linearity (*r*) [a)] | Recovery (%) [b)] | Repeatability (%) [b)] | LOQ (μg/L) | Retention time (min) |
|---|---|---|---|---|---|
| AFB1 | 0.995 | 93 | 6.9 | 1 | 2.89 |
| AFB2 | 0.992 | 96 | 9.9 | 0.25 | 2.75 |
| AFG1 | 0.997 | 88 | 7.3 | 1 | 2.61 |
| AFG2 | 0.992 | 97 | 9.7 | 0.25 | 2.46 |
| AFM1 | 0.993 | 102 | 5.6 | 1 | 2.50 |
| PAT | 0.994 | 86 | 10.7 | 5 | 0.81 |
| NIV | 0.993 | 70 | 4.6 | 5 | 0.93 |
| DON | >0.999 | 94 | 5.5 | 5 | 1.27 |
| HT-2 | 0.997 | 102 | 9.6 | 1 | 3.52 |
| T-2 | 0.996 | 104 | 5.3 | 1 | 3.83 |
| ZEN | 0.993 | 92 | 4.8 | 1 | 4.03 |
| FB1 | 0.996 | 105 | 14.6 | 5 | 0.78 |
| FB2 | 0.995 | 111 | 13.0 | 5 | 1.36 |
| FB3 | 0.997 | 108 | 12.3 | 5 | 1.09 |
| OTA | 0.997 | 110 | 8.1 | 1 | 1.28 |

[a)] The coefficient of linearity was determined using beer samples spiked with each mycotoxin at the following concentrations: 5, 10, 25, 50, and 100 μg/L for PAT, NIV, DON, ZEN, FB1, FB2, and FB3; 1, 2, 5, 10, and 20 μg/L for AFB1, AFG1, AFM1, HT-2, T-2, and OTA; and 0.25, 0.5, 1.25, 2.5, and 5 μg/L for AFB2 and AFG2.

[b)] Recovery and repeatability involved five replicate measurements that were carried out on the same day using beer samples spiked with each mycotoxin at the following concentrations: 50 μg/L for PAT, NIV, DON, ZEN, FB1, FB2, and FB3; 10 μg/L for AFB1, AFG1, AFM1, HT-2, T-2, and OTA; and 2.5 μg/L for AFB2 and AFG2.

**Table 1.4** Performance of the method used for determination of mycotoxins in wine.

| Mycotoxin | Linearity (*r*) [a)] | Recovery (%) [b)] | Repeatability (%) [b)] | LOQ (μg/L) | Retention time (min) |
|---|---|---|---|---|---|
| AFB1 | 0.995 | 96 | 4.4 | 0.2 | 6.00 |
| AFB2 | 0.994 | 90 | 9.4 | 0.2 | 5.78 |
| AFG1 | 0.996 | 91 | 11.8 | 0.2 | 5.58 |
| AFG2 | 0.994 | 82 | 7.4 | 0.2 | 5.36 |
| AFM1 | 0.994 | 94 | 5.7 | 0.2 | 5.40 |
| PAT | 0.996 | 76 | 3.9 | 5 | 2.61 |
| NIV | 0.994 | 43 | 8.1 | 5 | 2.97 |
| DON | 0.999 | 96 | 7.6 | 5 | 3.63 |
| HT-2 | 0.999 | 99 | 5.2 | 1 | 6.88 |
| T-2 | 0.999 | 93 | 3.4 | 1 | 7.27 |
| ZEN | >0.999 | 78 | 4.2 | 5 | 7.58 |
| FB1 | 0.999 | 76 | 4.1 | 1 | 2.42 |
| FB2 | >0.999 | 82 | 6.0 | 1 | 3.96 |
| FB3 | >0.999 | 94 | 5.1 | 1 | 3.25 |
| OTA | 0.990 | 105 | 8.6 | 0.2 | 3.43 |

[a)] The coefficient of linearity was determined using red wine samples spiked with each mycotoxin at the following concentrations: 5, 10, 20, 50, and 100 μg/L for PAT, NIV, DON, and ZEN; 0.2, 0.5, 1, 2, and 5 μg/L for AFB1, AFB2, AFG1, AFG2, AFM1, and OTA; and 1, 2, 4, 10, and 20 μg/L for HT-2, T-2, FB1, FB2, and FB3.

[b)] Recovery and repeatability involved five replicate measurements that were carried out on the same day using red wine samples spiked with each mycotoxin at the following concentrations: 20 μg/L for PAT, NIV, DON, and ZEN; 1 μg/L for AFB1, AFB2, AFG1, AFG2, AFM1, and OTA; 4 μg/L for HT-2 and T-2; and 5 μg/L for FB1, FB2, and FB3.

### 1.3.4 Analysis of commercially available samples

The newly developed method was applied to 24 commercially available beer-based drinks. The results of the analysis are summarized in Table 1.5. PAT, AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, ZEN, and OTA were not detected in any of the beer-based drink samples. A half of the samples (an incidence of 12/24) were found to be contaminated with DON at concentrations less than the LOQ (5 μg/L), while a few (an incidence of 2/24 to 5/24) were found to be contaminated with NIV, FB1, FB2, and FB3 at concentrations less than their respective LOQs (each 5 μg/L). The amounts of a mycotoxin detected in all samples were less than 5 μg/L, which corresponds to less than 1.75 μg per 350 mL (volume of a beer bottle). The provisional maximum tolerable daily intake (PMTDI) levels for mycotoxins established by the Joint FAO/WHO Export Committee on Food Additives (JECFA) is 1 μg/kg-bw/day for DON, and 2 μg/kg-bw/day for FB1, FB2, and FB3, alone or in combination [11]. Similarly, the TDI levels for DON and NIV defined by the FSCJ are 1 and 0.4 μg/kg-bw/day, respectively [12]. The intake of DON, FB1, FB2, FB3, and NIV from these samples would be no more than 7% of the PMTDI or TDI, even if an individual weighing 60 kg drank one of these beer-based drinks every day. Therefore, these results suggest that the health risk to consumers that is posed

**Table 1.5** Mycotoxins detected in the analyzed beer samples.

| Type of beer-based drink | Concentration of mycotoxin (μg/L) | | | | |
|---|---|---|---|---|---|
| | **NIV** | **DON** | **FB1** | **FB2** | **FB3** |
| Beer (7 samples) | [a] | <5 (6/7) | <5 (1/7) | <5 (2/7) | <5 (1/7) |
| Low-malt-beer (8 samples) | <5 (2/8) | <5 (4/8) | <5 (3/8) | <5 (1/8) | <5 (1/8) |
| New genre (7 samples) | <5 (2/7) | <5 (1/7) | <5 (1/7) | | |
| Nonalcoholic (2 samples) | <5 (1/2) | <5 (1/2) | | | |
| Total (Incidence) [b] | <5 (5/24) | <5 (12/24) | <5 (5/24) | <5 (3/24) | <5 (2/24) |

[a] No mycotoxins were detected. [b] This corresponds to the number of samples in which each mycotoxin was detected.

by ingestion of beer-based drinks is relatively low.

Twenty-seven domestic and imported wines available in Japan were analyzed using this newly developed method, and the results are summarized in Table 1.6. No mycotoxins except FB1, FB2, FB3 and OTA were detected in any of the wine samples. FB1, FB2, FB3, and/or OTA were detected in six samples of the red wines. The concentrations of FB1, FB2, and FB3 detected in samples were less than LOQ (1 μg/L). The maximal OTA concentration detected was 0.20 μg/L, which is less than its regulatory level for wine set in the EU (2 μg/L). This result indicates that the health risk posed to consumers by red wine is relatively low. Nonetheless, it will be necessary to keep monitoring wines in the future regarding other mycotoxins because I observed co-occurrence of different fumonisins in one sample and co-occurrence of fumonisins and OTA in three samples. Moreover, FB1 and OTA were detected in two and one samples of white wines, respectively; however, co-occurrence of fumonisins and/or OTA was not observed in any of the white-wine samples. This result clearly indicates that white-wine samples are less prone to mycotoxin contamination in comparison with red wines.

The newly developed methods revealed that beer and wine are at risk of co-contamination with mycotoxins, in particular with NIV, FB1, FB2, FB3, and OTA, whose regulatory levels have still not been set in Japan. Therefore, it is necessary to control the

**Table 1.6** Mycotoxins detected in the analyzed wine samples.

| Sample | Concentration of mycotoxin (µg/L) | | | | Sample | Concentration of mycotoxin (µg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **FB1** | **FB2** | **FB3** | **OTA** | | **FB1** | **FB2** | **FB3** | **OTA** |
| Red-1 | <1.0 | <1.0 | <1.0 | <0.20 | White-1 | | | | 0.42 |
| Red-2 | <1.0 | [a] | | <0.20 | White-2 | <1.0 | | | |
| Red-3 | <1.0 | | | | White-3 | <1.0 | | | |
| Red-4 | | | | 0.20 | White-4 | | | | |
| Red-5 | <1.0 | | | <0.20 | White-5 | | | | |
| Red-6 | | <1.0 | | | White-6 | | | | |
| Red-7 | | | | | White-7 | | | | |
| Red-8 | | | | | White-8 | | | | |
| Red-9 | | | | | White-9 | | | | |
| Red-10 | | | | | White-10 | | | | |
| Red-11 | | | | | White-11 | | | | |
| Red-12 | | | | | White-12 | | | | |
| Red-13 | | | | | White-13 | | | | |
| Red-14 | | | | | | | | | |

[a] No mycotoxins were detected.

risk of contamination with mycotoxins and to estimate the total intake of mycotoxins in food available in Japan, while monitoring the domestic and foreign regulatory trends.

## 1.4 Summary

In this chapter, I developed LC-MS/MS methods for determination of multi-mycotoxin (those that have gained international attention) in beers and wines. The highlights are as follows:

- Remarkable carryover of FB1, FB2, FB3, and OTA was observed during the LC experiments. Two types of LC conditions were used to prevent the carryover and made analysis of multiple mycotoxins possible.
- The QuEChERS methodology, which was originally developed for analysis of multiple pesticide residues, was applied here to preparation of multiple mycotoxins in beer samples. The sample preparation procedure, which was used for extraction in MeCN by means of the QuEChERS extraction kit and for purification in a C18 cartridge, made it possible to remove matrices such as pigments from beer and to ensure good results of validation testing for beer samples. Thus, I successfully designed a rapid method for accurate determination of the 15 mycotoxins in beer samples.
- The method for preparation of beer samples was applied to red wine, but pigments were removed from red wine insufficiently. Thus, for preparation of wine samples,

extraction and purification using Oasis HLB cartridge were performed, followed by purification using MultiSep 229 Ochra cartridge which is an MFC for OTA. The preparation procedure allowed me to remove highly polar matrices and pigments and to obtain sharp peaks in chromatograms. According to the results of method validation, I successfully developed a rapid method for accurate determination of 14 mycotoxins (with the exception of NIV) in wine samples.

- Commercially available beers and wines were analyzed using these methods. NIV, DON, FB1, FB2, and FB3 were detected in beer samples, whereas FB1, FB2, FB3, and OTA were detected in wine samples. The newly developed methods revealed that the detected mycotoxins were present in trace amounts, posing a low risk to human health; however, beer and wine are at risk of co-contamination with various mycotoxins.

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# Chapter 2

# Simultaneous determination of mycotoxins in corn grits by LC-MS/MS with minimization of carryover

## 2.1 Introduction

In the previous chapter, two types of LC conditions were used for analyses of studied mycotoxins in beers and wines. Under these LC conditions, I was unable to determine multiple mycotoxins in a single run on account of the carryovers of FB1, FB2, FB3, and OTA, whereas the studied mycotoxins in a sample (beers and wines) were prepared for analysis simultaneously. In the present chapter, therefore, LC condition for minimization of carryover was investigated in order to determine multiple mycotoxins in a single run. Additionally, I examined the method for analysis of corn samples, which are frequently contaminated with mycotoxins at high concentrations.

## 2.2 Experimental section

### 2.2.1 Samples and reagents

Twelve corn grit samples (CG-1–12) were purchased at local supermarkets in Japan in 2013. All the samples were stored at room temperature until the sample preparation procedure.

MeOH (LC-MS grade), MeCN (LC-MS grade), formic acid (guaranteed reagent grade), ammonium acetate (analytical grade), and isopropanol (IPA, analytical grade) were purchased from Kanto Chemical Inc. Water was purified using a Millipore Milli-Q system. Trisodium citrate (guaranteed reagent grade) and acetic acid (LC-MS grade) were purchased from Wako Pure Chemicals Ind., Ltd. A stainless steel (SUS) powder (60–80 nm, 99.9%), iron (Fe) powder (60–80 nm, 99.9%), nickel (Ni) powder (60–80 nm, 99.8%), and platinum (Pt) powder (100 nm, 99.9%) were purchased from Ionic Liquids Technologies (Denzlingen, Germany). A Q-sep Q110 QuEChERS extraction kit containing NaCl, $MgSO_4$, and citrate buffer was acquired from RESTEK (Bellefonte, PA, USA). A MultiSep 229 Ochra cartridge was acquired from Romer Labs Corp. PTFE filters

(0.20-μm mesh pores) were purchased from Advantec Toyo Kaisha.

The following standard solutions were used for each mycotoxin: Japanese aflatoxin mixture (AFB1, AFB2, AFG1, and AFG2; each at 25 μg/mL in MeCN) from Supelco; PAT (100 μg/mL in MeCN), ZEN (100 μg/mL in MeCN), FB1 [50 μg/mL in water/MeCN (1:1, v/v)], FB2 [50 μg/mL in water/MeCN (1:1, v/v)], and FB3 [50 μg/mL in water/MeCN (1:1, v/v)] from Romer Labs Corp.; and NIV (100 μg/mL in MeCN), DON (100 μg/mL in MeCN), HT-2 (100 μg/mL in MeCN), and T-2 (100 μg/mL in MeCN) from Wako Pure Chemical Ind., Ltd.

### 2.2.2 LC-MS/MS analysis

LC-MS/MS analysis was conducted on a Nexera ultra high performance liquid chromatography (UHPLC) system coupled to an LCMS-8040 tandem quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). LabSolutions LCMS software (Shimadzu) was used to control the instruments and to process the data. The Nexera UHPLC system that I used in the analysis consisted of a system controller (CBM-20A), two pumps (LC-30AD), an autosampler (SIL-30AC), a column heater (CTO-20AC), and a degasser

(DGU-20As).

Optimized LC conditions were as follows: Solvent A was 10 mM ammonium acetate in water, and solvent B was 2% acetic acid in MeOH. The gradient profile was as follows: 2% B (0–2.0 min), 55% B (3.0–4.0 min), 70% B (4.1 min), 80% B (7.0 min), 95% B (7.01–8.0 min), and 2% B (8.01–11.0 min). The flow rate was set to 0.4 mL/min, and the column temperature was 40°C. The chromatographic separation was carried out on a stainless-free Mastro C18 (2.1 × 100 mm, 3 μm) from Shimadzu GLC (Tokyo, Japan). The injection volume was 5 μL. The autosampler (SIL 30AC) that I used in this experiment rinsed both the inner and outer surfaces of the injection needle with solvents differing from the mobile phases, and four lines of rinse solvents (R0, R1, R2, and R3) were used. The inner surface of the injection needle was rinsed with three solvents (R0, R1, and R2), whereas the outer surface was rinsed with two solvents (R3 and one of R0, R1, or R2). The following solvents were selected to rinse the injection needle: R0, 10 mM ammonium acetate; R1, 10 mM trisodium citrate; R2 and R3, 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v). The profile was designed to rinse the inner surface with R1, R0, R2, and R0, and the outer surface with R3 and R0, in that order.

An LCMS-8040 tandem quadrupole mass spectrometer was operated in both positive and negative mode with an ESI source. Optimized operating parameters were as

follows: Nebulizer gas flow, 3 L/min; drying gas flow, 15 L/min; desolvation line temperature, 300°C; heat block temperature, 500°C. The other parameters were tuned automatically. The MRM transitions are summarized in Table 2.1.

### 2.2.3 Adsorption of fumonisins onto metals

Each metal powder (4 mg; SUS, Fe, Ni, and Pt) was placed into a 1.5-mL centrifuge tube, and 1 mL of a 5,000-μg/L fumonisin standard solution in 10 mM ammonium acetate/MeCN (85:15, v/v; solvent A) was added. Each mixture was vortexed for 1 min and then centrifuged at 10,000 rpm for 1 min. The supernatant of each mixture was filtered and analyzed by LC-MS/MS. This procedure is illustrated in Figure 2.1.

### 2.2.4 Solvents used to desorb fumonisins from metals

Each metal powder (4 mg; SUS, Fe, Ni, and Pt) was placed into a 1.5-mL centrifuge tube, and 1 mL of a 5,000-μg/L fumonisin standard solution in 10 mM

**Table 2.1** MRM transitions of 14 mycotoxins.

| Mycotoxin | Polarity | Precursor ion (*m/z*) | Product ion (*m/z*) | Collision energy (eV) [a)] |
|---|---|---|---|---|
| AFB1 | Positive | 313.1 | 285.1 | −25 |
| | | $[M+H]^+$ | 241.1 | −38 |
| AFB2 | Positive | 315.2 | 287.1 | −26 |
| | | $[M+H]^+$ | 259.0 | −30 |
| AFG1 | Positive | 329.0 | 243.1 | −29 |
| | | $[M+H]^+$ | 311.1 | −23 |
| AFG2 | Positive | 331.0 | 313.1 | −26 |
| | | $[M+H]^+$ | 245.0 | −30 |
| OTA | Positive | 404.2 | 239.1 | −25 |
| | | $[M+H]^+$ | 358.2 | −17 |
| PAT | Negative | 153.1 | 109.2 | 10 |
| | | $[M–H]^-$ | 81.2 | 11 |
| NIV | Negative | 371.2 | 281.2 | 14 |
| | | $[M+CH_3COO]^-$ | 311.1 | 11 |
| DON | Negative | 355.2 | 295.1 | 10 |
| | | $[M+CH_3COO]^-$ | 59.2 | 20 |
| HT-2 | Positive | 442.2 | 263.2 | −15 |
| | | $[M+NH_4]^+$ | 105.1 | −30 |
| T-2 | Positive | 484.2 | 305.2 | −16 |
| | | $[M+NH_4]^+$ | 215.2 | −19 |
| FB1 | Positive | 722.4 | 334.4 | −43 |
| | | $[M+H]^+$ | 352.3 | −35 |
| FB3 | Positive | 706.4 | 336.4 | −39 |
| | | $[M+H]^+$ | 318.3 | −45 |
| FB2 | Positive | 706.4 | 336.4 | −39 |
| | | $[M+H]^+$ | 318.3 | −45 |
| ZEN | Negative | 317.2 | 131.1 | 29 |
| | | $[M–H]^-$ | 175.1 | 25 |

[a)] The unit input in LCMS operating software.

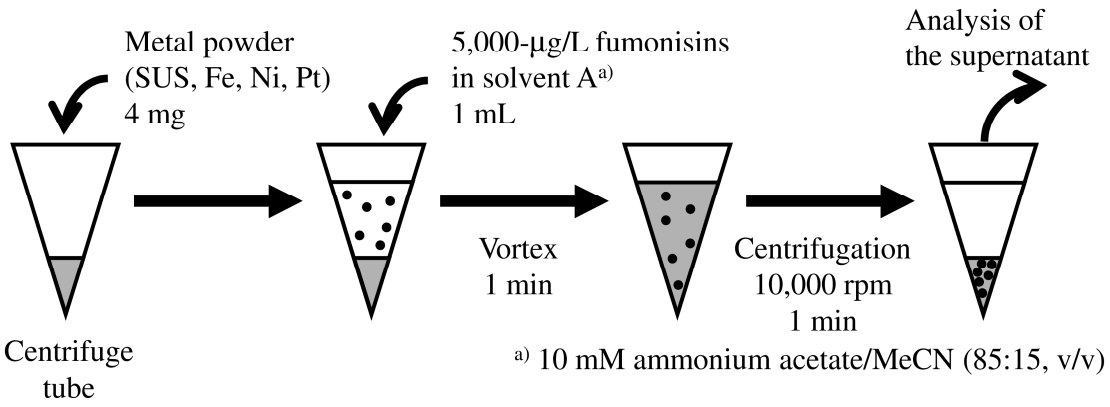


**Figure 2.1** The procedure for verification of fumonisin adsorption onto metals (in powder form).

ammonium acetate/MeCN (85:15, v/v; solvent A) was added. Each mixture was vortexed for 1 min and then centrifuged at 10,000 rpm for 1 min. The supernatant of each mixture was removed from the tube, and the following rinse solvents were added: Water/MeCN (1:1, v/v; rinse solvent B), 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v; rinse solvent C), and 10 mM trisodium citrate (rinse solvent D). Each mixture was further vortexed for 1 min and then centrifuged at 10,000 rpm for 1 min. The supernatant of each mixture was filtered and analyzed by LC-MS/MS. The procedure is illustrated in Figure 2.2.

### 2.2.5 Comparison of carryover among different analytical columns

The carryover of fumonisins was compared among the following analytical columns: Mastro C18 (2.1 × 100 mm, 3 μm), YMC-Triart C18 (2.0 × 100 mm, 3 μm; YMC, Kyoto, Japan), Inertsil ODS-4 (2.1 × 100 mm, 3 μm; GL Sciences) Zorbax Eclipse XDB-C18 (2.1 × 100 mm, 3.5 μm; Agilent Technologies, Geneva, Switzerland), Cadenza CD-C18 (2 × 100 mm, 3 μm; Imtakt, Kyoto, Japan), Xbridge $C_{18}$ (2.1 × 100 mm, 3.5 μm; Waters), and L-column 2 ODS (2.1 × 100 mm, 3 μm; Chemicals Evaluation and Research

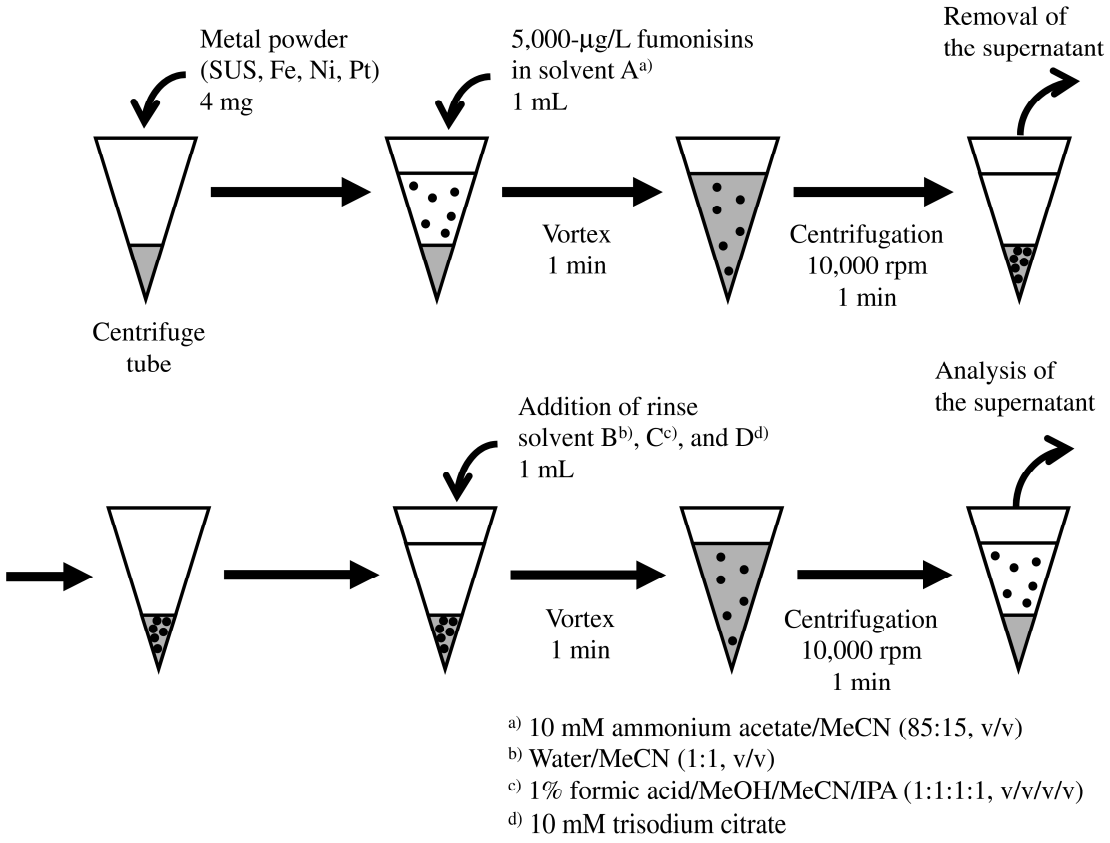


**Figure 2.2** The evaluation procedure for the ability of solvents to desorb fumonisins from a metal (in powder form).

Institute, Tokyo, Japan). The other LC-MS/MS conditions that I used in this experiment are described in the previous section.

### 2.2.6 Sample preparation

A 2.5-g sample of corn grits crushed in a mill (Labo Milser LM-PLUS; Iwatani, Tokyo, Japan) was placed into a 50-mL polypropylene centrifuge tube. Then, 20 mL of 2% acetic acid/MeCN (1:1, v/v) was added to the sample, which was mixed at 250 rpm on a shaker (SR-2 DS; Taitec, Saitama, Japan) for 1 h. Next, the contents of Q-sep Q110 were added to the tube, and the mixture was vortexed for 20 s and centrifuged at 1,580 × *g* for 5 min. The supernatant (MeCN phase) was frozen at −30°C for 1 h and was again centrifuged at 1,580 × *g* for 5 min. Then, 5 mL of the supernatant, 1 mL of water, and 60 μL of acetic acid were mixed and loaded onto a MultiSep 229 Ochra cartridge. Four milliliters of the eluate was evaporated completely at 40°C under a nitrogen stream, and the dry residue was dissolved with 400 μL of 10 mM ammonium acetate/MeCN (85:15, v/v). Each sample was filtered with a 0.20-μm PTFE filter immediately before LC-MS/MS analysis.

### 2.2.7 Method validation

The method was validated by evaluating the linearity, recovery, and repeatability. The coefficient of linearity was determined by means of calibration curves prepared by the standard addition method and constructed by plotting the peak areas of the prepared samples spiked with mycotoxins versus the concentrations of the analytes. The following concentrations of mycotoxins were added to the samples: 0.2, 0.5, 1, 2, 5, 10, and 20 μg/kg for AFB1, AFB2, AFG1, AFG2, and OTA; 2, 5, 10, 20, 50, 100, and 200 μg/kg for PAT; 2, 10, 20, 100, 200, 1,000, and 2,000 μg/kg for NIV, DON, and ZEN; 0.5, 2, 5, 20, 50, 200, and 500 μg/kg for HT-2 and T-2; and 5, 10, 50, 100, 500, 1,000, and 5,000 μg/kg for FB1, FB2, and FB3. The recovery was assessed using samples spiked with mycotoxins. The measurements were repeated five times on the same day. The repeatability was assessed by calculating the RSD of five assays on a single day. The following concentrations of mycotoxins were added to the samples: 1 μg/kg for AFB1, AFB2, AFG1, AFG2, and OTA; 5 μg/kg for PAT; 20 μg/kg for NIV, DON, and ZEN; 5 μg/kg for HT-2 and T-2; and 50 μg/kg for FB1, FB2, and FB3.

## 2.3 Results and Discussion

### 2.3.1 Optimization of LC-MS/MS conditions

First, the MRM transitions were optimized for the 14 mycotoxins. For AFB1, AFB2, AFG1, AFG2, FB1, FB2, FB3, OTA, PAT, and ZEN, $[M+H]^+$ or $[M-H]^-$ was selected as the precursor ion. Acetate adduct ions ($[M+CH_3COO]^-$) were selected as the precursor ions for NIV and DON, and ammonium adduct ions ($[M+NH_4]^+$) as precursor ions for HT-2 and T-2.

The mobile phase was then optimized using a YMC Triart C18 column. PAT was detected as $[M-H]^-$ with good intensity and shape of the peaks under neutral conditions. The sodium adduct ions ($[M+Na]^+$) of AFB1, AFB2, AFG1, and AFG2 were inhibited by addition of ammonium acetate to the mobile phase. In contrast, FB1, FB2, FB3, and OTA were detected at lower intensity of peaks under neutral condition. It was assumed that $[M+H]^+$ became less abundant because these mycotoxins have carboxyl groups, which are in dissociated state under neutral conditions. Therefore, acetic acid was added to the mobile phase to acidify it, and as a result, the intensity of peaks dramatically improved.

These findings indicate that good separation and simultaneous detection may be achieved by gradient elution with 10 mM ammonium acetate (A) and 2% acetic acid in MeOH (B) as the mobile phases.

### 2.3.2 The assay of carryover of fumonisins

Significant carryover of fumonisins was observed when a blank solution (10 mM ammonium acetate/MeCN; 85:15, v/v) was injected after injection of a standard solution of mycotoxins under the LC condition examined. The carryover concentrations were estimated as follows: 305 μg/L for FB1, 376 μg/L for FB2, and 389 μg/L for FB3 (Figure 2.3). These concentrations were calculated from the ratio of the peak area (obtained from the 5,000-μg/L standard solution of fumonisins) to that obtained from the blank solution. In general, ionic compounds and hydrophobic compounds tend to cause carryover because they can be adsorbed to materials via known interactions: ionic interaction with a metal and hydrophobic interaction with plastic, in the sample flow path [1]. The cause of fumonisin carryover were hypothesized as follows. Because the mobile phase that I initially used in the LC gradient program was nearly neutral, the carboxyl groups in the

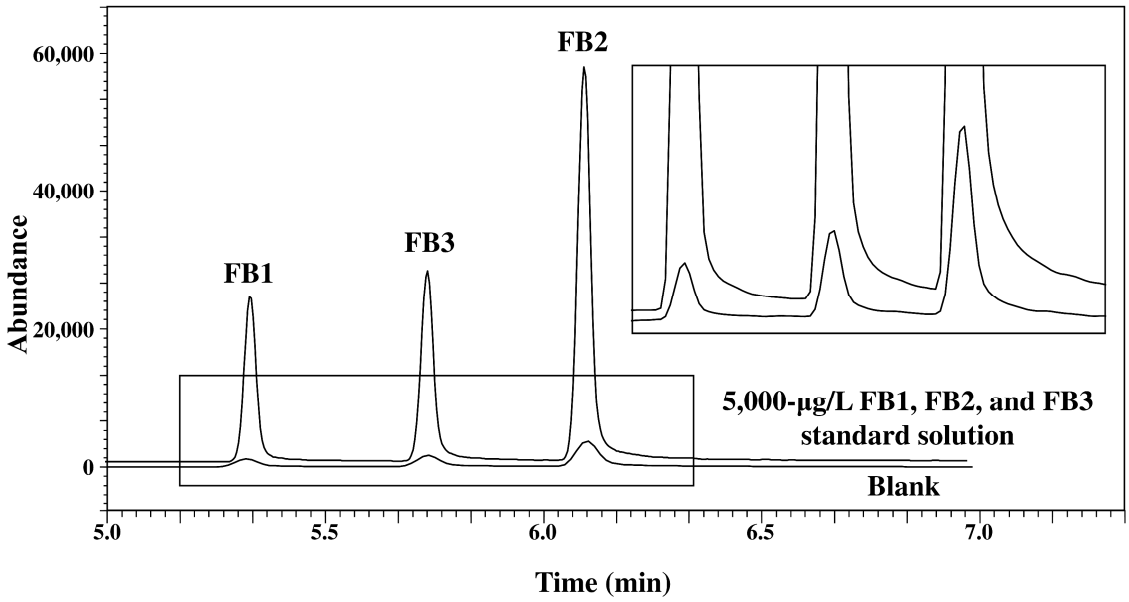


**Figure 2.3** A chromatogram showing carryover.

fumonisins were presumably in a dissociated state when injected. Thus, the dissociated carboxyl groups in the fumonisins chelated with trace metals in the sample flow path and remained inside the LC-MS/MS system, causing carryover into the subsequent analysis (Figure 2.4). Therefore, a standard solution of fumonisins was used to test whether fumonisins adsorb onto metals.

### 2.3.3 Testing whether fumonisins adsorb onto metals

Carryover occurs most often in the injection needle and in the analytical column [1–3], both of which are made of SUS. A standard solution of fumonisins was mixed with powdered SUS, and powdered Fe and Ni were also analyzed because they are the main ingredients of SUS. The amount of each fumonisin in the supernatant of the mixture was measured. The same measurement was performed for powdered Pt, which is chemically inert. Abundance of each fumonisin in the supernatant is shown in Figure 2.5 (A). Almost no fumonisins were detected in the supernatants of powdered SUS, Fe, or Ni, suggesting that the carboxyl groups in the fumonisins under the neutral condition are in dissociated states and adsorb onto the metals by chelation. In contrast, 80% of the fumonisins were

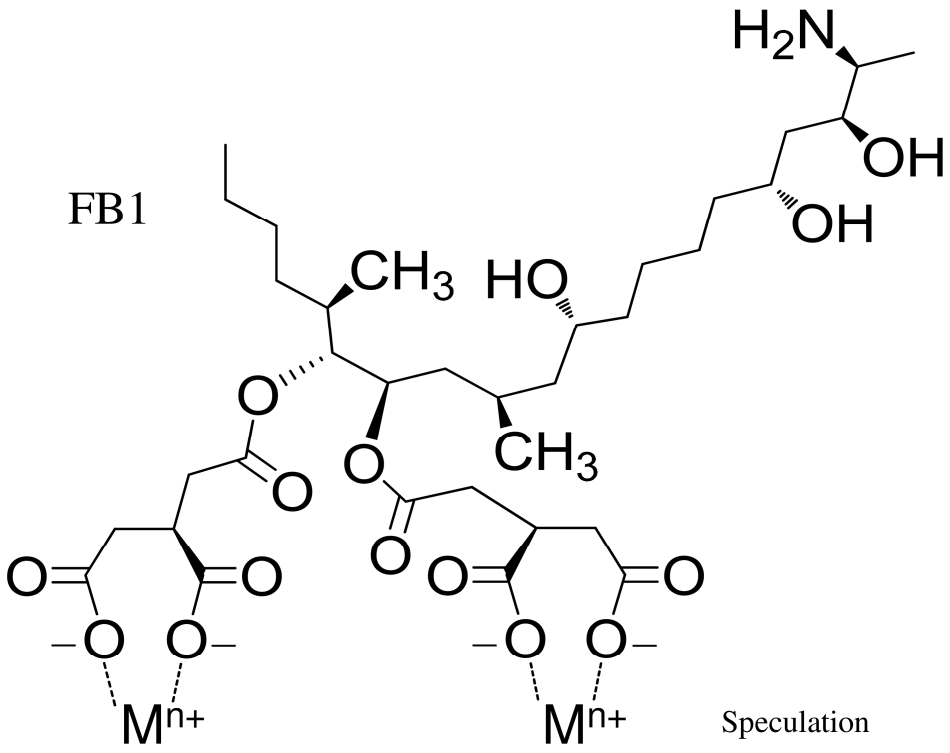


**Figure 2.4** Possible coordination interaction of metal ions with FB1.

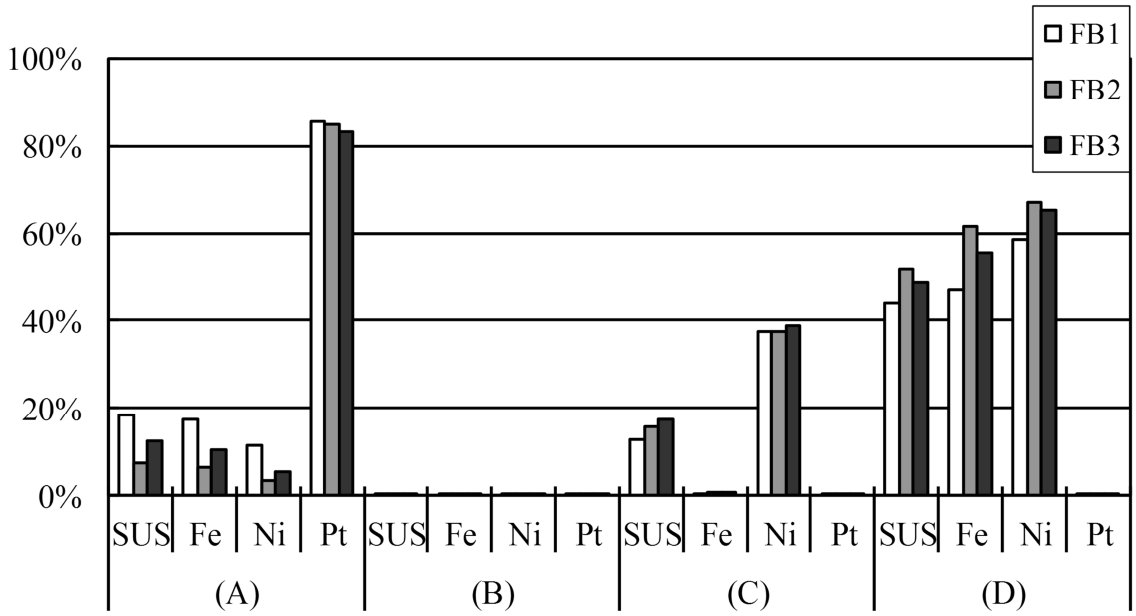


**Figure 2.5** Abundance of fumonisins in supernatants. (A) Supernatants after addition of each metal powder, (B) water/MeCN (1:1, v/v), (C) 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v), and (D) 10 mM trisodium citrate.

detected in the supernatant of powdered Pt, indicating that fumonisins seldom adsorb onto Pt.

### 2.3.4 Solvents used to desorb fumonisins from metals

The rinse solvents used to detach fumonisins from metals were then studied. Fumonisins have four carboxyl groups and a long hydrocarbon chain. I therefore assumed that fumonisins would desorb when the carboxyl groups become undissociated form under acidic conditions and would dissolve in the organic solvents. Therefore, 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v; rinse solvent C) was selected to elute the fumonisins from the metals. A chelating agent was also used in the rinse solvent. It binds readily to metals and was expected to compete with the fumonisins, promoting desorption of the fumonisins from the metals. Therefore, 10 mM trisodium citrate (rinse solvent D) was selected as a chelating agent for the rinse solvent. To compare the rinse effects, water/MeCN (1:1, v/v; rinse solvent B) was used as the other solvent. The rinse solvents were mixed with each metal powder onto which the fumonisins were adsorbed, and the amount of each fumonisin in the supernatant was measured. The level of each fumonisin in the rinse solvents is shown in Figure 2.5.

Very low levels of fumonisins were detected in water/MeCN (1:1, v/v) and thus did not detach from the metals in this rinse solvent. In contrast, the fumonisins were effectively desorbed from the metals by 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v) or by 10 mM trisodium citrate [Figure 2.5 (C) and (D)], with the latter being particularly effective. It was assumed that the fumonisins desorbed because the citrate ions in the solvents chelate with the metals (Figure 2.6).

### 2.3.5 Application of the rinse solvents to injection needles

The inner surface of the injection needle is a possible carryover site. As mentioned in the previous section, 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v) and 10 mM trisodium citrate efficiently detached the fumonisins that had adsorbed onto the metals in the flow path. These solvents were therefore selected for rinsing of the injection needle.

The autosampler (SIL-30AC) that I used in this experiment is capable of rinsing both the inner and outer surfaces of the injection needle with solvents other than the mobile phases. The following solvents were used as rinse solutions: R0, R1, R2, and R3,

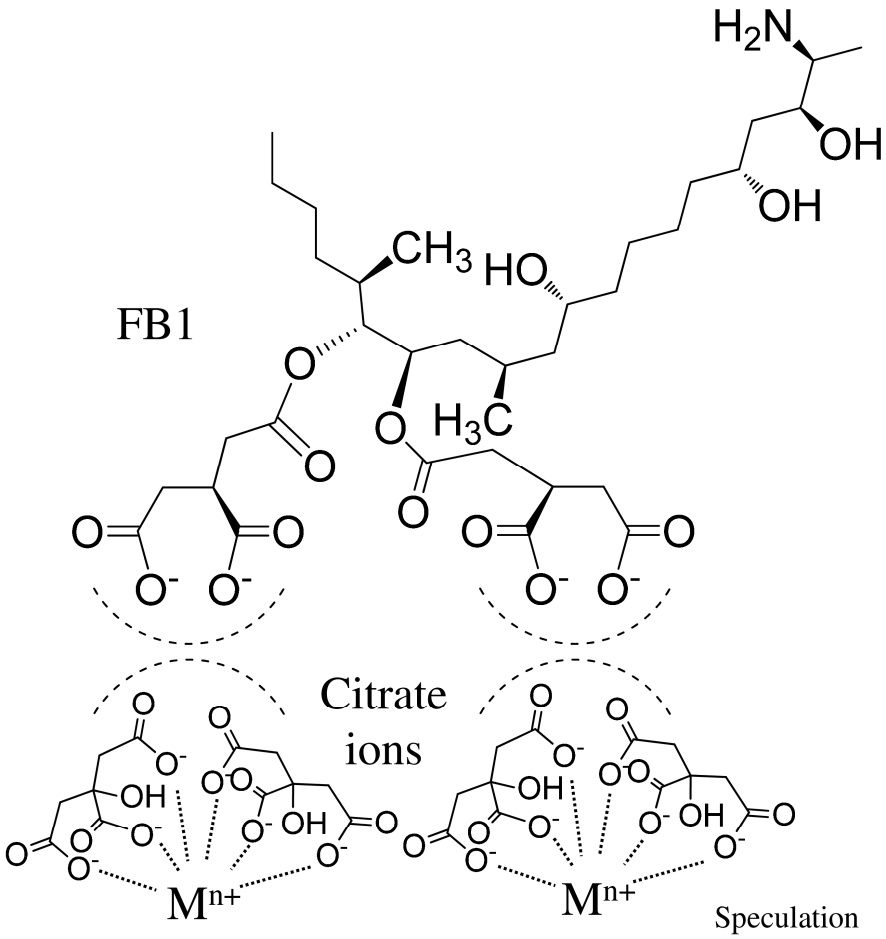


**Figure 2.6** Possible coordination interaction between metal ions and citrate ions.

where R0 was 10 mM ammonium acetate, R1 was 10 mM trisodium citrate, and R2 and R3 were 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v). The inner surface of the injection needle was sequentially rinsed with R1, R0, R2, and R0, and the outer surface was rinsed with R3 and R0, in that order. I compared the fumonisin carryover between the two rinse conditions, with one condition involving rinsing of the outer surface only and the other condition involving rinsing of both the inner and outer surfaces. Five microliters of a 5,000-μg/L fumonisin standard solution was injected, followed by 5 μL of a single blank solution (10 mM ammonium acetate/MeCN; 85:15, v/v). The concentration of each fumonisin carryover was calculated from the ratio of each peak area.

The concentrations of carryover in the first condition (rinsing of the outer surface) were 255, 308, and 294 μg/L for FB1, FB2, and FB3, respectively. The concentrations in the second condition (rinsing of both the inner and outer surfaces) were 119, 142, and 130 μg/L for FB1, FB2, and FB3, respectively. This result revealed that some carryover occurs when fumonisins adsorb onto the inner surface of the injection needle. In addition, the carryover is reduced efficiently when the inner surface of the injection needle is rinsed with 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v) and 10 mM trisodium citrate.

### 2.3.6 Comparison of carryover among different analytical columns

Although carryover was reduced by rinsing of the inner and outer surfaces of the injection needle, ~100-μg/L carryover was still present. Therefore, carryover was also compared among several analytical columns, which represent other possible sources of carryover.

In many cases, columns for LC analysis are made of SUS to make the columns pressure resistant. Thus, I hypothesized that SUS columns cause some carryover of fumonisins. A Mastro C18, in which SUS is inactivated because of polymer frits and polymer lining of the column body, and six C18 columns that have SUS frits and body were selected to test whether carryover occurs. The results are summarized in Table 2.2.

Negligible carryover was observed when the Mastro C18 was used. This finding indicates that the use of columns with polymer frits and polymer lining of the column body helps to reduce carryover. In contrast, some carryover was observed when the analysis was performed on the six C18 columns, with carryover concentrations ranging from 10 to 100 μg/L. This result suggests that carryover of fumonisins occurs when they adsorb onto the surface of SUS frits or body. These findings indicate that the LC conditions least conducive to carryover of fumonisins involve a Mastro C18 for the

**Table 2.2** Concentrations of fumonisin carryover for various columns.

| Column | Concentration of carryover (µg/L) | | |
|---|---|---|---|
| | **FB1** | **FB2** | **FB3** |
| Mastro C18 | <5 | <5 | <5 |
| YMC-Triart C18 | 119 | 142 | 130 |
| Inertsil ODS-4 | 65 | 74 | 82 |
| L-column 2 ODS | 37 | 49 | 51 |
| Xbridge $C_{18}$ | 38 | 42 | 45 |
| Cadenza CD-C18 | 17 | 13 | 20 |
| Zorbax Eclipse XDB-C18 | 14 | 17 | 20 |

analysis and 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v) and 10 mM trisodium citrate for rinsing of the inner surface of the injection needle. A chromatogram of the standard solutions obtained under these LC conditions is shown in Figure 2.7.

### 2.3.7 Sample preparation

The preparation of corn grit samples involved extraction by means of the QuEChERS extraction kit followed by purification with MultiSep 229 Ochra cartridge. This cartridge is effective at recovering the mycotoxins under study, as described in Chapter 1; therefore, the corn grit samples were also purified by means of MultiSep 229 Ochra cartridge. As a result, the pigments and lipids were removed from the samples effectively. Good recovery, ranging from 70% to 120%, was attained with this preparation procedure; thus, the method was evaluated further.

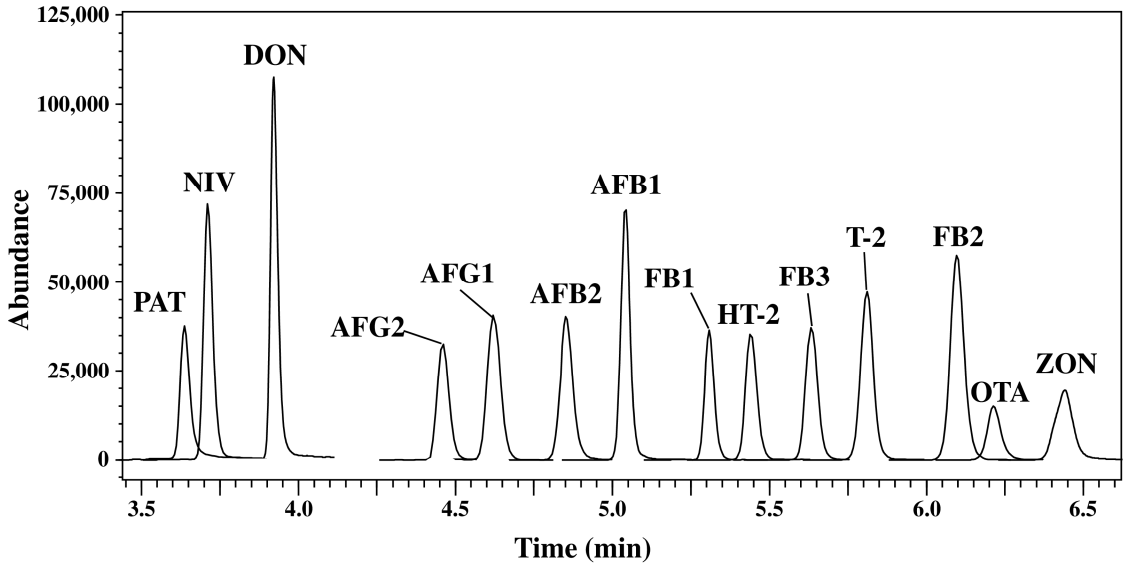


**Figure 2.7** A chromatogram of the 14 mycotoxins for a Mastro C18.

### 2.3.8 Method validation

Performance of the method was evaluated using corn grit samples (Table 2.3). The coefficient of linearity was >0.991, recovery was 73–117%, and good repeatability (4.0–12.4%) was observed. LOQs for the mycotoxins were defined as the lowest concentration values visible on the calibration curves: 2 μg/kg for PAT, NIV, DON, and ZEN; 0.2 μg/kg for AFB1, AFB2, AFG1, AFG2, and OTA; 0.5 μg/kg for HT-2 and T-2; and 5 μg/kg for FB1, FB2, and FB3. Validation of the standard addition method yielded good results for the purposes of this project, and it was decided that internal standards need not be used. As a result, simultaneous analysis across a wide range of concentrations was now possible because of minimization of the carryover that occurs during the analysis of highly concentrated samples.

### 2.3.9 Determination of 14 mycotoxins in corn grits purchased in local markets

The method was applied to the analysis of corn grit samples purchased locally (Table 2.4). The simultaneous determination revealed that the corn samples are co-

**Table 2.3** Performance of the method used for determining mycotoxins.

| Mycotoxin | Linearity (*r*) [a)] | Recovery (%) [b)] | Repeatability (%) [b)] | LOQ (μg/kg) | Retention time (min) |
|---|---|---|---|---|---|
| AFB1 | 0.993 | 117 | 8.6 | 0.2 | 5.05 |
| AFB2 | 0.999 | 82 | 4.0 | 0.2 | 4.85 |
| AFG1 | 0.991 | 92 | 7.1 | 0.2 | 4.63 |
| AFG2 | 0.998 | 89 | 6.6 | 0.2 | 4.45 |
| OTA | 0.995 | 78 | 6.0 | 0.2 | 6.21 |
| PAT | 0.996 | 108 | 12.4 | 2.0 | 3.64 |
| NIV | >0.999 | 73 | 4.4 | 2.0 | 3.71 |
| DON | 0.999 | 75 | 6.7 | 2.0 | 3.92 |
| HT-2 | >0.999 | 90 | 6.5 | 0.5 | 5.45 |
| T-2 | >0.999 | 90 | 5.1 | 0.5 | 5.81 |
| FB1 | 0.998 | 89 | 8.4 | 5.0 | 5.30 |
| FB3 | 0.996 | 89 | 4.0 | 5.0 | 5.64 |
| FB2 | 0.994 | 88 | 8.1 | 5.0 | 6.10 |
| ZEN | 0.991 | 95 | 9.9 | 2.0 | 6.45 |

[a)] The coefficient of linearity was determined using corn grit samples spiked with each mycotoxin at the following concentrations: 0.2, 0.5, 1, 2, 5, 10, and 20 μg/kg for AFB1, AFB2, AFG1, AFG2, and OTA; 2, 5, 10, 20, 50, 100, and 200 μg/kg for PAT; 2, 10, 20, 100, 200, 1,000, and 2,000 μg/kg for NIV, DON, and ZEN; 0.5, 2, 5, 20, 50, 200, and 500 μg/kg for HT-2 and T-2; and 5, 10, 50, 100, 500, 1,000, and 5,000 μg/kg for FB1, FB2, and FB3.

[b)] Recovery and repeatability assays involved five replicate measurements that were carried out on the same day using corn grit samples spiked with each mycotoxin at the following concentrations: 1 μg/kg for AFB1, AFB2, AFG1, AFG2, and OTA; 5 μg/kg for PAT; 20 μg/kg for NIV, DON, and ZEN; 5 μg/kg for HT-2 and T-2; and 50 μg/kg for FB1, FB2, and FB3.

**Table 2.4** Mycotoxins detected in the analyzed samples.

| Sample | Mycotoxin (μg/kg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **NIV** | **DON** | **HT-2** | **T-2** | **FB1** | **FB2** | **FB3** | **ZEN** |
| CG-1 | [a] | 107 | 0.79 | 0.91 | 595 | 110 | 67.1 | 8.47 |
| CG-2 | | 113 | | <0.5 | 344 | 59.5 | 23.6 | 7.10 |
| CG-3 | | 62.5 | | | 103 | 16.9 | 5.54 | 2.06 |
| CG-4 | <2 | 149 | 20.7 | 67.2 | 62.4 | <5 | | 21.4 |
| CG-5 | | 629 | | | 44.4 | 7.35 | <5 | 12.3 |
| CG-6 | <2 | 221 | | | 26.4 | <5 | 7.85 | 5.44 |
| CG-7 | 2.51 | 167 | | | 458 | 65.4 | 56.6 | 2.55 |
| CG-8 | | 231 | 0.70 | 0.90 | 1,100 | 237 | 125 | 15.3 |
| CG-9 | <2 | 1,260 | | | 26.2 | <5 | 6.63 | 74.1 |
| CG-10 | 8.09 | 5.47 | | | 39.0 | 10.3 | 7.50 | 4.86 |
| CG-11 | | | | <0.5 | 298 | 41.0 | 34.8 | |
| CG-12 | | 15.1 | 1.01 | 2.69 | 142 | 5.28 | 5.57 | |
| Incidence [b] | 5/12 | 11/12 | 4/12 | 6/12 | 12/12 | 12/12 | 11/12 | 10/12 |

[a] Blank, no mycotoxins were detected. [b] This corresponds to the number of samples in which each mycotoxin was detected.

contaminated with "*Fusarium* toxins," which include trichothecenes, fumonisins, and ZEN. To be precise, NIV, DON, ZEN, FB1, FB2, FB3, HT-2, and T-2 were detected in the samples. DON, ZEN, FB1, FB2, and FB3 were detected more frequently than the other mycotoxins. Compared to the other mycotoxins tested, DON and FB1 showed the highest levels, with the maxima of 1.26 and 1.10 mg/kg, respectively. The levels of these mycotoxins are strictly regulated in the EU, with the regulatory level of DON in corn grits being 750 μg/kg and that of fumonisins 1,000 μg/kg for the total amount of FB1 and FB2 [4]. Thus, the amounts of DON and FB1 detected in this experiment exceed the regulatory levels of the EU. Similarly, the CODEX set the maximum level of DON at 1 mg/kg and that of fumonisins (FB1 + FB2) at 2 mg/kg for corn grits [5]. Thus, one of samples showed a DON concentration above this maximum level. These results revealed that the samples are contaminated with several mycotoxins and suggest that these levels need to be controlled constantly, and the international standards need to be monitored.

## 2.4 Summary

In this chapter, identification of the sources of carryover and minimization of carryover were studied, and simultaneous determination of mycotoxins was accomplished by minimizing the carryover. The highlights are as follows:

- The verification assays revealed that fumonisins adsorb onto SUS and its raw materials (Fe and Ni) and that they can be desorbed with 1% formic acid/MeOH/MeCN/IPA (1:1:1:1, v/v/v/v) and 10 mM trisodium citrate. The carryover was minimized by rinsing of the inner surface of the injection needle with these solvents and by the use of a stainless-free Mastro C18. Thus, a method for simultaneous analysis of 14 mycotoxins was successfully developed.
- The protocol for preparation of corn grit samples was examined, and the matrices were removed from the samples when the analytes were extracted by means of a QuEChERS kit and purified on a MultiSep 229 Ochra cartridge. The results of method validation showed that simultaneous determination across a wide range of concentrations was made possible by minimization of the carryover that occurs during analysis of highly concentrated samples.

- The proposed method was then applied to analysis of 12 corn grit samples purchased in the market. The results revealed that NIV, DON, ZEN, FB1, FB2, FB3, HT-2, and T-2 were present in the samples. DON, ZEN, FB1, FB2, and FB3 were detected more frequently than the other mycotoxins.
- The simultaneous determination indicated that the corn samples are co-contaminated with "*Fusarium* toxins," which include trichothecenes, fumonisins, and ZEN.

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# Chapter 3

# Identification and quantification of fumonisin A1, fumonisin A2, and fumonisin A3 in corn by LC-Orbitrap MS

## 3.1 Introduction

In addition to fumonisin B-series, which have been detected in corn samples at high concentrations and at frequencies described in Chapter 2, several derivatives of these fumonisins (Figure 3.1), including the fumonisin A-series (*N*-acetyl derivatives), fumonisin C-series (demethyl derivatives), and fumonisin P-series (*N*-3-hydroxypiridinium derivatives), have been detected in the culture medium of the genus *Fusarium*. These compounds are produced by *Fusarium moniliforme*, *F. verticillioides*, *F. proliferatum*, *F. nygami*, and *F. oxysporum* [1–6]. Toxicity reports have suggested that, similar to the fumonisin B-series, the fumonisin A-series can also inhibit sphingosine *N*-acyltransferase [7]. In addition, the fumonisin C-series and P-series are known to be both phytotoxic and cytotoxic [8]. Because there are few reports of detection of various fumonisins in foods and feeds that are directly ingested by humans and animals, the extent to which these

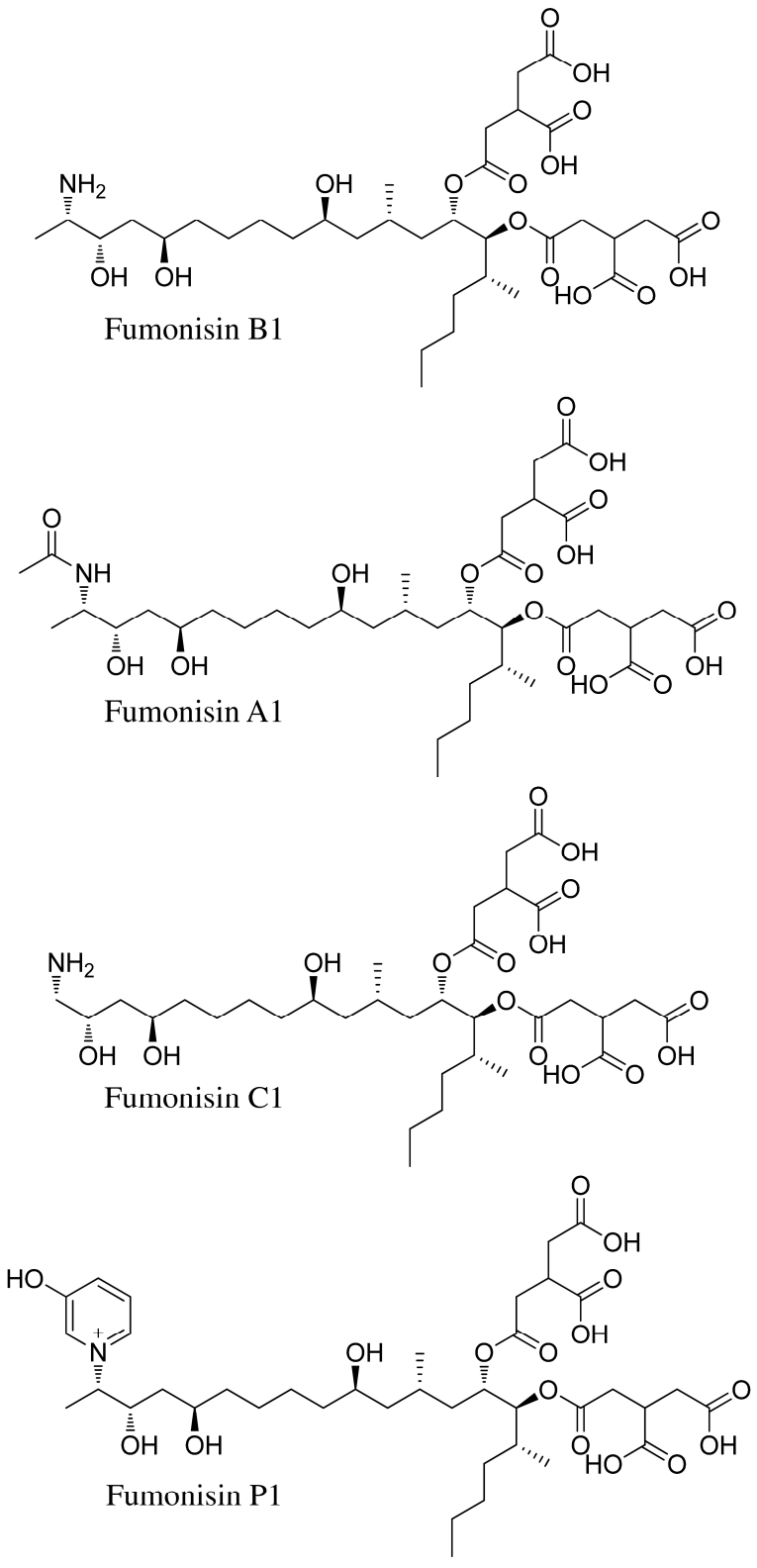
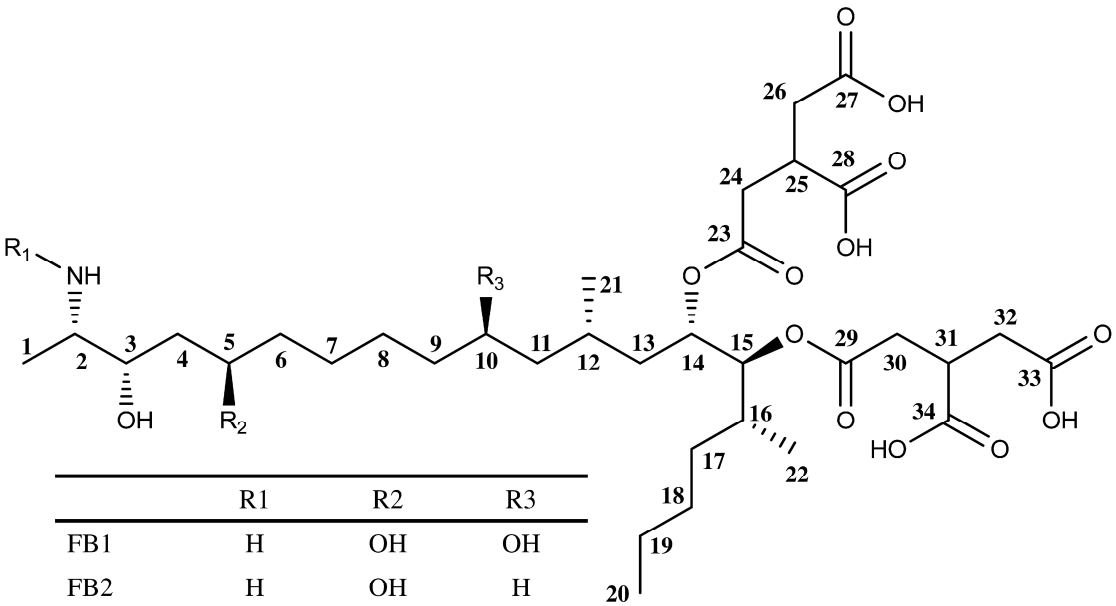



**Figure 3.1** Chemical structures of various fumonisins.

compounds in cereals pose the risks of toxicity and contamination remains unclear.

Therefore, I attempted to detect fumonisins in commercially available reference corn sample (MTC-9999E) that is naturally contaminated with mycotoxins including FB1, FB2, and FB3. The sample was analyzed using Q-Exactive, which is an Orbitrap MS equipped with a quadrupole mass filter and a collision cell. Structures of the compounds detected were estimated by fragment analysis using mass spectra of those product ions. Additionally, a method for determining the amount of six fumonisins [fumonisin A1 (FA1), fumonisin A2 (FA2), fumonisin A3 (FA3), FB1, FB2, and FB3] was developed and applied to corn samples. The chemical structures of FA1, FA2, FA3, FB1, FB2, and FB3 are shown in Figure 3.2.



| | R1 | R2 | R3 |
|---|---|---|---|
| FB1 | H | OH | OH |
| FB2 | H | OH | H |
| FB3 | H | H | OH |
| FA1 | $CH_3CO$ | OH | OH |
| FA2 | $CH_3CO$ | OH | H |
| FA3 | $CH_3CO$ | H | OH |

**Figure 3.2** Chemical structures of fumonisin B-series and fumonisin A-series.

## 3.2 Experimental section

### 3.2.1 Samples and reagents

Mycotoxin reference materials (MTC-9999E, MTC-9990, and FC-443) from the Trilogy Analytical Laboratory (Washington, DC, USA) were used as corn samples naturally contaminated with mycotoxins including FB1, FB2, and FB3. The acceptance limits of FB1, FB2, and FB3 in the reference materials, with incorporated uncertainties, are shown in Table 3.1. Seven corn samples were also purchased at local supermarkets in Japan in 2013.

FB1, FB2, and FB3 standards were acquired from Cayman Chemical Co. (Ann Arbor, MI, USA), LKT Laboratories, Inc. (St. Paul, MN, USA), and Medical Research Council (Swindon, Wiltshire, UK), respectively. Standard solutions containing 50 μg/mL FB1, FB2, and FB3 in MeCN/water (1/1, v/v) were purchased from Romer Labs Corp. MeOH (LC/MS grade), MeCN (analytical grade), acetic acid (guaranteed reagent grade), ammonium acetate (analytical grade), dipotassium hydrogen phosphate (guaranteed reagent grade), *N*,*N*-dimethylformamide (guaranteed reagent grade), and acetic anhydride

**Table 3.1** Acceptance limits of FB1, FB2, and FB3 in mycotoxin reference materials.

| Sample | Acceptance limit (mg/kg) | | |
|---|---|---|---|
| | **FB1** | **FB2** | **FB3** |
| MTC-9999E | 20.7–32.9 | 5.2–9.0 | 1.2–.2 |
| MTC-9990 | 1.0–1.6 | 0.1–0.3 | – |
| FC-443 | 2.2–5.0 | 0.5–1.1 | 0.2–0.4 |

(guaranteed reagent grade) were purchased from Kanto Chemical Co., Inc. MeOH-$d_4$ (NMR grade) and Supelpak 2 were acquired from Merck KGaA (Darmstadt, Germany) and Sigma-Aldrich, respectively. Water was purified using a Millipore Milli-Q system. The Q-sep Q 110 QuEChERS extraction kit was purchased from RESTEK. A MultiSep 229 Ochra cartridge was purchased from Romer Labs Corp. A PTFE filter (mesh pore size 0.20 μm) was acquired from Advantec Toyo Kaisha, Ltd. A Pierce LTQ Velos ESI Positive Ion Calibration Solution for positive mode calibration of the Orbitrap MS was acquired from Thermo Fisher Scientific (Bremen, Germany).

### 3.2.2 Sample preparation

Sample preparation was carried out as described in Chapter 2. In particular, a 2.5-g sample was placed in a 50-mL polypropylene centrifuge tube, and 20 mL of 2% acetic acid/MeCN (1:1, v/v) was added. The samples were mixed at 250 rpm for 1 h on a shaker (SR-2 DS; Taitec). The contents of Q-sep Q110 were then added to the centrifuge tube. The mixture was vortexed for 20 s and centrifuged at 1,580 × $g$ for 5 min. The supernatant (MeCN phase) was frozen at −30°C for 1 h and was then centrifuged at 1,580

× *g* for 5 min. Next, 5 mL of the supernatant, 1 mL of water, and 60 μL of acetic acid were mixed, and the mixture was loaded onto MultiSep 229 Ochra cartridge. The eluate (4 mL) was dried at 40°C under a nitrogen stream, and the dry residue was dissolved in 400 μL of 10 mM ammonium acetate/MeCN (85:15, v/v). Each sample was passed through a 0.20-μm PTFE filter immediately prior to LC-Orbitrap MS analysis.

### 3.2.3 LC-Orbitrap MS analysis

LC-Orbitrap MS analysis was performed on an Ultimate 3000 system coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). The Xcalibur 2.2 software (Thermo Fisher Scientific) was used to control the instruments and to process the data. LC was conducted using 10 mM ammonium acetate as solvent A and 2% acetic acid in MeOH as solvent B. The gradient profile was 2% B (0–2.0 min), 55% B (3.0–4.0 min), 70% B (4.1 min), 80% B (7.0 min), 95% B (7.01–8.0 min), and 2% B (8.01–11.0 min). The flow rate was set to 0.4 mL/min, and the column temperature was maintained at 40°C. Chromatographic separation was carried out on a Mastro C18 (2.1 × 100 mm, 3 μm; Shimadzu GLC) with the injection volume of 5 μL.

The Q-Exactive mass spectrometer was operated in positive mode with a heated ESI source (HESI-II) and the spray voltage of 3.00 kV. The capillary and heater temperatures were 350°C and 300°C, respectively. The sheath gas and auxiliary gas flow rates were 40 and 10 arbitrary units, respectively. Mass calibration for analysis was performed as follows: (1) instrument calibration was performed before each sequence using a calibration solution; (2) lock masses (*m/z* values of 188.98461 and 537.87906) were typically detected during the entire chromatographic run and were used for mass correction during the sequence. Precursor ion scanning was carried out in Full MS mode at the resolution of 70,000 for the *m/z* value of 200 (3 scans/s), with an auto gain control (AGC) target of 3e6, maximum injection time (IT) of 100 ms, and the scan range of 100–1,000 *m/z*. Product ion scanning was conducted in data-dependent $MS^2$ mode (dd-$MS^2$) at the resolution of 17,500 for the *m/z* value of 200, AGC target of 2e5, maximum IT of 200 ms, normalized collision energy (NCE) of 30 eV, stepped NCE of 50%, and the scan range of 50–800 *m/z*.

### 3.2.4 Synthesis of FA1, FA2, and FA3 and identification of their structures by nuclear magnetic resonance spectroscopy analysis

FA1 was synthesized from FB1 as follows [9, 10]. FB1 (4.61 mg) was placed in a 50-mL recovery flask and was dissolved in 0.2 mL of *N*,*N*-dimethylformamide. Next, 1.5 mL of a 3 M aqueous solution of dipotassium hydrogen phosphate and 1.5 mL of acetic anhydride were added to the FB1 solution and stirred with a magnetic stirrer for 10 min. After that, 3 mL of water was added to the reaction mixture, and the solution was stirred for 30 min. To this solution, 50 mL of water was added, and the whole reaction mixture was loaded onto Supelpak 2, which had been packed into an open column beforehand. The column loaded with the reaction solution was washed five times with 15 mL of water and once with 10 mL of a 50% MeCN solution. The compounds were then extracted with 60 mL of a fresh 50% MeCN solution, and the extract was evaporated to obtain 2.69 mg of FA1. A portion of the FA1 was dissolved again in MeOH-$d_4$ and analyzed by nuclear magnetic resonance spectroscopy (NMR). Similarly, 0.29 mg of FA2 and 0.46 mg of FA3 were obtained from 2 mg of FB2 and FB3, respectively.

Each portion of FA1, FA2, and FA3 dissolved in MeOH-$d_4$ was analyzed by NMR. $^1$H NMR (600 MHz) and heteronuclear single-quantum coherence (HSQC) spectra

were recorded on a Bruker AV 600 instrument (Bruker, Karlsruhe, Germany). Chemical shifts were expressed in δ (ppm) relative to the solvent signal (MeOH-$d_4$, $\delta^H$ 3.31, $\delta^C$ 49.0).

### 3.2.5 Method validation

The method was validated by evaluating the linearity, recovery, and repeatability using a corn grit sample containing 9.3 μg/kg FB1 (FB2, FB3, FA1, FA2, and FA3 were not detected). The coefficient of linearity was calculated from the calibration curves, which were constructed by plotting the peak areas of the prepared samples (spiked with FA1, FA2, FA3, FB1, FB2, and FB3 standards) against the concentrations of the analyte. The concentrations of FA1, FA2, FA3, FB1, FB2, and FB3 added to the samples were 5, 10, 50, 100, 500, 1,000, and 5,000 μg/kg. To the sample, FA1, FA2, FA3, FB1, FB2, and FB3 were added (50 μg/kg final concentration) for recovery and repeatability evaluations. Repeatability was calculated from five measurements on the same day (RSD). The definitions of the limit of detection and LOQ are not applicable to high-resolution mass spectrometric methods because the high mass accuracy yields no or limited noise [11].

On the other hand, for reliability of quantification, a certain degree of confidence is required. Therefore, the LOQ in this method was defined as the lowest calibration level (i.e., 5 μg/kg).

## 3.3 Results and Discussion

### 3.3.1 Detection of fumonisins by LC-Orbitrap MS

Figure 3.3 shows chromatograms of the prepared MTC-9999E obtained by full mass scanning. The retention time of the FB1, FB2, and FB3 peaks detected in the corn samples was quite similar to that of the standard solutions. The peaks of FB1, FB2, FB3, and of three unknown compounds (referred to as compounds I, II, and III) were simultaneously detected, and the measured mass, theoretical mass, mass error, and calculated formulae are shown in Table 3.2. The only difference between the calculated formulae of i) compounds I, II, and III and ii) FB1, FB2, and FB3 was that set “i)” contained an additional $C_2H_2O$ group. This result suggests that compounds I, II, and III were likely to be FA1, FA2, and FA3, which are *N*-acetyl derivatives of FB1, FB2, and FB3, respectively. Therefore, the product ion spectra for each chromatographic peak were recorded, and the fragment ions were structurally characterized as described below.

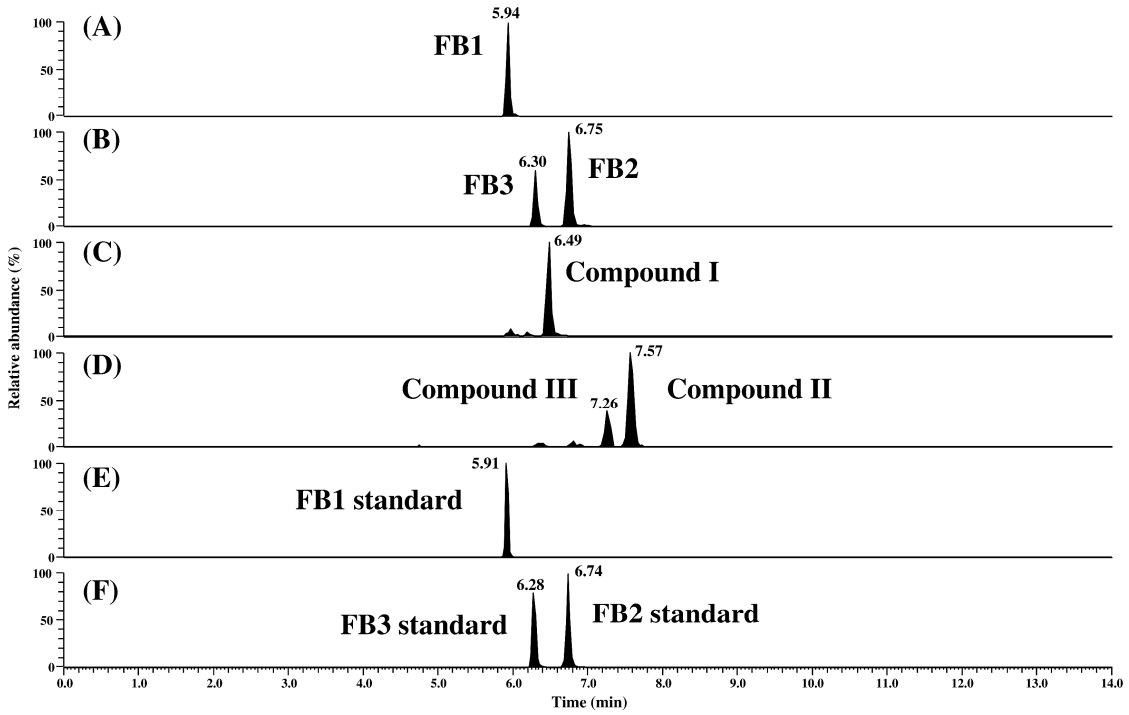



**Figure 3.3** Chromatograms of compounds in MTC-9999E and standard solutions of FB1, FB2, and FB3: (A) FB1 in the sample, (B) FB2 and FB3 in the sample, (C) compound I, (D) compounds II and III, (E) FB1 standard, and (F) FB2 and FB3 standards.

**Table 3.2** Characteristic peaks of FB1, FB2, FB3, and of unknown compounds in MTC-9999E.

| **Compound** | **Measured mass (*m/z*)** | **Theoretical mass (*m/z*)** | **Calculated formula $[M+H]^+$** | **Mass error (ppm)** |
|---|---|---|---|---|
| FB1 | 722.3973 | 722.3958 | $C_{34}H_{60}NO_{15}$ | 1.59 |
| FB2 | 706.4020 | 706.4008 | $C_{34}H_{60}NO_{14}$ | 1.21 |
| FB3 | 706.4015 | 706.4008 | $C_{34}H_{60}NO_{14}$ | 0.66 |
| Compound I | 764.4059 | 764.4063 | $C_{36}H_{62}NO_{16}$ | −0.48 |
| Compound II | 748.4123 | 748.4114 | $C_{36}H_{62}NO_{15}$ | 1.20 |
| Compound III | 748.4118 | 748.4114 | $C_{36}H_{62}NO_{15}$ | 0.54 |

### 3.3.2 Characterization of fragment ions of FB1, FB2, and FB3

Figure 3.4 shows mass spectra of the product ions from standard solutions of FB1, FB2, and FB3. The signals in the spectra are labeled with identification (ID) numbers corresponding to the numbers in Table 3.3, which summarizes the measured mass, calculated formula, and mass error for each signal. The mass spectra of product ions of FB1, FB2, and FB3 in the corn sample were similar to those of the standard solutions.

Fragment ions with *m/z* values of 200–800 are likely to be formed by cleavage of the tricarballylic acids (TCAs) and the hydroxyl groups from the precursor ions; these characteristic fragmentation patterns were common for FB1, FB2, and FB3. In contrast, at *m/z* values of 50–200, different fragment ions seemed to form depending on the positions of the hydroxyl groups in the compound. In the case of fragment ions of FB1, ID 4 of the 10-carbon chain was formed by C–C cleavage at C-10, while ID 1 of 2-amino-1-propanol (APA) was formed by cleavage at C-5. In the case of FB2, ID 1 of APA was formed by cleavage at C-5, as in the fragment ions of FB1, whereas an ion of a 10-carbon chain, such as that in ID 3 and ID 4, could not be formed because of the lack of a hydroxyl group at C-10. In contrast, in the case of FB3, ID 1 was not present because of the lack

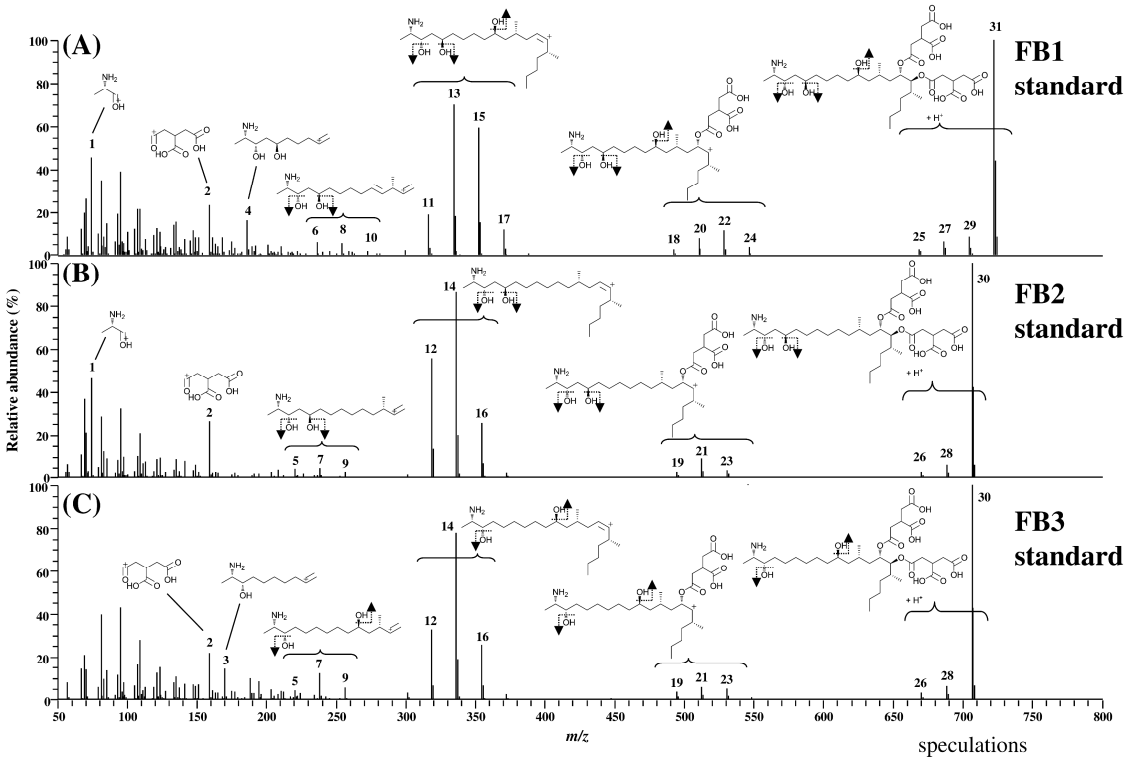



**Figure 3.4** Mass spectra of product ions from standard solutions of FB1, FB2, and FB3. (A) The FB1 standard, (B) FB2 standard, and (C) FB3 standard.

**Table 3.3** Characteristic signal assignment of product ions from mass spectra of standard solutions of FB1, FB2, and FB3.

| ID | FB1 | | | FB2 | | | FB3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Measured mass (*m/z*)** | **Calculated formula $[M+H]^+$** | **Mass error (ppm)** | **Measured mass (*m/z*)** | **Calculated formula $[M+H]^+$** | **Mass error (ppm)** | **Measured mass (*m/z*)** | **Calculated formula $[M+H]^+$** | **Mass error (ppm)** |
| 1 | 74.0601 | $C_3H_8NO$ | 1.17 | 74.0601 | $C_3H_8NO$ | 1.17 | | | |
| 2 | 159.0290 | $C_6H_7O_5$ | 1.40 | 159.0290 | $C_6H_7O_5$ | 0.92 | 159.0290 | $C_6H_7O_5$ | 1.21 |
| 3 | | | | | | | 170.1540 | $C_{10}H_{20}NO$ | 0.39 |
| 4 | 186.1492 | $C_{10}H_{20}NO_2$ | 1.77 | | | | | | |
| 5 | | | | 220.2059 | $C_{15}H_{26}N$ | -0.35 | 220.2058 | $C_{15}H_{26}N$ | −1.03 |
| 6 | 236.2013 | $C_{15}H_{26}NO$ | 1.77 | | | | | | |
| 7 | | | | 238.2168 | $C_{15}H_{28}NO$ | 0.96 | 238.2167 | $C_{15}H_{28}NO$ | 0.56 |
| 8 | 254.2118 | $C_{15}H_{28}NO_2$ | 1.44 | | | | | | |
| 9 | | | | 256.2276 | $C_{15}H_{30}NO_2$ | 1.93 | 256.2272 | $C_{15}H_{30}NO_2$ | 0.26 |
| 10 | 272.2226 | $C_{15}H_{30}NO_3$ | 0.88 | | | | | | |
| 11 | 316.3001 | $C_{22}H_{38}N$ | 0.83 | | | | | | |
| 12 | | | | 318.3157 | $C_{22}H_{40}N$ | 0.65 | 318.3158 | $C_{22}H_{40}N$ | 0.85 |
| 13 | 334.3106 | $C_{22}H_{40}NO$ | 0.59 | | | | | | |
| 14 | | | | 336.3263 | $C_{22}H_{42}NO$ | 0.51 | 336.3262 | $C_{22}H_{42}NO$ | 0.42 |
| 15 | 352.3213 | $C_{22}H_{42}NO_2$ | 0.72 | | | | | | |
| 16 | | | | 354.3369 | $C_{22}H_{44}NO_2$ | 0.56 | 354.3369 | $C_{22}H_{44}NO_2$ | 0.56 |
| 17 | 370.3318 | $C_{22}H_{44}NO_3$ | 0.58 | | | | | | |
| 18 | 492.3330 | $C_{28}H_{46}NO_6$ | 2.18 | | | | | | |
| 19 | | | | 494.3478 | $C_{28}H_{48}NO_6$ | 0.27 | 494.3480 | $C_{28}H_{48}NO_6$ | 0.76 |
| 20 | 510.3431 | $C_{28}H_{48}NO_7$ | 1.14 | | | | | | |
| 21 | | | | 512.3592 | $C_{28}H_{50}NO_7$ | 1.98 | 512.3593 | $C_{28}H_{50}NO_7$ | 2.10 |
| 22 | 528.3538 | $C_{28}H_{50}NO_8$ | 1.38 | | | | | | |
| 23 | | | | 530.3693 | $C_{28}H_{52}NO_8$ | 0.98 | 530.3691 | $C_{28}H_{52}NO_8$ | 0.63 |
| 24 | 546.3630 | $C_{28}H_{52}NO_9$ | −1.13 | | | | | | |
| 25 | 668.3648 | $C_{34}H_{54}NO_{12}$ | 1.04 | | | | | | |
| 26 | | | | 670.3806 | $C_{34}H_{56}NO_{12}$ | 1.27 | 670.3789 | $C_{34}H_{56}NO_{12}$ | −1.27 |
| 27 | 686.3731 | $C_{34}H_{56}NO_{13}$ | −2.29 | | | | | | |
| 28 | | | | 688.3909 | $C_{34}H_{58}NO_{13}$ | 0.87 | 688.3903 | $C_{34}H_{58}NO_{13}$ | −0.01 |
| 29 | 704.3867 | $C_{34}H_{58}NO_{14}$ | 2.18 | | | | | | |
| 30 | | | | 706.4016 | $C_{34}H_{60}NO_{14}$ | 1.02 | 706.4016 | $C_{34}H_{60}NO_{14}$ | 1.10 |
| 31 | 722.3966 | $C_{34}H_{60}NO_{15}$ | 1.11 | | | | | | |

of a hydroxyl group at C-5, whereas ID 3 of the 10-carbon chain was formed by cleavage at C-10. ID 2 was present in all three product ion spectra, and the calculated formula was $C_6H_7O_5$, which may represent TCA.

These results indicate that the fragmentation of FB1, FB2, and FB3 follows characteristic patterns, such as formation of fragment ions via cleavage of TCAs (depending on the position of the hydroxyl group) and formation of TCA present in each compound.

### 3.3.3 Analysis of fragment ions of compounds I, II, and III

Figure 3.5 shows the product ion spectra of compounds I, II, and III, whereas Table 3.4 summarizes the measured mass, calculated formula, and mass error for the fragment ions of these compounds. Compounds I, II, and III showed signals with the same calculated formulae as those of product ions of FB1, FB2, and FB3 and hydroxyl groups from the precursor ion; I also observed formation of different fragment ions in addition to product ions which differed by $C_2H_2O$ from the product ions of FB1, FB2, and FB3. Notably, ions with the same calculated formulae are labeled with the same ID numbers as

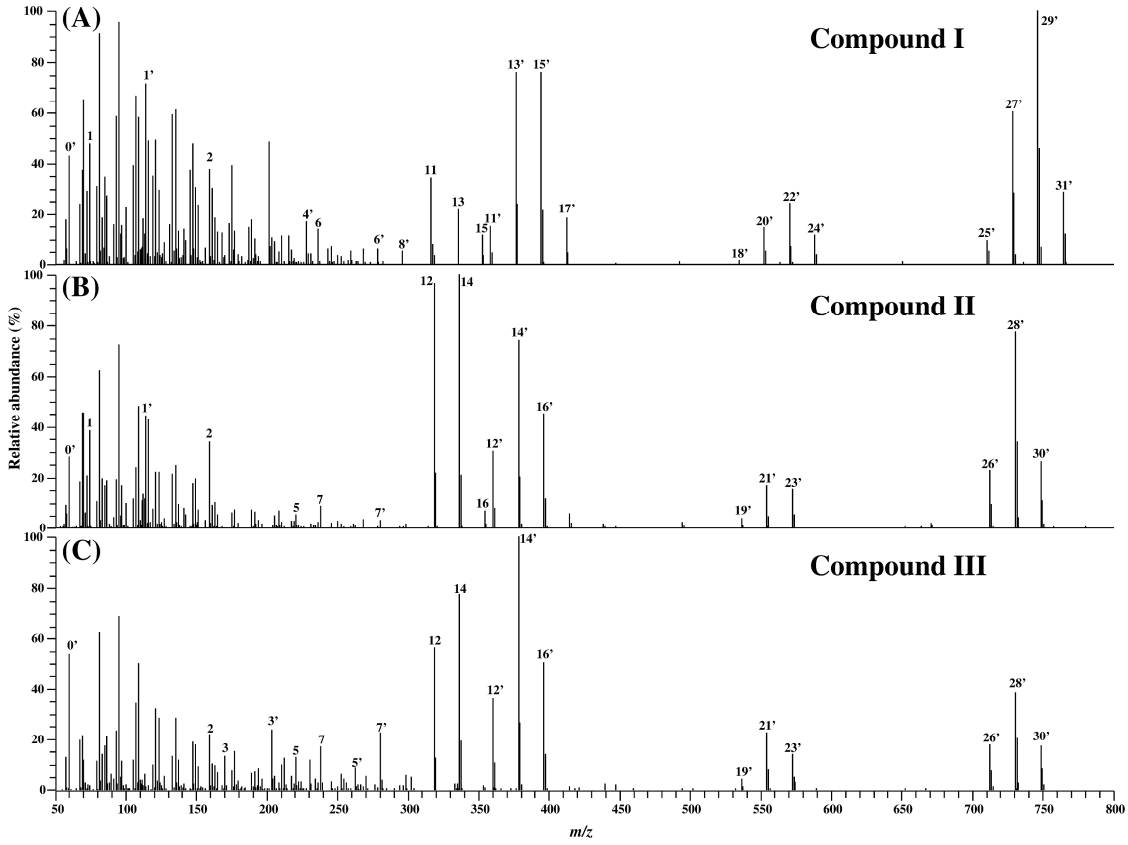



**Figure 3.5** Mass spectra of product ions of compounds I, II, and III. (A) Compound I, (B) compound II, and (C) compound III.

**Table 3.4** Characteristic signal assignment in the mass spectra of product ions of compounds I, II, and III.

| ID | Compound I | | | Compound II | | | Compound III | | |
|---|---|---|---|---|---|---|---|---|---|
| | Measured mass (*m*/*z*) | Calculated formula $[M+H]^+$ | Mass error (ppm) | Measured mass (*m*/*z*) | Calculated formula $[M+H]^+$ | Mass error (ppm) | Measured mass (*m*/*z*) | Calculated formula $[M+H]^+$ | Mass error (ppm) |
| 0' | 60.0444 | $C_2H_6NO$ | 0.21 | 60.0445 | $C_2H_6NO$ | 1.42 | 60.0444 | $C_2H_6NO$ | 0.72 |
| 1 | 74.0601 | $C_3H_8NO$ | 0.25 | 74.0601 | $C_3H_8NO$ | 0.97 | | | |
| 1' | 116.0706 | $C_5H_{10}NO_2$ | −0.35 | 116.0707 | $C_5H_{10}NO_2$ | 0.70 | | | |
| 2 | 159.0288 | $C_6H_7O_5$ | −0.04 | 159.0289 | $C_6H_7O_5$ | 0.53 | 159.0289 | $C_6H_7O_5$ | 0.44 |
| 3 | | | | | | | 170.1541 | $C_{10}H_{20}NO$ | 0.75 |
| 3' | | | | | | | 212.1646 | $C_{12}H_{22}NO_2$ | 0.21 |
| 4' | 228.1594 | $C_{12}H_{22}NO_3$ | −0.13 | | | | | | |
| 5 | | | | 220.2060 | $C_{15}H_{26}N$ | 0.29 | 220.2061 | $C_{15}H_{26}N$ | 0.42 |
| 5' | | | | | | | 262.2168 | $C_{17}H_{28}NO$ | 0.99 |
| 6 | 236.2003 | $C_{15}H_{26}NO$ | −2.38 | | | | | | |
| 6' | 278.2114 | $C_{17}H_{28}NO_2$ | −0.20 | | | | | | |
| 7 | | | | 238.2167 | $C_{15}H_{28}NO$ | 0.84 | 238.2164 | $C_{15}H_{28}NO$ | −0.78 |
| 7' | | | | 280.2264 | $C_{17}H_{30}NO_2$ | −2.70 | 280.2274 | $C_{17}H_{30}NO_2$ | 0.89 |
| 8' | 296.2205 | $C_{17}H_{30}NO_3$ | −2.07 | | | | | | |
| 11 | 316.2997 | $C_{22}H_{38}N$ | −0.61 | | | | | | |
| 11' | 358.3106 | $C_{24}H_{40}NO$ | 0.39 | | | | | | |
| 12 | | | | 318.3156 | $C_{22}H_{40}N$ | 0.08 | 318.3155 | $C_{22}H_{40}N$ | −0.02 |
| 12' | | | | 360.3261 | $C_{24}H_{42}NO$ | −0.12 | 360.3260 | $C_{24}H_{42}NO$ | −0.37 |
| 13 | 334.3102 | $C_{22}H_{40}NO$ | −0.78 | | | | | | |
| 13' | 376.3208 | $C_{24}H_{42}NO_2$ | −0.46 | | | | | | |
| 14 | | | | 336.3261 | $C_{22}H_{42}NO$ | 0.15 | 336.3260 | $C_{22}H_{42}NO$ | −0.21 |
| 14' | | | | 378.3367 | $C_{24}H_{44}NO_2$ | 0.12 | 378.3367 | $C_{24}H_{44}NO_2$ | 0.12 |
| 15 | 352.3202 | $C_{22}H_{42}NO_2$ | −2.40 | | | | | | |
| 15' | 394.3315 | $C_{24}H_{44}NO_3$ | −0.07 | | | | | | |
| 16 | | | | 354.3371 | $C_{22}H4_4NO_2$ | 1.34 | | | |
| 16' | | | | 396.3475 | $C_{24}H_{46}NO_3$ | 0.64 | 396.3473 | $C_{24}H_{46}NO_3$ | 0.25 |
| 17' | 412.3418 | $C_{24}H_{46}NO_4$ | −0.75 | | | | | | |
| 18' | 534.3431 | $C_{30}H_{48}NO_7$ | 1.14 | | | | | | |
| 19' | | | | 536.3582 | $C_{30}H_{50}NO_7$ | 0.08 | 536.3591 | $C_{30}H_{50}NO_7$ | 1.66 |
| 20' | 552.3516 | $C_{30}H_{50}NO_8$ | −2.67 | | | | | | |
| 21' | | | | 554.3691 | $C_{30}H_{52}NO_8$ | 0.72 | 554.3691 | $C_{30}H_{52}NO_8$ | 0.61 |
| 22' | 570.3637 | $C_{30}H_{52}NO_9$ | −0.02 | | | | | | |
| 23' | | | | 572.3793 | $C_{30}H_{54}NO_9$ | −0.06 | 572.3794 | $C_{30}H_{54}NO_9$ | 0.15 |
| 24' | 588.3752 | $C_{30}H_{54}NO_{10}$ | 1.63 | | | | | | |
| 25' | 710.3760 | $C_{36}H_{56}NO_{13}$ | 2.00 | | | | | | |
| 26' | | | | 712.3914 | $C_{36}H_{58}NO_{13}$ | 1.53 | 712.3902 | $C_{36}H_{58}NO_{13}$ | −0.10 |
| 27' | 728.3850 | $C_{36}H_{58}NO_{14}$ | −0.32 | | | | | | |
| 28' | | | | 730.4010 | $C_{36}H_{60}NO_{14}$ | 0.23 | 730.4014 | $C_{36}H_{60}NO_{14}$ | 0.82 |
| 29' | 746.3956 | $C_{36}H_{60}NO_{15}$ | −0.24 | | | | | | |
| 30' | | | | 748.4123 | $C_{36}H_{62}NO_{15}$ | 1.20 | 748.4118 | $C_{36}H_{62}NO_{15}$ | 0.54 |
| 31' | 764.4059 | $C_{36}H_{62}NO_{16}$ | −0.48 | | | | | | |

in Table 3.4, and the calculated formulae for ID numbers that are marked with an apostrophe in Table 3.4 contain an additional $C_2H_2O$ moiety.

For *m/z* values of 700–800, IDs 25′–31′ were observed at equal intervals. Because the difference between the calculated formulae pointed to the presence of hydroxyl groups, I concluded that compound I contained three hydroxyl groups and that compounds II and III contained two hydroxyl groups each. The same results were obtained for *m/z* values of 500–600 (IDs 18′–24′).

At *m/z* values of 300–450, the same signals (IDs 11–16) for the product ions of FB1, FB2, and FB3 were observed in addition to signals corresponding to FB1, FB2, and FB3 with an additional $C_2H_2O$ moiety (IDs 11′–17′). It was assumed that cleavage of $C_2H_2O$ in compounds I, II, and III produced the same fragment ions as in FB1, FB2, and FB3. This assumption was also made for *m/z* values of 50–300, where IDs 1–7 as well as IDs 1′–7′ (that were generated by the cleavage of $C_2H_2O$) were observed. In addition, ID 2, which was a product ion common to FB1, FB2, and FB3, was observed in the case of compounds I, II, and III. Because ID 2 represented TCA in the product ion mass spectra of FB1, FB2, and FB3, it was presumed that TCA was also a part of compounds I, II, and III. Furthermore, different fragment ions depending on the positions of the hydroxyl groups were observed in the spectra of compounds I, II, and III. This pattern

ischaracteristic of the fragmentation of FB1, FB2, and FB3. Spectra of compound I revealed that IDs 1′ and 4′ were formed, presumably via cleavage at C-10 and C-5, respectively.

Compound II contained ID 1′ but not spectra such as IDs 3′ and 4′, whereas compound III contained ID 3′ but not ID 1′. According to these observations, it was assumed that the hydroxyl groups were bound to compound I at C-5 and C-10, to compound II at C-10, and to compound III at C-5.

I hypothesized that compounds I, II, and III contained TCA moieties, hydroxyl groups, and $C_2H_2O$ moiety and that the fragmentation of compounds I, II, and III would be similar to that of FB1, FB2, and FB3, respectively. Because compounds I, II, and III may have structure similar to that of the fumonisin B-series, containing an additional $C_2H_2O$ moiety, these compounds may have been FA1, FA2, and FA3, which are *N*-acetyl derivatives of FB1, FB2, and FB3, respectively. In order to confirm this hypothesis, I synthesized FA1, FA2, and FA3 from the standards of FB1, FB2, and FB3, respectively, and compared the product ions of compounds I, II, and III with those of the synthesized FA1, FA2, and FA3, respectively.

### 3.3.4 Characterization of compounds I, II, and III using FA1, FA2, and FA3 standards

The acetylated derivative of FB1 (acetyl-FB1), which was synthesized from the FB1 standard, was analyzed by LC-Orbitrap MS. The measured mass, theoretical mass, calculated formulae, and mass error were 764.4087, 764.4063, $C_{36}H_{62}NO_{16}$, and 3.11 ppm, respectively. NMR analysis of the synthesized product indicated a ~3.9 ppm chemical shift of the proton at C-2. Because the chemical shift of the proton at C-2 was found to be ~3.1 ppm for FB1, this result confirmed that the synthesized compound was an *N*-acetyl derivative of FB1. The chemical shifts (δ) for other protons in the $^{1}$H NMR (MeOH-$d_4$) data were 1.002 (t, $J$ = 0.012 Hz, 3H), 1.025–1.100 (m, 6H), 1.235 (d, $J$ = 0.012 Hz, 3H), 1.323–1.632 (m, 18H), 1.690–1.852 (m, 2H), 1.917 (brs, 1H), 2.052 (s, 3H), 2.573–2.944 (m, 8H), 3.254–3.335 (m, 2H), 3.719 (brs, 1H), 3.852–3.909 (m, 2H), 3.957–4.020 (m, 1H), 5.069 (dd, $J$ = 0.005, 0.014 Hz, 1H), and 5.259 (td, $J$ = 0.005, 0.018 Hz, 1H). These results were in agreement with the values observed for FB1 and those determined in previous studies [2, 12]. On the basis of these findings, the acetyl-FB1 was identified as FA1. Purity of the synthesized FA1 was found to be 87.0%.

The acetyl-FB2 was analyzed by LC-Orbitrap MS; a measured mass of 748.4120

was obtained, and the calculated formula was $C_{36}H_{62}NO_{15}$, with theoretical mass of 748.4114 and the mass error 0.77 ppm. FB2 and acetyl-FB2 were analyzed by NMR; a peak corresponding to the C-2 proton of FB2 was observed at 3.2 ppm, while a peak for the C-2 proton of acetyl-FB2 was observed at 3.9 ppm. This chemical shift was similar to that reported for FB1 and FA1, which is the acetylated form of FB1. The NMR results suggested that an *N*-acetyl group was bound to C-2 of acetyl-FB2. Additionally, other chemical shifts [$^1$H NMR (MeOH-$d_4$)] were observed at 0.790–1.010 (m, 9H), 1.139 (d, $J$ = 0.012 Hz, 3H), 1.160–1.490 (m, 20H), 1.452–1.608 (m, 2H), 1.671 (brs, 1H), 1.959 (s, 3H), 2.473–2.819 (m, 8H), 3.120–3.220 (m, 2H), 3.747–3.820 (m, 2H), 3.850–3.925 (m, 1H), 5.181 (d, $J$ = 0.021 Hz, 1H), and 5.349 (t, $J$ = 0.008 Hz, 1H). Thus, acetyl-FB2 was identified as FA2. The purity of FA2 was 60.4%.

Similarly, the acetyl-FB3 was analyzed by Orbitrap MS and the following data were obtained: measured mass of 748.4122, theoretical mass of 748.4114, calculated formula $C_{36}H_{62}NO_{15}$, and the mass error 1.03 ppm. In $^1$H NMR data, a chemical shift of the C-2 proton appeared at 3.1 ppm for FB3 and at 3.9 ppm for acetyl-FB3. Additional chemical shifts [$^1$H NMR (MeOH-$d_4$)] were observed at 0.875–0.980 (m, 9H), 1.127 (d, $J$ = 0.011 Hz, 3H), 1.160–1.520 (m, 20H), 1.650–1.750 (m, 2H), 1.834 (brs, 1H), 1.954 (s, 3H), 2.430–2.830 (m, 8H), 3.130–3.215 (m, 2H), 3.630–3.720 (m, 2H), 3.875–3.950

(m, 1H), 5.151 (td, $J$ = 0.005, 0.018 Hz, 1H), and 5.349 (t, $J$ = 0.008 Hz, 1H). Thus, acetyl-FB3 was identified as FA3. Its purity was 66.5%.

The chromatograms and product ion spectra for compounds I, II, and III in MTC-9999E as well as the standards of FA1, FA2, and FA3, respectively, obtained by LC-Orbitrap MS are shown in Figures 3.6–3.9. Significant signals in the spectra are labeled with ID numbers corresponding to the numbers in Tables 3.5–3.7, which show the measured mass, theoretical mass, calculated formula, and mass error for each key signal in those spectra. The retention time and product ion spectra for compound I and FA1, compound II and FA2, and compound III and FA3 were in good agreement; therefore, compounds I, II, and III were identified as *N*-acetyl derivatives of FB1, FB2, and FB3, to be precise, as FA1, FA2, and FA3, respectively.

### 3.3.5 Method validation

Extraction with a QuEChERS kit followed by purification using a MultiSep 229 Ochra cartridge was performed for sample preparation. This method was previously used for the determination of FB1, FB2, and FB3 (in Chapter 2) and thus was considered a

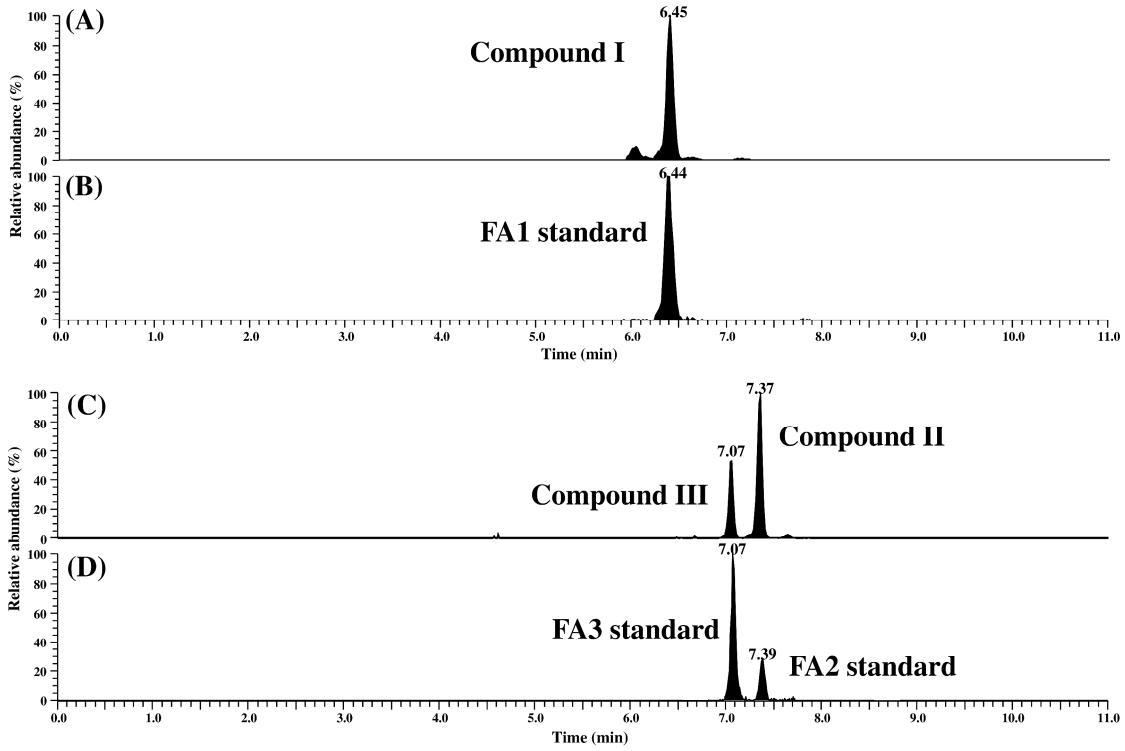



**Figure 3.6** Chromatograms of compounds I, II, and III, and standards of FA1, FA2, and FA3. (A) Compound I, (B) FA1 standard, (C) compounds II and III, and (D) standards of FA2 and FA3.

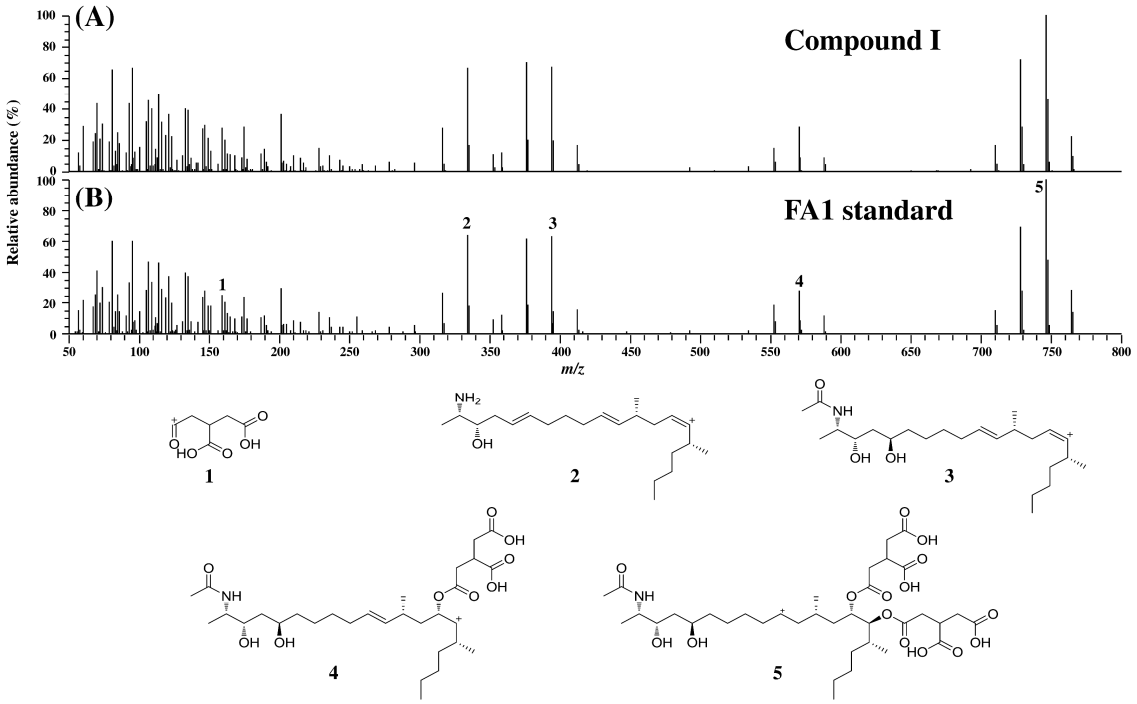



**Figure 3.7** Product ion spectra of compound I and FA1, and characteristic assignment of signals for FA1. (A) A product ion spectrum of compound I and (B) product ion spectrum of FA1.

**Table 3.5** Characteristic assignment of signals of the product ion spectrum of FA1.

| ID | Measured mass (*m/z*) | Theoretical mass (*m/z*) | Calculated formula $[M+H]^+$ | Mass error (ppm) |
|---|---|---|---|---|
| 1 | 159.0289 | 159.0288 | $C_6H_7O_5$ | 0.05 |
| 2 | 334.3103 | 334.3104 | $C_{22}H_{40}NO$ | −0.17 |
| 3 | 394.3318 | 394.3316 | $C_{24}H_{44}NO_3$ | 0.25 |
| 4 | 570.3637 | 570.3637 | $C_{30}H_{52}NO_9$ | 0.05 |
| 5 | 746.3961 | 746.3966 | $C_{36}H_{60}NO_{15}$ | 0.31 |

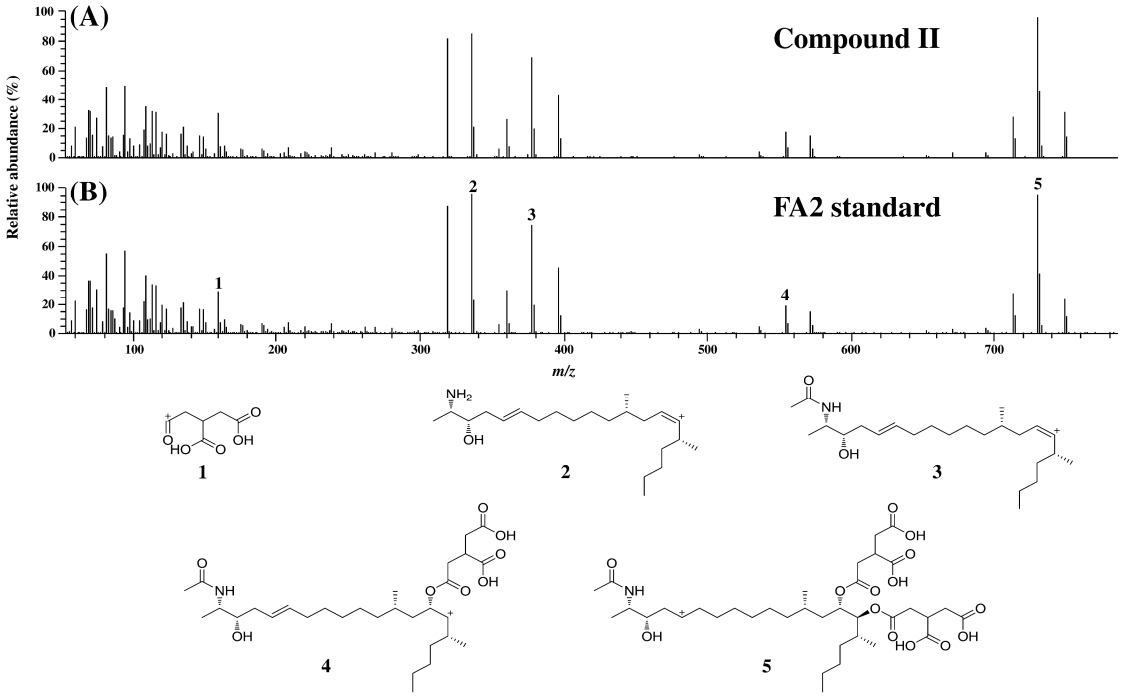



**Figure 3.8** Product ion spectra of compound II and FA2, and characteristic assignment of signals for FA2. (A) Product ion spectrum of compound II; (B) product ion spectrum of FA2.

**Table 3.6** Characteristic assignment of signals of the product ion spectrum of FA2.

| ID | Measured mass (*m/z*) | Theoretical mass (*m/z*) | Calculated formula $[M+H]^+$ | Mass error (ppm) |
|---|---|---|---|---|
| 1 | 159.0285 | 159.0288 | $C_6H_7O_5$ | −1.77 |
| 2 | 336.3266 | 336.3261 | $C_{22}H_{42}NO$ | 1.60 |
| 3 | 378.3369 | 378.3367 | $C_{24}H_{44}NO_2$ | 0.68 |
| 4 | 554.3693 | 554.3687 | $C_{30}H_{52}NO_8$ | 1.05 |
| 5 | 730.4014 | 730.4008 | $C_{36}H_{60}NO_{14}$ | −0.32 |

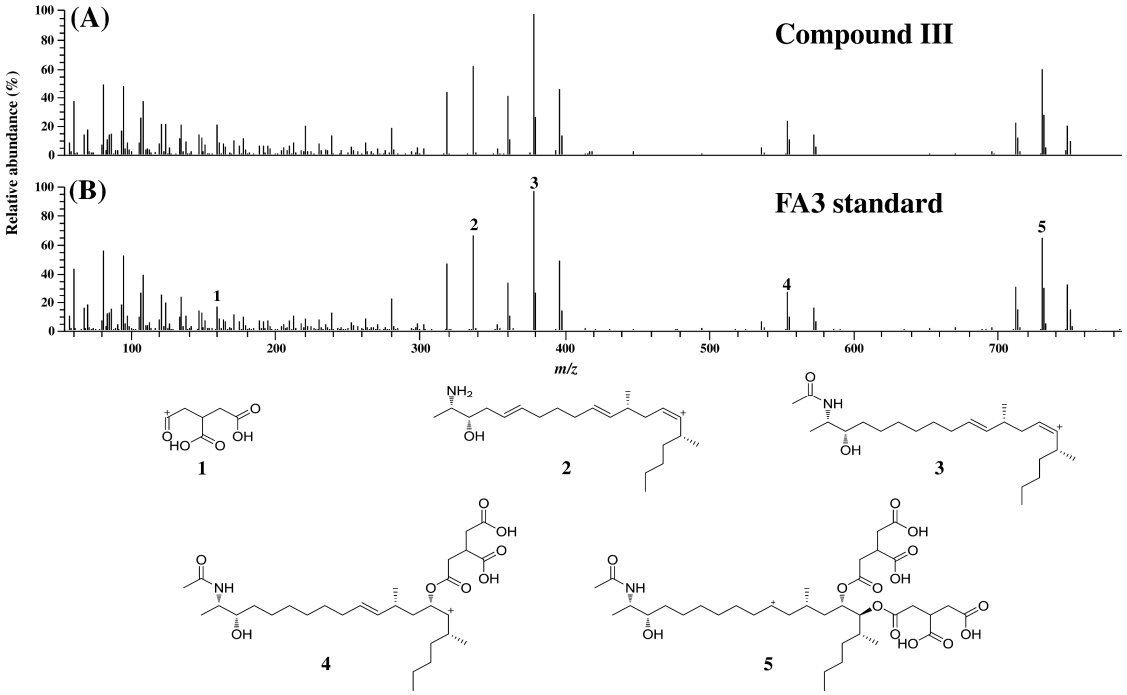



**Figure 3.9** Product ion spectra of compound III and FA3 and characteristic assignment of signals for FA3. (A) A product ion spectrum of compound III and (B) product ion spectrum of FA3.

**Table 3.7** Characteristic assignment of signals of the product ion spectrum of FA3.

| ID | Measured mass (*m/z*) | Theoretical mass (*m/z*) | Calculated formula $[M+H]^+$ | Mass error (ppm) |
|---|---|---|---|---|
| 1 | 159.0290 | 159.0288 | $C_6H_7O_5$ | 1.21 |
| 2 | 336.3259 | 336.3261 | $C_{22}H_{42}NO$ | −0.67 |
| 3 | 378.3366 | 378.3367 | $C_{24}H_{44}NO_2$ | −0.12 |
| 4 | 554.3700 | 554.3687 | $C_{30}H_{52}NO_8$ | 2.26 |
| 5 | 730.4009 | 730.4008 | $C_{36}H_{60}NO_{14}$ | 0.06 |

valid method for determination of FA1, FA2, and FA3. Accuracy of the method for quantification of FA1, FA2, FA3, FB1, FB2, and FB3 was evaluated using the prepared corn sample. The results are shown in Table 3.8. The linearity, recovery, and repeatability were acceptable: >0.994, 83–105%, and 3.7–9.5%, respectively. The LOQs of target fumonisins with this method were defined as the lowest calibration levels (i.e., 5 μg/kg). These results suggested that I successfully developed an acceptable method for simultaneous quantification of FA1, FA2, FA3, FB1, FB2, and FB3 in corn.

### 3.3.6 Quantification of FA1, FA2, FA3, FB1, FB2, and FB3 in corn

Concentrations of FA1, FA2, FA3, FB1, FB2, and FB3 in corn samples were determined by a simultaneous analysis. MTC-9999E, MTC-9990, and FC-443, which are contaminated with mycotoxins (including FB1, FB2, and FB3), were selected as the analytical samples. Because the individual concentrations of FB1 and FB2 in MTC-9999E exceeded the range of the calibration curves, they were diluted 10-fold. Additionally, seven samples of commercially available corn contaminated with FB1, FB2, and FB3 (C-1 to C-7) were evaluated as reported in Chapter 2. The results are shown in

**Table 3.8** Performance of the method.

| Mycotoxin | Linearity (*r*) [a)] | Recovery (%) [b)] | Repeatability (%) [b)] | LOQ (μg/kg) | Retention time (min) |
|---|---|---|---|---|---|
| FA1 | 0.9996 | 83 | 2.7 | 5 | 6.44 |
| FA2 | 0.9999 | 86 | 9.5 | 5 | 7.39 |
| FA3 | 0.9993 | 95 | 6.3 | 5 | 7.07 |
| FB1 | 0.9960 | 102 | 5.3 | 5 | 5.91 |
| FB2 | 0.9946 | 105 | 3.7 | 5 | 6.68 |
| FB3 | 0.9962 | 104 | 7.1 | 5 | 6.26 |

[a)] The concentration range of linearity, 5–5,000 μg/kg. [b)] $n$ = 5; the samples were spiked with mycotoxins at 50 μg/kg.

Table 3.9. The analysis revealed that the 10 corn samples that are contaminated with FB1, FB2, and FB3 are also contaminated with FA1, FA2, and FA3.

In MTC-9999E, which contained the largest amounts of FB1, FB2, and FB3, the contaminants belonging to the fumonisin A-series were also observed at relatively high concentrations, particularly 4.18 mg/kg for FA1, 4.03 mg/kg for FA2, and 269 μg/kg for FA3. Additionally, 7.99–62.5 μg/kg FA1, (<5) to 84.2 μg/kg FA2, and (<5) to 30.6 μg/kg FA3 were detected in commercially available corn. This result confirmed that samples contaminated with fumonisin B-series were also contaminated with the fumonisin A-series. Because fumonisin A-series are produced by *Fusarium moniliforme, F. verticillioides, F. proliferatum,* and *F. nygami* [1–5], the analyzed corn samples were likely contaminated with these fungi. Although some researchers have demonstrated the presence of the fumonisin A-series in *Fusarium* cultures, this thesis is the first report to describe identification and quantification of FA1, FA2, and FA3 in corn samples. Because the link between the toxicity and mechanism of action of fumonisins is unknown, further studies on fumonisins and their derivatives are needed.

**Table 3.9** Concentrations of fumonisins in corn samples.

| Sample | Concentration of fumonisins (μg/kg) | | | | | |
|---|---|---|---|---|---|---|
| | **FA1** | **FA2** | **FA3** | **FB1** | **FB2** | **FB3** |
| MTC-9999E | 4.18 [a)] | 4.03 [a)] | 269 | 28.6 [a)] | 8.87 [a)] | 2.03 [a)] |
| MTC-9990 | 256 | 222 | 30.2 | 1.23 [a)] | 320 | 189 |
| FC-443 | 501 | 489 | 85.2 | 2.66 [a)] | 715 | 358 |
| C-1 | 62.5 | 45.0 | 30.6 | 661 | 115 | 53.4 |
| C-2 | 10.6 | 6.64 | <5 | 309 | 37.2 | 19.8 |
| C-3 | <5 [b)] | <5 | <5 | 90.4 | 21.3 | 11.5 |
| C-4 | 42.4 | 23.4 | 8.73 | 462 | 86.4 | 52.1 |
| C-5 | 59.7 | 84.2 | 23.4 | 1.18 [a)] | 276 | 182 |
| C-6 | 17.9 | 11.9 | 5.27 | 385 | 43.3 | 32.9 |
| C-7 | 7.99 | 5.90 | <5 | 151 | 16.4 | 12.2 |

[a)] Concentration unit, mg/kg. [b)] "<5" means a peak detected under the LOQ (i.e., 5 μg/kg).

### 3.4 Summary

Identification of three compounds detected in a corn sample contaminated with mycotoxins (MTC-9999E) was performed by high-resolution LC-Orbitrap MS. The highlights are as follows:

- Because the compounds were hypothesized to be FA1, FA2, and FA3 (which are *N*-acetyl derivatives of the fumonisin B-series), FA1, FA2, and FA3 were synthesized by acetylating FB1, FB2, and FB3, respectively. Comparative analysis of the retention time and product ion spectra of the detected compounds and of the synthesized FA1, FA2, and FA3 confirmed the compounds to be *N*-acetyl derivatives of FB1, FB2, and FB3, to be precise: FA1, FA2, and FA3.
- A method for simultaneous quantification of the six fumonisins—FA1, FA2, FA3, FB1, FB2, and FB3—was examined. Corn samples were prepared using a QuEChERS kit for extraction and MultiSep 229 Ochra cartridge for purification. The linearity, recovery, and repeatability were found to be >0.994, 83–105%, and 3.7–9.5%, respectively. Thus, I successfully developed a valid method for simultaneous quantification of FA1, FA2, FA3, FB1, FB2, and FB3 in corn.
- The simultaneous quantification of the six fumonisins revealed that the 10 corn

samples that are contaminated with FB1, FB2, and FB3 are also contaminated with FA1, FA2, and FA3. Although some researchers have detected fumonisin A-series in *Fusarium* cultures, this is the first report to describe identification and quantification of FA1, FA2, and FA3 in corn samples.

- According to the results of this study, corn marketed for consumption may be contaminated not only with fumonisin B-series but also with the fumonisin A-series. Because the relation between the toxicity and mechanism of action of fumonisins is unknown, further studies on fumonisins and their derivatives are needed.

## 3.5 References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# Chapter 4

# The method for simultaneous determination of 20 *Fusarium* toxins in cereals by LC-Orbitrap MS with a pentafluorophenyl (PFP) column

## 4.1 Introduction

In Chapters 2 and 3, various *Fusarium* toxins (trichothecenes, fumonisins, and zearalenone) were detected in corn samples. It is known that there are derivatives of *Fusarium* toxins with equal or higher toxicity and similar structure. Among trichothecenes, derivatives of DON, i.e., 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), are converted to DON (by deacetylation) *in vivo* and exert toxic effects comparable to those of DON. Therefore, PMTDI was set to 1 μg/kg-bw/day for DON and its acetylated derivatives (3-ADON and 15-ADON) by the JECFA in 2011 [1]. In the zearalenone-group, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), and β-zearalanol (β-ZAL) are known to be reduced metabolites of ZEN [2, 3]. Their affinity for estrogenic receptors is ranked in the following order: α-ZAL＞α-ZEL＞β-ZAL＞ZEN＞β-ZEL, implying that the metabolism of ZEN to α-ZEL

and α-ZAL results in a stronger estrogenic effect. A fungus of the *Rhizopus* species, which was found in cereals during storage [4], is able to transform ZEN to α-ZEL [5]. Thus, there is a risk that derivatives of ZEN may be present in cereals.

Co-eluting isomers are hardly distinguished by MS/MS because they share similar structures and the same molecular weight. As described in this doctoral thesis, *Fusarium* toxins include three pairs of regioisomers, namely 3-ADON/15-ADON, FB2/FB3, and FA2/FA3, and two pairs of stereoisomers, i.e., α-ZEL/β-ZEL and α-ZAL/β-ZAL. For accurate determination, it is necessary to separate these isomers by LC. The existing analytical methods for *Fusarium* toxin isomers are based on determination of only a limited number of isomeric pairs {e.g., 3-ADON/15-ADON and FB2/FB3 [6], FB2/FB3 and FA2/FA3 (Chapter 3), α-ZEL/β-ZEL and α-ZAL/β-ZAL [7], and 3-ADON/15-ADON, FB2/FB3, and α-ZEL/β-ZEL [8]}. To date, there is no method for simultaneous determination of the three groups of *Fusarium* toxins (trichothecenes, fumonisins, and the zearalenone-group) including five pairs of isomers, namely, 3-ADON/15-ADON, FB2/FB3, FA2/FA3, α-ZEL/β-ZEL, and α-ZAL/β-ZAL. Such a simultaneous determination method is highly desirable because of the risk of co-contamination of cereals with *Fusarium* toxins of different groups.

Hence, in this chapter, a method for simultaneous determination of 20 *Fusarium*

toxins, including the isomers, is examined by using LC-Orbitrap MS. The mycotoxins tested are NIV, fusarenon-X (FUX), DON, 3-ADON, 15-ADON, HT-2, T-2, neosolaniol (NEO), diacetoxyscirpenol (DAS), FB1, FB2, FB3, FA1, FA2, FA3, ZEN, α-ZEL, β-ZEL, α-ZAL, and β-ZAL (Figure 4.1). In addition, the newly developed method was used to determine the 20 *Fusarium* toxins in cereal samples purchased in the market.

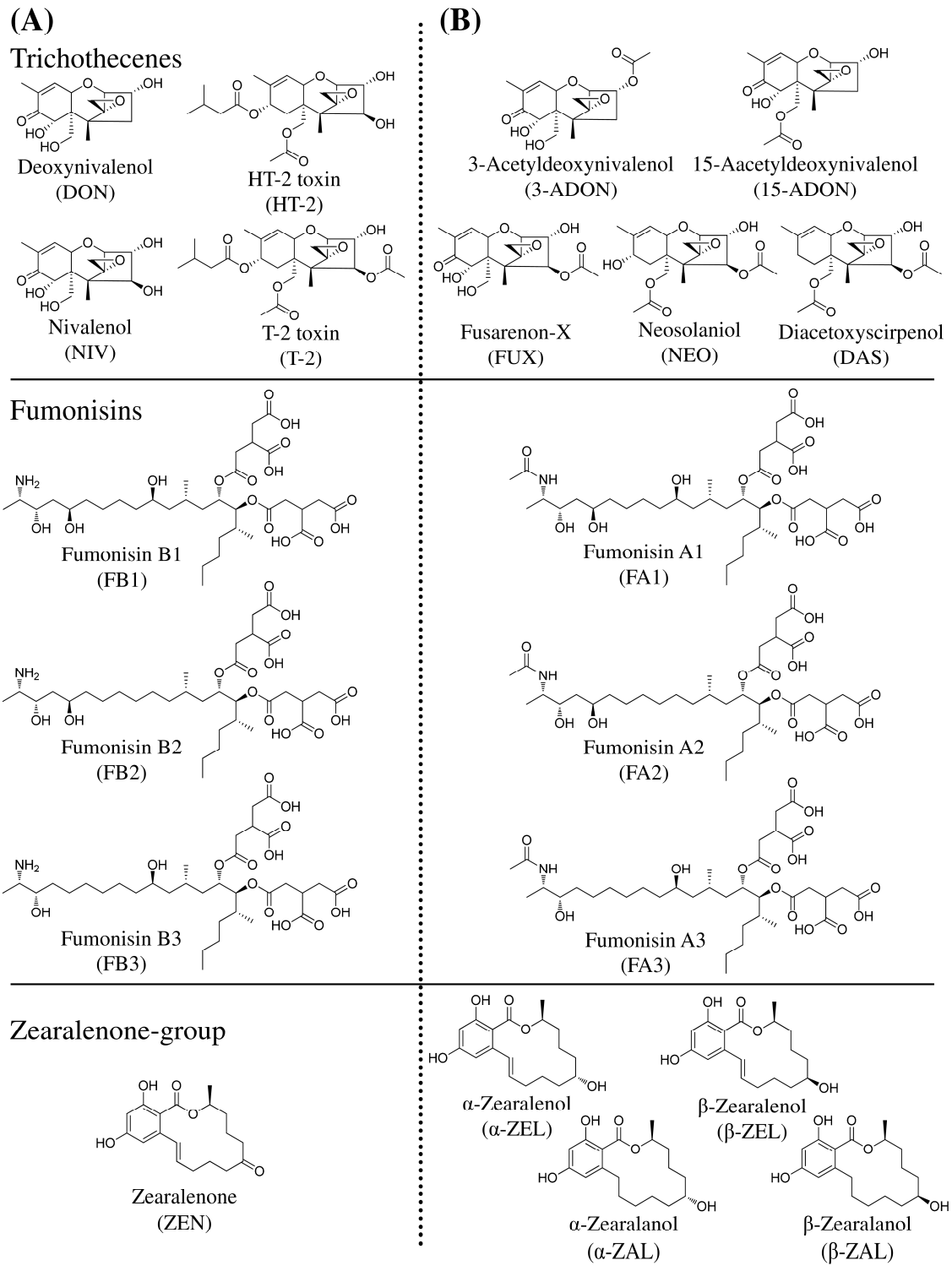


**Figure 4.1** Structures of *Fusarium* toxins. (A) Key *Fusarium* toxins and (B) derivatives of the key *Fusarium* toxins.

## 4.2 Experimental section

### 4.2.1 Samples and reagents

Thirty-four cereal samples, including 13 corn samples (grits and flour; C-1 to C-13), 12 wheat samples (polished grains and flour; W-1 to W-12), and nine barley samples (polished grains and flour, B-1 to B-9), were purchased at local supermarkets in Japan in 2015. Reference corn samples (DC-617, FC-443, ZC-327, MTC-9990, and MTC-9999E), which are naturally contaminated with mycotoxins, were acquired from Trilogy Analytical Laboratory. The acceptance limits of FB1, FB2, and FB3 in the reference materials, with the incorporated uncertainties, are shown in Table 4.1.

MeOH (LC/MS grade), MeCN (analytical grade), acetic acid (guaranteed reagent grade), and ammonium acetate (analytical grade) were purchased from Kanto Chemical Co., Inc. Water was purified using a Millipore Milli-Q system. The Q-sep Q 110 QuEChERS extraction kit was purchased from RESTEK. A MultiSep 229 Ochra cartridge was acquired from Romer Labs Corp. A PTFE filter (mesh pore size 0.20 μm) was purchased from Advantec Toyo Kaisha, Ltd. A Pierce LTQ Velos ESI Positive Ion

**Table 4.1** Acceptance limits of *Fusarium* toxins in mycotoxin reference materials.

| Sample | Acceptance limit (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **DON** | **HT-2** | **T-2** | **FB1** | **FB2** | **FB3** | **ZEN** |
| DC-617 | 4.2–6.4 | | | | | | |
| FC-443 | [a] | | | 2.3–4.9 | 0.5–0.1 | 0.2–0.4 | |
| ZC-327 | | | | | | | 1.1–1.9 |
| MTC-9990 | 1.6–2.2 | | | 1.0–1.6 | 0.1–0.3 | | |
| MTC-9999E | 2.2–3.0 | 0.3–0.7 | 0.2–0.4 | 20.7–35.9 | 5.2–9.0 | 1.2–2.3 | 0.3–0.5 |

[a] The blank cells indicate that no mycotoxin was certified.

Calibration Solution for positive mode calibration of the Orbitrap MS was acquired from Thermo Fisher Scientific.

The chromatographic separations of the 20 *Fusarium* toxins using the following analytical columns were compared: Mastro C18 (2.1 × 150 mm, 3 μm; Shimadzu GLC, Ltd.), Mastro PFP (2.1 × 150 mm, 3 μm; Shimadzu GLC, Ltd.), ACQUITY UPLC CSH Fluoro-Phenyl (2.1 × 150 mm, 1.7 μm; Waters), and Discovery HS F5 (2.1 × 150 mm, 3 μm; Supelco).

The standard solutions of NIV (100 μg/mL in MeCN), FUX (100 μg/mL in MeCN), DON (100 μg/mL in MeCN), 3-ADON (100 μg/mL in MeCN), 15-ADON (100 μg/mL in MeCN), HT-2 (100 μg/mL in MeCN), T-2 (100 μg/mL in MeCN), NEO (100 μg/mL in MeCN), and DAS (100 μg/mL in MeCN) were purchased from Wako Pure Chemical Ind., Ltd., whereas those of FB1 (50 μg/mL in MeCN/water, 1:1 v/v), FB2 (50 μg/mL in MeCN/water, 1:1 v/v), FB3 (50 μg/mL in MeCN/water, 1:1 v/v), ZEN (100 μg/mL in MeCN), α-ZEL (10 μg/mL in MeCN), β-ZEL (10 μg/mL in MeCN), α-ZAL (10 μg/mL in MeCN), and β-ZAL (10 μg/mL in MeCN) were acquired from Romer Labs Corp. FA1, FA2, and FA3 were prepared by acetylation of the FB1, FB2, and FB3 standards, respectively, as described in Chapter 3.

### 4.2.2 Sample preparation

Sample preparation was carried out as previously described (in Chapters 2 and 3). In particular, corn grits and polished grains were ground beforehand in a Labo Milser LM-PLUS (Iwatani). A 2.5-g sample was placed in a 50-mL polypropylene centrifuge tube, and 20 mL of 2% acetic acid/MeCN (1:1, v/v) was added. The samples were mixed at 250 rpm on a shaker (SR-2 DS; Taitec) for 1 h. The contents of Q-sep Q110 were then added to the centrifuge tube. The mixture was vortexed for 20 s and centrifuged at 1,580 × $g$ for 5 min. The supernatant (MeCN phase) was frozen at −30 °C for 1 h and then centrifuged at 1,580 × $g$ for 5 min. Next, 5 mL of the supernatant, 1 mL of water, and 60 μL of acetic acid were mixed, and the mixture was applied to the MultiSep 229 Ochra cartridge. The eluate (4 mL) was dried at 40°C under a nitrogen stream and dissolved in 400 μL of 10 mM ammonium acetate/MeCN (85:15, v/v). Each sample was passed through a 0.20-μm PTFE filter immediately prior to LC-Orbitrap MS analysis.

### 4.2.3 LC-Orbitrap MS analysis

LC-Orbitrap MS analysis was performed on an Ultimate 3000 system coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). The Xcalibur 2.2 software (Thermo Fisher Scientific) was used to control the instruments and to process the data.

LC was performed using 10 mM ammonium acetate as solvent A and 2% acetic acid in MeOH as solvent B. The gradient profile was 20% B (0 min), 40% B (1–2 min), 60% B (2 min), 70% B (9 min), 95% B (9–12 min), and 20% B (12–15 min). The flow rate was set to 0.3 mL/min, and the column temperature was maintained at 40°C. The chromatographic separation was conducted on a Mastro PFP (2.1 × 150 mm, 3 μm) with the injection volume of 5 μL.

The Q-Exactive mass spectrometer was operated in positive mode with a heated ESI source (HESI-II) and the spray voltage of 3.00 kV. Capillary and heater temperatures were fixed at 350°C and 300°C, respectively. The sheath gas and the auxiliary gas flow rates were set to 40 and 10 arbitrary units, respectively. The mass calibration was performed as described in Chapter 3, namely, (1) calibration of the instrument was performed before each sequence using a calibration solution; (2) the lock masses (*m/z* values of 188.98461 and 537.87906) were usually detected during the whole

chromatographic run and were used for mass correction during the sequence. The precursor ion scan was carried out in full MS mode at the resolution of 140,000 for the *m/z* value of 200 (3 scans/s), with an AGC target of 3e6, maximum IT of 100 ms, and a scan range of 100–1,000 *m/z*. For quantification, ammonium adduct ions $[M+NH_4]^+$ were selected for HT-2, T-2, NEO, and DAS, whereas proton adduct ions $[M+H]^+$ were selected for the other mycotoxins under study because of the high sensitivity in positive mode. To evaluate the presence of the mycotoxins in question, a product ion scan was conducted in targeted $MS^2$ mode at the resolution of 140,000 for the *m/z* value of 200, AGC target of 2e5, maximum IT of 200 ms, NCE of 30 eV, stepped NCE of 50%, and a scan range of 50–800 *m/z*. Table 4.2 shows the parameters used for quantification and certification of the 20 *Fusarium* toxins by LC-Orbitrap MS.

### 4.2.4 Method validation

The method was validated by evaluating the linearity, recovery, and repeatability. The coefficient of linearity was calculated from the calibration curves of the standard addition method; they were constructed by plotting the areas of the prepared samples

**Table 4.2** Parameters for LC-Orbitrap MS analysis of the 20 *Fusarium* toxins.

| ***Fusarium* toxin** | **Analyzed ion** | **Precursor ion for quantification (*m/z*)** | **Product ion for certification (*m/z*)** | **Retention time (min)** |
|---|---|---|---|---|
| NIV | $[M+H]^+$ | 313.12818 | 137.05971/295.11761 | 2.99 |
| FUX | $[M+H]^+$ | 355.13874 | 137.05971/247.09649 | 4.55 |
| DON | $[M+H]^+$ | 297.13326 | 203.10666/249.11214 | 3.85 |
| 3-ADON | $[M+H]^+$ | 339.14383 | 203.10666 /231.10157 | 5.50 |
| 15-ADON | $[M+H]^+$ | 339.14383 | 137.05971 /321.13326 | 5.35 |
| HT-2 | $[M+NH_4]^+$ | 442.24354 | 215.10666/263.12779 | 7.88 |
| T-2 | $[M+NH_4]^+$ | 484.25411 | 185.09609/215.10666 | 9.88 |
| NEO | $[M+NH_4]^+$ | 400.19659 | 215.10666/305.13835 | 4.64 |
| DAS | $[M+NH_4]^+$ | 384.20168 | 247.13287/307.15400 | 6.49 |
| FB1 | $[M+H]^+$ | 722.39575 | 334.31044/352.32101 | 8.73 |
| FB2 | $[M+H]^+$ | 706.40083 | 318.31553/336.32609 | 11.57 |
| FB3 | $[M+H]^+$ | 706.40083 | 318.31553/336.32609 | 10.67 |
| FA1 | $[M+H]^+$ | 764.40631 | 728.38518/746.39575 | 8.21 |
| FA2 | $[M+H]^+$ | 748.41140 | 318.31553/730.40083 | 11.11 |
| FA3 | $[M+H]^+$ | 748.41140 | 336.32609/378.33666 | 10.42 |
| ZEN | $[M+H]^+$ | 319.15400 | 187.07536/283.13287 | 11.95 |
| α-ZEL | $[M+H]^+$ | 321.16965 | 189.09101/303.15909 | 11.78 |
| β-ZEL | $[M+H]^+$ | 321.16965 | 285.14852/303.15909 | 10.77 |
| α-ZAL | $[M+H]^+$ | 323.18530 | 123.04406/305.17474 | 11.44 |
| β-ZAL | $[M+H]^+$ | 323.18530 | 189.09101/305.17474 | 9.61 |

(spiked with the 20 *Fusarium* toxins) versus the analyte concentrations. The concentrations of *Fusarium* toxins added to the test samples were 5, 10, 50, 100, 500, 1,000, and 5,000 μg/kg. Recovery was assessed using samples spiked with each of the 20 *Fusarium* toxins. Repeatability was assessed by calculating the RSD of five measurements on a single day. For recovery and repeatability studies, the test samples were spiked with each *Fusarium* toxin (final concentration 100 μg/kg) before the extraction process. As described in Chapter 3, the limit of detection and LOQ are not applicable to high-resolution mass spectrometric methods because high mass accuracy yields only limited noise [9], which is sometimes not detectable. Nevertheless, to ensure proper quantification, a certain degree of confidence is required. Thus, in this method, the LOQ was defined as the lowest calibration level (i.e., 5 μg/kg).

## 4.3 Results and Discussion

### 4.3.1 Separation of 20 *Fusarium* toxins on the PFP column

Initially, LC separation was examined using a C18 column, Mastro C18, as described in Chapter 2. Separation of the 20 *Fusarium* toxins was attempted by means of 10 mM ammonium acetate and 2% acetic acid in MeOH as mobile phases. The chromatograms of 200-μg/L standards in a neat solvent on the C18 column are shown in Figure 4.2. In the LC condition, 3-ADON and 15-ADON were not completely separated. Thus, the PFP column (Mastro PFP) was tested. The PFP column enables separation of regio- and stereoisomers by electrostatic interactions with the fluorine atoms in the functional groups on the support [10, 11]. The chromatograms of 200-μg/L standards in a neat solvent for the PFP column are shown in Figure 4.3. All 20 *Fusarium* toxins were completely separated with good peak shapes. Separation of 3-ADON and 15-ADON on the PFP column can be attributed to the different position of their hydroxyl groups: 3-ADON, in which the hydroxyl groups are closer to each other, showed a stronger electrostatic interaction with the PFP functional group, as compared to 15-ADON [12].

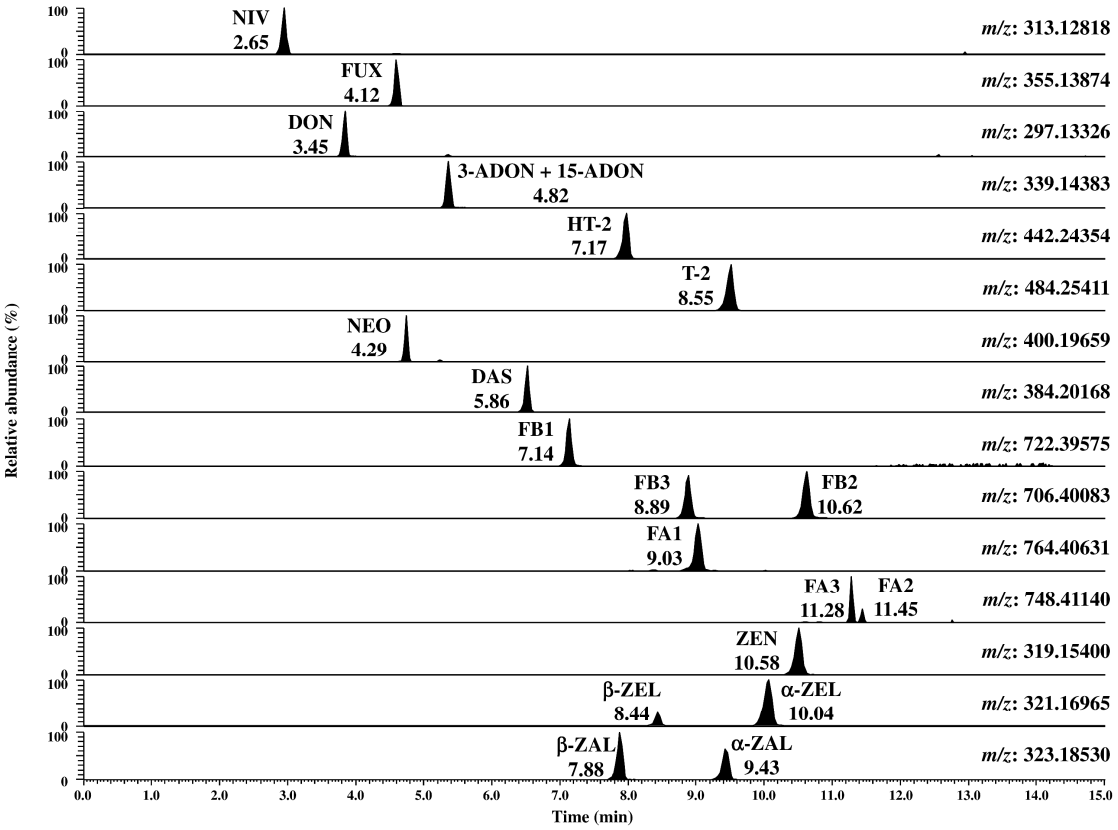


**Figure 4.2** Chromatograms of the 20 *Fusarium* toxins for the Mastro C18. The analytical sample consisted of a 200-µg/L standard in a neat solvent. The extraction mass window was ±5 ppm.

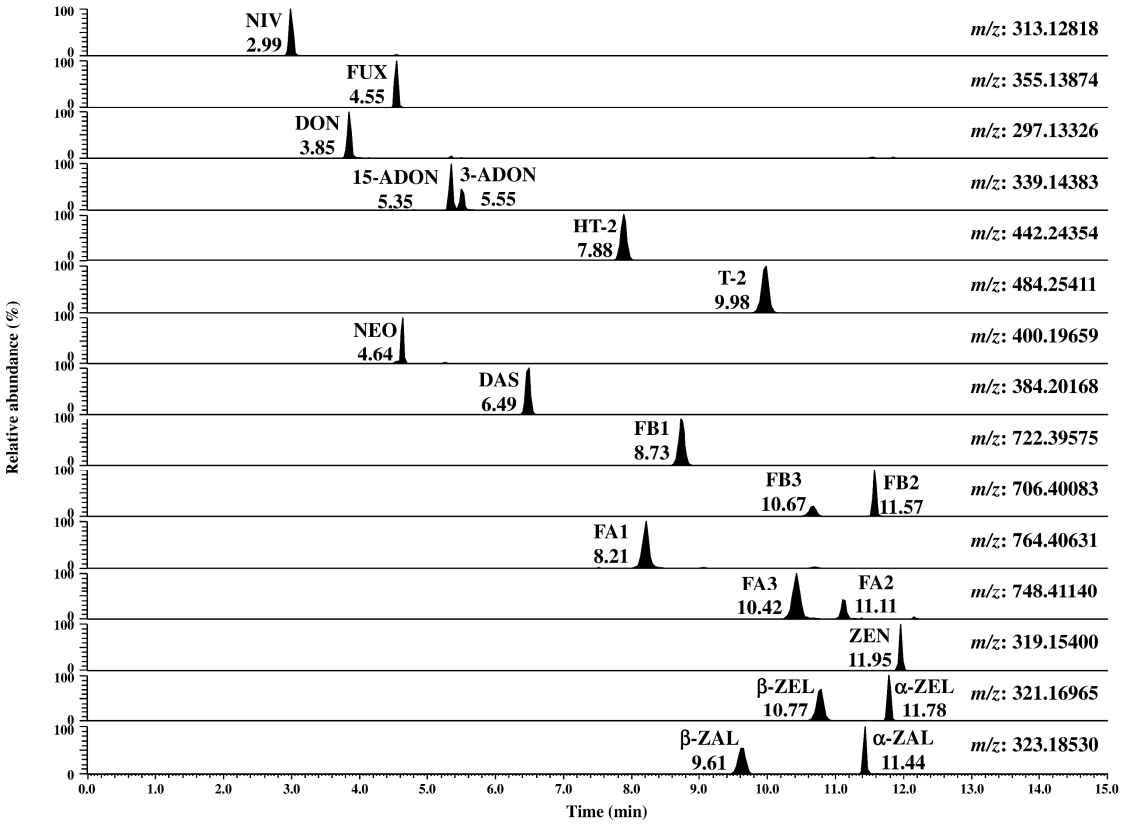


**Figure 4.3** Chromatograms of the 20 *Fusarium* toxins for the Mastro PFP. The analytical sample consisted of 200-µg/L standards in a neat solvent. The extraction mass window was ±5 ppm.

In addition, the separation of the isomers was compared with that obtained by means of an ACQUITY UPLC CSH Fluoro-Phenyl and a Discovery HS F5, under the same gradient conditions. The characteristic chromatograms of 3-ADON/15-ADON, FB2/FB3, and FA2/FA3 are shown in Figure 4.4. Separation of α-ZEL/β-ZEL and α-ZAL/β-ZAL on both PFP columns was relatively good. Nonetheless, with the ACQUITY UPLC CSH Fluoro-phenyl, the separation of 3-ADON/15-ADON and FA2/FA3 was not satisfactory, and minor peak tailing was observed. Moreover, fumonisin peaks showed severe tailing with the Discovery HS F5. Although a multianalyte method does not provide ideal conditions for all compounds, according to these results, Mastro PFP, which allowed for separation of the 20 *Fusarium* toxins with good peak shapes, was selected as the optimal column.

### 4.3.2 Detection of the 20 *Fusarium* toxins by LC-Orbitrap MS

Next, the detection of known compounds in cereal matrices was confirmed via accurate mass measurement by Orbitrap MS. The extracted accurate mass chromatograms and nominal mass chromatograms were compared using a corn sample spiked with 100-

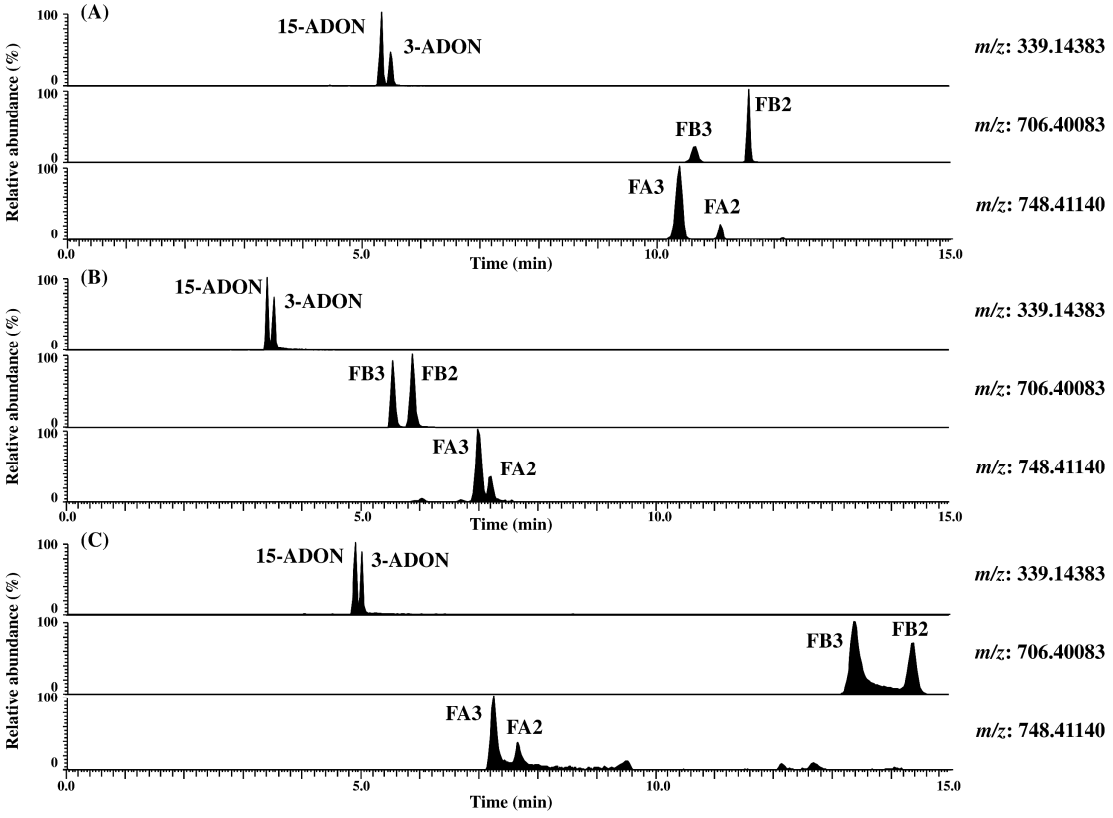


**Figure 4.4** Chromatograms of 3-ADON/15-ADON, FB2/FB3, and FA2/FA3 for (A) a Mastro PFP, (B) an ACQUITY UPLC CSH Fluoro-Phenyl, and (C) a Discovery HS F5. The analytical sample consisted of 200-µg/L standards in a neat solvent. The extraction mass window was ±5 ppm.

μg/kg *Fusarium* toxin standards (Figure 4.5). In the total ion chromatogram (TIC), contaminating compounds from all matrix components were detected within the retention time of each *Fusarium* toxin. The corn sample was prepared by the method described in subsection 4.2.2. In the extracted nominal mass chromatograms, NIV, 3-ADON, and 15-ADON could not be distinguished from the matrix components [Figure 4.5 (A)]. In contrast, the extracted accurate mass chromatography showed clear peaks for all 20 *Fusarium* toxins in the corn sample. These results suggested that the accurate mass measurements were suitable for detection of *Fusarium* toxins in food [Figure 4.5 (B)].

The mass error is the difference between measured and theoretical mass. A small value of the mass error indicates that the measured mass is closer to the theoretical mass and that known compounds can be detected with high accuracy. The mass error was determined for 200-μg/L *Fusarium* toxin standards in a neat solvent, for a corn sample spiked with 100-μg/kg standards of *Fusarium* toxins, and for a reference corn sample (MTC-9999E) naturally contaminated with mycotoxins (DON, HT-2, T-2, FB1, FB2, FB3, and ZEN). Table 4.3 summarizes the measured masses and the mass errors. The mass errors were within ±0.30 ppm for the standard and within ±0.77 ppm for the corn samples. In accordance with the guidelines established by the EC [13], a mass error within ±5 ppm is used as a criterion for compound identification. Thus, high-resolution

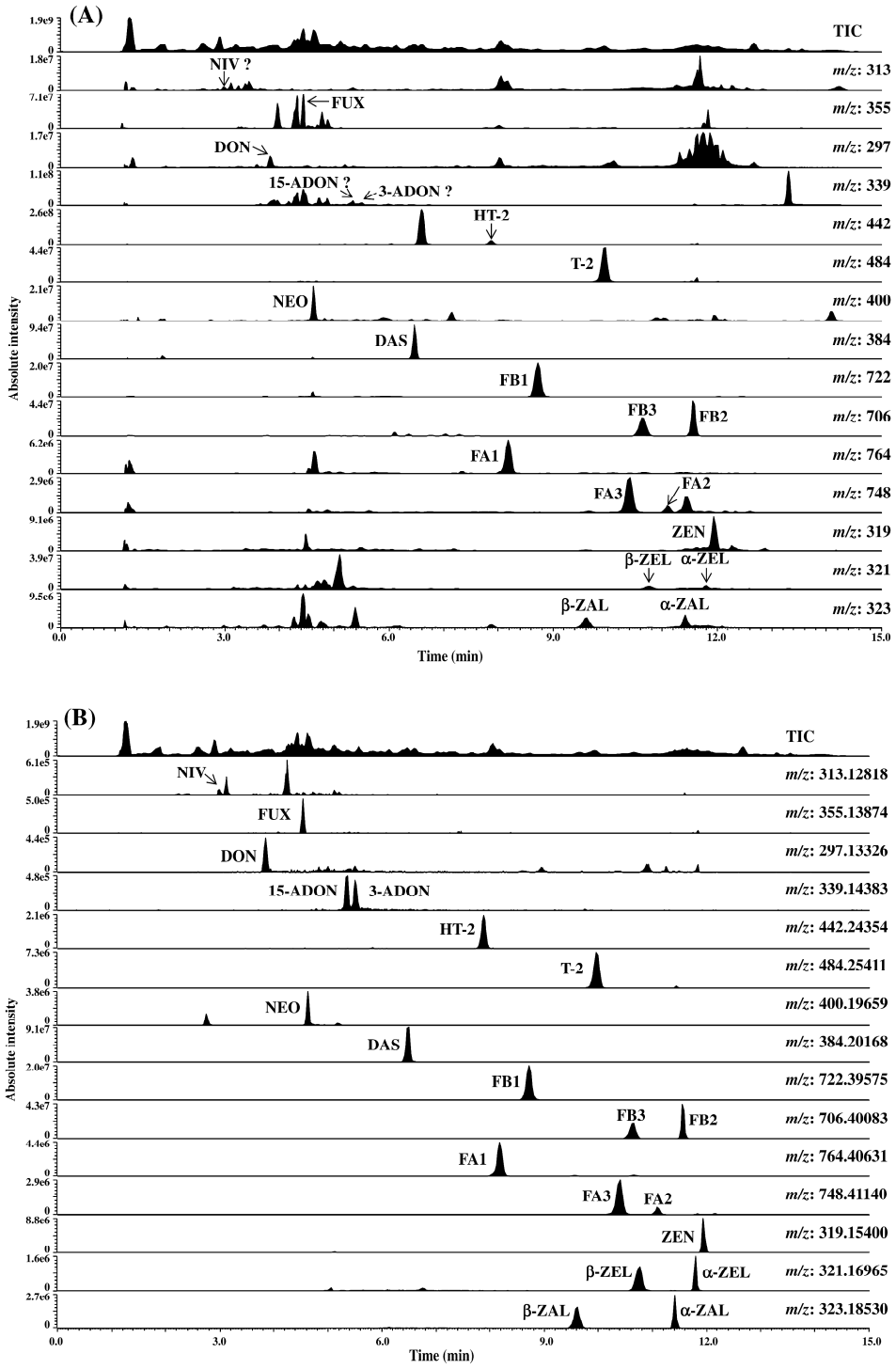


**Figure 4.5** Extracted ion chromatograms of the 20 *Fusarium* toxins by means of (A) nominal mass (extraction mass window ±0.5 units) and (B) accurate mass (extraction mass window ±5 ppm). The analytical sample consisted of corn spiked with 100-µg/kg standards.

**Table 4.3** Measured mass and mass error of *Fusarium* toxins detected in the standards, in the corn sample spiked with the 20 *Fusarium* toxins, and in MTC-9999E.

| *Fusarium* toxins | Measured ion | Formula | Theoretical mass (*m/z*) | Standard in neat solvent [a] | | Corn sample spiked with the 20 *Fusarium* toxins [b] | | MTC-9999E | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Measured mass (*m/z*) | Mass error (ppm) | Measured mass (*m/z*) | Mass error (ppm) | Measured mass (*m/z*) | Mass error (ppm) |
| NIV | $[M+H]^+$ | $C_{15}H_{21}O_7$ | 313.12818 | 313.12815 | −0.11 | 313.12799 | −0.60 | | |
| FUX | $[M+H]^+$ | $C_{17}H_{23}O_8$ | 355.13874 | 355.13868 | −0.18 | 355.13894 | −0.77 | | |
| DON | $[M+H]^+$ | $C_{15}H_{21}O_6$ | 297.13326 | 297.13326 | −0.01 | 297.13313 | −0.44 | 297.13328 | 0.04 |
| 3-ADON | $[M+H]^+$ | $C_{17}H_{23}O_7$ | 339.14383 | 339.14382 | −0.04 | 339.14371 | −0.36 | | |
| 15-ADON | $[M+H]^+$ | $C_{17}H_{23}O_7$ | 339.14383 | 339.14383 | 0.02 | 339.14382 | −0.02 | | |
| HT-2 | $[M+NH_4]^+$ | $C_{22}H_{36}O_8N$ | 442.24354 | 442.24361 | 0.14 | 442.24357 | 0.07 | 442.24356 | 0.04 |
| T-2 | $[M+NH_4]^+$ | $C_{24}H_{38}O_9N$ | 484.25411 | 484.25425 | 0.30 | 484.25405 | −0.12 | 484.25423 | 0.25 |
| NEO | $[M+NH_4]^+$ | $C_{19}H_{30}O_8N$ | 400.19659 | 400.19666 | 0.15 | 400.19683 | 0.60 | | |
| DAS | $[M+NH_4]^+$ | $C_{19}H_{30}O_7N$ | 384.20168 | 384.20164 | −0.09 | 384.20145 | −0.77 | | |
| FB1 | $[M+H]^+$ | $C_{34}H_{60}O_{15}N$ | 722.39575 | 722.39562 | −0.18 | 722.39551 | −0.32 | 722.39559 | −0.22 |
| FB2 | $[M+H]^+$ | $C_{34}H_{60}O_{14}N$ | 706.40083 | 706.40062 | −0.30 | 706.40047 | −0.51 | 706.40058 | −0.36 |
| FB3 | $[M+H]^+$ | $C_{34}H_{60}O_{14}N$ | 706.40083 | 706.40066 | −0.25 | 706.40064 | −0.27 | 706.40070 | −0.18 |
| FA1 | $[M+H]^+$ | $C_{36}H_{62}O_{16}N$ | 764.40631 | 764.40634 | 0.04 | 764.40631 | −0.01 | | |
| FA2 | $[M+H]^+$ | $C_{36}H_{62}O_{15}N$ | 748.41140 | 748.41151 | 0.15 | 748.41135 | −0.07 | | |
| FA3 | $[M+H]^+$ | $C_{36}H_{62}O_{15}N$ | 748.41140 | 748.41136 | −0.05 | 748.41131 | −0.12 | | |
| ZEN | $[M+H]^+$ | $C_{18}H_{21}O_4$ | 319.15400 | 319.15397 | −0.11 | 319.15384 | −0.52 | 319.15399 | −0.02 |
| α-ZEL | $[M+H]^+$ | $C_{16}H_{25}O_5$ | 321.16965 | 321.16971 | 0.17 | 321.16954 | −0.34 | | |
| β-ZEL | $[M+H]^+$ | $C_{16}H_{25}O_5$ | 321.16965 | 321.16965 | −0.01 | 321.16962 | −0.10 | | |
| α-ZAL | $[M+H]^+$ | $C_{18}H_{27}O_5$ | 323.18530 | 323.18543 | −0.13 | 323.18551 | 0.63 | | |
| β-ZAL | $[M+H]^+$ | $C_{18}H_{27}O_5$ | 323.18530 | 323.18532 | 0.07 | 323.18542 | 0.38 | | |

[a] The analytical sample consisted of 200-μg/L standard in neat solvent. [b] The analytical sample consisted of a corn spiked with 100-μg/kg standards.

Orbitrap MS analysis proved to be suitable for accurate detection of the 20 *Fusarium* toxins in cereal matrices.

### 4.3.3 Method validation

Extraction with a QuEChERS kit followed by purification using MultiSep 229 Ochra cartridge was used for sample preparation. As shown in the above experiments (Chapters 1, 2, and 3), this procedure is also useful for simultaneous purification of *Fusarium* toxins, including NIV, DON, HT-2, T-2, FB1, FB2, FB3, ZEN, FA1, FA2, and FA3. Thus, this is a viable method for analysis of the other *Fusarium* toxins in this study, namely, FUX, 3-ADON, 15-ADON, NEO, DAS, α-ZEL, β-ZEL, α-ZAL, and β-ZAL. The method for determination of the 20 *Fusarium* toxins was evaluated using prepared corn, wheat, and barley samples spiked with *Fusarium* toxin standards. I selected the samples in which *Fusarium* toxins were not detected or were detected at very low concentrations as confirmed by the preparation method and the LC-Orbitrap MS analysis (subsections 4.2.2 and 4.2.3). The results are shown in Table 4.4. The linearity, repeatability, and recovery were acceptable: >0.996, 71–106%, and

**Table 4.4** Performance of the method.

| *Fusarium* toxins | | Corn | | | Wheat | | | Barley | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Linearity** (*r*)[a] | **Recovery** (%)[c] | **Repeatability** (%)[c] | **Linearity** (*r*) | **Recovery** (%)[c] | **Repeatability** (%)[c] | **Linearity** (*r*) | **Recovery** (%)[c] | **Repeatability** (%)[c] |
| Trichothecenes | NIV | 0.9997 | 76 | 2.1 | 0.9999 | 71 | 5.4 | 0.9996 | 78 | 5.1 |
| | FUX | 0.9995 | 87 | 4.4 | 0.9998 | 93 | 6.2 | 0.9995 | 102 | 7.8 |
| | DON | 0.9993 | 80 | 4.2 | 0.9996 | 89 | 6.0 | 0.9968 | 82 | 4.6 |
| | 3-ADON | 0.9995 | 92 | 5.7 | 0.9992 | 89 | 5.6 | 0.9978 | 88 | 5.8 |
| | 15-ADON | 0.9989 | 95 | 2.4 | 0.9990 | 99 | 5.0 | 0.9982 | 96 | 6.8 |
| | HT-2 | 0.9999 | 98 | 1.4 | 0.9998 | 101 | 1.0 | 0.9991 | 98 | 3.4 |
| | T-2 | 0.9998 | 93 | 0.9 | 0.9994 | 95 | 1.7 | 0.9989 | 94 | 1.0 |
| | NEO | 0.9986 | 98 | 4.7 | 0.9987 | 99 | 2.9 | 0.9964 | 100 | 7.0 |
| | DAS | 0.9989 | 97 | 1.0 | 0.9979 | 97 | 1.5 | 0.9967 | 97 | 2.5 |
| Fumonisins | FB1 | 0.9999 | 96 | 1.6 | 0.9992 | 85 | 2.7 | 0.9998 | 93 | 2.6 |
| | FB2 | 0.9994 | 102 | 2.4 | 0.9985 | 91 | 3.8 | 0.9991 | 94 | 3.6 |
| | FB3 | 0.9998 | 104 | 0.8 | 0.9998 | 93 | 3.4 | 0.9997 | 94 | 3.1 |
| | FA1 | 0.9991 | 100 | 1.5 | 0.9997 | 97 | 2.3 | 0.9997 | 96 | 1.6 |
| | FA2 | 0.9999 | 93 | 11.9 | 0.9999 | 106 | 6.7 | 0.9986 | 98 | 14.7 |
| | FA3 | 0.9999 | 97 | 2.6 | 0.9996 | 97 | 2.6 | 0.9992 | 97 | 2.0 |
| Zearalenone group | ZEN | 0.9998 | 82 | 3.4 | 0.9995 | 90 | 4.0 | 0.9997 | 84 | 7.3 |
| | α-ZEL | 0.9998 | 86 | 6.1 | 0.9994 | 91 | 2.4 | 0.9979 | 79 | 4.3 |
| | β-ZEL | 0.9993 | 95 | 3.1 | 0.9997 | 84 | 5.6 | 0.9988 | 92 | 7.6 |
| | α-ZAL | 0.9998 | 78 | 2.7 | 0.9998 | 97 | 6.4 | 0.9984 | 82 | 12.4 |
| | β-ZAL | 0.9990 | 86 | 6.8 | 0.9999 | 99 | 4.1 | 0.9984 | 78 | 6.2 |

[a] Concentration range of linearity = 5–5000 μg/kg. [b] $n = 5$, the samples were spiked with 100 μg/kg. [c] $n = 1$, the samples were spiked with 100 μg/kg.

0.8–14.7%, respectively. The LOQs were identical to the lowest calibration levels (i.e., 5 μg/kg). Moreover, the analytical levels of DON, HT-2, T-2, FB1, FB2, FB3, and ZEN in the reference corn samples (DC-617, FC-443, ZC-327, MTC-9990, and MTC-9999E) were within the acceptance limits. Because the individual concentrations of FB1 and FB2 in the MTC-9999E sample exceeded the range of the calibration curve, the prepared sample was diluted 10-fold with 10 mM ammonium acetate/MeCN (85:15, v/v) prior to the analysis.

The “dilute-and-shoot” approach to multi-mycotoxin analysis, as reported by Sulyok *et al.* [8], is easily implemented because it requires only extraction with a solvent (e.g., water/MeCN); however, large amounts of matrix components also get extracted simultaneously. Although target peaks can be distinguished from matrix components by Orbitrap MS, sample preparation, especially the purification process, is important for stable and consecutive quantification. In addition, although the reported method is suitable for quantification of 87 analytes, including 3-ADON/15-ADON, FB2/FB3, and α-ZEL/β-ZEL, it does not yield sufficient separation of 3-ADON/15-ADON in terms of retention time. Moreover, in this method, because 3-ADON is detected in negative mode and 15-ADON in positive mode, two chromatographic runs per sample (in positive and negative mode) are needed. Hence, 3-ADON and 15-ADON could not be simultaneously

analyzed. In comparison with the method reported by Sulyok, the method proposed here has the following advantages: (1) the possibility of simultaneous analysis in positive mode because 3-ADON and 15-ADON are completely separated; (2) wide calibration ranges for the toxins under study, with similar recovery and repeatability; (3) contamination of the instrument by matrix components is less likely because of the sample preparation step. Thus, I successfully developed a method for simultaneous determination of 20 *Fusarium* toxins in corn, wheat, and barley samples.

### 4.3.4 Determination of the 20 *Fusarium* toxins in cereal samples

Concentrations of the 20 *Fusarium* toxins in commercial cereal samples, including 13 corn samples, 12 wheat samples, and 9 barley samples, were analyzed by the simultaneous determination method. The reference corn samples (DC-617, FC-443, ZC-327, MTC-9990, and MTC-9999E), which are contaminated with various *Fusarium* toxins, were selected for the analysis. The concentrations were calculated by the standard addition method, in order to compensate for the losses during sample preparation and for adjustment of matrix effects. Therefore, it was not necessary to separately correct the

values for recovery. The results are shown in Tables 4.5 and 4.6.

In the commercial corn samples (C-1 to C-13 in Table 4.5), FUX, DON, 3-ADON, 15-ADON, T-2, DAS, FB1, FB2, FB3, FA1, FA2, FA3, and ZEN were detected. DON, 15-ADON, FB1, FB2, FB3, FA1, FA2, FA3 and ZEN were detected in more than a half of the samples, whereas FB1, FB2, and FB3 in all corn samples. FB1 showed the highest concentrations, with a maximum of 1.30 mg/kg. High concentrations of DON, 15-ADON, and ZEN were detected more frequently in corn samples than in wheat and barley samples, with maximal concentrations of 1.11 mg/kg, 145 μg/kg, and 148 μg/kg, respectively. NIV, HT-2, NEO, α-ZEL, β-ZEL, α-ZAL, and β-ZAL were not detected. The reference corn samples showed the same trend as the commercial ones did. DON, 15-ADON, FB1, FB2, FB3, FA1, FA2, FA3, and ZEN were detected in all samples, whereas α-ZEL, β-ZEL, α-ZAL, and β-ZAL were not detected. These results revealed that corn samples are at a high risk of co-contamination with various groups of *Fusarium* toxins.

In wheat samples (W-1 to W-12 in Table 4.6), trichothecenes were detected, and NIV, FUX, DON, HT-2, and T-2 were detected in more than a half of the samples. The concentration of DON was particularly high, with a maximum of 451 μg/kg. FB1, FB2, and ZEN were detected in some samples, but their concentrations were relatively low. FB3, FA1, FA2, FA3, α-ZEL, β-ZEL, α-ZAL, and β-ZAL were not detected in any wheat

**Table 4.5** Concentration of *Fusarium* toxins in corn samples.

| Sample | Concentration of *Fusarium* toxins (μg/kg) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Trichothecenes | | | | | | | | | Fumonisins | | | | | | Zearalenone -groups |
| | NIV | FUX | DON | 3-ADON | 15-ADON | HT-2 | T-2 | NEO | DAS | FB1 | FB2 | FB3 | FA1 | FA2 | FA3 | ZEN |
| C-1 | [a] | | 63.7 | | 10.6 | | <5 | | | 373 | 70.9 | 54.8 | 34.7 | 53.7 | 5.44 | <5 |
| C-2 | | <5 | 15.3 | | <5 | | | | <5 | 229 | 41.4 | 19.0 | 17.6 | 23.9 | <5 | |
| C-3 | | | 16.0 | | <5 | | | | | 32.3 | 8.66 | <5 | <5 | <5 | | |
| C-4 | | <5 | | | | | | | <5 | 154 | 30.6 | 13.8 | 11.0 | 13.3 | <5 | |
| C-5 | | | 10.5 | | <5 | | | | | 67.3 | 12.5 | 11.5 | 8.29 | 11.0 | <5 | <5 |
| C-6 | | | 53.3 | | 14.8 | | <5 | | <5 | 924 | 171 | 122 | 87.8 | 168 | 11.6 | 13.7 |
| C-7 | | | 96.1 | | 18.0 | | <5 | | | 526 | 82.9 | 60.2 | 38.5 | 66.6 | <5 | 30.2 |
| C-8 | | <5 | 401 | | 145 | | <5 | | | 38.3 | <5 | <5 | | | | 81.7 |
| C-9 | | | 154 | <5 | 34.9 | | | | | 40.8 | 10.9 | <5 | <5 | <5 | | 19.9 |
| C-10 | | 8.39 | 135 | 5.57 | 38.1 | | | | | 413 | 45.6 | 60.5 | 34.7 | 47.3 | 7.52 | 6.79 |
| C-11 | | | 214 | <5 | 26.1 | | <5 | | | 1.30[c] | 291 | 193 | 106 | 93.9 | 6.76 | 65.8 |
| C-12 | | 5.62 | 1.11 [c] | 12.6 | 47.4 | | | | | 54.8 | 14.9 | <5 | <5 | | | 148 |
| C-13 | | | | | | | | | | 466 | 85.0 | 77.3 | 51.4 | 42.6 | 5.09 | |
| DC-617 | <5 [b] | | 4.82 [c] | | 372 | 29.5 | 13.9 | <5 | | 2.48 [c] | 486 | 263 | 563 | 677 | 71.0 | 592 |
| FC-443 | | | 99.8 | | 16.3 | <5 | <5 | <5 | | 3.69 [c] | 786 | 244 | 522 | 705 | 74.9 | <5 |
| ZC-327 | | | 2.57 [c] | 27.5 | 241 | | | | | 1.56 [c] | 291 | 150 | 262 | 378 | 30.2 | 1.72 |
| MTC-9990 | | <5 | 1.78 [c] | 10.5 | 153 | 16.2 | 6.73 | <5 | <5 | 1.14 [c] | 181 | 125 | 257 | 370 | 40.8 | 284 |
| MTC-9999E | 23.0 | <5 | 2.16 [c] | 7.01 | 168 | 368 | 153 | 19.4 | <5 | 28.3 [c] | 5.39 [c] | 1.36 [c] | 2.37 | 2.61 | 182 | 323 |

[a] The blank cells indicate that no peak was detected. [b] "<5" is a peak detected under the LOQ (i.e., 5 μg/kg). [c] Concentration unit, mg/kg.

**Table 4.6** Concentration of *Fusarium* toxins in wheat and barley samples.

| Sample | | Concentration of *Fusarium* toxins (µg/kg) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Trichothecenes | | | | | | | | | Fumonisins | Zearalenone-groups |
| | | NIV | FUX | DON | 3-ADON | 15-ADON | HT-2 | T-2 | NEO | DAS | FB1 | FB2 | ZEN |
| Wheat | W-1 | <5 [a] | | 102 | <5 | | <5 | <5 | <5 | <5 | <5 | | |
| | W-2 | [b] | | 35.1 | | | | <5 | | | | | |
| | W-3 | <5 | <5 | 19.0 | | | | | | | | | |
| | W-4 | <5 | | 71.6 | | | <5 | <5 | | | | | |
| | W-5 | <5 | | 405 | 21.3 | 16.9 | 10.1 | <5 | | | | | <5 |
| | W-6 | | | 110 | | | 7.08 | <5 | <5 | | | | |
| | W-7 | <5 | | 352 | | | <5 | <5 | | | | | |
| | W-8 | <5 | 6.76 | 451 | 9.88 | | <5 | | | | <5 | | 33.1 |
| | W-9 | <5 | | 12.2 | | | | | | | | | |
| | W-10 | 11.6 | 15.3 | 198 | | 13.1 | 5.22 | <5 | <5 | <5 | | <5 | 5.95 |
| | W-11 | 6.99 | 25.5 | 271 | | | <5 | | | | | | |
| | W-12 | 10.6 | 16.0 | 272 | | | 12.4 | <5 | | | | <5 | 9.85 |
| Barley | B-1 | | | 7.41 | | | | | | | | | |
| | B-2 | <5 | | 6.56 | 34.1 | | | | | | | | |
| | B-3 | | <5 | | | | | | | | | | |
| | B-4 | | <5 | | | | | | | | | | |
| | B-5 | | <5 | | | | | | | | | | |
| | B-6 | <5 | <5 | 116 | | 8.32 | <5 | <5 | | | | | 60.9 |
| | B-7 | <5 | | | | | | | | | | | |
| | B-8 | | | 5.93 | | <5 | <5 | <5 | <5 | <5 | | | |
| | B-9 | | | 77.7 | | <5 | <5 | <5 | | <5 | 0.43 | | |

[a] "<5" is a peak detected under the LOQ (i.e., 5 µg/kg). [b] The blank cells indicate that no peak was detected.

sample. These results confirmed that wheat samples are co-contaminated with trichothecenes.

Co-contamination with trichothecenes was also detected in barley samples (B-1 to B-9 in Table 4.6), but the rates of detection and the concentrations were relatively low. The maximal concentration was observed for DON (116 μg/kg). α-ZEL, β-ZEL, α-ZAL, and β-ZAL were not detected in any cereal samples. Because these compounds are derivatives (reduced metabolites) of ZEN, this finding indicates that the risk of ZEN being metabolized and reduced by microorganisms during cereal storage is low.

These results confirmed that cereals are susceptible to co-contamination with *Fusarium* toxins. Corn is at a particularly high risk of co-contamination with various *Fusarium* toxins, namely, trichothecenes, fumonisins, and ZEN, at high concentrations.

## 4.4 Summary

I successfully developed a method for simultaneous determination of 20 *Fusarium* toxins (including five pairs of isomers) in cereal products by LC-Orbitrap MS with a PFP column. The highlights are as follows:

- Complete separation of 20 *Fusarium* toxins was achieved using a Mastro PFP. Additionally, the *Fusarium* toxins in cereal matrices could be accurately detected by Orbitrap MS with a mass error within ±0.77 ppm.
- Corn, wheat, and barley samples were prepared using a QuEChERS kit for extraction and MultiSep 229 Ochra cartridge for purification. Validation of the newly developed method was successful. Additionally, analytical levels of *Fusarium* toxins in the reference corn samples were within the acceptance limits. Thus, the development of the method for simultaneous determination of 20 *Fusarium* toxins was successful.
- Commercially available corn, wheat, and barley samples were analyzed using the method, and the results revealed that *Fusarium* toxins, namely trichothecenes, fumonisins, and ZEN, were detected at high concentrations and with a high frequency in the corn samples. Fumonisin B-series, in particular, were detected

at high concentrations. Trichothecenes were detected in the wheat and barley samples. In particular, DON was detected at a high frequency. On the other hand, α-ZEL, β-ZEL, α-ZAL, and β-ZAL, which are derivatives of ZEN, were not detected in all the samples.

## 4.5 References


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## Conclusions

In this thesis, simultaneous determination methods for mycotoxins in food by LC-MS/MS and LC-Orbitrap MS are proposed as new official methods. The development of simple and easy protocols for sample preparations and optimization of LC conditions were performed for mycotoxins with different properties in various food products. Commercially available samples of beer, wine, corn, wheat, and barley were analyzed by these methods. As a result, *Fusarium* toxins were detected frequently, i.e., were found to be frequent contaminants of food. In particular, corn samples were found to be contaminated not only with the key *Fusarium* toxins but also with their derivatives.

1. Methods for the multiple determinations of 15 key mycotoxins (which have gained international attention) in beers and wines by LC-MS/MS were developed here.
    - Carryover of FB1, FB2, FB3, and OTA was observed during the LC experiments. To prevent the carryover, two types of LC conditions were used.
    - The beer samples were prepared for extraction with MeCN by the QuEChERS method, followed by purification by means of a C18 cartridge. The preparation

procedure was able to recover the mycotoxins in question and to remove matrices such as beer pigments.

- The wine samples were prepared for the second purification with MultiSep 229 Ochra cartridge after extraction and the first purification by means of Oasis HLB cartridge. The sample preparation procedure allowed me to remove the pigments and highly polar matrices from wines, and chromatograms with good peak shapes were obtained.
- Commercially available beers and wines were analyzed by these methods. NIV, DON, FB1, FB2, and FB3 were detected in the beer samples, whereas FB1, FB2, FB3, and OTA were detected in the wine samples. The newly developed methods revealed that beer and wine are at risk of co-contamination with mycotoxins, whereas the identified mycotoxins were detected under the LOQ, thus posing a low risk to human health.

2. A method was developed for the simultaneous determination of key mycotoxins with minimization of carryover in a single run.

- Because the carryover of FB1, FB2, and FB3 was confirmed to be caused by

adsorption to metals, minimization of carryover was achieved by using appropriate solvents for washing in an injection needle and by using an analytical column with low activity of metals.

- Corn samples were prepared for extraction with MeCN by the QuEChERS method followed by purification by means of MultiSep 229 Ochra cartridge. Matrix components such as pigments and lipids (present in the corn samples) were adequately removed.
- Method validation yielded good results. Simultaneous determination across a wide range of concentrations was accomplished by minimizing the carryover that occurs with highly concentrated samples.
- Analysis of commercially available corn samples by these methods revealed the presence of trichothecenes (NIV, DON, HT-2, and T-2), fumonisins (FB1, FB2, and FB3), and ZEN. In particular, DON, FB1, FB2, FB3, and ZEN were detected at high concentrations and with a high frequency. These results mean that the samples are co-contaminated with *Fusarium* toxins, which were found to be trichothecenes, fumonisins, and ZEN.

3. Fumonisin A-series, which represent derivatives of the fumonisin B-series, were identified by LC-Orbitrap MS, and a simultaneous quantification for these fumonisins in corn samples was developed successfully.

   - Three unknown compounds were detected by LC-Orbitrap MS in a corn sample contaminated with fumonisins B-series. Those compounds were hypothesized to be FA1, FA2, and FA3, which are *N*-acetylated derivatives of the fumonisin B-series. Comparison with synthesized fumonisin A-series revealed that the three unknown compounds are FA1, FA2, and FA3.
   - A method for simultaneous quantification of six fumonisins (FA1, FA2, FA3, FB1, FB2, and FB3) in corn samples was examined. The samples were prepared for extraction with MeCN by the QuEChERS method followed by purification by means of MultiSep 229 Ochra cartridge. Ten corn samples that are contaminated with mycotoxins (including FB1, FB2, and FB3) were analyzed using the method; FA1, FA2, and FA3 were detected in all the samples.
   - This result represents the first identification and quantification of FA1, FA2, and FA3 in corn samples.

4. A method for simultaneous determination of 20 *Fusarium* toxins by LC-Orbitrap MS with a PFP column was developed.

- Twenty *Fusarium* toxins including isomers were separated completely on the PFP column. Additionally, the *Fusarium* toxins in cereal matrices could be accurately detected by Orbitrap MS with a mass error within ±0.77 ppm.
- The samples of corn, wheat, and barley were prepared for extraction with MeCN by the QuEChERS method followed by purification using MultiSep 229 Ochra cartridge. The method was validated for each sample, and good results were obtained.
- Analysis of 34 commercially available cereals revealed that they are highly susceptible to co-contamination with *Fusarium* toxins. Corn is at a particularly high risk of co-contamination with various *Fusarium* toxins at high concentrations. Thus, in the future, continuous control and monitoring of *Fusarium* toxins will be necessary to ensure food safety and to prevent economic losses. The method reported herein proved to be suitable for this purpose.

The simultaneous determination of mycotoxins should strengthen regulations related to mycotoxins in Japan in the near future and enable their stringent management.

I believe that this thesis will help to reduce the risks associated with food contamination and can publicize the importance of simultaneous determination mass spectrometry and thereby may pave the way for its adoption as a new official method.

## List of Publications

Reference theses


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Sub-theses

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