# Studies on the development of analytical methods for quantification of mycotoxins in feed and pet foods

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# Contents

# Abbreviations

| | |
|---|---|
| 3-AcDON | 3-acetyldeoxynivalenol |
| 15-AcDON | 15-acetyldeoxynivalenol |
| $AFB_1$ | aflatoxin $B_1$ |
| APCI | atmospheric pressure chemical ionization |
| bw | body weight |
| CDL | curved desolvation line |
| CERI | Chemicals Evaluation and Research Institute, Japan |
| DAS | diacetoxyscirpenol |
| DON | deoxinivalenol |
| D3G | deoxinivalenol-3-glucoside |
| EFSA | European Food Safety Authority |
| ESI | electrospray ionization |
| EU | European Union |
| FAMIC | Food and Agricultural Materials Inspection Center |
| FAO | Food and Agriculture Organisation |
| FBs | $FB_1$, $FB_2$ and $FB_3$ |
| $FB_1$ | fumonisin $B_1$ |
| $FB_2$ | fumonisin $B_2$ |
| $FB_3$ | fumonisin $B_3$ |
| FDA | Food and Drug Administration |
| HorRat | Horwitz ratio |
| HT-2 | HT-2 toxin |
| JECFA | Joint FAO/WHO Expert committee on Food Additives |
| LC-MS | liquid chromatography mass spectrometry |
| LC-MS/MS | liquid chromatography tandem mass spectrometry |
| LOD | limit of detection |
| LOQ | limit of quantification |
| MAFF | Ministry of Agriculture, Forestry and Fisheries |
| MFC | multifunctional column |

| | |
|---|---|
| NES | neosolaniol |
| NIV | nivalenol |
| PMTDI | provisional maximum tolerable daily intake |
| $RSD_r$ | relative standard deviation of repeatability |
| $RSD_R$ | relative standard deviation of reproducibility |
| SIM | selected ion monitoring |
| STC | sterigmatocystin |
| SRM | selected reaction monitoring |
| T-2 | T-2 toxin |
| ZEA | zearalenone |

# General Introduction

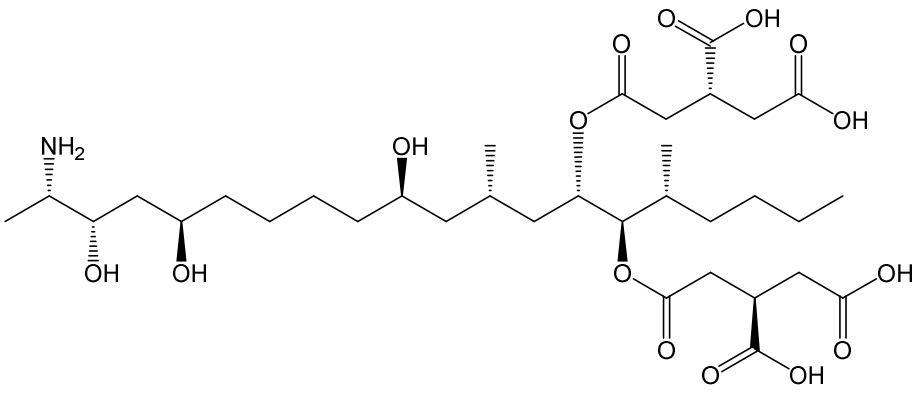
While some molds are useful, such as those used in foods such as blue cheese, miso, and soy sauce, those used in antibiotics, and those that decompose fallen leaves to clean up the environment, there are others that are harmful to humans and animals, such as those that spoil food and those that produce mycotoxins. Mycotoxins are toxic compounds produced by molds (Kruska et al. 2012). Mycotoxins can damage the liver, kidneys, and other organs of humans and animals, and in severe cases can cause death (Creppy 2002). Some fungal toxins have been reported to be carcinogenic and teratogenic. Food contaminated with mold is subject to trade restrictions in some countries, leading to economic losses (Wu 2015). In Japan, research on yeast fungi has been underway since the first half of the 19th century (Murakami 1971). In 1937, *Penicillium* spp. was discovered in yellow rice imported from Taiwan (Miyake et al. 1940). The mycotoxin produced by *Penicillium* spp. causes liver cirrhosis and other liver dysfunctions (Kobayashi 1958). During the postwar food shortage, there was a problem that some of the rice imported from other countries was yellowish rice (Ichinoe 1994). The first time that mycotoxins came to the attention of the public was in 1960, when a large number of turkeys in the United Kingdom died from poisoning, and until the causative agent was identified as aflatoxin, it was thought to be turkey X disease (Kruska et al. 2012). Currently, more than 300 mycotoxins have been identified, the majority of which are produced by the genera *Aspergillus*, *Penicillium* and *Fusarium* (Goto 1990; Sweeney and Dobson 1998). The genus *Aspergillus* is distributed especially in warm tropical and temperate zones and produces aflatoxin, sterigmatocystin (STC), and other toxins (Manabe et al. 1976). Aflatoxin $B_1$ ($AFB_1$) is highly toxic and recognized as the most carcinogenic natural product (IARC 1993; Creppy 2002). Compared to $AFB_1$, STC is generally less toxic. Nevertheless, $AFB_1$ and STC have similar cytotoxicity (Terao 1980). *Penicillium* spp. are distributed in colder regions than *Aspergillus* spp. and are known to produce ochratoxin A (Khoury and Atoi 2010). Ochratoxin mainly damages the kidneys, but can also damage the liver (Pfohl- Leszkowic and Manderville 2007). It is a carcinogen in rats and mice (Walker and Larsen 2005). The genus *Fusarium*, which is widely distributed throughout the world as a soil fungus (Dall'Asta and Battilani 2016), is also

known to produce the mycotoxins including fumonisin, zearalenone (ZEA), and trichothecenes (Gardiner et al. 2010).

**Mycotoxins derived from *Fusarium***

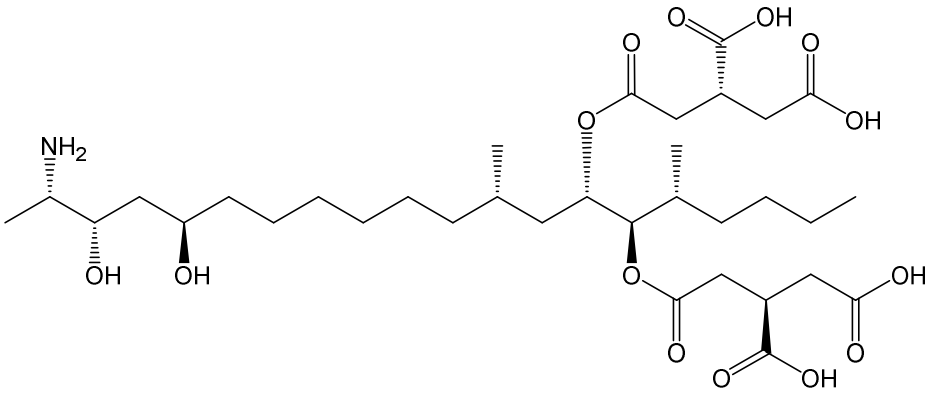
Fumonisin $B_1$, $B_2$ and $B_3$ (FBs) are mycotoxins produced by fungi of the genus *Fusarium* (Thiel et al. 1991). $FB_1$ and $FB_2$ were first isolated from cultures of *Fusarium moniliforme* MRC 826 (Gelderblom et al. 1988). Fumonisins $B_3$, $B_4$, $A_1$ and $A_2$ were then isolated, followed by fumonisins C and P groups, and their structures were determined (Cawood et al. 1991; Seo and Lee 1999; Rheeder et al. 2002). FBs have a sphingolipid-like chemical structure (Figure G-1) and inhibit ceramide synthase, which plays an important role in the sphingolipid biosynthesis pathway, suggesting that this action is involved in fumonisin toxicity (Wang et al. 1991; Norred et al. 2001). FBs have been linked to esophageal cancer in humans (Sydenham et al. 1990). $FB_1$ is carcinogenic to mice and rats (National Toxicology Program 2001). FBs cause pulmonary edema in pigs (Harrison et al. 1990) and leukoencephalomalacia in horses (Lazicka and Orzechowski 2010).

ZEA is a mycotoxin with estrogen-like effects produced by fungi of the genus *Fusarium* (Boermans and Leung 2007; Lazicka and Orzechowski 2010; European Food Safety Authority 2011). ZEA is a macrocyclic lactone compound that binds to estrogen receptors, causing hyperestrogenism (Figure G-2) (Mochizuki 2012; Tashiro 2000). ZEA has attracted attention as an endocrine disrupting chemical, one of the so-called environmental hormones (Li et al. 2012). Pigs are particularly sensitive to ZEA that causes reproductive disorders such as infertility and miscarriage in pigs (Lazicka and Orzechowski 2010). In pets, testicular atrophy (Lazicka and Orzechowski 2010) and suppression of spermatogenesis (Boermans and Leung 2007) have been reported in male dogs, and ovarian cell lesions (Gajecka et al. 2008) and uterine edema and enlargement in female dogs (Boermans and Leung 2007). ZEA is frequently found in high concentrations in maize, wheat, barley, sorghum, and oats used in food and pet food (Zinedine et al. 2007; European Food Safety Authority 2011). Co-contamination of ZEA with deoxynivalenol (DON), fumonisin, and nivalenol (NIV) produced by *Fusarium*, is observed frequently (Mankevičienė et al. 2011; Tanaka et al. 2010).



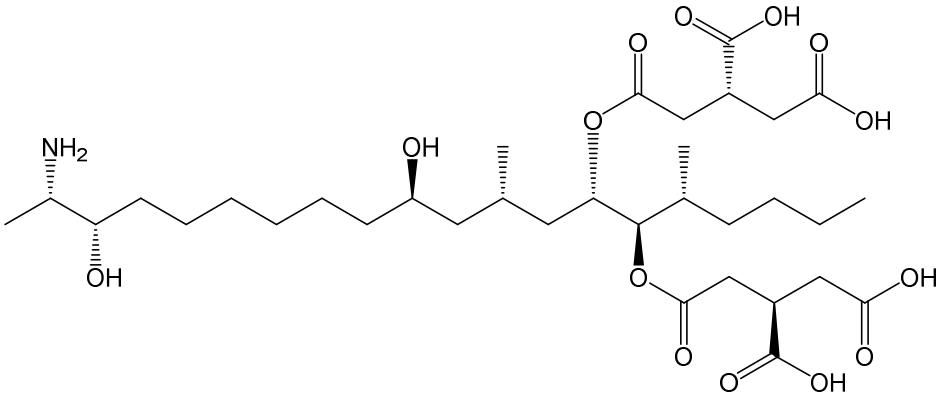

Fumonisin $B_1$

$C_{34}H_{59}NO_{15}$, MW: 721.83, CAS No. 116355-83-0




Fumonisin $B_2$

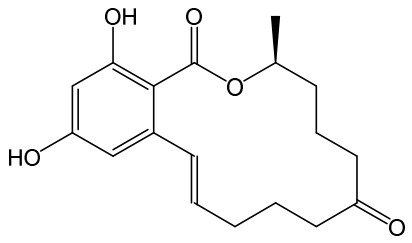
$C_{34}H_{59}NO_{14}$, MW: 705.83, CAS No. 116355-84-1




Fumonisin $B_3$

$C_{34}H_{59}NO_{14}$, MW: 705.83, CAS No. 116379-59-4

**Figure G-1 Chemical structures of fumonisins**




Zearalenone

$C_{18}H_{22}O_5$, MW: 318.36, CAS No. 17924-92-4

**Figure G-2 Chemical structure of zearalenone**

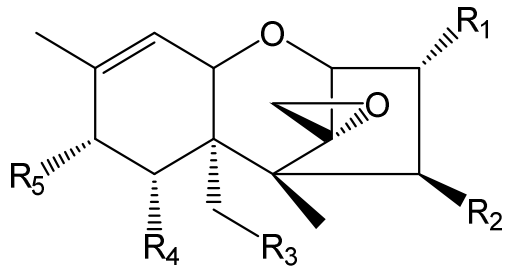
Trichothecene mycotoxins are produced primarily by multiple species of fungi in the genus *Fusarium* (Desjardins et al. 1993), and are a generic term for fungal toxins that have a trichothecene skeleton with epoxy rings at the C-12 and C-13 positions (Figure G-3). Modified mycotoxins have recently attracted attention as new risk factors. These mycotoxins include mycotoxin glucosides that are undetectable by conventional analytical methods. Such mycotoxin glucosides, including deoxynivalenol-3-glucoside (D3G), are metabolized *in vivo* to free mycotoxins and should be considered when assessing mycotoxin toxicity (Berthiller et al. 2003; Berthiller et al. 2011). Trichothecene mycotoxins are classified as types A-D according to their structures (WHO 1990). Type A trichothecenes (HT-2 toxin (HT-2), T-2 toxin (T-2), diacetoxyssylpenol (DAS), etc.) and type B trichothecenes (DON, NIV, etc.) are said to be particularly involved in human and livestock poisoning and cause acute toxicity includes diarrhea, vomiting, and inflammation (WHO 2001).

**Safety Regulation for Feed and Pet Foods in Japan**

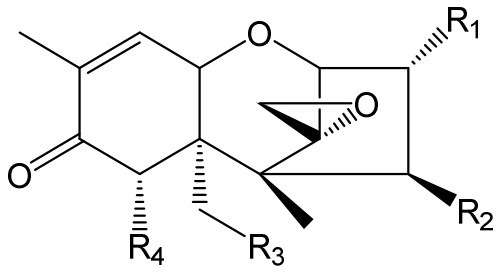
Feed for livestock feeding is a food that can be used for the nutrition of livestock. The Law on Safety Assurance and Quality Improvement of Feeds (Feed Safety Law) has been stipulated since 1953 to assure the safety of feed in Japan (FAMIC 1953). The purpose of this law is to ensure the safety and improve the quality of feed, and to contribute to public safety and the stability of livestock production. Based on this law, standard values are set for pesticide residues, heavy metals, mold toxins, melamine, and cyanuric acid in feed

(FAMIC 1988).

Type A




Type B




| Type | Trichothecene (Abbreviation) | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Chemical formula | MW | CAS |
|---|---|---|---|---|---|---|---|---|---|
| A | HT-2 toxin (HT-2) | OH | OH | OAc | H | $OCOCH_2CH(CH_3)_2$ | $C_{22}H_{32}O_8$ | 424.48 | 26934-87-2 |
| | T-2 toxin (T-2) | OH | OAc | OAc | H | $OCOCH_2CH(CH_3)_2$ | $C_{24}H_{34}O_9$ | 466.52 | 21259-20-1 |
| | Diacetoxyscirpenol (DAS) | OH | OAc | OAc | H | H | $C_{19}H_{26}O_7$ | 366.41 | 2270-40-8 |
| | Neosolaniol (NES) | OH | OAc | OAc | H | OH | $C_{19}H_{26}O_8$ | 382.40 | 36519-25-2 |
| B | 3-Acethyldeoxynivalenol (3-AcDON) | OAc | H | OH | OH | — | $C_{17}H_{22}O_7$ | 338.35 | 50722-38-8 |
| | 15-Acethyldeoxynivalenol (15-AcDON) | OH | H | OAc | OH | — | $C_{17}H_{22}O_7$ | 338.35 | 88337-96-6 |
| | Deoxynivalenol (DON) | OH | H | OH | OH | — | $C_{15}H_{20}O_6$ | 296.32 | 51481-10-8 |
| | Deoxynivalenol-3-glucoside (D3G) | O-Glc | H | OH | OH | — | $C_{15}H_{20}O_6$ | 296.32 | 51481-10-8 |
| | Nivalenol (NIV) | OH | OH | OH | OH | — | $C_{15}H_{20}O_7$ | 312.32 | 23282-20-4 |
| | Fusarenon-X (FUS-X) | OH | OAc | OH | OH | — | $C_{17}H_{22}O_8$ | 354.35 | 23255-69-8 |

**Figure G-3 Chemical structures of trichothecenes**

In 2007, a large-scale health hazard to dogs and cats occurred in the United States due to pet food manufactured using vegetable protein from China that was contaminated with melamine, a raw material for plastics (MAFF 2014). This led to the enactment of the "Law for Ensuring the Safety of Pet Food" (Law No.83, FAMIC 2008a). Hazardous

materials that may be contained in pet food include mycotoxins, heavy metals, pesticides, additives, and others (melamine), and standard values have been set for pet food (FAMIC 2009a).

In Japan, the method used to establish a standard for mycotoxins in feed is basically as follows (Hayashi et al. 2014). First, a survey on mycotoxins contamination in feed ingredients and formula feed is conducted by FAMIC or other organizations by using official methods. Next, the Ministry of Agriculture, Forestry and Fisheries (MAFF), applies the ALARA principle to the survey results, takes processing factors, and so on into account, and then establishes a draft standard value by assuming the worst case in contamination status. Finally, the proposed standard values are compared with safety data (effects on livestock and transferability to livestock products, and human intake via foods containing livestock products) and then set as standard values. The standard values of pet food are set in the same way as feed. (Safety data: effects on pets). Therefore, in order to set standard values for mycotoxins in feed or pet food, it is essential to develop an official method and to accumulate data on contamination of mycotoxins by the official method. Even when a standard value has been set, the standard value will be revised based on the actual contamination surveys by using the official method. The development of analytical methods is also essential in the pursuit of the causes of health problems in livestock and pets. The official method is also used to test for for mycotoxins in feed and pet food that exceed standard values.

Among the fumonisins, $FB_1$, $FB_2$, and $FB_3$ are the most common natural contaminants (Cawood et al. 1991; Boermans and Leung 2007; Lazicka and Orzechowski 2010). FBs contamination is frequently observed in corn. In JECFA, the provisional maximum tolerable daily intake (PMTDI) was set at 2 μg/kg-body weight (bw) as FBs, using red blood cells in hepatocytes in a long-term toxicity study in mice as an indicator (JECFA 2011). In Japan, the tolerable daily intake (TDI) of FBs was set as 2 μg/kg-bw/day by applying an uncertainty coefficient of 100 (10 for species differences and 10 for individual differences) to the no-observed-adverse-effect level (NOAEL) (subacute toxicity study in rats) of 0.21 mg/kg-bw/day obtained at the lowest dosage in the risk assessment of the Food Safety Commission (Food Safety Commission of Japan 2017). Since the transfer of FBs to livestock products in feed is small, it was reported that FBs

in feed is not a food safety issue for humans (Food Safety Commission of Japan 2017). In Japan, the maximum allowable concentration of FBs in feed for livestock and poultry is set at 4 mg/kg (FAMIC 1988). Currently, no guidance values for FBs in pet food have been established in Japan.

ZEA is reported to occur in corn, wheat, barley, rye, and other crops used as ingredients in foods and pet foods, and its contamination has spread to many countries around the world. In JECFA, based on the acceptable daily intake (ADI) of 0-0.5 μg/kg-bw of α-zearalenol evaluated as a veterinary drug, the PMTDI for the total amount of ZEA and its metabolites was set at 0.5 μg/kg-bw by using the effects on hormones in short-term toxicity studies in pigs (no-observed-effect level (NOEL): 40 μg/kg-bw/day; lowest-observed-effect level (LOEL): 200 μg/kg-bw/day) and by applying an uncertainty coefficient of 100) (JECFA 2000). In Japan, the maximum allowable concentration of ZEA in feed (excluding compound feed) for livestock and poultry is set at 1 mg/kg (FAMIC 1988). Currently, no guidance value is set for pet food, but the developed analytical method is intended to determine the amount of ZEA in pet food.

Natural contamination of grains by the type B trichothecene DON and NIV has been widely reported (Yazar and Omuetag 2008; Milicevic et al. 2015). Both DON and NIV have been repeatedly detected in wheat products such as flour and bran, and in barley and corn products (Yoshizawa and Jin 1995). In JECFA, the PMTDI was set at 1 μg/kg-bw as DON, 3-AcDON and 15-AcDON groups, based on body weight loss in long-term toxicity studies in mice (JECFA 2010). The acute reference dose (ARfD) was set at 8 μg/kg-bw as DON, 3-AcDON and 15-AcDON groups, based on the results of a study on emesis in pigs (JECFA 2010). In Japan, the Food Safety Commission of the Cabinet Office set an acceptable intake of 1 μg/kg-bw/day for DON alone in the risk assessment (Food Safety Commission of Japan 2010). It was reported that intake of DON from foods is unlikely to have adverse effects on health (Food Safety Commission of Japan 2010). DON is the only trichothecene mycotoxin with an advisory level in the Japanese feed safety system. In a risk assessment conducted by JECFA in 2001, the PMTDI was set at 0.06 μg/kg-bw for the mixture of T-2 and HT-2, based on variability of white blood cell and red blood cell counts in pigs (JECFA 2002). DAS was evaluated by JECFA in 2016 (JECFA 2017). Because DAS is structurally similar to T-2 and HT-2 and has similar toxic effects, the

PMTDI was set at 0.06 μg/kg-bw for T-2, HT-2 and DAS alone or in total (JECFA 2017).

Japan relies on imports for the majority of corn, soybeans, and other crops used to make feed and pet feed (MAFF 2021), but it is often difficult to control and monitor the occurrence of mycotoxins during the cultivation and transportation stages in exporting countries (FAMIC 2008b, Hayashi et al. 2014). Feed for livestock must not only be safe for livestock, but also the livestock products produced from the livestock that consume the feed must be safe for humans. In addition, dogs and cats have become important companion animals, and from the perspective of animal welfare, it is necessary to ensure the safety of feed for pet animals. To ensure the safety of livestock and feed for pet animals, it is necessary to eliminate raw materials and feed products contaminated with mycotoxins, and for this purpose, it is essential to develop quantitative analytical methods to detect mycotoxins in raw materials and feed products.

**Requirements for Analytical Methods of Mycotoxins**

Mycotoxin contamination is widespread worldwide (Binder et al. 2007). Food and Agriculture Organisation (FAO) estimates that 25% of the world's food is heavily contaminated with mycotoxins (Adams and Motarjemi 1999). FB contamination occurs very frequently in corn (Sydenham et al. 1991), and that of ZEA occurs in corn, wheat, barley, sorghum, and oats used in food and pet food (Zinedine et al. 2007; European Food Safety Authority 2011). In addition, there have been reports of grains contaminated with multiple fungal toxins. Co-contamination of grains with ZEA, deoxynivalenol, fumonisin, and nivalenol produced by *Fusarium* is frequently found (Mankevičienė et al. 2011; Tanaka et al. 2010). Therefore, simultaneous determination methods for multiple mycotoxins need to be developed. After the discovery of aflatoxin, thin-layer chromatography was often used for analysis, but liquid chromatography is now the mainstream (Kruska et al. 2012). Detectors for liquid chromatography include ultraviolet detectors, fluorescence detectors, mass spectrometers, and tandem mass spectrometers (Scudamore et al. 1996; Nakagawa et al. 2013). Mass spectrometers and tandem mass spectrometers, which are highly sensitive and selective, are used to analyse multiple components of mycotoxins simultaneously (Scudamore et al. 1996; Nakagawa et al. 2013).

After an analytical method is developed, results validated within a single laboratory may differ from results from other laboratories. Therefore, it is advisable to conduct inter-laboratory collaborative testing to confirm the validity of the method (AOAC International 2019; MAFF 2020). This is the best way to ensure that the method is sufficiently accurate and robust. Because pet foods have particularly high lipid and protein content, analytical methods for corn and other grains may not be applicable, and there are very few analytical methods for mycotoxins in pet foods themselves. Furthermore, there are no effective methods that use quantitative methods for mycotoxins in pet foods up to validation by inter-laboratory collaborative testing. Therefore, a method that has been validated that way is desired.

When setting the standard values for mycotoxins in pet foods in Japan, MAFF sets the draft standard values based on the results of the contamination survey in pet foods conducted by FAMIC and other organizations using official methods. (Hayashi et al. 2014). The proposed standard values are compared with safety data (effects on pets) and then set as the standard values (Hayashi et al. 2014). Therefore, in order to set a standard value for mycotoxins in pet foods in Japan, data from the actual contamination survey of mycotoxins in pet foods using the official method must be accumulated. However, until now, there was no official method for measuring FBs and ZEA in pet food, and thus the contamination status could not be determined, and we had a strong request from MAFF for such a method. The development of an analytical method is also essential in the pursuit of the cause of pet health problems. Currently, we have no standard value for FBs and ZEA in pet food in Japan, and no evaluation of the safety of FBs and ZEA in pet food for pets has been conducted.

For trichothecenes, JECFA set for DON, 3-AcDON, and 15-AcDON as the same group and considered the inclusion of D3G (JECFA 2010), and added DAS to the PMTDI for T-2 and HT-2 (JECFA 2017). Therefore, an analytical method for simultaneous measurement of T -2, HT-2, DAS, DON, 3-AcDON, 15-AcDON, and D3G was required but there was no official method for feed (FAMIC 2008c), MAFF strongly requested to develop an analytical method for simultaneous measurement.

In Chapter 1, the author developed an analytical method to determine $FB_1$, $FB_2$ and $FB_3$ in pet foods using a liquid chromatography-electrospray ionization-mass

spectrometer. An inter-laboratory collaborative study of a method for the determination of FBs in pet foods using liquid chromatography-mass spectrometry was conducted and applied to the investigation of contamination of commercially available pet foods. In Chapter 2, the author describes the development of a method for the determination of ZEA in pet foods using liquid chromatography-mass spectrometry and the implementation of an inter-laboratory collaborative study. In Chapter 3, the author describes the development of a simultaneous determination method using a liquid chromatography-tandem mass spectrometer for 10 trichothecene mycotoxins in feed, and the implementation of an inter-laboratory collaborative study.

The method for determination of FBs in pet foods, the method for determination of ZEA in pet foods, and the simultaneous determination method for 10 trichothecene mycotoxins in feeds in this thesis were all studied to make them official analytical methods and were included in the inspection methods for the Pet Food Inspection Method or Feed Analysis Standard (FAMIC 2008c; FAMIC 2009b). These studies are expected to contribute to the formulation of official analytical methods and to the safety of pet foods and feeds against mycotoxins (FAMIC 2008c; FAMIC 2009b).

# Chapter 1

# Development and inter-laboratory study of a method for quantification of fumonisins $B_1$, $B_2$ and $B_3$ in pet foods*

*This Chapter presents a revised version of the paper published in World Mycotoxin Journal, 2015, 8:1, 55-61 with the following co-authors:
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## 1.1 Introduction

$FB_1$ and $FB_2$ were detected in 42% of the commercial dry dog food samples, with a median of 122 µg/kg, and a maximum of 568 µg/kg (Böhm et al. 2010). In addition, the pollution of FBs occurs very frequently in maize, used as raw materials for food and pet food (Sydenham et al. 1991).

For these reasons, it is desirable to measure the amount of FBs in pet foods. However, there are very few validated methods to measure FBs in pet foods which were subjected to validation by an inter-laboratory study. FBs have been conventionally analysed using methods based on the AOAC Official Methods of Analysis 995.15 for maize and maize products (AOAC International 2016). Using this method, FBs are extracted with aqueous methanol, then purified by an anion exchange cartridge and analysed by high performance liquid chromatography with fluorescence detection. However, Aoyama et al. (2010) reported that for food with high lipid content (e.g., maize snacks), this method is not applicable. Instead, they extracted FBs from maize snacks with a water-methanol-acetonitrile (2:1:1, v/v/v) mixture and removed the fat from the samples with acetonitrile-saturated hexane. The FBs were further purified by a $C_{18}$ column and analysed by liquid chromatography mass spectrometry (LC-MS) (Aoyama et al. 2010). In pet foods, dietary protein and lipid contents are high in many cases. In our pilot studies, we found that recoveries of FBs from many pet food samples using the combination of water-methanol-acetonitrile (2:1:1, v/v/v) extraction and purification using SAX or $C_{18}$ column or immunoaffinity columns clean-up were not sufficient. Therefore, in this study, the author developed a method for the analysis of FBs using a multifunctional column (MFC), Multi MultiSep 221 Fum cartridge for pet food with high protein and lipid contents. The author chose LC-MS, since liquid chromatography tandem mass spectrometry (LC-MS/MS) is not prevalent in pet food and feed manufacturing laboratories. The method was subsequently validated through inter-laboratory collaborative study with eleven laboratories.

## 1.2 Materials and methods

### 1.2.1 Chemicals and samples

$FB_1$ and $FB_2$ standards were purchased from Sigma Aldrich Chemicals Co. (St. Louis,

MO, USA), and the $FB_3$ standard was obtained from PROMEC (Medical Research Council, Tygerberg, South Africa). Fumonisin stock solutions were prepared in a concentration of 50 μg/mL in acetonitrile-water (1:1, v/v). Spiking and calibration solutions were also prepared in acetonitrile-water (1:1, v/v). Formic acid was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan), and acetonitrile and methanol (LC-MS grade) were purchased from Kanto Chemical Co. Ltd (Tokyo, Japan). Analytical grade acetic acid and 28% ammonia water were purchased from Kanto Chemical Co. Ltd and Wako Pure Chemical Industries, Ltd, respectively. Ultrapure water was prepared by Milli-Q Integral 5 (Millipore; Molsheim, France) and used throughout the experiments.

All pet foods used in this study were purchased from local markets and small retail shops in major cities in Japan such as Sendai, Saitama, Tokyo, Nagoya, Kobe in 2012. Dry and semi-dry pet foods were crushed by a centrifuge mill ZM200 (with 1 mm mesh screen) (Retsch Co., Ltd, Hahn, Germany). Wet pet foods were homogenized by a food processor MK-K58 (Panasonic Co., Osaka, Japan). All samples were stored at 4°C until analysed.

### 1.2.2 Extraction and clean-up of samples

A 20.0 g sample of pet food was measured into a 200 mL Erlenmeyer flask, and extracted with 100 mL of acetonitrile-water (1:1, v/v) for 30 min using a SR-2W shaker (TAITEC, Saitama, Japan) set at 250 rpm. The sample was filtered using a Buchner funnel (Kiriyama Glass Works Co., Tokyo, Japan) with glass-fiber filter paper (GFP-60, Kiriyama). The residue was extracted with 50 mL of acetonitrile-water (1:1, v/v) and filtrated in the same way. This extract was combined to previous extract. The pH of the combined extracts was adjusted to 6-9 by ca 5% ammonia water, and the volume was adjusted to 200 mL by adding acetonitrile-water (1:1, v/v). A MultiSep 221 Fum cartridge (Romer Labs, Union, MO, U.S.) was fitted with an SPE manifold (GL-SPE, GL Science, Tokyo, Japan), and prewashed with 5 mL of methanol, followed by 5 mL of methanol-water (3:1, v/v). A sample solution (3 mL) was placed in a 20 mL test tube, and mixed with 8 mL of methanol-water (3:1, v/v). The mixture was applied to the MFC, while maintaining a flow rate of about 1 drop/sec by the use of the vacuum manifold. Then the MFC was washed with 8 mL of methanol-water (3:1, v/v), followed by 3 mL of methanol.

FBs were eluted with 20 mL of methanol-acetic acid (99:1, v/v) and collected into a 50 mL eggplant-shaped flask. The eluent was evaporated to dryness at 40°C. The resulting residue was dissolved in 1 mL of acetonitrile-water (1:1, v/v), then centrifuged at 5,000 ×g for 5 min, and collected in a 1.5 mL amber glass vial for LC-MS analysis. Scheme 1 shows the flow of this analytical method.

20 g of sample
- extracted by acetonitrile–water (1:1, v/v) (100 mL and 50 mL, twice)
- neutralized pH and filled up to 200 mL with acetonitrile–water (1:1, v/v)

Extract

3 mL of extract
- mixed with 8 mL of methanol–water (3:1,v/v)
- MultiSep 221 Fum cartridge (cartridge size 3 mL)
- washed with 8 mL of methanol–water (3:1, v/v)
- washed with 3 mL of methanol
- eluted by 20 mL of methanol–acetic acid (99:1, v/v)

Eluate (20 mL)
- evaporated to dryness at below 40°C

Residue
- dissolved in 1 mL of acetonitorile–water (1:1, v/v)
- centrifuged at 5,000×*g* for 5min

Supernatant
- analyzed by LC-ESI-MS (m/z 722 ($FB_1$), m/z 706 ($FB_2$ and $FB_3$))

**Scheme 1 Analytical procedure for fumonisins in pet foods**

### 1.2.3 LC-MS analysis

FBs were analysed by LC-MS (MSD SL, Agilent Technologies, Santa Clara, CA, USA). FBs were separated by an ODS column (ZORBAX Eclipse XDB-C18, 5 μm, 2.1×150 mm, Agilent Technologies) maintained at 40°C. The mobile phases were 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B). The initial gradient condition was 75% A and 25% B. Solvent B was increased linearly to 50% in 5 min, and was held for 3 min. Then solvent B was returned to 25% in 2 min and was held for 4 min before next injection. The flow rate was 0.2 mL/min and the injection volume was 5 μL.

For the detection of FBs, the sample was introduced to the MS in the electrospray

ionization (ESI) positive mode, and $FB_1$ was detected at *m*/*z* 722, while $FB_2$ and $FB_3$ were detected at *m*/*z* 706 by the selected ion monitoring mode. The fragment voltage was 220 V and the capillary voltage was +3000 V. $N_2$ gas at a flow rate of 10 L/min was used as the drying gas at 350°C and the nebulizer pressure was set at 50 psi.

The positive results were confirmed by LC-MS/MS. The ACQUITY UPLC System and ACQUITY Xevo TQ Detector (Waters, USA) were used for LC-MS/MS analysis. Chromatography conditions for LC-MS/MS were same as for LC-MS analysis. FBs were analysed by ESI with the positive ion mode. The quantifier and qualifier ions used were as follows.

Quantifier ions of $FB_1$: *m*/*z* 722 > 334

Quantifier ions of $FB_2$ and $FB_3$: *m*/*z* 706 > 336

Qualifier ions of $FB_1$: *m*/*z* 722 > 352

Qualifier ions of $FB_2$ and $FB_3$: *m*/*z* 706 > 354

### 1.2.4 Linearity of standard curve

To check the linearity of the calibration curves of FBs, 5 μL of a mixture of FBs (0. 02, 0.2, 0.4, 0.6, 0.8, 1.0 μg/mL each) was injected into the LC-MS, and the peak area was measured.

### 1.2.5 Limit of quantification and lower detection limit

To estimate the minimum quantitation limit of FBs, dry and semi-dry pet foods were spiked with 0.2 mg/kg of FBs, and wet pet foods were spiked with 0.1 mg/kg of FBs. Then, dry and semi-dry pet foods were left in the dark at room temperature overnight, while wet pet foods were left in the dark at room temperature for 30 min and then analysed. The limit of quantification (LOQ) and the limit of detection (LOD) were estimated according to the “guidelines on analytical terminology” (CAC/GL 72-2009) of Codex (Codex Alimentarius Commission 2009).

### 1.2.6 Elution of FBs from MultiSep 221 Fum cartridge

Extracts from the dry dog foods and wet cat foods were spiked with 0.2 mg/kg of $FB_1$, $FB_2$ and $FB_3$, according to the method described previously. Those extracts were applied to the MultiSep 221 Fum cartridge and 5 mL of the elution fractions from the MultiSep

221 Fum cartridge were collected in separate 50 mL eggplant-shaped flasks, and then analysed to determine which fractions contained FBs.

### 1.2.7 Spike and recovery test

A spike and recovery test was conducted using five types of pet foods spiked with 1.0 and 0.2 mg/kg FBs for dry dog food, dry cat food and semi-dry dog food, and 0.2 and 0.1 mg/kg FBs for wet dog food and cat food.

### 1.2.8 Matrix effect for quantification

The matrix effect was confirmed by measuring the peak area ratio of the standard solution and the sample solution in which the same concentration of the standard solution was added to the blank sample solution. According to this sample preparation method, blank sample solutions of dry dog food, semi-dry dog food and wet cat food were obtained. Then each sample was spiked with FB standards equivalent to 2 mg/kg and 0.4 mg/kg for dry and semi-dry pet food, and 1 mg/kg and 0.2 mg/kg for wet pet food. They were measured by LC-MS and compared with the standard solutions. A matrix effect ±20% was evaluated to be quantifiable without significant matrix effects.

### 1.2.9 Inter-laboratory study

Homogenized blank dry and semi-dry dog foods and a wet cat food samples were distributed to eleven laboratories, along with standard solutions to spike the samples. Each sample was sealed in aluminium pouch bags, and transported to each laboratory with a refrigerant. The samples were refrigerated until use. After the samples were spiked, dry and semi-dry pet foods, were stored overnight in dark at room temperature, while the wet cat food was stored in the dark at room temperature for 30 min before analysis. All the laboratories were requested to follow the same procedure. A standard curve was created using the standard solutions supplied, and then each sample was quantified. Each laboratory was required to provide significant results in 3-digit numbers. With these results, according to the protocol of IUPAC (AOAC International 2019), a Cochran and Grubbs single and Grubbs pair outlier test was conducted. After outliers were rejected, recovery, repeatability ($RSD_r$) and reproducibility ($RSD_R$) were calculated. The Horwitz ratio (HorRat) was also calculated according to a modified version of the Horwitz

equation (Thompson 2000).

## 1.3 Results

### 1.3.1 Linearity of standard curve

As shown in Figure 1-1, the calibration curve was in the range of 0.1-5 ng (0.02-1.0 μg/mL), the coefficient of determination was at least 0.9995 for $FB_1$, $FB_2$ and $FB_3$, and the calibration curves were linear. Calibration curve was liner in the range from 0.1 ng (0.067 mg/kg) to 5 ng (3.3 mg/kg).

### 1.3.2 Limit of quantification and lower detection limit

FBs (0.2 mg/kg) were added to dry and semi-dry pet foods, and 0.1 mg/kg of FBs was added to wet pet foods and then the samples were analysed. From the concentration of 10 times the standard deviation (σ) shown in Table 1-1, the LOQ was estimated to be 0.2 mg/kg for dry and semi-dry pet foods, and 0.1 mg/kg for wet pet foods. The LOD was also estimated to be 0.1 mg/kg for dry and semi-dry pet foods, and 0.06 mg/kg for wet pet foods.

### 1.3.3 FBs elution from MultiSep 221 Fum cartridge

In dry dog food, the recovery rate was 97% in the fraction of 0-5 mL and 6% in the fraction of 5-10 mL for $FB_1$. These values were 96% and 4% for $FB_2$ and 98% and 5% for $FB_3$, and 1% in the fraction of 10-15 mL for $FB_3$. In wet cat food, the recovery rate was 55% in the fraction of 0-5 mL, 29% in the fraction of 5-10 mL, 8% in the fraction of 10-15 mL and 1% in the fraction of 15-20 mL for $FB_1$. These values were 62%, 27%, 7% and 1% for $FB_2$ and 64%, 23%, 5% and 1% for $FB_3$. Because FBs were eluted from the MFC in the first 20 mL solvent, the amount of the elution solvent was set at 20 mL.

### 1.3.4 Spike and recovery test

As shown in Table 1-2, mean recoveries and relative standard deviations of $FB_1$ were in the range of 93.3-107% and below 7.9%, respectively. For $FB_2$, those values were 87.3-102% and 8.6%, and for $FB_3$, the values were 90.8-102% and 8.6%, respectively. Figure 1-2 shows an example of spike and recovery test SIM (selected ion monitoring)

chromatograms.

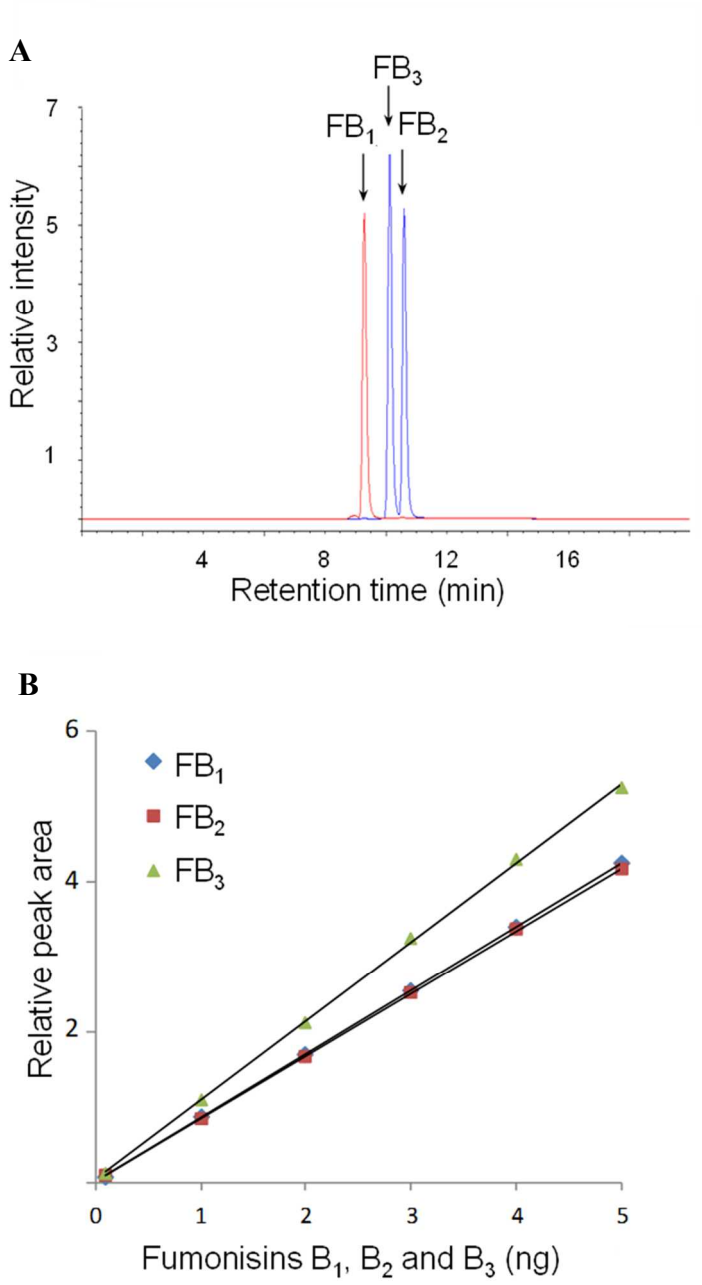


**Figure 1-1 (A) LC-MS SIM chromatogram of standard solution for fumonisins $B_1$, $B_2$ and $B_3$.**
**(B) Calibration curves of fumonisins $B_1$, $B_2$ and $B_3$.**

(A) Standard solution (2 ng) of FBs (B) 5 µL of mixture of FBs (0. 02, 0.2, 0.4, 0.6, 0.8, 1.0 µg/mL each) were injected into the LC-MS.
LC-MS: MSD SL, Agilent Technologies. LC column: ZORBAX Eclipse XDB-C18, 5 µm, 2.1×150 mm, Agilent Technologies. Column temperature: 40°C. Mobile phases (gradient): 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B). Flow rate: 0.2 mL/min. Injection volume: 5 µL. Ionization: ESI, positive mode. Monitor ion: *m/z* 722 ($FB_1$), *m/z* 706 (FB2 and FB3). The fragment voltage: 220 V. Capillary voltage: +3000 V. Drying gas: $N_2$ (10 L/min, 350°C). Nebulizer pressure: 50 psi.

**Table 1-1 Fumonisin recoveries in spiked pet foods at levels near the limit of quantification**

| | Sample | Dry dog food | Dry cat food | Semi-dry dog food | Wet dog food | Wet cat food |
|---|---|---|---|---|---|---|
| | Spiked level (mg/kg) | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 |
| $FB_1$ | Recovery[a] (%) | 98.7 | 107 | 101 | 103 | 107 |
| | σ [b] (mg/kg) | 0.0099 | 0.0167 | 0.0084 | 0.0036 | 0.0065 |
| | $RSD_r$[c] (%) | 5.0 | 7.9 | 4.2 | 3.5 | 6.1 |
| $FB_2$ | Recovery[a] (%) | 99.8 | 96.0 | 97.8 | 101 | 102 |
| | σ [b] (mg/kg) | 0.0121 | 0.0165 | 0.0096 | 0.0053 | 0.0030 |
| | $RSD_r$[c] (%) | 6.1 | 8.6 | 4.9 | 5.3 | 3.0 |
| $FB_3$ | Recovery[a] (%) | 97.3 | 97.9 | 101 | 101 | 101 |
| | σ [b] (mg/kg) | 0.0113 | 0.0168 | 0.0054 | 0.0055 | 0.0021 |
| | $RSD_r$[c] (%) | 5.8 | 8.6 | 2.7 | 5.5 | 2.1 |

[a] Mean ($n$=5), [b] Standard deviation, [c] Relative standard deviation of repeatability

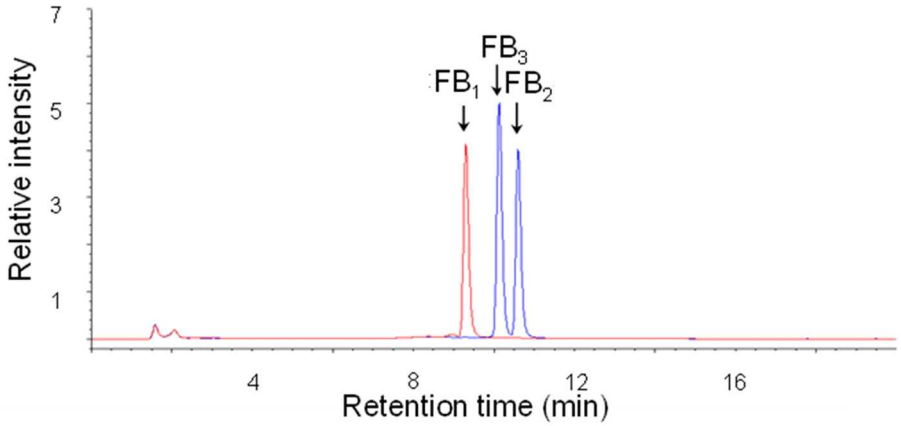


**Figure 1-2 LC-MS SIM Chromatogram of dry dog food (spiked fumonisins).**

(A) Dry dog food extract (spiked at 1 mg/kg fumonisins) was injected into the LC-MS.

LC-MS: MSD SL, Agilent Technologies. LC column: ZORBAX Eclipse XDB-C18, 5 μm, 2.1×150 mm, Agilent Technologies. Column temperature: 40°C. Mobile phases (gradient): 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B). Flow rate: 0.2 mL/min. Injection volume: 5 μL. Ionization: ESI, positive mode. Monitor ion: $m/z$ 722 ($FB_1$), $m/z$ 706 ($FB_2$ and $FB_3$). The fragment voltage: 220 V. Capillary voltage: +3000 V. Drying gas: $N_2$ (10 L/min, 350°C). Nebulizer pressure: 50 psi.

**Table 1-2 Recovery of fumonisins from spiked pet foods (*n*=5)**

| | Sample | Dry dog food | | Dry cat food | | Semi-dry dog food | | Wet dog food | | Wet cat food | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Spiked level (mg/kg) | Recovery (%) | $RSD_r$[a] (%) | Recovery (%) | $RSD_r$[a] (%) | Recovery (%) | $RSD_r$[a] (%) | Recovery (%) | $RSD_r$[a] (%) | Recovery (%) | $RSD_r$[a] (%) |
| $FB_1$ | 1 | 93.3 | 6.3 | 102 | 5.8 | 103 | 3.9 | - | - | - | - |
| | 0.2 | 98.7 | 5.0 | 107 | 7.9 | 101 | 4.2 | 103 | 1.3 | 98.4 | 4.4 |
| | 0.1 | -[b] | - | - | - | - | - | 103 | 3.5 | 107 | 6.1 |
| $FB_2$ | 1 | 91.9 | 4.9 | 87.3 | 5.5 | 98.0 | 3.8 | - | - | - | - |
| | 0.2 | 99.8 | 6.1 | 96.0 | 8.6 | 97.9 | 4.9 | 102 | 1.8 | 96.8 | 1.8 |
| | 0.1 | - | - | - | - | - | - | 101 | 5.3 | 102 | 3.0 |
| $FB_3$ | 1 | 90.8 | 6.1 | 90.9 | 6.4 | 101 | 5.0 | - | - | - | - |
| | 0.2 | 97.3 | 5.8 | 97.9 | 8.6 | 101 | 2.7 | 102 | 1.4 | 101 | 1.6 |
| | 0.1 | - | - | - | - | - | - | 101 | 5.5 | 101 | 2.1 |

[a] Relative standard deviation of repeatability, [b] Not tested

### 1.3.5 Matrix effect for quantification

The matrix effect was about 10% for most samples and the enhancement of the signal was up to 18%. Since the matrix effect was less than 20%, it was evaluated not to affect the determination of FBs.

### 1.3.6 Inter-laboratory study

The results of the inter-laboratory collaborative study are shown in Table 1-3. The data from two laboratories were excluded from the Cochran test for $FB_1$, and the data from one laboratory were excluded from the Grubbs test for $FB_2$. The rejected outliers were attributable to a low recovery rate and variability, but the cause of this was not clear. The mean recovery, $RSD_r$, $RSD_R$, and HorRat for $FB_1$ were 92.9-98.9%, 2.6-4.6%, 6.8-10%, and 0.41-0.54, respectively. For $FB_2$, the values were 91.5-94.7%, 2.7-5.9%, 6.8-8.9% and 0.33-0.55, respectively. For $FB_3$, the values were 90.1-94.3%, 3.3-5.9%, 7.3-9.5% and 0.44-0.57, respectively. From these results, the developed method is considered to be suitable for the analysis of FBs in pet foods.

### 1.3.7 Analysis in commercial pet foods

Eleven dry dog foods, three dry cat foods, three semi-dry dog foods, three wet dog foods, and two wet cat foods were analysed. FBs were frequently detected by LC-MS in five of the eleven dry dog foods (45%) and one dry cat food (33%). FBs were not detected in the other 16 pet food samples. All peaks detected from these samples by LC-MS were also analysed and confirmed as FBs by LC-MS/MS analysis. Therefore, LC-MS was adequate to analyse the FB content in pet food samples. Only values of FB content higher than LOD were calculated. The maximum level of $FB_1$ contamination in dry dog food was 1.4 mg/kg with a mean value of 0.72 mg/kg. These values were 0.33 mg/kg and 0.21 mg/kg for $FB_2$ and 0.24 mg/kg and 0.13 mg/kg for $FB_3$. In dry cat food, the maximum level of $FB_1$ contamination was 0.80 mg/kg with a mean value of 0.45 mg/kg. These values were 0.26 mg/kg and 0.14 mg/kg for $FB_2$ and 0.13 mg/kg for $FB_3$. These results were identical with those of the eleven samples measured by LC-MS/MS. The quantifier ion/qualifier ion ratios were same for standard solutions and positive samples (1.1 for $FB_1$,

**Table 1-3 Results of inter-laboratory study of FBs in pet foods**

| | $FB_1$ | | | $FB_2$ | | | $FB_3$ | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample[a] | Dry | Semi-dry | Wet | Dry | Semi-dry | Wet | Dry | Semi-dry | Wet |
| Participant labs[b] | 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 |
| Statistical labs[c] | 10[f] | 11 | 10[f] | 11 | 11 | 10[g] | 11 | 11 | 11 |
| Spiked level (mg/kg) | 1.0 | 0.8 | 0.2 | 1.0 | 0.8 | 0.2 | 1.0 | 0.8 | 0.2 |
| Recovery (%) | 95.0 | 98.9 | 92.9 | 91.5 | 94.7 | 92.2 | 91.2 | 94.3 | 90.1 |
| $RSD_r$ [d] (%) | 3.5 | 2.6 | 4.6 | 5.9 | 2.7 | 5.5 | 5.9 | 3.3 | 5.3 |
| $RSD_R$ [e] (%) | 8.8 | 6.8 | 10 | 8.9 | 7.6 | 6.7 | 9.3 | 7.3 | 9.5 |
| HorRat | 0.54 | 0.41 | 0.49 | 0.55 | 0.45 | 0.33 | 0.57 | 0.44 | 0.46 |

[a] Fumonisins were spiked at 1.0 mg/kg for dry dog food, 0.8 mg/kg for semi-dry dog food, 0.2 mg/kg for wet cat food. [b] Number of participant laboratories, [c] Number of laboratories included in statistical analysis. [d] Relative standard deviation for repeatability, [e] Relative standard deviation for reproducibility, [f] The data from one laboratory was excluded by the Cochrane test, [g] The data from one laboratory was excluded by the Grubbs test

3.0 for $FB_2$, 2.5 for $FB_3$) thereby confirming that the positive samples were naturally contaminated.

## 1.4 Discussion

Few analytical methods for FBs in pet food have been reported, and $FB_3$ is not included in the analysis (Scudamore et al. 1997; Pagliuca et al.2011). In Japan and overseas we did not have any method validated by inter-laboratory studies to determine FBs in pet foods, and therefore, had no choice but to develop an official method for FBs in feeds as a reference (FAMIC 2008c). However, pet foods have high lipid and protein contents, and the purification method using an anion exchange cartridge used in the official method for FBs in feeds could not be applied because the recovery rate of FBs in spike and recovery tests was low.

FBs are extracted in feed with methanol-water (3:1), purified with an anion exchange cartridge Bond Elut LRC SAX cartridge (Varian, Palo Alto, CA, USA), and measured by LC-MS (FAMIC 2008c).

However, in pet food, the ionic strength in the extracted solution is high due to counter ions such as salts and organic acids contained in the sample, making it difficult for FBs to be retained in the ion exchange column, resulting in low recovery rates in spike and recovery tests. Therefore, the author had an experience of not being able to apply the official feed method (feed analysis standard listing method) (FAMIC 2008c). In addition, it has been reported that methanol-water (3:1) as an extractant is not sufficiently extractable for samples such as rice (Kushiro et al. 2008). Bennett et al. reported that acetonitrile-water (1:1) was the best extraction solvent for fumonisins $B_1$ and $B_2$ in corn (Bennett and Richard 1994), and in pet food, the recovery rate was better in acetonitrile-water (1:1). Therefore, acetonitrile-water (1:1) was used in this method. Here, however, wet-type pet food contains about 70% (or more) water, so the ratio of extraction solvent changes. In order to prevent the influence of water, the official method for pet food often involves two extractions, the first time to remove water and the second time for the original extraction. Therefore, the author decided this two-extraction method. The sample solution was extracted with acetonitrile-water (1:1) and submitted to an InertSepC18 mini-column (1 g/column volume: 6 mL, GL Science, Tokyo, Japan) for lipid removal.

The column was then acidified with formic acid and subjected to InertSepC18 mini column to retain fumonisin. After removal of foreign components, the sample was purified on a Bond Elut LRC SAX mini-column, resulting in low recovery, especially for samples with high lipid and protein content. To improve purification efficiency, the loading volume of the InertSepC18 mini-column was doubled to 2 g, and the sample volume loaded on the mini-column was reduced by one-half to 2.5 mL. As a result, the recovery was slightly increased, but the recovery was still low, so the method using the InertSepC18 mini-column was abandoned. SAX column (Yoshinari et al. 2013a) used in analytical methods for FBs as well as immunoaffinity columns fumoniprep (R-Biopharm AG, Germany) (Castegnaro et al. 2006) and immunoaffinity columns FumoniStar (Romer Labs, Union, MO, U.S.) (Rodrigues et al. 2011), were used to examine purification methods, but could not applied to FBs in pet food due to low recovery of $FB_3$ in spike and recovery tests. This method is a valuable method that can be applied to FBs in pet foods with high lipid and protein content, which are considerably difficult to purify by other analytical methods.

Finally, the author was able to develop a method for the determination of FBs using a MFC, which requires short purification time and allows rapid determination for pet foods with high lipid content, which are difficult to purify. In Japan, in order to set a standard value for FBs in pet food, it is necessary to accumulate data of a survey on the contamination of FBs in pet food using an official method (Hayashi et al. 2014).

The added concentrations in the spike and recovery tests were performed at two different concentrations. Since the standard value of FBs in pet food in Japan has not been established, we set the assumed standard value of $FB_1$, $FB_2$ and $FB_3$ at 1 mg/kg, respectively, referring to the indicated value in EU (total of 5 mg/kg for $FB_1$ and $B_2$) (Commission of the European Communities 2006a), and confirmed that this concentration is sufficiently measurable. The high concentration side was performed at 1 mg/kg each of $FB_1$, $B_2$, and $B_3$ as the assumption standard value. For wet products with approximately 90% water content, the 1 mg/kg addition concentration was converted to 10% water content, which is the water content of dry products, at an addition concentration of 0.2 mg/kg. The low concentration side of the spike and recovery tests was performed near the LOQ. In order to make the standard values measurable, the

official method for pet food stipulates the following target values for the limit of quantification (LOQ) and the limit of detection (LOD) with respect to the standard values (FAMIC 20009b).

Target value of LOQ: 1/5 or less of the standard value for dry and semi-dry products, and 1/2 or less of the standard value for wet products

Target value of LOD: 1/10 or less of the standard value for dry and semi-dry products, and 1/3 or less of the standard value for wet products

Although no standard value for FBs in pet foods in Japan has been established, the sensitivity of this analytical method is sufficient for the determination of FBs in pet foods because both the LOQ and the LOD for the assumed standard value of FBs were satisfied.

After being consulted by the official methods review committee, the results of the single-laboratory and inter-laboratory collaborative validity studies met all the requirements of the Official Methods for Pet Food (FAMIC 2009b) and were therefore made Official Methods. The following points were very highly evaluated at the review meeting. 1) The extractant and purification column are different from those of the official method for feed (the method listed in the Standard for Feed Analysis), and it is a completely new analytical method. 2) The analytical method was developed with great difficulty for pet food, which has a high lipid content and is difficult to purify. 3) It is a simple quantitative method using a multifunctional column, highly repeatable and reproducible, and has a recovery rate close to 100%. 4) The results of inter-laboratory collaborative studies have been favorable. Furthermore, the limit of quantitation is sufficient for the determination of FBs in pet food.

Since this method has been listed in an official method for pet food (FAMIC 2009b), the safety of FBs in pet food will be evaluated, including whether the standard value of FBs in pet food is necessary when data under actual contamination conditions are accumulated by this method in the future. Therefore, this method will directly contribute to the safety of pet food.

In conclusion, for the detection of FBs in pet foods, the author developed a quantitative analytical method using an MFC, MultiSep 211 Fum cartridge. Spike and recovery studies showed that the recovery of FBs was more than 87.3% for all the samples. The LOQ and the LOD of each fumonisin were 0.2 mg/kg and 0.1 mg/kg, respectively, for dry and semi-

dry pet foods, and 0.1 mg/kg and 0.06 mg/kg for wet pet food. The mean recovery, repeatability, reproducibility and HorRat of FBs from the inter-laboratory study were 90.1-98.9%, 2.6-5.9%, 6.8-10% and 0.33-0.57, respectively. From these results, we conclude that this method is suitable to analyse FBs in pet foods.

# Chapter 2

# Development and inter-laboratory study of a method for quantification of zearalenone in pet foods*

*This Chapter presents a revised version of the paper published in World Mycotoxin Journal, 2016, 9:4, 497-503 with the following co-authors:
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## 2.1 Introduction

ZEA was detected in 47% of the commercial dry dog food samples, with a median of 80 μg/kg, and a maximum of 298 μg/kg. For this reason, it is desirable to measure the amount of ZEA in pet foods (Böhm et al. 2010). However, there are no methods that use a LC-MS or LC-MS/MS to measure ZEA in pet foods that have been validated by an inter-laboratory study. In previous studies, ZEA in food and feed grains was extracted by solvents such as methanol/water or acetonitrile/water, and then purified using an immunoaffinity column (Fazekas and Tar 2001; Campbell and Armstrong 2007) and a MultiSep226® column (Tanaka et al. 2010). Since pet foods contain more protein and fat than other foods, it is expected to be difficult to purify ZEA from pet foods. Whether a MFC or an immunoaffinity column was applicable for the analysis of ZEA present in pet food had not been confirmed. Therefore, in this study, the author developed a method to analyse the quantity of ZEA, using an MFC for pet food with a high protein and lipid content. The author choses the simpler LC-MS method since LC-MS/MS instruments are not readily available in pet food and feed manufacturing laboratories. The method was subsequently validated through inter-laboratory collaborative study with eleven laboratories.

## 2.2 Materials and methods

### 2.2.1 Chemicals and samples

The ZEA standard was purchased from Sigma Aldrich Chemicals Co. (St. Louis, MO, USA), and a ZEA stock solution was prepared in a concentration of 200 μg/mL in acetonitrile. Spiking and calibration solutions were prepared in acetonitrile/water (acetonitrile-water (1:1, v/v)). A 1 mol/L solution of ammonium acetate was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan), and acetonitrile and methanol (LC-MS grade) were purchased from Kanto Chemical Co. Ltd (Tokyo, Japan). Ultrapure water was prepared by a Milli-Q Integral 5 system (Millipore, Molsheim, France) and used throughout the experiments. All pet foods used in this study were purchased randomly from local markets and small retail shops in major cities in Japan such as Sendai, Saitama, Tokyo, Nagoya, and Kobe in 2012. Dry and semi-dry pet foods were crushed using a centrifuge mill ZM200 (with 1 mm mesh screen) (Retsch, Hahn,

Germany). Wet pet foods were homogenized by an MK-K58 food processor (Panasonic, Osaka, Japan). All samples were stored at 4°C until analysed. After the samples were crushed or homogenized, they were spiked with the ZEA standard solution. Before spiking, all samples were confirmed to be below the LOD of ZEA by a 'Study of interfering substances'. All spiked samples for the study of 'LOD and LOQ', 'Method validation', and dry and semi-dry pet foods were left in the dark at room temperature overnight, while wet pet foods were left in the dark at room temperature for 30 min, and then analysed.

### 2.2.2 Extraction and clean up samples

A 25.0 g sample of pet food was measured into a 300 mL Erlenmeyer flask, and extracted with 75 mL of acetonitrile-water (21:4, v/v) for 30 min using an SR-2W shaker (TAITEC, Saitama, Japan) set at 250 rpm. The sample was then filtered using a Buchner funnel (Kiriyama Glass Works Co., Tokyo, Japan) with glass-fibre filter paper (GFP-60, Kiriyama). The residue was extracted with 50 mL of acetonitrile-water (21:4, v/v) by shaking for 30 min and was then filtered again using the previous procedure. The total volume of the combined extracts was adjusted to 200 mL by adding acetonitrile-water (21:4, v/v). Fifteen mL of the extract solution was purified using a MultiSep 226 Aflazon+® cartridge (Romer Labs), discarding the first 4 mL of eluate. The following 3 mL of eluate from the cartridge was collected and exactly 2 mL was transferred into a 50 mL eggplant-shaped flask and evaporated to dryness at 50°C. The resulting residue was dissolved in 1 mL of acetonitrile-water (1:1, v/v), then centrifuged at 5,000 × g for 5 min, and collected in a 1.5 mL amber glass vial for LC-MS analysis. Scheme 2 shows the flow chart for this analytical method.

### 2.2.3 LC-MS analysis

ZEA was analysed by LC-MS (LCMS-2010EV, Shimadzu Co. Ltd, Kyoto, Japan). ZEA was separated by an ODS column (L-column2 ODS, 5 μm, 3×250 mm, Chemicals Evaluation and Research Institute, Japan (CERI)), which was maintained at a temperature of 40°C. The mobile phases were a 10 mmol/L aqueous ammonium acetate solution (mobile phase A) and acetonitrile-methanol (4:7, v/v) (mobile phase B). The initial

25 g of sample
- extraction by acetonitrile–water (21:4, v/v) (75 mL and 50 mL, twice)
- filled up 200 mL with acetonitrile–water (21:4, v/v)

Extract
- MultiSep226 AflaZon$^{+}$ cartridge (cartridge size 6 mL)

Eluate (discarded the first 4 mL and collected the following 3 mL of eluate)

2 mL of eluate
- evaporated to dryness at below 50°C
- dissolved in 1 mL of acetonitrile–water (1:1, v/v)
- centrifuge at 5,000×*g* for 5 min

Supernatant
- analyzed by LC-APCI-MS (*m/z* : 317)

**Scheme 2 Analytical procedure for ZEA in pet foods**

solvent gradient was set to 45% A and 55% B. Solvent B was increased linearly from 55% to 95% over a period of 10 min, and was held at 95% B for an additional 5 min. The flow rate was 0.5 mL/min and the injection volume was 10 µL. For the detection of ZEA, the sample was introduced to the MS in the atmospheric pressure chemical ionization (APCI) detector in negative mode, and ZEA was detected at *m/z* 317 in the selected ion-monitoring mode. The temperatures of the interface, heat-block, and curved desolvation line (CDL) were 400°C, 200°C, and 200°C, respectively. Nitrogen was used as a nebulizer gas with a flow rate of 2.5 L/min.

### 2.2.4 Standard curve, LOD and LOQ

To check for linearity in the calibration curves of ZEA, 10 µL of a ZEA standard solution (0.01, 0.2, 0.4, 0.6, 0.8, 1.0 µg/mL each) was injected into the LC-MS, and the corresponding ZEA peak height or peak area was measured. In this study, the peak area was measured for quantification of ZEA. To estimate the minimum quantitation limit of ZEA, dry and semi-dry pet foods were spiked with 0.2 mg/kg of ZEA, and wet pet foods were spiked with 0.1 mg/kg of ZEA. The LOQ and the LOD were estimated according to the ‘Guidelines on Analytical Terminology’ (CAC/GL 72-2009) of Codex (Codex Alimentarius Commission 2009).

### 2.2.5 Matrix effect for quantification

The matrix effect was confirmed by measuring the peak area ratio of the standard solution and the sample solution in which the same concentration of the standard solution was added to the blank sample solution. According to this sample preparation method, blank sample solutions of dry dog food, semi-dry dog food, and wet cat food were obtained. Then each sample was spiked with ZEA standards, equivalent to 2 mg/kg and 0.4 mg/kg for dry and semi-dry pet food, and 1 mg/kg and 0.2 mg/kg for wet pet food. These samples were analysed by LC-MS and compared to the standard solutions. A matrix effect ±20% was evaluated to be quantifiable without significant matrix effects.

### 2.2.6 Method validation

A spike and recovery test was conducted using five types of pet foods: dry dog food, dry cat food, semi-dry dog food, wet dog food, and wet cat food. The pet food samples were spiked with 1.0 and 0.2 mg/kg ZEA for dry and semi-dry food, and 0.2 and 0.1 mg/kg ZEA for wet food. For the inter-laboratory studies, a homogenized blank dry and semi-dry dog food and a wet cat food sample were distributed to eleven laboratories, along with standard solutions to spike the samples. Each sample was sealed in an aluminium pouch bag, and transported to each independent laboratory in a refrigerated container. The samples were refrigerated at 4°C until use. After the samples were spiked with the ZEA standard solution that was distributed by us, dry and semi-dry pet foods, were stored overnight in the dark at room temperature, while the wet cat food was stored in the dark at room temperature for 30 min before analysis. All laboratories were requested to follow the same procedure by using the same mobile phase, flow rate, column oven temperature, APCI mode (negative), quantifier ion, column injection volume, column, and to confirm that the S/N was over 10 at 0.05 μg/mL standard solution for their instruments. Table 2-1 shows the instruments used in the inter-laboratory study. A standard curve was created using the standard solutions supplied, and then each sample was quantified. Each laboratory was required to provide results with an accuracy of three significant digits. With these results, according to the protocol of IUPAC (AOAC International 2019), Cochran and Grubbs single and Grubbs pair outlier tests were conducted. After outliers were rejected, the recovery, $RSD_r$, and $RSD_R$ were calculated. The HorRat was also calculated according to a modified version of the Horwitz equation

(Thompson 2000).

**Table 2-1 The liquid chromatography (LC) and mass spectrometry (MS) instruments used in the inter-laboratory study**

| Lab.No. | Instrument |
|---|---|
| 1 | LC: Prominence, Shimadzu<br>MS: LCMS2010EV, Shimadzu |
| 2 | LC: Prominence, Shimadzu<br>MS: LCMS2010EV, Shimadzu |
| 3 | LC: UFLC XR CBM-20A, Shimadzu<br>MS: TSQ Quantum Ultra, Thermo fisher scientific |
| 4 | LC: Prominence, Shimadzu<br>MS: LCMS2010EV, Shimadzu |
| 5 | LC: ACQUITY, Waters<br>MS: TQD, Waters |
| 6 | LC: UPLC-Acquity, Waters<br>MS: Quattro Premier XE, Waters |
| 7 | LC: 2695, Waters<br>MS: Quattro micro API, Micromass |
| 8 | LC: 1100 Series, Agilent Technologies<br>MS: LC/MSD SL G1956B, Agilent Technologies |
| 9 | LC: Nexera, Shimadzu<br>MS: LCMS2020, Shimadzu |
| 10 | LC: Prominence, Shimadzu<br>MS: LCMS2010EV, Shimadzu |
| 11 | LC: LC-10ADvp, Shimadzu<br>MS: LCMS2010A, Shimadzu |

## 2.3 Results

### 2.3.1 LC-MS analysis

This study used APCI in negative ion mode for the detection of the molecular ion of ZEA, as this method is commonly used for this purpose. The ZEA standard was measured in scan mode, and the mass spectrum is shown in Figure 2-1. The most sensitive ion (*m*/*z* 317 ($[MH]^-$)) was used. No other ions that could be used to confirm the presence of ZEA

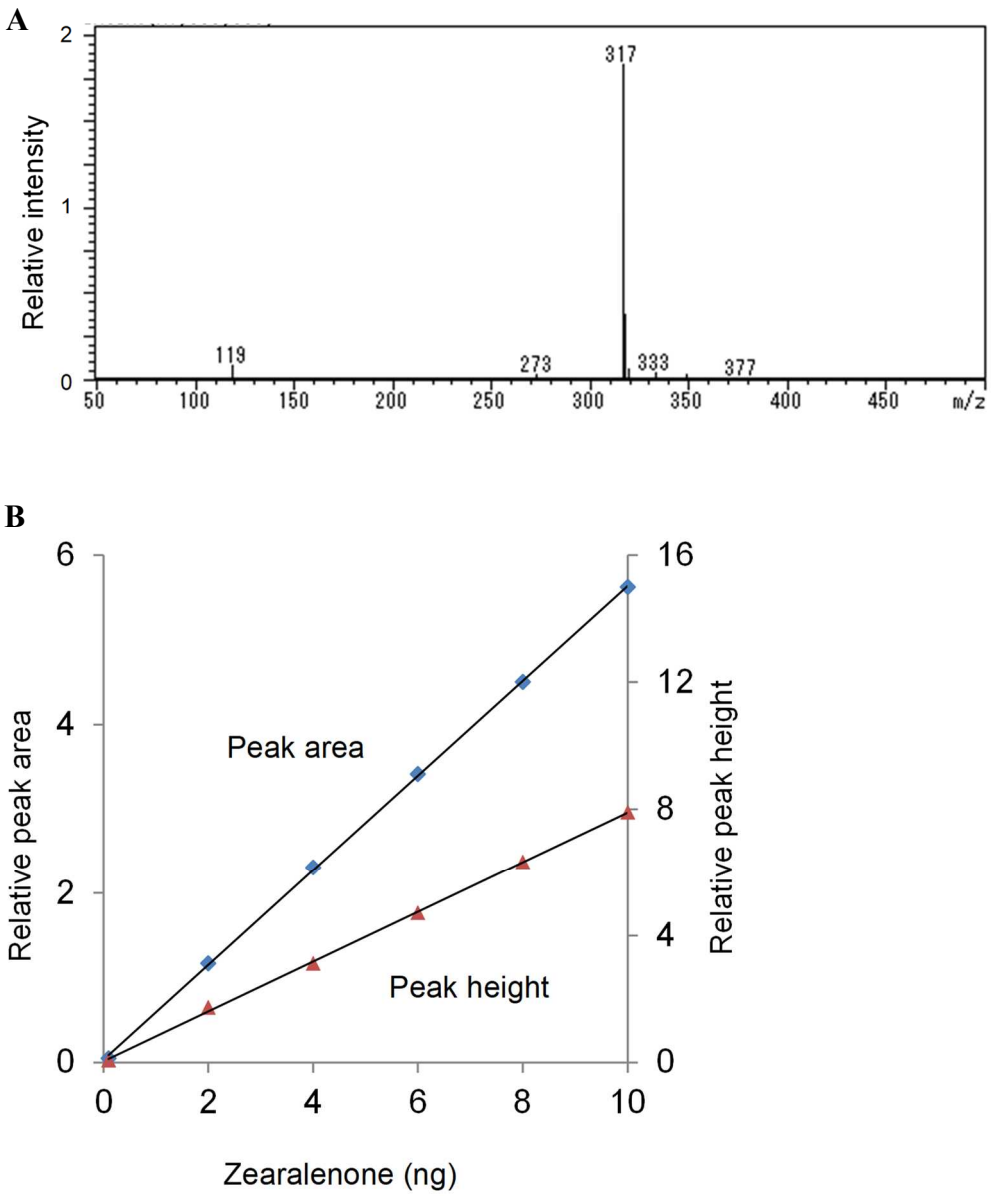


**Figure 2-1 (A) Mass spectrum of ZEA standard solution by LC-APCI-MS. (B) Calibration curves for ZEA**

(A) Standard solution (10 ng) of ZEA (B) 5 µL of ZEA (0. 02, 0.2, 0.4, 0.6, 0.8, 1.0 µg/mL each) were injected into the LC-MS (left axis: area, right axis: height).

LC-MS: LCMS-2010EV, Shimadzu Co. Ltd. LC column: L-column2 ODS, 5 µm, 3×250 mm, Chemicals Evaluation and Research Institute, Japan (CERI). Column temperature: 40°C. Mobile phases (gradient): 0.1% (v/v) 10 mmol/L aqueous ammonium acetate solution (mobile phase A) and acetonitrile-methanol (4:7, v/v) (mobile phase B). Flow rate: 0.5 mL/min. Injection volume: 10 µL. Ionization: APCI, negative mode. Monitor ion: *m/z* 317. Interface temperature: 400°C. Heat block temperature: 200°C. CDL temperature: 200°C. Nebulizer gas: $N_2$ (2.5 L/min).

were found. As shown in Figure 2-1, the calibration curve for ZEA was linear in the range from 0.1 ng (0.04 mg/kg) to 10 ng (4 mg/kg) and the coefficient of determination was at least 0.9995. ZEA (0.2 mg/kg) was added to dry and semi-dry pet foods, and 0.1 mg/kg of ZEA was added to wet pet foods, and then the samples were analysed. The LOQ was estimated from the concentration of 10 times the standard deviation (σ) shown in Table 2-2, to be 0.2 mg/kg for dry and semi-dry pet foods, and 0.1 mg/kg for wet pet foods. The LOD was also estimated to be 0.1 mg/kg for dry and semi-dry pet foods, and 0.06 mg/kg for wet pet foods.

### 2.3.2 Study of interfering substances

Eleven dry dog foods, three dry cat foods, three semi-dry dog foods, three wet dog foods, and two wet cat foods were used to determine the presence of interfering substances. ZEA was detected by LC-MS in eleven dry dog foods and three dry cat foods. All the peaks observed by LC-MS were small, below the LOD of ZEA. As for all the samples used, they were confirmed that the content of ZEA is below LOD.

### 2.3.3 Matrix effect for quantification

Some signal enhancement was observed with the matrix. However, this enhancement effect was less than 9%, except for one wet cat feed at 15%. Therefore, we decided to generate the calibration curve using non-matrixed standard solutions.

### 2.3.4 Method validation

As shown in Table 2-2, the mean recovery of ZEA was in the range of 90.8-108% and the relative standard deviations were below 8.0%. Figure 2-2 shows an example of spike and recovery test SIM chromatograms of ZEA. As shown in the results of the inter-laboratory collaborative study (Table 2-3), the mean recovery of ZEA was in the range of 99.0-102%, and $RSD_r$ and reproducibility $RSD_R$ and HorRat were 2.2-3.0%, 5.6-6.6%, and 0.33-0.36, respectively. These results show that the method we have developed is suitable for the analysis of ZEA in pet foods.

**Table 2-2 Recoveries of Zearalenone (*n*=5)**

| Sample | Dry dog food 1 | | | Dry dog food 2 | | | Dry cat food 1 | | | Dry cat food 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spiked level (mg/kg) | Recovery (%) | $RSD_r$[a] (%) | σ[b] (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) |
| 1 | 102 | 1.6 | 0.0167 | 108 | 1.1 | 0.0122 | 102 | 2.3 | 0.0230 | 104 | 2.0 | 0.0205 |
| 0.2 | 103 | 1.6 | 0.0032 | 103 | 4.3 | 0.0089 | 100 | 3.5 | 0.0069 | 106 | 2.3 | 0.0049 |

| Sample | Semi-dry dog food 1 | | | Semi-dry dog food 2 | | |
|---|---|---|---|---|---|---|
| Spiked level (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) |
| 1 | 102 | 3.0 | 0.0308 | 95.3 | 1.0 | 0.0093 |
| 0.2 | 98.7 | 1.5 | 0.0030 | 104 | 1.2 | 0.0026 |

| Sample | Wet dog food 1 | | | Wet dog food 2 | | | Wet cat food 1 | | | Wet cat food 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spiked level (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) |
| 1 | 105 | 0.8 | 0.0019 | 106 | 2.6 | 0.0030 | 96.7 | 6.6 | 0.0127 | 96.7 | 8.0 | 0.0154 |
| 0.2 | 100 | 1.9 | 0.0018 | 104 | 2.9 | 0.0054 | 90.8 | 7.2 | 0.0066 | 100 | 3.7 | 0.0037 |

| Sample | Overall | | |
|---|---|---|---|
| Spiked level (mg/kg) | Recovery (%) | $RSD_r$ (%) | σ (mg/kg) |
| 1 | 102 | 1.8 | 0.0188 |
| 0.2 | 102 | 3.2 | 0.0045 |
| 0.1 | 98.7 | 3.9 | 0.0088 |

[a] Relative standard deviation of repeatability, [b] Standard deviation

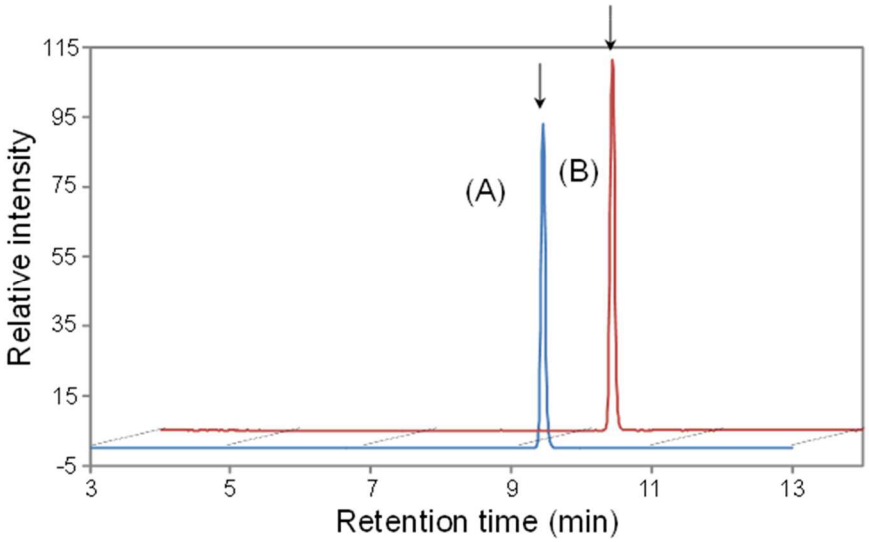


**Figure 2-2 SIM chromatograms of ZEA**

(A) Standard solution (amount of ZEA = 2.5 ng)
(B) Sample solution of dry dog food (spiked with ZEA at 1 mg/kg)
(Arrows indicate retention times for ZEA. The arrows in (A) and (B) are at the same retention time.)

LC-MS: LCMS-2010EV, Shimadzu Co. Ltd. LC column: L-column2 ODS, 5 µm, 3×250 mm, Chemicals Evaluation and Research Institute, Japan (CERI). Column temperature: 40°C. Mobile phases (gradient): 0.1% (v/v) 10 mmol/L aqueous ammonium acetate solution (mobile phase A) and acetonitrile-methanol (4:7, v/v) (mobile phase B). Flow rate: 0.5 mL/min. Injection volume: 10 µL. Ionization: APCI, negative mode. Monitor ion: *m/z* 317. Interface temperature: 400°C. Heat block temperature: 200°C. CDL temperature: 200°C. Nebulizer gas: $N_2$ (2.5 L/min).

**Table 2-3 Results of inter-laboratory study of ZEA in pet foods**

| Laboratory | Dry type for dogs | Semi-dry type for dogs | Wet type for cats |
|---|---|---|---|
| Participant labs[a] | 11 | 11 | 11 |
| Spiked level（mg/k | 1.0 | 0.8 | 0.2 |
| Recovery（%） | 99.0 | 101 | 102 |
| $RSD_r$ [b]（%） | 3.0 | 2.2 | 2.7 |
| $RSD_R$ [c]（%） | 5.6 | 5.9 | 6.6 |
| HorRat | 0.35 | 0.36 | 0.33 |

[a] Number of laboratories included in statistical analysis. [b] Relative standard deviation of repeatability. [c] Relative standard deviation of reproducibility

## 2.4 Discussion

In Japan and overseas, we did not have any method validated by interlaboratory tests for the determination of ZEA in pet food. Since there is an official method for ZEA in feed, in which samples are extracted with acetonitrile-water (21:4) and then purified in a multifunctional column and analysed by LC-MS (FAMIC 2008c), the study was

conducted with reference to this method. However, when the official method for feed (Method listed in the Standard for Analysis of Feed) was used in the spike and recovery tests, the recovery rate for dry products was high and exceeded 100%, whereas the recovery rate for wet products was relatively low. This trend suggests that the moisture content in the sample affects the recovery rate. In other words, the high recovery of the dry product was attributed to the sample swelling due to absorption of water from the extraction solvent, resulting in concentration of the extraction solvent. Wet products, on the other hand, contain more than 80% water, which may conversely result in dilution. In the feed analysis standard listing method, the amount of extraction solvent was 150 mL for 50 g of analysis sample, and the small amount of solvent relative to the sample volume was considered to be one of the causes. Therefore, in this method, the sample was extracted in two batches with the volume of 25 g, which is one half of the sample volume, and the volume was fixed at 200 mL after filtration. In liquid chromatography, the feed analysis standard listing method uses isocratic elution of acetonitrile-methanol-10 mmol/L ammonium acetate solution (4:7:9), with the analogous compound zearalanone is used as the internal standard (Figure 2-3). Zearalanone is commonly used as an internal standard because it is susceptible to ionization suppression when measuring ZEA by LC-MS or LC-MS/MS as well as other analytical methods (Zöllner et al. 2002; Laganà et al. 2003). However, since there is a possibility of natural contamination of zearalanone, it was necessary for this method to be quantitative by absolute calibration curve. Since the peak shape was broad in the feed analysis standard listing method because of isocratic elution,

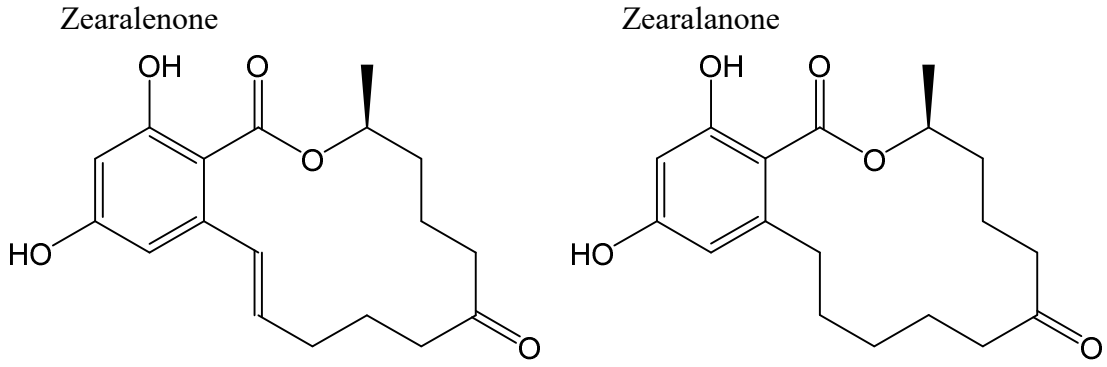


**Figure 2-3 Chemical structures of zearalenone and zearalanone**

gradient elution was considered, and a sharp peak was obtained, improving absolute sensitivity and signal-to-noise ratio. As a result, it was found that the absolute calibration curve method was sufficient to meet the requirements of this method.

After developing the analytical method, as part of the validation of the analytical method, we examined interfering substances in 22 pet feed products. Sample solutions prepared according to the method were injected into LC-MS and analysed for the presence of peaks that interfere with the determination of ZEA. As a result, peaks that interfered with the quantification of ZEA were observed in the cat wet product, which posed a challenge; when ZORBAX Eclipse XDB-C18 (Agilent Technologies, Santa Clara, CA, USA) was used as the column for LC, the standard solution of ZEA peak was observed at the elution position of ZEA in the wet cat product without the addition of ZEA (Figure 2-4A, B). On the other hand, no peak was observed when L-column 2 ODS column

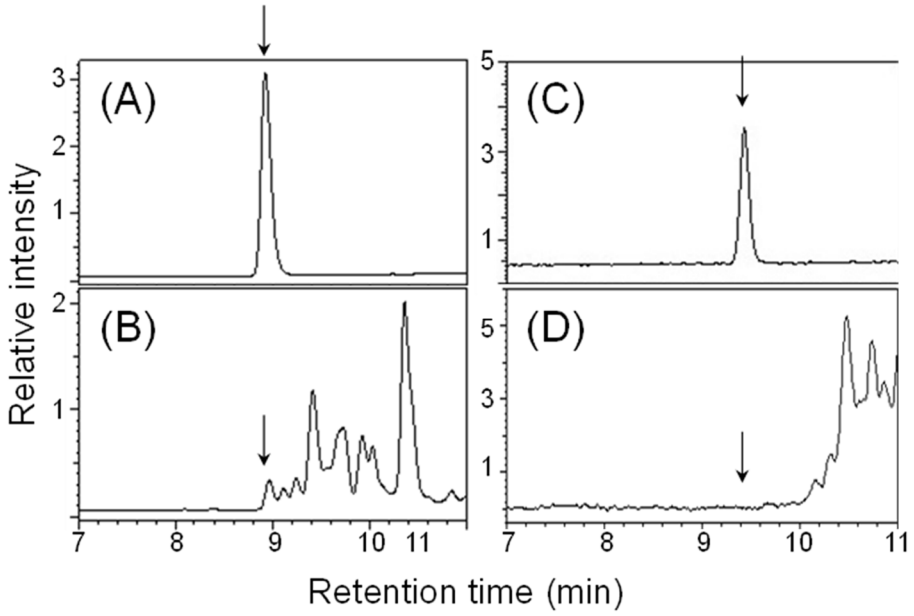


**Figure 2-4 SIM chromatograms of standard solution and wet type pet food for cats**

(A) Standard solution (The amount of zearalenone 2.5 ng)
(B) Blank sample of wet type pet food for cats
(A, B): Measurement using ZORBAX Eclipse
(C) Standard solution (The amount of zearalenone 0.05 ng)
(D) Blank sample of wet type pet food for cats
(C, D): Measurement using L-column2 ODS

(Chemicals Evaluation and Research Institute, Japan (CERI)) was used (Figure 2-4C, D). The peaks detected in the cat wet product in question were confirmed using liquid chromatography tandem mass spectrometry (LC-MS/MS) and were considered to be interfering peaks, and therefore, L-column 2 ODS was used in this method.

Finally, the author were able to develop a method for the determination of ZEA using a MFC, which requires short purification time and allows rapid determination for pet foods. This method has been modified to eliminate the use of zearalanone, which is used as an internal standard in other analytical methods. This method is an improved method that avoids the use of zearalanone, which is used as an internal standard in other common analytical methods for ZEA, and it is also applicable to measure ZEA in pet food with high lipid content and high protein content, which may be difficult to purify by other analytical methods.

The added concentrations in the spike and recovery tests were performed at two concentrations. Although the standard value of ZEA in pet food in Japan has not been established, the standard value of ZEA in feed (1 mg/kg) (FAMIC 1988) was assumed as the pet food standard value. In order to confirm that this concentration was sufficiently measurable, it was used as the concentration on the high concentration side for spike and recovery study. For the wet product with approximately 90% moisture content, the 1 mg/kg added concentration was converted to 10% moisture content, which is the moisture content of the dry product, at an added concentration of 0.2 mg/kg. The low concentration side was performed near the LOQ.

Although a standard value for ZEA in pet foods in Japan has not been established, the sensitivity of this analytical method is sufficient for the determination of ZEA in pet foods because both the LOQ and the LOD for the assumed standard value of ZEA were satisfied.

After being consulted by the review committee, the results of the single laboratory and inter-laboratory collaborative validity testing met all the requirements of the official pet food method (FAMIC 2009b), and the method was made official. The following points were highly evaluated at the review meeting. 1) The method is a simple quantitative method using a multifunctional column. 2) High repeatability and reproducibility were obtained with a recovery rate close to 100%. 3) The results of the inter-laboratory

collaborative study were favorable. 4) Furthermore, the limit of quantification should be sufficient for the determination of ZEA in pet food.

In conclusion, for the detection of ZEA in pet foods, we have developed a quantitative analytical method using an MFC, MultiSep226 Aflazon$^{+®}$ cartridge, to separate ZEA from the liquid extracts. Spike and recovery studies showed that the recovery of ZEA was more than 99.0% for all samples analysed. The LOQ and LOD for ZEA in the samples were 0.2 mg/kg and 0.1 mg/kg, for dry and semi-dry pet foods, and 0.1 mg/kg and 0.06 mg/kg for wet pet food, respectively. The mean recoveries, repeatabilities, reproducibilities, and HorRats of ZEA from the inter-laboratory study were 99.0-102%, 2.2-3.0%, 5.6-6.6%, and 0.33-0.36, respectively. From these results, the author concluded that this method is suitable for the analysis of ZEA in pet foods.

# Chapter 3

# Development and inter-laboratory study of a simultaneous quantification method for ten trichothecenes including deoxynivalenol-3-glucoside in feed*

*This Chapter presents a revised version of the paper published in Mycotoxin Research, 2020, 36, 353-360 with the following co-authors:
Kenji Shidara, Iyo Yasuda, Koji Aoyama, Akiko Takahashi, Takayuki Ishibashi

In this Chapter presents a revised version of the paper of inter laboratory study submitted to Mycotoxin Research, with the following co-authors:
Kenji Shidara, Iyo Yasuda

## 3.1 Introduction

Some reports have indicated that toxicity is higher for type A than for type B trichothecenes (Rotter et al. 1996), but cases for type A contamination are less than that of type B. Natural contamination of cereals with the type B trichothecenes DON and NIV, have been widely reported (Yazar and Omuetag 2008; Milicevic et al. 2015). Modified mycotoxin have recently attracted attention as new risk factors. These mycotoxins are include mycotoxin glucosides that are undetectable by conventional analytical methods. Such mycotoxin glucosides, including D3G, can be metabolized into free mycotoxins *in vivo*, and therefore need to be considered during the evaluations of maternal mycotoxin toxicity (Berthiller et al. 2003; Berthiller et al. 2011). Therefore, when considering the effects of DON, those of D3G also should be included. However, DON is the only trichothecene mycotoxin with advisory levels in the Japanese feed safety system. Both DON and NIV have been repeatedly detected in wheat products, such as flour and bran, as well as in barley and maize products (Yoshizawa and Jin 1995). Grains such as wheat, barley, and maize, and their products such as bran, are important ingredients for feed, so concerns have arisen regarding the dangers of these mycotoxins to the health and safety of livestock. Therefore, we developed a method to simultaneously confirm the contamination levels of 10 trichothecenes (both type A and type B, including D3G), in feed.

Mycotoxins are usually extracted from samples for analytical purposes using a specific solvent in a single process. However, some mycotoxins in naturally contaminated feed might not be sufficiently extracted by a one-time extraction (Numanoğlu et al. 2011; Turner et al. 2009). Current methods of trichothecene analysis focus on regulated mycotoxins, and often target only a limited range of matrices and limited variety trichothecenes. More complicated matrices, such as those of formula feed, are susceptible to ionization suppression during analyses using LC-MS/MS. A comparison of LC-MS/MS with APCI and ESI found that APCI was less susceptible to ionization suppression, and delivered good results (Tanaka et al. 2009). Here, extraction of trichothecenes was performed twice to improve the extraction efficiency in naturally contaminated samples, and to reduce ionization suppression using APCI with LC-MS/MS. In addition, clean-up columns were used to reduce effects on ionization efficiency.

In this study, we developed and validated a method of simultaneously quantifying 10 trichothecenes using LC-APCI-MS/MS. These are type B trichothecenes [3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), D3G, DON, NIV and fusarenon-X (FUS-X)] and type A trichothecenes (HT-2, T-2, DAS, and NES). Numerous methods for analysing trichothecenes such as LC-MS/MS (De Girolamo et al. 2020; Zhao et al. 2015; Habler and Rychlik 2016; Zhang et al. 2017; Tittlemier et al. 2021) have been reported. However, the range of samples and types of mycotoxins are often limited (Bessaire et al. 2019). Previous reports on inter-laboratory studies on a wide range of mycotoxins (Zhang et al. 2017; Bessaire et al. 2019; De Girolamo et al. 2020) did not include D3G. Therefore, methods for the simultaneous analyses of type A and type B trichothecenes including D3G for feed, further validated through an inter-laboratory study are necessary. A simultaneous analysis method for ten trichothecenes, including type A, type B, and D3G for a feed developed by our group previously, was validated by conducting an inter-laboratory study with eight laboratories.

## 3.2 Materials and methods

### 3.2.1 Chemicals and samples

A mixed standard stock solution (100 µg/mL each) of trichothecenes (HT-2, T-2, DAS, NES, 3-AcDON, 15-AcDON, DON, NIV and FUS-X; 200 µg each) was prepared by adding 2 mL of acetonitrile to the trichothecenes standard (Trilogy Dried Standard-Trichothecenes A & B, Trilogy Analytical Laboratory, Washington, MO, USA). D3G standard solution (50 µg/mL in acetonitrile solution) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deoxynivalenol as a solid mycotoxin standard was purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan). Acetonitrile was added to dissolve 10 mg of a DON standard in a 10 mL amber glass volumetric flask. This was further adjusted to 10 mL with the same solvent to prepare DON standard stock solution (1 mL of this solution contained 1 mg of DON). All standard stock solutions were stored below −18°C. Spiking and calibration solutions were prepared in acetonitrile-water (1:9, v/v). Acetonitrile (LC-MS grade) was purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan), and ammonium acetate (1 mol/L) was purchased from Fujifilm Wako Pure Chemical Co. Ltd. (Osaka, Japan). Ultrapure water was prepared using a Milli-Q Integral

5 water purification system (Millipore Sigma Co., Ltd., Burlington, MA, USA). Feed and ingredients were randomly obtained from Japanese feed factories and silos. The samples used herein were crushed in a centrifuge mill ZM200 (with 1 mm mesh screen) (Retsch Co., Ltd, Hahn, Germany) and stored at 4°C.

### 3.2.2 Chemicals and samples for inter-laboratory study

LC-MS/MS adjustment standard solutions were prepared at a concentration of 2 ng/mL for each mycotoxin by dilution of the mixed standard stock solution and the D3G standard stock solution with acetonitrile-water (1:9, v/v). The aim of distributing the LC-MS/MS adjustment standard solution were to let the participants become to optimize the LC-MS/MS conditions for mycotoxin detection.

Spiking solutions were prepared as follows.

(a) Spiking solution for maize: Ten mL each of the mixed standard stock solution and the D3G standard stock solution were added to a 100 mL amber glass volumetric flask. This was further adjusted to 100 mL by acetonitrile to prepare a spiking solution for maize (1 mL of this solution contained 5 μg of D3G and 10 μg of other mycotoxins).

(b) Spiking solution for soybean meal: Spiking solution for maize was diluted two-fold with acetonitrile to prepare a spiking solution for soybean meal (One mL of this solution contained 2.5 μg of D3G and 5 μg of other mycotoxins).

(c) Spiking solution for formula feed for suckling pigs: Twenty five mL of spiking solution for maize and 2 mL of DON standard solution were added to a 50 mL amber glass volumetric flask, then adjusted to 50 mL by acetonitrile. This resulted in a spiking solution for formula feed for suckling pigs (One mL of this solution contained 50 μg of DON, 2.5 μg of D3G, and 5 μg of other mycotoxins).

Acetonitrile (LC-MS grade) purchased from Kanto Chemical Co. Ltd (Tokyo, Japan) and ultrapure water prepared using a Milli-Q Integral 5 (Millipore, Molsheim, France) were used in standard stock solutions, calibration solutions, LC-MS/MS adjustment standard solutions, and spiking solutions.

### 3.2.3 Preparation of samples for inter-laboratory study

Maize, soybean meal, and formula feed for suckling pigs were crushed in a centrifuge mill ZM200 (with a 1 mm mesh screen) (Retsch Co., Ltd, Hahn, Germany). After mixing,

the samples were homogenised using a V-type mixer (volume capacity 2 L, Tsutsui Physics and Chemistry Instrument, Tokyo, Japan). Approximately 55 g of each sample was packed into a bag, and kept at 4°C until they were distributed to collaborators. The homogeneity of each test sample of packed bags was assessed using the F-test by analysing DON and NIV in maize, DON in soybean meal, and DON in the formula feed for suckling pigs. Samples with confirmed homogeneity were randomly numbered and distributed to each laboratory. They received the following samples/solutions in an inter-laboratory study:

(a) Sample for spike and recovery test (sample name was not specified); (b) Spiking solution for each sample (concentration of each mycotoxin was not notified); (c) Calibration solution for preparing calibration curve; (d) Standard solution for LC-MS/MS adjustment (2 ng/mL); (e) InertSep VRA-3 (GL Sciences, Tokyo, Japan) and HybridSPE®-Phospholipid (Sigma-Aldrich; St. Louis, MO, USA). Simultaneously, each laboratory received the method, an instruction form for participants, a sample delivery form, a reporting sheet, and a sample receipt. Participants were advised to store (a)-(d) in the dark at 4°C, store (e) at room temperature, and not open until analysis.

### 3.2.4 Fortification procedure for inter-laboratory study

The test materials (50.0 g each) were weighed into 6 different 500 mL amber glass Erlenmeyer flask marked A-F. One mL of the corresponding spiking solution was added to each sample, and the samples were stored overnight in the dark at room temperature. Furthermore, individual mycotoxin were extracted from each added sample and quantified according to the protocol.

### 3.2.5 Sample extraction and clean-up

Samples (50.0 g each) were extracted with 200 mL of acetonitrile-water (17:3, v/v) for 60 min using an SR-2W shaker (TAITEC, Saitama, Japan) at 250 rpm (sample solution A), then passed through filter paper (No. 5B, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) in a Buchner funnel (Kiriyama Glass Works Co., Tokyo, Japan). The residue was extracted with 200 mL of acetonitrile-water (17:3, v/v) by shaking for 60 min and then filtered. The total volume of the combined extracts was adjusted to 500 mL with acetonitrile-water (17:3, v/v) (sample solution B). Sample solution B was purified using an InertSep VRA-

3 column (GL Sciences Inc., Tokyo, Japan), and the first 5 mL of eluate was discarded. The following 2 mL of eluate from the column was collected and 1.0 mL was transferred to a 10 mL eggplant-shaped flask (Sample solution C) and evaporated to dryness at 50°C. The resulting residue was dissolved in 1 mL of acetonitrile-water (1:9, v/v) (Sample solution D). Sample solution D was applied to HybridSPE®-Phospholipid plates (Sigma-Aldrich., St. Louis, MO, USA) on an SPE manifold (GL-SPE, GL Sciences Inc., Tokyo, Japan), at a flow rate of about 1 drop/sec, then passed through a vacuum manifold, and collected into 10 mL eggplant-shaped flasks (Sample solution E). The flask containing sample solution D was washed with 1 mL of acetonitrile, and the wash solution was added to the previous HybridSPE®-Phospholipid columns and collected into the same 10 mL eggplant-shaped flask. Sample solution E was evaporated to dryness at 50°C. The resulting residues were dissolved in 1 mL of acetonitrile-water (1:9, v/v), centrifuged at 5,000 × *g* for 5 min. Then the supernatant was decanted into 1.5 mL amber glass vials for LC-MS/MS analysis. Scheme 3 shows the flow of this analytical method.

### 3.2.6 LC-MS/MS analysis

Each mycotoxin was separated on an Inertsil ODS-3 (3 μm) 2.1×150-mm analytical column (GL Sciences Inc.), maintained at 40°C. The mobile phases comprised 10 mmol/L aqueous ammonium acetate (mobile phase A) and acetonitrile (mobile phase B). The initial solvent gradient comprised 95% A and 5% B held for 4 min. Mobile phase B was linearly increased from 5% to 80% over a period of 16 min, then held at 80% for 8 min. The flow rate was 0.2 mL/min and the injection volume was 5 μL. Each mycotoxin was analysed by LC-MS/MS using an LCMS-8040 instrument (Shimadzu Co., Ltd., Kyoto, Japan). Samples were loaded into the MS using APCI, and mycotoxins were detected in the selected reaction monitoring (SRM) mode. Table 3-1 shows the measurement mode, monitor ion and collision energy. The temperatures of the interface, heat-block, and desolvation line were 350°C, 200°C, and 250°C, respectively. The nebulizer gas was air at a flow rate of 4 L/min, and nitrogen was the drying gas at a flow rate of 5 L/min.

50.0 g of sample
- extracted by acetonitrile–water (17:3, v/v) (200 mL, twice)
- filled up to 500 mL with acetonitrile–water (17:3, v/v)

Extract
- InertSep VRA-3 cartridge (cartridge size 6 mL)

Eluate (discarded the first 5 mL and collected the following 2 mL of eluate)

1 mL of eluate
- evaporated to dryness at below 50°C
- dissolved in 1 mL of acetonitrile–water (1:9, v/v)
- HybridSPE-Phospholipid cartridge (cartridge size 1 mL)
- added 1 mL of acetonitrile and collected the eluate

Eluate
- evaporated to dryness at below 50°C
- dissolve in 1 mL of acetonitrile–water (1:9, v/v)
- centrifuge at 5,000×*g* for 5 min

Supernatant
- analyzed by LC-MS/MS (APCI)

**Scheme 3 Analytical procedure for simultaneous determination of trichothecenes in feeds using LC-MS/MS**

### 3.2.7 LC-MS/MS conditions for inter-laboratory study

Each mycotoxin was separated on an octadecyl silica C18 column. The appropriate LC-MS/MS conditions and the recommended gradient program are as shown in 3.2.6. In each laboratory, all other conditions were set such that a peak with an SN ratio of 10 or more for 3-AcDON and 15-AcDON, 20 or more for T-2, DON, and FUS-X, 40 or more for HT-2, DAS, NES, D3G, and NIV could be obtained by injecting 5 µL of the standard solution (2 ng/mL) for LC-MS/MS adjustment in each laboratory. Table 3-2 shows the LC-MS/MS models used.

### 3.2.8 Linearity of standard curve

We confirmed the linearity of the mycotoxin calibration curves by analysing 5 µL of standards (0.1, 0.5, 1, 5, 10, 25, 50, 75, 100, 250, 500, 750, 1,000 ng/mL) by LC-MS/MS. Peak height was measured, because 15-AcDON from 3-AcDON might not be properly separated depending on the type of LC-MS/MS or LC column.

**Table 3-1 MS/MS parameters of each trichothecenes for this method**

| Target | Mode | Precursor ion (m/z) | Product ion Quantifier (m/z) | Qualifier (m/z) | Collision energy (eV) |
|---|---|---|---|---|---|
| HT-2 | – | 483.20 | 59.15 | - | 21 |
| | + | 442.20 | - | 215.10 | 13 |
| | | | - | 263.10 | 15 |
| T-2 | + | 484.20 | 185.10 | - | 23 |
| | | | - | 305.10 | 16 |
| | | | - | 215.10 | 21 |
| DAS | + | 384.20 | 307.10 | - | 12 |
| | | | - | 247.10 | 14 |
| NES | + | 400.20 | 305.10 | - | 14 |
| | | | - | 215.10 | 18 |
| | | | - | 185.10 | 22 |
| 3-AcDON | – | 397.15 | 337.10 | - | 8 |
| | | | - | 307.10 | 13 |
| 15-AcDON | – | 397.15 | 59.10 | - | 21 |
| | | | - | 277.10 | 7 |
| DON | – | 355.10 | 265.20 | - | 13 |
| | | | - | 295.20 | 10 |
| D3G | – | 517.20 | 427.20 | - | 21 |
| | | | - | 457.20 | 14 |
| NIV | – | 371.10 | 281.10 | - | 13 |
| | | | - | 311.10 | 10 |
| FUS-X | – | 413.10 | 263.10 | - | 16 |
| | | | - | 353.10 | 10 |

### 3.2.9 Calibration curve for inter-laboratory study

In each laboratory, a standard solution for preparing a calibration curve was diluted with acetonitrile-water (1:9, v/v). Each mycotoxin's concentration and peak signal (height) were plotted for five different concentrations. The concentration of each mycotoxin in individual samples was calculated using a calibration curve.

### 3.2.10 Comparison of one-step and repeated extractions

Mycotoxins were extracted as described above but from 20 g of samples using 80 mL of solvent.

Sample solution A (extracted once) was centrifuged at 650 × *g* for 5 min, and sample solution B (extracted twice) was the sample for further experiments. Both samples were

applied to multifunctional InertSep VRA-3 clean-up columns, and the first 5 mL of eluate was discarded. The next 2 mL eluted from the column was collected and 1 mL was

**Table 3-2 Instruments used in the inter-laboratory study**

| Lab.No | Instrument | LC column |
|---|---|---|
| A | LC: Nexera X2, Shimadzu<br>MS/MS: LCMS-8040, Shimadzu | Inertsil ODS-3<br>(150 × 2.1 mm i.d., 3 μm particle size)<br>GL Sciences |
| B | LC: Nexera X2, Shimadzu<br>MS/MS: LCMS-8040, Shimadzu | Inertsil ODS-3<br>(150 × 2.1 mm i.d., 3 μm particle size)<br>GL Sciences |
| C | LC: Nexera X2, Shimadzu<br>MS/MS: QTRAP 5500, AB SCIEX | Inertsil ODS-3<br>(150 × 2.1 mm i.d., 3 μm particle size)<br>GL Sciences |
| D | LC: Nexera X2, Shimadzu<br>MS/MS: TRIPLE QUAD 4500, AB SCIEX | Inertsil ODS-3<br>(150 × 2.1 mm i.d., 3 μm particle size)<br>GL Sciences |
| E | LC: Prominence, Shimadzu<br>MS/MS: API 4000 QTRAP, AB SCIEX | Inertsil ODS-3<br>(150 × 2.1 mm i.d., 3 μm particle size)<br>GL Sciences |
| F | LC: Prominence, Shimadzu<br>MS/MS: QTRAP 5500, AB SCIEX | Inertsil ODS-3<br>(150 × 2.1 mm i.d., 3 μm particle size)<br>GL Sciences |
| G | LC: Nexera X2, Shimadzu<br>MS/MS: LCMS-8050, Shimadzu | Shim-pack XR-ODS Ⅲ<br>(150 × 2.0 mm i.d., 2.2 μm particle size)<br>Shimadzu |
| H | LC: Prominence, Shimadzu<br>MS/MS: LCMS-8030, Shimadzu | Inertsil ODS-3<br>(150 × 2.1 mm i.d., 3 μm particle size)<br>GL Sciences |

evaporated to dryness at 50°C. The resulting residue was dissolved in 2 mL of acetonitrile-water (1:9, v/v) and centrifuged at 5,000 × *g* for 5 min. The supernatant was then quantified by LC-MS/MS.

### 3.2.11 Phospholipid removal column

To remedy T-2 and HT-2 signal enhancement in barley and formula feed on spike and recovery tests, use of the phospholipid removal column was investigated. A sample of formula feed for dairy cattle was spiked with 10 or 100 μg/kg of D3G and 20 or 200 μg/kg

of the remaining mycotoxins and extracted as described above in “Sample extraction and clean-up”. Thereafter, the following groups were tested:

(1) Without phospholipid removal column: sample solution D was placed in a plastic tube, centrifuged at 5,000 × *g* for 5 min, and then the supernatant was quantified by LC-MS/MS.

(2) With phospholipid removal column, and without acetonitrile wash: sample solution E was placed in a plastic tube, centrifuged at 5,000 × *g* for 5 min, and then the supernatant was quantified by LC-MS/MS.

(3) With phospholipid removal column, and with 1 mL of acetonitrile wash: sample solution C was spiked with 10 ng of D3G and 20 ng of the remaining mycotoxins as described above in “Sample extraction and clean-up”

### 3.2.12 Matrix effect for quantification

The matrix effect was confirmed by measuring the peak area ratio of the standard solution and the sample solution in which the same concentration of the standard solution was added to the blank sample solution. Each solution was spiked with mycotoxin standards equivalent to 100 μg/kg of D3G and 1,000 μg/kg of DON in formula feed of starting broiler chicks and suckling pigs, 4,000 μg/kg of DON in that for beef cattle, and 200 μg/kg of DON in maize, barley, soybean meal and rapeseed meal, and 200 μg/kg of the remaining mycotoxins. The ratio (peak height of the matrix standard/ peak height of standard × 100) is defined here generally as a matrix effect. A matrix effect 80-120% was evaluated to be quantifiable without significant matrix effects.

### 3.2.13 Limit of detection and limit of quantification

To estimate the LOQ for all tested mycotoxins, barley and/or rapeseed meals were spiked with 20 μg/kg of 3-AcDON and 15-AcDON, 10 μg/kg of T-2, DON and FUS-X, and 5 μg/kg of NIV and the remaining mycotoxins. The samples were placed in darkness at room temperature overnight and then analysed. The LOQ was calculated using the signal-to-noise ratio of 10 from the peaks obtained by the spike and recovery tests, and LOD was calculated using the signal-to-noise ratio of 3.

### 3.2.14 Spike and recovery tests

Recovery was assessed by spiking maize, barley, soybean meal, rapeseed meal, and three formula feeds for starting broiler chicks, suckling pigs, and beef cattle with each mycotoxin. The samples were placed in darkness at room temperature overnight and then analysed.

### 3.2.15 Inter-laboratory study design

In order to evaluate this analytical method, an inter-laboratory study was performed using the following three samples in duplicate:

(A) One hundred μg/kg equivalent of D3G and 200 μg/kg equivalent of other mycotoxins were spiked into maize (maize with blank value 21.2 μg/kg for DON and blank value 159 μg/kg for NIV) (One mL of a standard solution for spiking maize containing 5 μg of D3G and 10 μg of other mycotoxins in 1 mL was spiked).

(B) Fifty μg/kg equivalent of D3G and 100 μg/kg equivalent of other mycotoxins were spiked into soybean meal (soybean meal with a blank value of 28.6 μg/kg for DON) (One mL of a standard solution for spiking soybean meal containing 2.5 μg of D3G and 5 μg of other mycotoxins in 1 mL was spiked).

(C) One thousand μg/kg equivalent of DON, 50 μg/kg equivalent of D3G, and 100 μg/kg of other mycotoxins were spiked into formula feed for suckling pigs (formula feed with a blank value of 15.8 μg/kg for DON) (1 mL of standard solution for spiking formula feed for suckling pig containing 50 μg of DON, 2.5 μg of D3G, and 5 μg of other mycotoxins in 1 mL was spiked).

Inter-laboratory studies were conducted in eight laboratories in Japan.

### 3.2.16 Statistics

Each laboratory was required to provide significant results using three-digit numbers. Based on these results, Cochran and Grubbs single and Grubbs pair outlier tests were conducted according to internationally harmonised collaborative study procedures (AOAC International 2019). After the rejecting of outliers, the recovery, $RSD_r$, and $RSD_R$ were calculated. The HorRat was calculated according to a modified version of the Horwitz equation (Thompson 2000).

Homogeneous and average concentrations of naturally contaminated DON in maize,

soybean meal, formula feed for suckling pigs and NIV of natural contamination in maize were tested before sample distribution. As the F-value was lower than the critical value, the F-test at a confidence level of 95% considered each naturally contaminated sample homogeneous.

## 3.3 Results

### 3.3.1 LC-MS/MS measurement conditions

Both 3-AcDON and 15-AcDON are structural isomers, and the precursor and product ions have the same *m*/*z*. Therefore, these mycotoxins must be separated by liquid chromatography columns. In the case of APCI, use of methanol as the mobile phase generally results in higher sensitivity (Berthiller et al. 2005). However, 3-AcDON and 15-AcDON are difficult to separate in this manner, but they were easily separated using acetonitrile as the mobile phase, then could be quantified (Berger et al. 1999; Tamura et al. 2014).

### 3.3.2 Linearity of standard curves

The calibration curves were linear in the ranges of 0.0025–0.5 ng (0.5~100 ng/mL) for HT-2, 3-AcDON, 15-AcDON, NIV, and FUS-X; 0.005–0.5 ng (1~100 ng/mL) for T-2; 0.0005–0.5 ng (0.1–100 ng/mL) for DAS, NES, and D3G; and 0.005–5 ng (1~1000 ng/mL) for DON. The coefficients of determination were at least 0.998 as shown in Table 3-3. When the linearity of the calibration curve was confirmed in the low concentration range, the coefficient of determination were at least 0.9960 as shown in Table 3-4.

**Table 3-3 Linearity of calibration curves for mycotoxins**

| Mycotoxins | Measured concentration (ng/mL) | y = ax+b | | $R^2$ |
|---|---|---|---|---|
| | | a | b | |
| HT-2 | 0.5, 1, 5, 10, 25, 50, 75, 100 | 199.8 | -114.9 | 0.999 |
| T-2 | 1, 5, 10, 25, 50, 75, 100 | 136.4 | -76.1 | 0.999 |
| DAS | 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100 | 552.5 | -324.0 | 0.999 |
| NES | 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100 | 690.8 | 164.0 | 0.999 |
| 3-AcDON | 0.5, 1, 5, 10, 25, 50, 75, 100 | 396.3 | 50.9 | 0.999 |
| 15-AcDON | 0.5, 1, 5, 10, 25, 50, 75, 100 | 102.1 | 13.3 | 1.000 |
| DON | 1, 5, 10, 25, 50, 75, 100, 250, 500, 750, 1000 | 189.0 | -358.6 | 1.000 |
| D3G | 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100 | 538.1 | 75.5 | 0.998 |
| NIV | 0.5, 1, 5, 10, 25, 50, 75, 100 | 340.0 | -66.8 | 0.999 |
| FUS-X | 0.5, 1, 5, 10, 25, 50, 75, 100 | 149.3 | 0.6 | 1.000 |

**Table 3-4 Linearity of low concentration calibration curves for mycotoxins**

| Mycotoxins | Measured concentration (ng/mL) | y = ax+b | | $R^2$ |
|---|---|---|---|---|
| | | a | b | |
| HT-2 | 0.5, 1, 5, 10, 25 | 184.1 | 21.6 | 1.000 |
| T-2 | 1, 5, 10, 25, 50 | 128.9 | 6.8 | 1.000 |
| DAS | 0.1, 0.5, 1, 5, 10 | 480.8 | 31.6 | 0.997 |
| NES | 0.1, 0.5, 1, 5, 10 | 675.4 | -4.0 | 1.000 |
| 3-AcDON | 0.5, 1, 5, 10, 25 | 384.0 | 10.4 | 1.000 |
| 15-AcDON | 0.5, 1, 5, 10, 25 | 102.5 | -3.5 | 1.000 |
| DON | 1, 5, 10, 25, 50 | 175.2 | 39.0 | 0.999 |
| D3G | 0.1, 0.5, 1, 5, 10 | 551.1 | -56.2 | 0.996 |
| NIV | 0.5, 1, 5, 10, 25 | 339.9 | -84.0 | 0.999 |
| FUS-X | 0.5, 1, 5, 10, 25 | 149.5 | -7.2 | 0.999 |

### 3.3.3 Comparison of one-step and repeated extractions

The recovery rate of D3G in soybean meal was low. Therefore, the author compared recovery rates between extraction by shaking once for 60 min and shaking for 60 min, followed by suction filtration, and then shaking the residue again for 60 min. The repeated extraction improved the recovery rate of D3G from soybean meal as Table 3-5. Mean recovery after one extraction was 38.6% and 52.4%, whereas that after repeated extractions was 91.6% and 96.8% at spike values of 10 and 100 μg/kg, respectively, for D3G. The mean recovery (84.8–117%) of the remaining mycotoxins was good. The mean recovery rates of D3G from soybean meal naturally contaminated with mycotoxins were 15.7 and 35.7 μg/kg, respectively after one and repeated extractions. The results were similar for 15-AcDON (66.2 and 89.1 μg/kg, respectively) and DON (335 and 434 μg/kg, respectively). The mean measured value was always higher, and the $RSD_r$ was always smaller after repeated extractions (15-AcDON, 3.4%; DON, 8.0%) than after one (15-AcDON, 32%; DON, 24%) extraction. Other mycotoxins were undetectable.

### 3.3.4 Study of phospholipid removal column

Table 3-6 shows higher recovery rates of HT-2, DAS, and NES from formula feed without phospholipid removal column (HT-2, 122%; DAS, 124%; NES, 130%), and that the signal enhancement remedied by phospholipid removal column led to recovery rates of 85.5%, 92.8%, and 99.7%, respectively. However, the recovery of T-2 was low (61.3%). When additional acetonitrile wash was added to the phospholipid removal column, improved the recovery of T-2 to 90.3% (Table 3-6).

**Table 3-5 Comparison of one-step and repeated extractions**

| Mycotoxins | Spiked test | | | | | Naturally contaminated sample | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Spiked level (μg/kg) | One-time extraction | | Repeated extraction | | One-time extraction | | Repeated extraction | |
| | | Recovery[a] (%) | $RSD_r$[b] (%) | Recovery[a] (%) | $RSD_r$[b] (%) | Measured value[a] (μg/kg) | $RSD_r$[b] (%) | Measured value[a] (μg/kg) | $RSD_r$[b] (%) |
| 15-AcDON | 20 | 108 | 1.9 | 117 | 8.5 | 66.2 | 32 | 89.1 | 3.4 |
| | 200 | 111 | 4.5 | 110 | 1.4 | | | | |
| DON | 20 | 101 | 14 | 84.8 | 11 | 335 | 24 | 434 | 8.1 |
| | 200 | 108 | 2.1 | 99.5 | 3.5 | | | | |
| D3G | 10 | 38.6 | 5.8 | 91.6 | 2.8 | 15.7 | 24 | 35.7 | 12 |
| | 100 | 52.4 | 2.2 | 96.8 | 1.5 | | | | |

[a] Mean ($n$=3), [b] Relative standard deviation of repeatability

**Table 3-6 Results comparing with and without phospholipid removal columns**

| Mycotoxins | Spiked level (μg/kg) | Without phospholipid removal column | | With phospholipid removal column | | |
|---|---|---|---|---|---|---|
| | | Recovery[a] (%) | $RSD_r$[b] (%) | Without acetonitrile wash | | Acetonitrile 1 mL |
| | | | | Recovery[a] (%) | $RSD_r$[b] (%) | Recovery[c] (%) |
| HT-2 | 200 | 122[d] | 2.2 | 85.5 | 3.6 | 99.8 |
| T-2 | 200 | 112 | 5.5 | 61.3[e] | 4.3 | 90.3 |
| DAS | 200 | 124[d] | 2.0 | 92.8 | 4.5 | 99.4 |
| NES | 200 | 130[d] | 1.9 | 99.7 | 5.9 | 107 |

[a] Mean ($n$=3), [b] Relative standard deviation of repeatability, [c] $n$=1,
[d] Recovery > 120% with signal enhancement, [e] Recovery < 70% with signal suppression

### 3.3.5 Confirmation of the matrix effect

The peak height ratio of the matrix standard for each mycotoxin standard was 91.5–110% as shown in Table 3-7, and each mycotoxin was measurable without being greatly affected by the sample matrix.

### 3.3.6 Spike and recovery tests

The spiked concentrations of mycotoxin in recovery tests included the advisory values in Japan (for example, 4 mg/kg of DON in feed for cattle aged ≥ 3 months, and 1 mg/kg of DON in feed for other livestock). Table 3-8 shows the results of the spike and recovery tests. Additional data of the chromatogram for spike and recovery tests are given on Figure 3-1. The estimated LOQ required to produce a signal-to-noise ratio of 10 in recovery tests, was 5 μg/kg for HT-2, DAS, NES, D3G, and NIV, 10 μg/kg for T-2, DON, and FUS-X, and 20 μg/kg for 3-AcDON and 15-AcDON. The estimated LOD required to achieve an SN ratio of 3 was 2 μg/kg for HT-2, DAS, NES, D3G, and NIV, 3 μg/kg for T-2, DON, and FUS-X, and 6 μg/kg for 3-AcDON and 15-AcDON. The expanded measurement uncertainties were 2.3 μg/kg at mean value of 15.1 μg/kg of HT-2 in barley, 2.4 μg/kg at a mean of 20.1 μg/kg in T-2 in formula feed for suckling pigs, 3.1 μg/kg at a mean of 19.7 μg/kg for DAS in soybean meal, 1.1 μg/kg at a mean of 18.9 μg/kg for NES in formula feed for beef cattle, 3.3 μg/kg at a mean of 19.4 μg/kg for 3-AcDON in rapeseed meal, 2.2 μg/kg at a mean of 18.6 μg/kg for 15-AcDON in formula feed for beef

**Table 3-7 Results of the matrix effect study by APCI**

| Mycotoxins | Spiked level (μg/kg) | Matrix effect[a](%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Maize | Barley | Soybean meal | Rapeseed meal | Formula feed for starting broiler chicks | Formula feed for suckling pigs | Formula feed for beef cattle |
| HT-2 | 200 | 98.5 | 97.5 | 101 | 101 | 98.0 | 101 | 109 |
| T-2 | 200 | 99.5 | 96.6 | 98.8 | 102 | 98.9 | 98.5 | 108 |
| DAS | 200 | 99.9 | 102 | 99.5 | 100 | 103 | 95.5 | 100 |
| NES | 200 | 92.2 | 103 | 97.3 | 106 | 99.3 | 98.1 | 97.0 |
| 3-AcDON | 200 | 99.5 | 99.8 | 103 | 106 | 106 | 96.7 | 101 |
| 15-AcDON | 200 | 98.5 | 108 | 107 | 109 | 106 | 103 | 107 |
| DON | 200 | 108 | 110 | 110 | 107 | - | - | - |
| | 1000 | -[b] | - | - | - | 106 | 103 | - |
| | 4000 | - | - | - | - | - | - | 109 |
| D3G | 100 | 106 | 100 | 106 | 91.5 | 109 | 108 | 104 |
| NIV | 200 | 103 | 99.3 | 96.6 | 100 | 98.2 | 96.6 | 92.9 |
| FUS-X | 200 | 96.9 | 104 | 104 | 108 | 99.7 | 96.8 | 102 |

[a] $n$=1, [b] Not tested

**Table 3-8 Results of recovery tests for each trichothecenes in feed**

| Mycotoxins | Spiked level (μg/kg) | Maize | | Soybean meal | | Rapeseed meal | |
|---|---|---|---|---|---|---|---|
| | | Recovery[a] (%) | $RSD_r$[b] (%) | Recovery[a] (%) | $RSD_r$[b] (%) | Recovery[a] (%) | $RSD_r$[b] (%) |
| HT-2 | 5 | -[c] | - | - | - | 106 | 5.9 |
| | 10 | - | - | - | - | 98.1 | 4.4 |
| | 20 | 97.7 | 5.4 | 108 | 1.3 | 101 | 7.1 |
| | 200 | 101 | 2.0 | 106 | 4.3 | 108 | 1.8 |
| T-2 | 10 | - | - | - | - | 95.2 | 8.9 |
| | 20 | 96.8 | 9.5 | 108 | 5.8 | 101 | 6.2 |
| | 200 | 111 | 6.9 | 109 | 12 | 111 | 4.8 |
| DAS | 5 | - | - | - | - | 107 | 5.8 |
| | 10 | - | - | - | - | 108 | 9.1 |
| | 20 | 90.9 | 3.0 | 98.4 | 14 | 113 | 4.5 |
| | 200 | 88.9 | 7.0 | 106 | 8.1 | 112 | 16 |
| NES | 5 | - | - | - | - | 109 | 7.4 |
| | 10 | - | - | - | - | 108 | 7.9 |
| | 20 | 110 | 2.0 | 102 | 1.4 | 115 | 2.0 |
| | 200 | 101 | 4.2 | 107 | 6.2 | 112 | 17 |
| 3-AcDON | 20 | 104 | 3.9 | - | - | 96.8 | 15 |
| | 40 | - | - | 78.5 | 2.9 | - | - |
| | 200 | 94.2 | 6.3 | 90.5 | 3.5 | 108 | 4.1 |
| 15-AcDON | 20 | 106 | 9.8 | - | - | 110 | 3.7 |
| | 40 | - | - | 102 | 3.6 | - | - |
| | 200 | 103 | 5.4 | 112 | 1.7 | 115 | 1.7 |
| DON | 10 | - | - | - | - | 91.0 | 8.3 |
| | 20 | - | - | 99.0 | 6.0 | 110 | 5.9 |
| | 200 | 90.3 | 7.7 | 93.8 | 6.2 | 104 | 3.5 |
| D3G | 5 | - | - | - | - | 90.2 | 8.9 |
| | 10 | - | - | - | - | 90.7 | 13 |
| | 20 | 86.0 | 16 | 91.1 | 17 | 71.4 | 6.4 |
| | 100 | 81.5 | 4.7 | 82.2 | 4.4 | 70.6 | 7.0 |
| NIV | 5 | - | - | - | - | 102 | 5.9 |
| | 10 | - | - | - | - | 90.0 | 14 |
| | 20 | - | - | 98.0 | 4.2 | 82.0 | 2.7 |
| | 200 | 108 | 1.9 | 97.2 | 4.3 | 107 | 5.2 |
| FUS-X | 10 | - | - | - | - | 111 | 13 |
| | 20 | 83.3 | 4.7 | 90.8 | 17 | 86.1 | 15 |
| | 200 | 91.1 | 8.0 | 97.2 | 9.4 | 101 | 8.6 |

**Table 3-8 (continued)**

| Mycotoxins | Spiked level (μg/kg) | Barley | | Formula feed for starting broiler | | Formula feed for suckling pigs | | Formula feed for beef cattle | |
|---|---|---|---|---|---|---|---|---|---|
| | | Recovery[a] (%) | $RSD_r$[b] (%) | Recovery[a] (%) | $RSD_r$[b] (%) | Recovery[a] (%) | $RSD_r$[b] (%) | Recovery[a] (%) | $RSD_r$[b] (%) |
| HT-2 | 10 | 104 | 17 | -[c] | - | - | - | - | - |
| | 20 | 75.4 | 13 | - | - | 91.6 | 9.9 | 89.3 | 10 |
| | 200 | 103 | 5.1 | 110 | 5.0 | 101 | 2.1 | 102 | 1.8 |
| T-2 | 10 | 116 | 11 | - | - | - | - | - | - |
| | 20 | 101 | 3.7 | - | - | 101 | 11 | 102 | 10 |
| | 200 | 104 | 4.0 | 108 | 6.5 | 105 | 5.6 | 94.3 | 4.9 |
| DAS | 10 | 98.2 | 9.3 | - | - | - | - | - | - |
| | 20 | 99.2 | 6.3 | - | - | 108 | 4.2 | 104 | 9.7 |
| | 200 | 109 | 3.0 | 110 | 1.2 | 103 | 4.5 | 116 | 2.0 |
| NES | 10 | 90.1 | 12 | - | - | - | - | - | - |
| | 20 | 110 | 4.3 | - | - | 107 | 4.2 | 94.4 | 5.0 |
| | 200 | 107 | 3.7 | 114 | 2.6 | 107 | 7.0 | 116 | 1.1 |
| 3-AcDON | 20 | - | - | - | - | 102 | 8.0 | - | - |
| | 40 | 95.8 | 7.2 | - | - | - | - | - | - |
| | 200 | 103 | 4.7 | 99.3 | 2.5 | 101 | 2.2 | 101 | 2.5 |
| 15-AcDON | 20 | - | - | - | - | 102 | 6.9 | 92.9 | 10 |
| | 40 | 100 | 4.3 | - | - | - | - | - | - |
| | 200 | 102 | 6.3 | 108 | 1.1 | 96.6 | 5.0 | 119 | 3.5 |
| DON | 20 | - | - | - | - | 94.4 | 9.4 | - | - |
| | 200 | 92.1 | 3.0 | - | - | - | - | - | - |
| | 1000 | - | - | 91.4 | 9.1 | 90.2 | 7.1 | - | - |
| | 4000 | - | - | - | - | - | - | 111 | 2.4 |
| D3G | 10 | 83.4 | 9.6 | - | - | - | - | - | - |
| | 20 | 119 | 5.3 | - | - | 95.8 | 3.1 | 104 | 15 |
| | 100 | 88.4 | 5.7 | 97.8 | 3.8 | 72.5 | 17 | 98.2 | 2.2 |
| NIV | 20 | - | - | - | - | 74.6 | 3.2 | - | - |
| | 200 | 98.8 | 3.0 | 95.5 | 1.6 | 89.4 | 3.5 | 108 | 4.4 |
| FUS-X | 10 | 108 | 11 | - | - | - | - | - | - |
| | 20 | 89.0 | 1.5 | - | - | 100 | 4.4 | 84.1 | 5.0 |
| | 200 | 107 | 3.2 | 101 | 1.5 | 104 | 8.6 | 118 | 1.5 |

[a] Mean ($n$=3), [b] Relative standard deviation of repeatability, [c] Not tested.

cattle, 2.0 μg/kg at a mean of 18.9 μg/kg for DON in formula feed for suckling pigs, 1.2 μg/kg at a mean of 18.2 μg/kg for D3G in soybean meal, 0.6 μg/kg at a mean of 19.6 μg/kg for NIV in soybean meal, and 3.5 μg/kg at a mean of 18.2 μg/kg for FUS-X in soybean meal. The expanded measurement uncertainties applied here were always $k = 2$.

### 3.3.7 Inter-laboratory study

The results of the inter-laboratory study are shown in Tables 3-9–3-11. The recovery, $RSD_r$, $RSD_R$, and HorRat of DON were 97.2–103%, 3.4–6.5%, 8.5–13%, and 0.42–0.70, respectively. The values for D3G were 78.2–96.7%, 3.5–6.4%, 13–22%, and 0.59–1.0. The values of NIV were 89.9–99.9%, 3.9–5.6%, 5.6–9.4%, and 0.25–0.43, and the values for other mycotoxins were 93.8–116%, 3.1–9.8%, 4.3–14%, and 0.19–0.65. HorRat values 0.5–1.5 may be taken to indicate that the performance value for the method corresponds to historical performance. The limits for performance acceptability are of 0.5–2 (AOAC International 2019). In this study, the HorRat values ranged from 0.19–1, these results similar to the HorRat values (HorRat values: 0.2-1.3, Bessaire et al. 2019; 0.16-1.65, Ye and Wu 2018) of recent inter-laboratory collaborative studies. Although, HorRat was below 0.50 in some cases, no problem was found in the inter-laboratory study. This might be explained by the analytical skills of participating laboratories.

## 3.4 Discussion

Standard value established for DON in feed, but the official method is used as a contamination survey when the standards are reviewed. In Japan, there are no standard values for mycotoxins other than DON in feed. In the EU, the indicative value of the combined amount of T-2 and HT-2 in the feed is given. DAS was added to the PMTDI for T -2 and HT -2 by JECFA. (JECFA 2017). Therefore, a method for simultaneously quantifying T-2, HT-2, and DAS was required. Although D3G was not included due to lack of information, PMTDI was established by JECFA as a group consisting of DON, 3-AcDON, and 15 AcDON (JECFA 2010). Therefore, an analytical method for simultaneously measuring not only DON but also 3-AcDON, 15-AcDON and D3G was required. There was no method for analysis of D3G and DAS, and no method for simultaneous determination of HT-2, 15-AcDON and 3-AcDON in the official method of

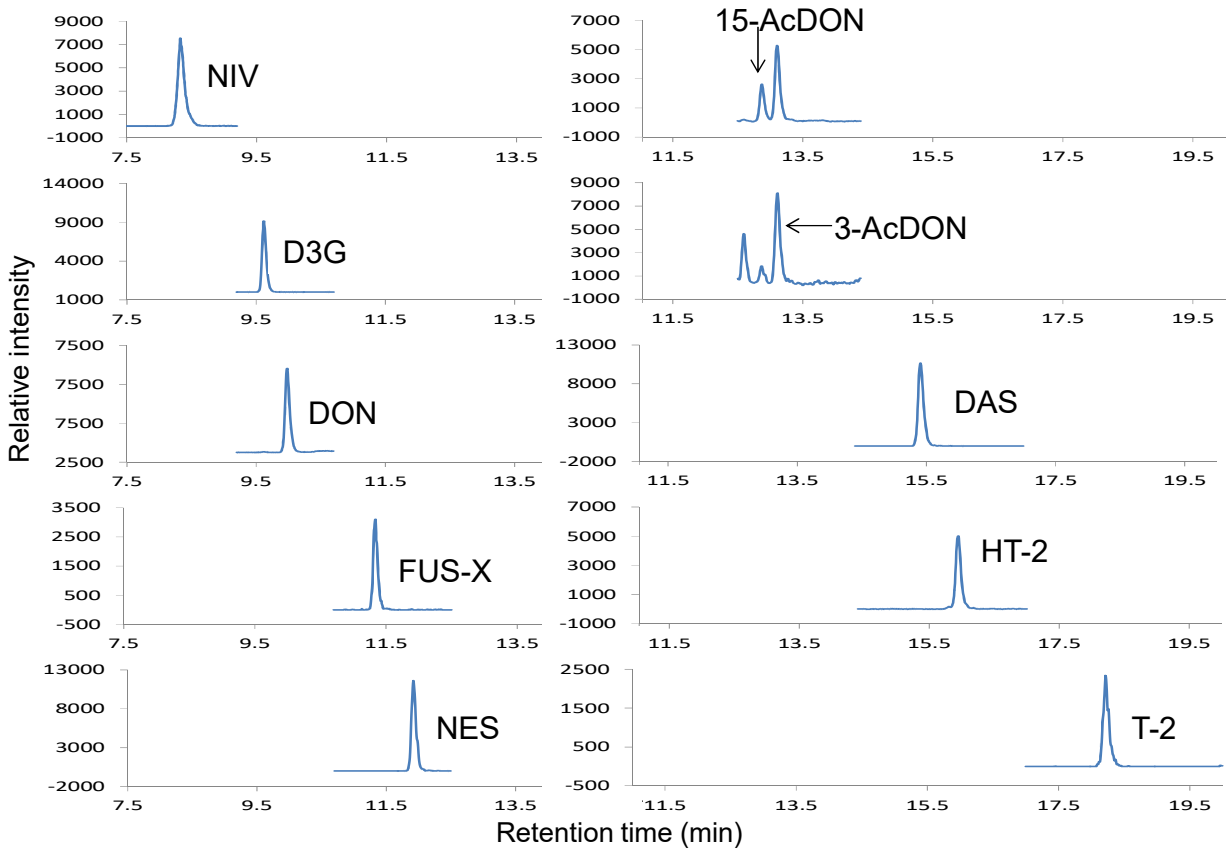


**Figure 3-1　Representative reaction monitoring chromatograms of formula feed for starting broiler chicks (spiked with 100 μg/kg of D3G, 1000 μg/kg of DON and 200 μg/kg of others)**

LC-MS/MS: LCMS-8040,　Shimadzu Co. Ltd. LC column: Inertsil ODS-3, 2.1×150 mm, GL Sciences Inc. Column temperature: 40°C. Mobile phases (gradient): 10 mmol/L aqueous ammonium acetate solution (mobile phase A) and acetonitrile (mobile phase B). Flow rate: 0.2 mL/min. Injection volume: 5 μL. Ionization: APCI. Interface temperature: 350°C. Heat block temperature: 200°C. DL temperature: 250°C. Nebulizer gas: Air (4L/min). Drying gas: $N_2$ (5 L/min).

**Table 3-9 Results of the inter-laboratory study of trichothecenes in maize**

| Trichothecenes | HT-2 | T-2 | DAS | NES | 3-AcDON | 15-AcDON | DON | D3G | NIV | FUS-X |
|---|---|---|---|---|---|---|---|---|---|---|
| Participant labs | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Statistical labs | 8 | 8 | 7 [c] | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Spiked level (µg/kg) | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 100 | 200 | 200 |
| Naturally contaminated (µg/kg) | | | | | | | 21.2 | | 159 | |
| Recovery (%) | 103 | 102 | 99.8 | 107 | 97.1 | 115 | 97.2 | 96.7 | 98.8 | 101 |
| $RSD_r$ [a] (%) | 8.3 | 7.5 | 4.5 | 8.6 | 9.1 | 7.8 | 5.2 | 3.5 | 4.1 | 8.8 |
| $RSD_R$ [b] (%) | 8.3 | 7.5 | 4.5 | 8.6 | 9.2 | 12 | 8.5 | 15 | 7.6 | 8.8 |
| HorRat | 0.41 | 0.37 | 0.22 | 0.43 | 0.45 | 0.58 | 0.42 | 0.68 | 0.40 | 0.43 |

[a] Relative standard deviation of repeatability, [b] Relative standard deviation of reproducibility, [c] Outlier of single Grubbs test



**Table 3-10 Results of the inter-laboratory study of trichothecenes in soybean**

| Trichothecenes | HT-2 | T-2 | DAS | NES | 3-AcDON | 15-AcDON | DON | D3G | NIV | FUS-X |
|---|---|---|---|---|---|---|---|---|---|---|
| Participant labs | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Statistical labs | 8 | 8 | 7 [c] | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Spiked level (µg/kg) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 50 | 100 | 100 |
| Naturally contaminated (µg/kg) | | | | | | | 28.6 | | | |
| Recovery (%) | 104 | 100 | 101 | 109 | 103 | 116 | 103 | 78.2 | 89.9 | 93.8 |
| $RSD_r$ [a] (%) | 9.8 | 7.1 | 4.3 | 3.1 | 7.6 | 9.0 | 3.4 | 6.4 | 5.6 | 8.5 |
| $RSD_R$ [b] (%) | 9.8 | 7.1 | 4.3 | 4.9 | 12 | 14 | 13 | 13 | 5.6 | 10 |
| HorRat | 0.45 | 0.32 | 0.19 | 0.22 | 0.57 | 0.65 | 0.59 | 0.59 | 0.25 | 0.48 |

[a] Relative standard deviation of repeatability, [b] Relative standard deviation of reproducibility, [c] Outlier of Cochran test

**Table 3-11 Results of the inter-laboratory study of trichothecenes in formula feed for suckling pig**

| Trichothecenes | HT-2 | T-2 | DAS | NES | 3-AcDON | 15-AcDON | DON | D3G | NIV | FUS-X |
|---|---|---|---|---|---|---|---|---|---|---|
| Participant labs | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Statistical labs | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Spiked level (μg/kg) | 100 | 100 | 100 | 100 | 100 | 100 | 1000 | 50 | 100 | 100 |
| Naturally contaminated (μg/kg) | | | | | | | 15.8 | | | |
| Recovery (%) | 104 | 105 | 103 | 106 | 102 | 109 | 98.2 | 84.3 | 99.9 | 105 |
| $RSD_r$[a] (%) | 8.5 | 5.2 | 6.6 | 6.8 | 8.8 | 4.3 | 6.5 | 4.6 | 3.9 | 5.1 |
| $RSD_R$[b] (%) | 8.5 | 9.2 | 8.8 | 6.8 | 11 | 8.1 | 11 | 22 | 9.4 | 14 |
| HorRat | 0.39 | 0.42 | 0.40 | 0.31 | 0.49 | 0.37 | 0.70 | 1.0 | 0.43 | 0.62 |

[a] Relative standard deviation of repeatability, [b] Relative standard deviation of reproducibility

feed. Therefore, the MAFF requested the development of an analytical method for simultaneous determination of T-2, HT-2, DAS, 3-AcDON, 15-AcDON, and D3G.

A comparison of the extraction methods in this study suggested that some mycotoxins in naturally contaminated feed are difficult to extract and might not be sufficiently extracted by a one-step process. This result shows the same tendency with the findings of Numanoğlu et al. (2011), who increased the yield of DON from maize using two extraction steps. Therefore, repeated extraction steps in our present study were considered suitable. Numanoğlu et al. (2011) did not confirm whether repeatability was improved by two extraction steps, but the present study found that it was. In addition, signal enhancement of HT-2, DAS, and NES in formula feed was improved by using a phospholipid removal column, there is a possibility that signal enhancement occurred due to the substances removed by the phospholipid removal column. Our analytical method can be applied to a wide range of ingredients such as maize, barley, soybean meal, rapeseed meal, and formula feed. The method can simultaneously analyse many trichothecenes, including NIV, 3-AcDON, 15-AcDON, and D3G. In addition, the repeated extraction improved the extraction efficiency in naturally contaminated samples, and ionization suppression during LC-MS/MS was minimized using APCI.

The USFDA (Food and Drug Administration) recommends that the maximum amount of DON contamination should be < 1 mg/kg in swine feed and ~30 mg/kg for distiller and brewer grains for cattle aged over 4 months (FDA 2010). The Commission of the European Communities (EC) recommends the guidance value of DON of 0.9 mg/kg in swine formula feed and ~12 mg/kg for maize by-products (Commission of the European Communities, 2006a). For T-2 and HT-2 in total, the indicative levels by the EC are 100 μg/kg for raw wheat, rye, and other cereals and ~2,000 μg/kg for oat flour from processed grain products used for feed and formula feed (including barley) (Commission of the European Communities 2013). The LOQ of 10 μg/kg for DON, 5 μg/kg for HT-2, and 10 μg/kg for T-2; thus, the LOQ in the present study was below the advisory levels of European guidelines for DON, HT-2, and T-2 (JECFA 2010; Commission of the European Communities 2006a; Commission of the European Communities 2013). Therefore, the sensitivity of our method was considered satisfactory. The expanded measurement uncertainty ($k$=2) of the method at 20 μg/kg of mycotoxins in feed was < 20%.

According to the ECFA criteria for food (Commission of the European Communities 2006b), the recovery rates and $RSD_r$ of T-2 and HT-2 at concentrations of 50~250 and 100~200 μg/kg are 60%~130% and ≤ 40%, respectively. The recovery rates and $RSD_r$ values of DON at 100–500 μg/kg are 60%–110% and ≤ 20%, and those of > 500 μg/kg DON are 70%–120% and ≤ 20%, respectively. The results of T-2, HT-2, and DON in the present study satisfied all these criteria. There are no available criteria for DAS, NES, 3-AcDON, 15-AcDON, D3G, but our results satisfied the criteria for DON. Therefore, the recovery rates and the $RSD_r$ indicated that our method could help monitor and control the mycotoxin contamination levels in feed.

In a previous study, Aoyama et al. (2012) conducted an inter-laboratory study in 11 laboratories for DON and NIV in wheat. In addition, Yoshinari et al. (2013b) reported that an inter-laboratory study was conducted in nine laboratories for DON, 3-AcDON, and 15-AcDON in wheat. Additionally, Nakagawa et al. (2014) reported that an inter-laboratory study was performed in 12 laboratories in NIV, DON, T-2, HT-2, and ZEA in wheat and barley. The current study exhibited similar results to those previously reported with an inter-laboratory study for simultaneous quantification of a greater number of mycotoxins than those reported. De Girolamo et al. (2020) conducted an inter-laboratory study in 15 laboratories using wheat and wheat products for detecting NIV, DON, 3-AcDON, 15-AcDON, T-2, HT-2, and ZEA. According to a report by De Girolamo et al., in NIV, the sample spiked with a standard solution for inter-laboratory study had a low and acceptable recovery rate. In addition, some laboratories reported unsuccessful separation of 3-AcDON and 15-AcDON which could not be quantified. In our inter-laboratory study, no problems were faced with NIV recovery and separation of 3-AcDON and 15-AcDON. In addition, some analytical methods use expensive isotope-labelled standard solutions for a wide range of mycotoxins (De Girolamo et al. 2020; Zhao et al. 2015; Habler and Rychlik 2016; Zhang et al. 2017; Tittlemier et al. 2021) and since our analytical method does not use this, it can also be used for routine analysis.

For those with a reference value for trichothecene mycotoxins, the spike and recovery tests were conducted at the reference value on the high concentration side. The method listed in the Feed Analysis Standard involves the simultaneous analytical method for mycotoxins (aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, aflatoxin $G_2$, sterigmatocystin, ZEA,

T-2 toxin, DON, NIV, NES, fusalenone-X, α-zearalanol, β-zearalanol, zearalanone, α-zearalenol and β-zearalenol (16 components)) (FAMIC 2008c, Fukunaka and Hiraoka 2006). However, the method uses MultiSep 226 AflaZon+ (Romer Labs) as the purification column and ESI as the ionization method, and the recovery rate of some mycotoxins, especially in oil meals (defatted rice bran, corn gluten meal, soybean meal and rapeseed meal), is very low and out of the scope of application (Suzuki et al. 2016). Therefore, the development of a simultaneous analytical method for mycotoxins in feeds that can also be used for oil meals have been desired. The method developed by the author uses APCI as the ionization method and two types of mini-columns for purification. The results of the spike and recovery tests showed that the recovery rate was satisfactory for the analysis of oil meals. This method is the only simultaneous analytical method for mycotoxins that can be used for oil meals in the feed analysis standard, is highly practical, and has been used for testing feed since it became an official method.

During the development of the analytical method, the extractant and four purification columns were examined and selected with reference to examples of the use of multifunctional columns and other reports (Nakagawa et al. 2013; Yoshinari et al. 2012; Kadota et al. 2011). As a result, InertSep VRA-3 column (GL Sciences Inc., Tokyo, Japan) was selected for this method because it gave the best results. The results of the spike and recovery tests showed poor recovery of D3G in soybean meal, so a comparison was made between single extraction and double extraction. As a result, the problem of poor recovery of D3G in soybean meal was solved by the second extraction, and good recovery rates were obtained for other mycotoxins. The $RSD_r$ was lower in the second extraction, although there are reports that the recovery rate was better in the second extraction (Numanoğlu et al. 2011), the fact that the $RSD_r$ was also smaller is a new finding in this study.

In addition, the recovery of HT-2 and other compounds exceeded 120% in the spike and recovery tests of cattle formula feed, and since ionization enhancement due to insufficient purification was considered, the addition of purification by a phospholipid removal mini-column was considered. The problem of ionization enhancement of some mycotoxins was resolved by adding purification using a phospholipid removal mini-column. The addition of purification by phospholipid removal mini-columns resulted in

a lower recovery of T-2. However, elution of the sample solution with acetonitrile-water (1:9) followed by additional elution with 1 mL of acetonitrile eliminated the problem of low T-2 recovery.

Phospholipid removal mini-columns HybridSPE®-Phospholipid plates (Sigma-Aldrich., St. Louis, MO, USA) are primarily used to remove phospholipids from serum and plasma in biological samples, but have not been used so far in feed, food or pet food analysis. In this study, it is a new finding that the problem of ionization enhancement of some mycotoxins in feed was ameliorated by the use of phospholipid removal mini-columns.

Although the distribution of mycotoxins in pet food as a product is considered to be uniform, mycotoxin localization is a concern, especially in cereal-based feed ingredients. Since trichothecene mycotoxins are considered to be relatively homogeneous due to sufficient infestation prior to harvest, it was considered that a sample volume of about 25 g would be acceptable if properly pretreated, as was the case with 1 mm mesh in the Retsch mill. However, several previous literature surveys (Sydenham et al. 1996; Bennett et al. 1985; Campbell and Armstrong 2007) used a sample volume of 50 g, so the sample volume was set at 50 g to account for the heterogeneous distribution of mycotoxins. Furthermore, by performing two extractions, mycotoxins from natural contamination can be extracted efficiently.

The method we developed was eventually made official because the validity test results of the single-laboratory and inter-laboratory collaborative studies met all the requirements of an official method for feed. The following points were particularly appreciated. 1) Development of a simultaneous analytical method for trichothecene mycotoxins in feed that can be used for oil meals, which is susceptible to ionization suppression. 2) No other analytical method was available for simultaneous analysis of type A and type B trichothecene mycotoxins, including D3G for feed, which was validated through inter-laboratory testing.

In conclusion, an inter-laboratory study of trichothecenes, including type A, type B, and D3G in feeds was conducted in eight laboratories using a simultaneous quantification method using a multifunctional column InertSep VRA-3 and a phospholipid-removal minicolumn HybridSPE®-Phospholipid for purification. As a result, the simultaneous

quantification method using LC-MS/MS for HT-2, T-2, DAS, NES, 3-AcDON, 15-AcDON, DON, D3G, NIV, and FUS-X in the feed was found to be promising. This optimized method could be useful for monitoring the concentration of trichothecene mycotoxins in the feed.

## Summary and conclusion

Until now, there was no official method for measuring FBs and ZEA in pet food, and thus the contamination status could not be determined. For trichothecenes, there was no method for analysis of D3G and DAS, and no method for simultaneous determination of HT-2, 15-AcDON and 3-AcDON in the official method of feed. Therefore, the author developed analytical methods.

In Chapter 1, an analytical method to determine fumonisins $B_1$ ($FB_1$), $B_2$ ($FB_2$) and $B_3$ ($FB_3$) in pet foods using a liquid chromatography-electrospray ionization-mass spectrometer (LC-ESI-MS) was developed, and an inter-laboratory study was conducted in eleven laboratories. $FB_1$, $FB_2$ and $FB_3$ (FBs) were extracted with aqueous acetonitrile. The extract was purified by a multifunctional column, MultiSep 211 Fum, and analysed with LC-ESI-MS. The limit of quantification of FBs by this method was estimated to be 0.2 mg/kg for dry and semi-dry pet foods, and 0.1 mg/kg for wet pet food. The calibration curve of FBs for this method showed linearity in the range of 0.1-5 ng of FBs (0.02-1.0 μg/mL). The values of the mean recovery for $FB_1$ at 0.1-1.0 mg/kg were 93.3-107% and those of relative standard deviation were less than 7.9%. These values were 87.3-102% and 8.6%, respectively, for $FB_2$ and 90.8-102% and 8.6%, respectively for $FB_3$. The mean recovery, repeatability, reproducibility and the Horwitz ratio for $FB_1$ from the inter-laboratory validation study were 92.9-98.9%, 2.6-4.6%, 6.8-10%, and 0.41-0.54, respectively. The values for $FB_2$ were 91.5-94.7%, 2.7-5.9%, 6.8-8.9%, and 0.33-0.55, respectively, and the values for $FB_3$ were 90.1-94.3%, 3.3-5.9%, 7.3-9.5%, and 0.44-0.57, respectively.

In Chapter 2, an analytical method for quantifying zearalenone (ZEA) in pet foods using a liquid chromatograph-atmospheric pressure chemical ionization-mass spectrometer (LC-APCI-MS) was developed, and an inter-laboratory study was conducted. ZEA was extracted from pet food samples with aqueous acetonitrile. The extract was purified using a multifunctional column, MultiSep 226 Aflazon$^{+®}$, and analysed with LC-APCI-MS. The limit of quantification of ZEA by this method was estimated to be 0.2 mg/kg for dry and semi-dry pet foods, and 0.1 mg/kg for wet pet food. The calibration curve of ZEA for this method showed linearity in the range of 0.1-10 ng of ZEA (0.01-1.0 μg/mL, 0.04-4.0 mg/kg matrix). The mean recoveries, repeatabilities,

reproducibilities, and the HorRats for ZEA from the inter-laboratory validation study were 99.0-102%, 2.2-3.0%, 5.6-6.6%, and 0.33-0.36, respectively.

In Chapter 3, an analytical method for the simultaneous quantitation of ten trichothecenes of type A (HT-2 toxin, T-2 toxin, diacetoxyscirpenol and neosolaniol) and type B (3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol, deoxynivalenol-3-glucoside, nivalenol and fusarenon-X) in feed has been developed using liquid chromatography with tandem mass spectrometry. Mycotoxins extracted twice from samples using aqueous acetonitrile were purified using a multifunctional clean-up column, followed by a phospholipid removal column. Trichothecenes were analysed using liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry. Extraction efficiency of the mycotoxins and the repeatability of some were improved by repeated extractions. Ionization enhancement (signal enhancement) of some mycotoxins was improved by using the phospholipid removal column at the clean-up step. Spike and recovery tests of trichothecenes were conducted on maize, barley, soybean meal, rapeseed meal, and formula feeds (for starting broiler chicks, suckling pigs, and beef cattle). The mean recovery values were 70.6%–119% with relative standard deviations <17%. The LOQ and the LOD of our method were 20 and 6 μg/kg, respectively, for 3- acetyldeoxynivalenol and 15-acetyldeoxynivalenol, 10 and 3 μg/kg, respectively, for T-2 toxin, deoxynivalenol and fusarenon-X, and 5 and 2 μg/kg, respectively, for nivalenol and the remaining mycotoxins. An inter-laboratory study was performed in eight laboratories to evaluate the simultaneous quantification method for ten trichothecenes in feed using a liquid chromatography tandem mass spectrometer. The mean recovery, repeatability, reproducibility, and HorRats for type A trichothecene from the inter-laboratory validation study were 99.8–109%, 3.1–9.8%, 4.3–9.8%, and 0.19–0.45, respectively. The values for type B trichothecene were 89.9–116%, 3.4–9.1%, 5.6–14%, and 0.25–0.70, respectively, and the values for modified mycotoxin were 78.2–96.7%, 3.5–6.4%, 13–22%, and 0.59–1.0, respectively.

The official method is used for the inspection of mycotoxins exceeding the standard value in feed and pet food, and is also indispensable for the contamination survey of mycotoxin when the standard value of mycotoxin in feed and pet food is set. The development of analytical methods is indispensable for the cause investigation of health

problems of domestic animals and pets.

The official method is used to test for mycotoxins in feed and pet food that exceed standard values. In order to set standard values for mycotoxins in feed or pet food, it is essential to develop an official method, and is also essential in the pursuit of the causes of health problems in livestock and pets. The analytical methods developed in this research contributes to the development of an official analytical method for mycotoxin in pet food and feed, and are considered to be an effective means for improving the safety of pet food and feed.

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