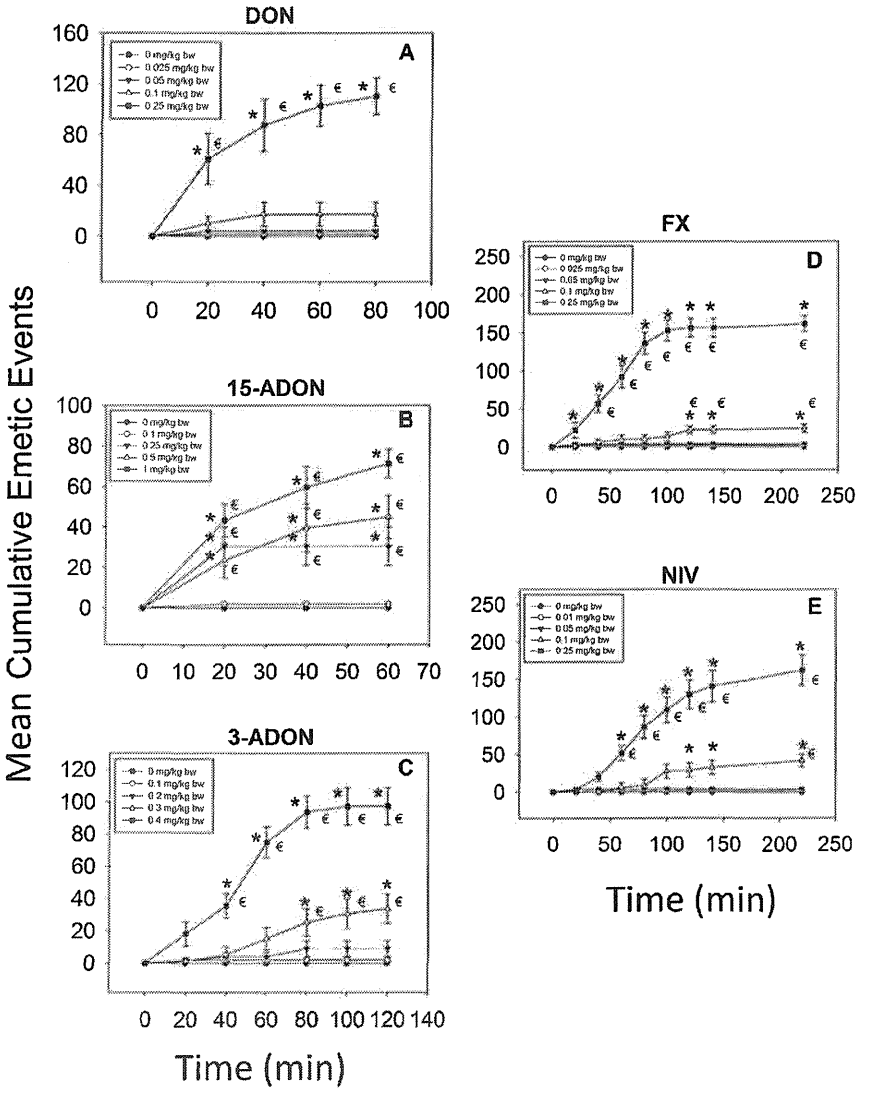

FIG. 5. Mean cumulative emetic events in mink following ip exposure to 8-ketotrichothecenes. Data are averages for both responders and nonresponders and represent mean ± SEM ($n = 6$ per group). Symbols: * indicates statistically significant differences in cumulative emetic episodes compared with the control ($p < 0.05$). € indicates a statistically significant difference relative to the 0-min time point within a given dose ($p < 0.05$).

to and duration of emesis were similar for the two chemicals after oral exposure (Pestka *et al.*, 1987). The duration of emesis after ip exposure was less for 15-ADON compared with DON, suggesting that the former might be metabolized at a faster rate than the latter.

3-ADON is generally considered to be equivalent to DON in terms of causing anorexia, growth retardation, and other similar effects (Yoshizawa and Morooka, 1977). However, the emetic response induced by 3-ADON here was very different compared with DON in that oral exposure to 3-ADON evoked an emetic effect with a longer latency as well as duration. Because 3-ADON must be deacetylated to DON before appreciable absorption occurs in the gut following consumption (Eriksen *et al.*, 2003), emesis induction might require more time due to the need for metabolic conversion of 3-ADON to DON.

Latency to emesis induced by FX was similar to that induced by DON. When radiolabeled FX was administered to mice by gavage, like DON, plasma radioactivity reached a maximum concentration at 30 min (Poapolathep *et al.*, 2003). NIV had a longer latency to emesis than DON. After oral exposure of mice to radiolabeled NIV, peak plasma radioactivity was detected after 60 min. Slower absorption of NIV might be a reason for a greater latency to emesis compared with DON.

The duration of emesis induced by both FX and NIV was longer compared with DON, which might relate to differences in their respective elimination rates. Clearance of radiolabeled DON in the mouse following administration follows

TABLE 2
Comparison of Emetic Responses in Mink Following Oral Exposure to 8-Ketotrichothecenes

Toxin	Dose (mg/kg bw)	Incidence (responding/tested)	Latency to emesis (min)[a,b]	Emesis duration (min)[a,b]	Emetic events[c]		
					Retching	Vomiting	Total
Control	0	0/30	-	-	0±0	0±0	0±0
DON	0.01	0/6	-	-	0±0	0±0	0±0
	0.05*	5/6	15±2	1±1	12±4	3±1	15±5
	0.25*	6/6	17±2	6±1	44±13	8±1	52±15
	0.5*	6/6	11±3	14±3	62±9	9±1	71±10
3-ADON	0.05	0/6	-	-	0±0	0±0	0±0
	0.25	1/6	62±0	1±0	2±2	1±1	3±3
	0.5*	5/6	53±9	12±9	16±8	4±1	20±9
	1*	6/6	19±5	44±9	65±11	14±2	79±13
15-ADON	0.01	0/6	-	-	0±0	0±0	0±0
	0.1*	5/6	20±4	1±1	9±3	2±1	11±4
	0.5*	6/6	18±3	3±1	24±5	6±1	30±5
	1*	6/6	17±4	14±7	26±5	6±1	32±6
FX	0.01	0/6	-	-	0±0	0±0	0±0
	0.05*	4/6	23±2	13±10	8±3	2±1	10±4
	0.25*	6/6	36±10	34±11	38±5	11±2	49±7
	0.5*	6/6	15±3	63±21	45±4	13±2	58±4
NIV	0.05	0/6	-	-	0±0	0±0	0±0
	0.1	0/6	-	-	0±0	0±0	0±0
	0.25*	4/6	31±4	15±10	21±14	4±2	25±16
	0.5*	6/6	29±4	32±10	55±14	9±2	64±15

[a] Average of positive responders only.
[b] If animals failed to retch or vomit, the latency and duration of emesis are shown as "-."
[c] Average of both responders and nonresponders. Data represent the mean ± SEM. Values with an asterisk indicate insignificant differences at $p < 0.05$ relative to the control for incidence, retching, vomits, and total emetic events.

two-compartment kinetics with an initial rapid phase of elimination ($t_{1/2\alpha}$ =0.36 h) and a slower terminal elimination phase ($t_{1/2\beta}$ = 7.62 h) (Azcona-Olivera *et al.*, 1995). A study employing ELISA similarly reported $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 0.29 h and 11.8 h, respectively. (Pestka *et al.*, 2008). In contrast, although following two-compartment kinetics clearance in mice, longer half lives were observed for FX ($t_{1/2\alpha}$ = 0.878 h, $t_{1/2\beta}$ = 37.63 h) and NIV ($t_{1/2\alpha}$ = 2.5 h, $t_{1/2\beta}$ = 14.34 h) (Poapolathep *et al.*, 2003). Longer elimination half-life may lead to greater durations of emetic events compared with DON. The duration of emesis induced by NIV was longer compared with FX following ip exposure, but shorter compared with FX via oral administration. Absorption of FX is more rapid and efficient compared with NIV, as a large proportion of NIV may have passed through the gastrointestinal lumen without being absorbed following oral exposure (Poapolathep *et al.*, 2003). This implies that limited absorption of NIV after oral exposure might have diminished its emetic effect.

Oral exposure to DON, 15-ADON, and FX evoked stronger emetic effects than ip exposure based on NOAEL, LOAEL, and ED_{50} values (Tables 1–3). This observation is not consistent with previous reports in pigs for DON and 15-ADON (Forsyth *et al.*, 1977; Pestka *et al.*, 1987), which was suggestive of less absorption of these toxins by the oral route. Similarly, ED_{50} values for DON based on emesis were 0.020 and 0.085 μg/kg bw following intravenous and oral exposure in pigs, respectively (Prelusky and Trenholm, 1993), again suggesting that oral exposure was less effective in emesis induction. It should be noted that, unlike previous studies in pigs that did not control food intake prior to toxin exposure, we fasted mink for 24 h in this study and provided a measured amount (50 g) of food, which might have facilitated efficient uptake. Other possible reasons might relate to species differences in absorption, metabolism, distribution, and bioavailability of 8-ketotrichothecenes for the different exposure routes (ip vs. oral). In addition, it is plausible that DON and its congeners bind to one or more unidentified chemoreceptors with varying affinities located in the gastrointestinal tract. Differences in the distribution of such receptors throughout the GI tract or systemically as well as differences in receptor–ligand affinity may be responsible for the observed differences in potency of the emetic response following oral and ip exposure to DON, 15-ADON, and FX. Our lab is currently conducting research to identify receptors through which DON and its congeners may act to elicit an emetic response.

It is further important to note that there were differences in environmental conditions under which the initial ip (Table 1) and oral (Table 2) studies were carried out. Specifically, the ip study was conducted in March and April, 2011, which had average temperatures of 1.1°C and 8.1°C, respectively, whereas the oral study was carried out in June and July, 2011, with average temperatures being 19.4°C and 21.4°C, respectively. Behavior of animals can be affected by heat stress, which can

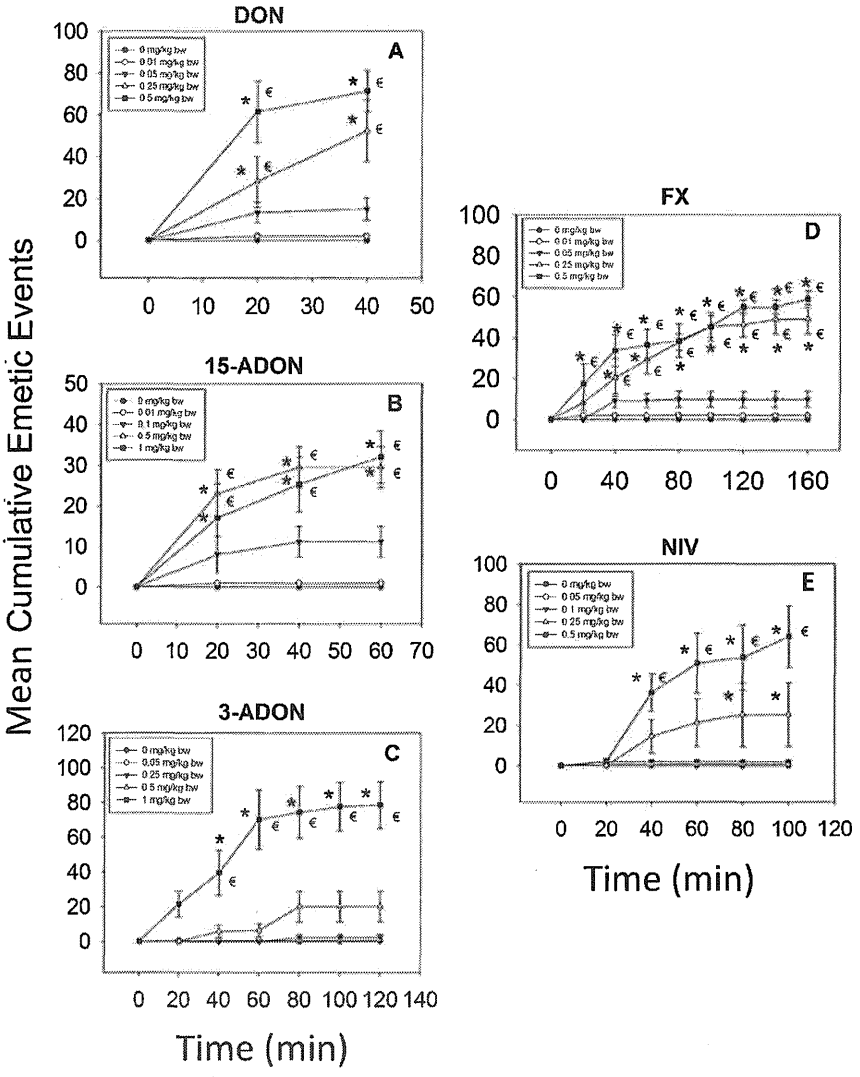

FIG. 6. Mean cumulative emetic events in mink following oral exposure to 8-ketotrichothecenes. Data are averages for both responders and nonresponders and represent mean ± SEM ($n = 6$ per group). Symbols: * indicates statistically significant differences in cumulative emetic episodes compared with the control ($p < 0.05$). € indicates a statistically significant difference relative to the 0-min time point within a given dose ($p < 0.05$).

cause effects such as feed refusal, emesis, nausea, and low blood pressure (Lugo-Amador *et al.*, 2004). As thermoregulation by mink at high ambient temperatures is difficult (Hansen and Jeppesen, 2003), conducting the oral study in the summer with mink being housed in an open-sided shed could possibly have enhanced the emetic response. To address this concern, we conducted a follow-up study in a new cohort of mink in June 2012 (19.5°C, average ambient temperature) in the latter environment and verified that emesis incidence was indeed greater for oral exposure than for ip exposure (Table 3). Thus differences in sensitivity to ip and oral challenges were not attributable to the temperature or housing conditions.

Conversely to the three aforementioned compounds, 3-ADON and NIV were more effective in inducing emesis following ip exposure compared with oral exposure. It is likely that 3-ADON and NIV were more completely absorbed following ip exposure. Although no data on absorption, distribution, excretion, and metabolism following oral administration of 3-ADON are available, a large proportion of NIV passes through the gastrointestinal tract without being absorbed after oral exposure in mice (Poapolathep *et al.*, 2003). This would likely reduce the emetic effect of NIV when administered by this route. Moreover, intestinal microorganisms further attenuate the emetic response by de-epoxidation of 8-ketotrichothecenes after oral exposure (Eriksen *et al.*, 2003; Onji *et al.*, 1989). These possibilities will require further study.

Interestingly, the average duration and intensity of emesis after oral exposure was generally less than that for ip exposure.

TABLE 3
Comparison of Emetic Responses in Mink Following ip and Oral Exposure

Toxin DON	Dose (mg/kg bw)	Incidence (responding/tested)	Latency to emesis (min)[a,b]	Emesis duration (min)[a,b]	Emetic events[c]		
					Retching	Vomiting	Total
IP	0	0/3	-	-	0±0[d]	0±0[d]	0±0[d]
	0.025	0/3	-	-	0±0[d]	0±0[d]	0±0[d]
	0.05	0/3	-	-	0±0[d]	0±0[d]	0±0[d]
	0.1	2/3	13±2	9±4	37±29[e]	7±5[e]	44±35[e]
	0.25	3/3	11±1	48±4	84±4[e]	20±1[e]	105±3[e]
Oral	0	0/3	-	-	0±0[d]	0±0[d]	0±0[d]
	0.025	0/3	-	-	0±0[d]	0±0[d]	0±0[d]
	0.05	1/3	8±0	7.5±0	24±24[e]	4±4[e]	28±28[e]
	0.1	2/3	14±6	5±0.3	23±14[e]	7±4[e]	30±16[e]
	0.25	3/3	11±3	5±2	44±23[e]	10±1[e]	55±24[e]

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis is shown as "-."
[c]Average of both responders and nonresponders. Data represent the mean ± SEM. Values without the same superscript within a column differ ($p < 0.05$).
[d,e]Different letters indicate a statistically significant difference between dose groups.

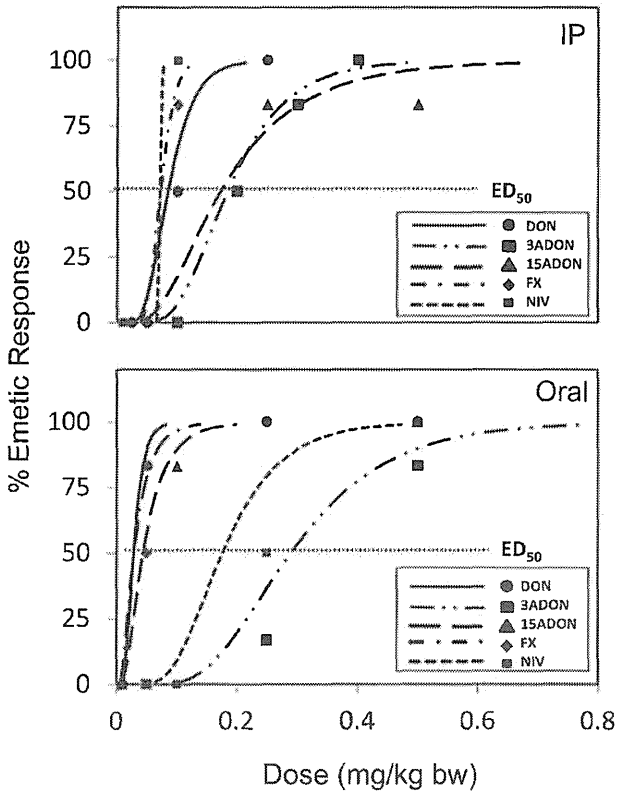

FIG. 7. Emetic dose curves for 8-ketotrichothecenes following ip and oral exposure.

Salivation and vomiting induced by 8-ketotrichothecenes at relatively high doses probably reduced the amount of toxin available for absorption, leading to an overall decrease in the average duration of emesis, particularly because absorption of orally administered doses may start in the stomach or from the upper part of the duodenum (Eriksen *et al.*, 2003; Hedman *et al.*, 1997). The average duration of emesis directly affected the average number of emetic events because the duration of the emetic response induced by oral exposure was less compared with ip exposure.

The neurologic and physiologic basis for emesis involves a complex reflex pathway involving neurotransmitters, hormones, and afferent fibers and coordination of neural, respiratory, and digestive systems (Carpenter, 1990). The mechanisms for trichothecene-induced vomiting are not fully understood but appear to involve activation of the chemoresponsive area postrema of the brain, as suggested by studies of T-2 toxin-induced emesis in the cat (Borison and Goodheart, 1989) and DON-induced conditioned taste aversion in the rat (Ossenkopp *et al.*, 1994). Furthermore, DON's emetic action is likely to be at least partially due to release of the neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) (Prelusky and Trenholm, 1993). 5-HT is synthesized and secreted by enterochromaffin cells and plays an important role in emesis caused by cytotoxic chemicals (Endo *et al.*, 2000). Increased 5-HT concentration will trigger the 5-HT_3 receptors on the vagal afferent nerves, activating the emesis reflex via vagal afferent nerve depolarization (Andrews *et al.*, 1990; Hillsley and Grundy, 1998; Hillsley *et al.*, 1998).

Although the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recognized that DON potentially causes human acute illness, this group concluded that there were insufficient data to determine an acute reference dose for this effect (Canady *et al.*, 2001). An even greater paucity of data on other 8-ketotrichothecenes makes such estimates for these congeners even more problematic (Pestka, 2010a). The findings of this study provide new insight into the relative potential of DON and its congeners to contribute to human food poisoning. The estimated mean and 95th percentile per capita grain intakes in the United States for 1- to 6-year olds are 6.3 and 12 g/kg bw/d and for 16- to 21-year olds are 2.5 and 5.1 g/kg bw/d, respectively (EPA, 2011). If it is assumed that humans have a similar threshold for oral DON-induced emesis (0.05 mg/kg

TABLE 4
Summary of NOAELs, LOAELs, and ED_{50}s for Emetic Effects of 8-Ketotrichothecenes

	Intraperitoneal			Oral		
Toxin	NOAEL[a]	LOAEL[b]	ED_{50}[c]	NOAEL[a]	LOAEL[b]	ED_{50}[c]
DON	0.05	0.1	0.08 (0.05–0.18)	0.01	0.05	0.03 (0–0.05)
15-ADON	0.1	0.25	0.17 (0.07–0.29)	0.01	0.1	0.04 (0–0.11)
3-ADON	0.1	0.2	0.18 (0.1–0.27)	0.05	0.25	0.29 (0.01–0.47)
FX	0.05	0.1	0.07 (0.04–0.1)	0.01	0.05	0.03 (0.01–0.08)
NIV	0.05	0.1	0.06 (0–0)	0.1	0.25	0.18 (0.1–0.29)

[a]NOAEL = no observed adverse effect level.
[b]LOAEL = lowest observed adverse effect level.
[c]ED_{50} = Dose causing emesis in 50% of the animals tested. ED_{50} values were determined using a Proc Probit model.

TABLE 5
Previously Reported Emetic Effects of 8-Ketotrichothecenes

Toxin	Species	Route	NOAEL (mg/kg bw)	LOAEL (mg/kg bw)	References
DON	Pig	ip	n.d.	0.05	Vesonder *et al.*, 1973
DON	Pig	oral	0.075	0.1	Forsyth *et al.*, 1977
		ip	0.025	0.05	
DON	Pig	ip	0.025	0.05	Pestka *et al.*, 1987
DON	Pig	oral	0.025	0.05	Prelusky *et al.*, 1993
		iv	0.015	0.02	
DON	Dog	sc	n.d.	0.1	Yoshizawa *et al.*, 1974
	Dog	iv	n.d.	0.1	
					Yoshizawa *et al.*, 1977
			0.42		
15-ADON	Pig	oral	0.05	0.075	Pestka *et al.*, 1987
		ip	0.05	0.075	
15-ADON	Dog	sc	n.d	0.2	Yoshizawa *et al.*, 1974
3-ADON	Dog	sc	n.d.	0.2	Yoshizawa *et al.*, 1977
3-ADON	Dog	iv	n.d.	0.2	
					Yoshizawa *et al.*, 1977
FX	Dog	iv	n.d	0.1	Matsuoka *et al.*, 1979
FX	Cat	sc	n.d.	1	Ueno *et al.*, 1971
NIV	Dog	iv	n.d.	0.3	Matsuoka *et al.*, 1979

bw) and a worst case scenario it is further assumed in which an entire day's grain intake is eaten at a single meal (e.g., breakfast) by those in the 95th percentile groups, it could be speculated that contamination levels of 4 and 10 ppm could cause vomiting episodes in the 1- to 6-year and the 16- to 21-year-old groups, respectively. Consistent with these estimates, a very large Chinese outbreak of gastrointestinal illness that included vomiting in 1984 was associated with consumption of maize contaminated with DON at levels between 3.8 and 93 mg/kg (Luo, 1994). Assuming consumption of 560 g of grain and 50 kg bw, as estimated in another Chinese study (Gao and Yoshizawa, 1997), the LOAEL could be expected to fall between 0.04 and 1.04 mg/kg bw. Similar calculations for high grain consumers can be extrapolated from the mink data for 15-ADON (8 and 20 ppm), 3-ADON (20 and 50 ppm), FX (4 and 10 ppm), and NIV (20 and 50 ppm). It must be cautioned that these estimates are preliminary at best because they do not take into account potential differences in absorption and metabolism between species or possible differences among humans in sensitivities to these toxins. Nevertheless, this research represents a first step in identifying threshold doses for DON and its congeners for induction of vomiting and noninfectious gastroenteritis.

Because emesis is stressful, it would be desirable to have an alternative rodent model based on anorexia or pica. In previous studies, the NOAELs and LOAELs for 8-ketotrichothecene-induced feed refusal in mice were approximately 10-fold greater than corresponding values based here on emesis (Flannery *et al.*, 2011; Wu *et al.*, 2012). Furthermore, the anorectic potencies of 8-ketotrichothecenes followed rank orders of NIV > FX > DON ≈ 3-ADON ≈ 15-ADON for ip exposure and FX > NIV > DON ≈ 3-ADON ≈ 15-ADON for oral exposure. Possible reasons for the observed species differences in sensitivity based on anorexia and emesis might be due to differences in sensitivity to and toxicokinetics of these toxins between mouse and mink, but also differences in the mechanisms involved in anorexia and emesis. Nevertheless, anorectic studies in the mouse cannot be fully relied on for prediction of emetic potencies of 8-ketotrichothecenes. It is further

notable that DON caused significant reduction of kaolin consumption, instead of increased consumption associated with pica behavior as observed in a recent study (Girardet *et al.*, 2011). Although mice displayed a trend toward pica behavior early after oral exposure to DON in that study, we found that it was rather difficult to measure kaolin as it was consumed in very small amounts. Other researchers have found difficulty in determining exact kaolin consumption in mice after exposure to emetic stimuli, suggesting that it is not useful to use pica to predict nausea in this species (Yamamoto *et al.*, 2002).

In conclusion, the mink emesis model described herein was robust and should be applicable to further investigation of the mechanisms of 8-ketotrichothecene-induced emesis. Such studies will improve our ability to predict specific thresholds for the emetic response during trichothecene food poisoning as well as the persistence and reversibility of the emetic effect in the human population. From a public health perspective, comparative emetic potency data derived from this model should be useful for establishing toxic equivalency factors for DON and other trichothecenes.

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Research Note

Development of a Purification Method for Simultaneous Determination of Deoxynivalenol and Its Acetylated and Glycosylated Derivatives in Corn Grits and Corn Flour by Liquid Chromatography–Tandem Mass Spectrometry

TOMOYA YOSHINARI,[1]* TAKAHIRO OHNISHI,[1] TOMOYUKI KADOTA,[2] AND YOSHIKO SUGITA-KONISHI[1]

[1]*National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; and* [2]*United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu City, Gifu, 501-1193, Japan*

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ABSTRACT

We developed a purification method based on liquid chromatography–tandem mass spectrometry for the identification of deoxynivalenol (DON), its acetylated derivatives (3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol), and a glycosylated derivative (deoxynivalenol-3-glucoside [D3G]) in corn-based products. The analytes were extracted from samples with acetonitrile-water (85:15, vol/vol) and then purified with multifunctional columns. Evaluation of five kinds of multifunctional columns revealed that DON and its acetylated derivatives were recovered well (96 to 120%) by all columns, but D3G was recovered adequately (93.5%) by only one column, InertSep VRA-3. Samples of corn grits and corn flour were analyzed using the purification method with InertSep VRA-3. DON, D3G, and 15-acetyl-deoxynivalenol were the major contaminants in the samples harvested in 2009, but only DON was detected in the samples harvested in 2010. These results suggest that the purification method using InertSep VRA-3 is effective for identification of DON and its derivatives in corn-based products.

Trichothecene mycotoxins such as deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15ADON), and 3-acetyl-deoxynivalenol (3ADON) are produced by *Fusarium* species. DON is the most frequently detected trichothecene in grain samples such as wheat, corn, and barley, but 3ADON and 15ADON also can be found *(6, 11)*. Contamination of cereals with these mycotoxins is associated with several diseases in humans and animals *(5)*. The main effects of exposure to trichothecenes are gastrointestinal disturbances and impairment of the immune system *(4, 13)*.

The Joint Food and Agriculture–World Health Organization Expert Committee on Food Additives established the provisional maximum tolerable daily intake of DON for humans at 1 μg/day/kg of body weight based on a reduction of body weight gain *(8)*. To reduce the intake of DON, many countries have established regulatory limits or guidance concentrations for DON in foods and feeds. The advisory concentrations intended for human consumption in cereals and finished cereal products range from 100 to 2,000 μg/kg *(7)*.

Contamination with both DON and its acetylated derivatives has been detected in cereals and cereal products. The toxicity of ADON against mammals has not been well established, but in a bromodeoxyuridine incorporation assay 3ADON and 15ADON were cytotoxic to mouse fibroblasts and 15ADON was as toxic as DON *(14)*. Therefore, information about the occurrence of acetylated forms of DON is also important, and a convenient method for detecting these forms is needed.

Deoxynivalenol-3-glucoside (D3G) has been reported as the major form of chemically altered DON *(2)*. D3G is formed by *Fusarium*-infected plants in response to the production of DON by fungi and can be detected in naturally contaminated wheat, maize, barley, and malt *(1, 10)*. Because D3G exhibited a dramatically reduced ability to inhibit protein synthesis of wheat ribosomes in vitro, it appears that glycosylation of DON is a detoxification process in plants *(12)*. These DON derivatives escape analytical detection tailored to DON because of changed polarities and masses. For example, D3G was more strongly retained than DON by a multifunctional column because of the high polarity of D3G. Some analytical methods for the determination of these derivatives have been reported, but efficient recovery was not obtained, especially for D3G *(15)*. The toxicity of D3G has not been clarified, but D3G may be toxic and evaluation of risks associated with this derivative is necessary, as has been performed for other DON derivatives. Therefore, information on its presence as a food contaminant is also important.

In this study, we evaluated a purification method using a new multifunctional column for identifying DON and its derivatives, 3ADON, 15ADON, and D3G, and used this

* Author for correspondence. Tel: 81-3-3700-1141; Fax: 81-3-3700-9852; E-mail: t-yoshinari@nihs.go.jp.

TABLE 1. *LC-MS/MS analysis parameters of each mycotoxin*

Analyte	Precursor ion (*m/z*)	Declustering potential (V)	Quantifier ion (*m/z*) (collision energy [eV])	Qualifier ion (*m/z*) (collision energy [eV])
Deoxynivalenol (DON)	294.8 $[M-H]^-$	−25	264.8 (−14)	138.1 (−26)
3-Acetyl-deoxynivalenol (3ADON)	336.9 $[M-H]^-$	−25	307.1 (−14)	173.0 (−14)
15-Acetyl-deoxynivalenol (15ADON)	336.9 $[M-H]^-$	−20	150.2 (−26)	218.9 (−12)
Deoxynivalenol-3-glucoside (D3G)	456.9 $[M-H]^-$	−50	426.9 (−18)	247.1 (−30)

method to collect data on the presence of these toxins in corn-based products.

MATERIALS AND METHODS

Chemicals and materials. Solid crystals of DON, 3ADON, and 15ADON and a standard solution of D3G (50 ng/ml in acetonitrile) were purchased from Sigma-Aldrich (St. Louis, MO). Each solid crystal was dissolved in acetonitrile (each 50 ng/ml) and stored at −20°C. Before use, the solutions were brought to room temperature. Liquid chromatography (LC)–grade acetonitrile and water were purchased from Wako Pure Chemicals (Osaka, Japan).

Extraction and sample cleanup. Corn grits and corn flour were purchased in Japan from a local retail market. Corn used to produce these products was imported from the United States or China. Samples were directly extracted without further processing; 25.0 g of sample was extracted with 100 ml of acetonitrile-water (85:15, vol/vol). Extractions were performed on a horizontal shaker for 30 min at 180 rpm. After filtration, 10 ml of extract was transferred into a multifunctional column. Five different multifunctional columns were tested: MultiSep 226 AflaZon+, MultiSep 227 Trich+, and MultiSep 230 NIV (RomerLabs, Union, MO), Autoprep MF-T 1500 (Showa Denko K.K., Tokyo, Japan), and InertSep VRA-3 (GL Sciences Inc., Tokyo, Japan). The cleanup procedure was identical for all columns. The first 4 ml of eluate was discarded, and the next 2 ml of eluate was collected and evaporated to dryness. Dried residues were dissolved in 500 μl of 5% acetonitrile for LC–tandem mass spectrometry (MS/MS) injection. To evaluate toxin recovery, the toxins were spiked into corn grits at 100 ng/g.

Method validation. The method was validated with corn grits spiked at two concentrations, 15 and 100 ng/g, in quintuplicate for each analyte. The spiking concentrations were chosen based on natural contaminated levels *(1, 10)*. Detected concentrations were calculated on a peak-area basis using Analyst version 1.5.1 software (AB Sciex, Foster City, CA). Limits of detection (LODs) and limits of quantification (LOQs) were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively.

LC-MS/MS conditions. LC-MS/MS analyses were performed with a 3200 Q TRAP LC-MS/MS system (AB Sciex) equipped with an atmospheric pressure chemical ionization (APCI) source and an LC-20A series high-performance (HP) LC system (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved at 40°C with an Inertsil ODS-3 column (2.1 by 150 mm, 3 μm; GL Sciences Inc., Tokyo, Japan). The injection volume was 10 μl, and the flow rate was set to 0.4 ml/min. Each HPLC run lasted 7 min, and gradient elution was conducted with mixtures of acetonitrile and water. Initial eluent composition was acetonitrile-water (5:95, vol/vol) as the mobile phase. A linear gradient was used to reach 65% acetonitrile after 7 min. The APCI source was operated at 300°C in the negative ionization mode. Other MS parameters were as follows: curtain gas at 15 psi, nebulizer gas (GS1) at 50 psi, corona discharge needle current at 2 μA, collision-activated dissociation gas at 3 (arbitrary units), multiple reaction monitoring, dwell time of 100 ms, and a 5-ms pause between mass ranges. Table 1 shows the selected mass transition, declustering potentials, and collision energies for each analyte.

RESULTS AND DISCUSSION

Examination of multifunctional columns. Cleanup of sample extracts was performed with five commercially available multifunctional columns. Each column in the MultiSep series contained a combination of absorbents that was designed for recovering mycotoxins. MultiSep 226 AflaZon+ is used for simultaneous determination of aflatoxins and zearalenone, whereas MultiSep 227 Trich+, MultiSep 230 NIV, and Autoprep MF-T 1500 are designed for cleanup of trichothecenes. MultiSep 230 has been effective for identification of DON and D3G in wheat *(2)*.

TABLE 2. *Recovery of each spiked mycotoxin with various multifunctional columns*

Column	% recovery[a]			
	DON	3ADON	15ADON	D3G
MultiSep 226 AflaZon+	96.0 ± 8.1	112.7 ± 1.2	110.2 ± 2.5	0[b,c,d]
MultiSep 227 Trich+	106.2 ± 13.8	111.0 ± 6.1	114.4 ± 13.0	0[b,c,d]
MultiSep 230 NIV	96.0 ± 5.7	116.3 ± 7.4	101.8 ± 5.1	52.3 ± 5.0[c,d]
Autoprep MF-T 1500	98.5 ± 12.0	106.0 ± 4.0[d]	98.0 ± 8.1	31.3 ± 1.8[d]
InertSep VRA-3	104.2 ± 4.9	120.0 ± 4.4	113.8 ± 8.4	93.5 ± 0.9

[a] Values are means ± standard deviations. Differences between groups were assessed with a nonparametric Tukey-type multiple comparison test.
[b] Significantly different when compared with MultiSep 230 ($P < 0.05$).
[c] Significantly different when compared with Autoprep MF-T 1500 ($P < 0.05$).
[d] Significantly different when compared with InertSep VRA-3 ($P < 0.05$).

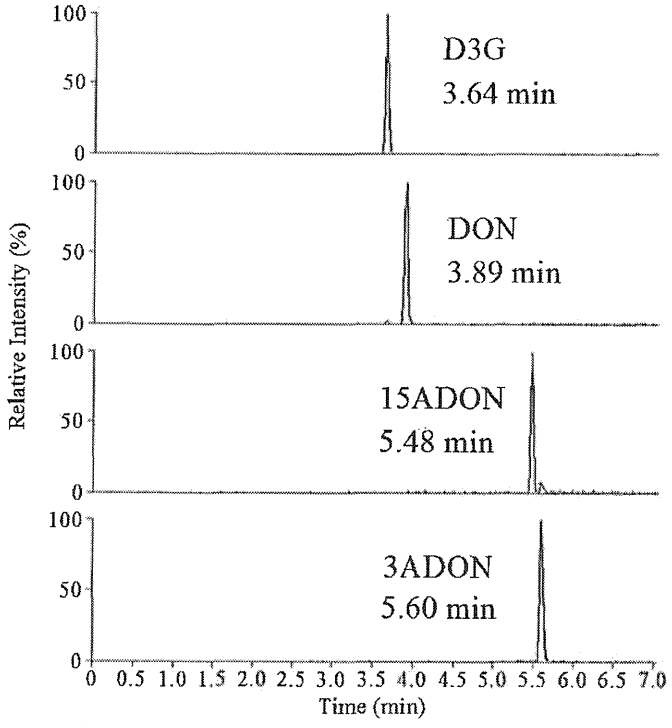

FIGURE 1. *Chromatogram of target mycotoxins (30 ng/ml concentrations of each analyte).*

InertSep VRA-3, which contains reverse-phase and ion exchange resins, is designed for purification of cereal and soil samples.

Results of the spike test are shown in Table 2. Recoveries of DON, 3DON, and 15ADON ranged between 90 and 120% with all five columns. However, different results were obtained for D3G. D3G was barely recovered in the MultiSep 226 and 227 cleanup. In contrast to the results presented here, high recoveries of D3G from wheat have been obtained when the MultiSep 227 column was used for purification *(9)*. The differences between corn and wheat may influence the recovery of D3G. MultiSep 230 and Autoprep MF-T 1500 cleanup resulted in better recovery but were inappropriate for the application. Only InertSep VRA-3 cleanup provided good recovery of all four analytes, at 90 and 120%. Consequently, we selected InertSep VRA-3 for subsequent experiments.

Chromatographic conditions. 3ADON and 15ADON are isomers, and the only difference in structure is the position of the acetyl group. Because most daughter ions are similar to the two compounds, it is necessary to separate them by HPLC to quantify them precisely. An HPLC chromatogram is shown in Figure 1. Retention times were 3.64 min for D3G, 3.89 min for DON, 5.48 min for 15ADON, and 5.60 min for 3ADON. 3ADON and 15ADON were eluted adjacent to one another, but no overlap was observed. Total analytical time was only 7 min, which allows efficient and rapid analysis of many samples.

Method validation. The results of the method validation are shown in Table 3. Ionization was slightly promoted when 15ADON was spiked at 15 ng/g, but almost all mean recoveries were 100 to 120% and standard deviations of recoveries were less than 10% for all the mycotoxins. These results indicate that the InertSep VRA-3 cleanup method is useful for the determination of the four kinds of trichothecenes tested: DON, 3ADON, 15ADON, and D3G.

TABLE 3. *Limits of detection and quantification, spiking concentrations, and recovery of toxins for validation of the LC-MS/MS method in corn grits*[a]

Analyte	LOD (ng/g)	LOQ (ng/g)	Spiking concn (ng/g)	Recovery (%)[b] Mean	RSD
DON	0.5	1.3	15	115.1	3.1
			100	106.4	3.6
3ADON	1.1	2.7	15	118.7	8.5
			100	114.8	1.9
15ADON	2.7	6.2	15	125.3	9.1
			100	117.4	5.7
D3G	1.2	3.6	15	103.9	7.0
			100	106.4	3.6

[a] LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.
[b] Mean and RSD of five experiments.

Occurrence of DON, 3ADON, 15ADON, and D3G in corn grits and corn flour. The DON, 3ADON, 15ADON, and D3G concentrations in corn grits and corn flour were determined using the InertSep VRA-3. Samples were collected over 2 years (2009 and 2010). The analysis results and an example chromatogram of a contaminated sample are shown in Table 4 and Figure 2, respectively. In the samples of the first year, DON, 15ADON, and D3G were the major contaminants. Among the three mycotoxins, the concentration of DON was highest and the relative concentrations of 15ADON and D3G as compared with DON were 16.2 and 14.5%, respectively. This result is consistent with those of a previous study in which D3G was detected in maize samples and its mean concentration was about 20% of the DON concentration *(1)*. Although 15ADON was detected in most samples, 3ADON was not detected at all. However, only DON was detected in the samples from the second year, and its concentration was about 25% of that of the first year.

The LC-MS/MS method that we developed in this study can be used to provide data concerning the state of food contamination by trichothecene mycotoxins. The toxicity of ADON and D3G is gradually becoming understood. Because these derivatives change into DON during the process of metabolism, it is important to determine their concentrations in cereals *(3)*. As the next step in this project, we will determine whether our method is applicable to other cereal samples in addition to corn grits and corn flour. More work is needed to improve our method for purifying the analytes from complex matrices. In parallel, we will continue to study food contamination by DON and its derivatives over several years. Information about the environmental factors that affect the kinds and

TABLE 4. *Concentrations of DON, D3G, 3ADON, and 15ADON in corn grits and corn flour harvested in 2009 and 2010*[a]

Analyte	No. of samples	No. of samples below LOQ	Maximum (ng/g)	Mean 1 (ng/g)	Mean 2 (ng/g)	Mean 3 (ng/g)	Ratio/DON (%)
2009							
DON	22	0	389			119.9	
3ADON	22	22		0	1.2		
15ADON	22	2	46.4			17.1	14.3
D3G	22	0	39.5			19.4	16.2
2010							
DON	20	2	192			30.1	
3ADON	20	20		0	1.2		
15ADON	20	16	34.0	4.0	6.6		
D3G	20	14	69.1	7.2	8.3		

[a] When 60% or more of the results were below the LOD, two estimates were produced by using 0 (mean 1) and the LOD (mean 2) for all results below the LOD. When less than 60% of the results were below the LOD, mean 3 was calculated using LOD/2 for all results below the LOD.

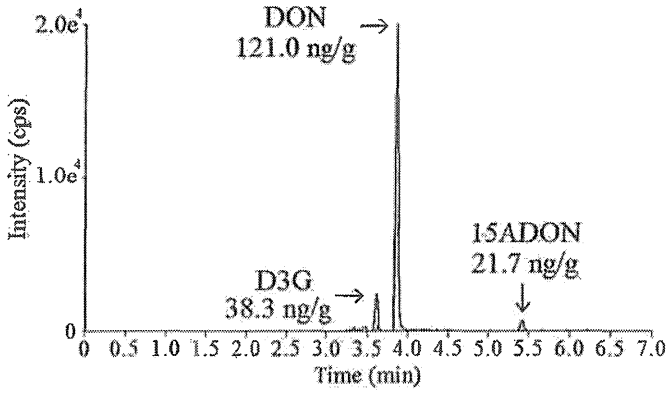

FIGURE 2. *Chromatogram of a contaminated corn grits sample.*

concentrations of trichothecenes in cereals might be revealed during the course of these studies.

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Validation Study

Interlaboratory Study of LC-UV and LC-MS Methods for the Simultaneous Determination of Deoxynivalenol and Nivalenol in Wheat

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Koji AOYAMA[1,*], Hajime AKASHI[2], Naoki MOCHIZUKI[3], Yuji ITO[4], Takashi MIYASHITA[5], Soohyung LEE[6], Motoki OGISO[7], Mamoru MAEDA[8], Shigemi KAI[9], Hiroki TANAKA[10], Hiroko NORIDUKI[11], Hisaaki HIRAOKA[1], Toshitsugu TANAKA[12], Eiichi ISHIKURO[7], Yoshinori ITOH[13], Toshihiro NAGAYAMA[14], Masahiro NAKAJIMA[15], Shigehiro NAITO[16] and Yoshiko SUGITA-KONISHI[13]

[1]Food and Agricultural Materials Inspection Center, Sendai Regional Center: 1–3–15 Gorin, Miyagino-ku, Sendai 983–0842, Japan;
[2]Nisshin Seifun Group Inc.: 5–3–1 Tsurugaoka, Fujimino-shi, Saitama 356–8511, Japan;
[3]Asahi Breweries Ltd.: 1–1–21 Midori, Moriya-shi, Ibaraki 302–0106, Japan;
[4]Kirin Group Office Company Ltd.: 1–17–1 Namamugi, Tsurumi-ku, Yokohama 230–8628, Japan;
[5]Kewpie Corporation: 5–13–1 Sumiyoshi-cho, Fuchu-shi, Tokyo 183–0034, Japan;
[6]Rural Development Administration: 249 Seodun-dong, Gwonseon-gu, Suwon-si, Gyeonggi-do 441–707, Republic of Korea;
[7]Japan Food Research Laboratories: 52–1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151–0062, Japan;
[8]Japan Frozen Foods Inspection Corporation: 2–4–6 Shibadaimon, Minato-ku, Tokyo 105–0012, Japan;
[9]Kanagawa Prefectural Institute of Public Health: 1–3–1 Shimomachiya, Chigasaki-shi, Kanagawa 253–0087, Japan;
[10]Suntory Business Expert Ltd.: 1–1–1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618–8503, Japan;
[11]Japan Grain Inspection Association: 2–17–3 Arai, Ichikawa-shi, Chiba 272–0144, Japan;
[12]Kobe Institute of Health: 4–6 Minatojimanakamachi, Chuo-ku, Kobe 650–0046, Japan;
[13]National Institute of Health Sciences: 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan;
[14]Tokyo Metropolitan Institute of Public Health: 3–24–1 Hyakunin-cho, Shinjuku-ku, Tokyo 169–0073, Japan;
[15]Nagoya City Public Health Research Institute: 1–11 Hagiyama-cho, Mizuho-ku, Nagoya 467–8615, Japan;
[16]National Food Research Institute: 2–1–12 Kannondai, Tsukuba-shi, Ibaraki 305–8642, Japan;
* Corresponding author

To evaluate LC methods with UV or MS detection for simultaneous analysis of deoxynivalenol (DON) and nivalenol (NIV) in wheat, an interlaboratory study was conducted in 11 laboratories. DON and NIV were purified using a multifunctional column, and their concentrations were determined using LC-UV or LC-MS(/MS). No internal standards were used. Three fortified wheat samples (0.1, 0.5 and 1 mg/kg), one naturally contaminated wheat sample, and one blank wheat sample were used. The recoveries ranged from 90% to 110% for DON and from 76% to 83% for NIV. For DON, the relative standard deviations for repeatability (RSDr) ranged from 1.1% to 7.6%. The relative standard deviations for reproducibility (RSDR) ranged from 7.2% to 25.2%. For NIV, the RSDr ranged from 2.0% to 10.7%, and the RSDR ranged from 7.0% to 31.4%. Regardless of sample and detector, the HorRat values for DON and NIV ranged from 0.4 to 1.4. Both LC-UV and LC-MS(/MS) methods were considered to be suitable for application as an official method.

Key words: deoxynivalenol; nivalenol; liquid chromatography (LC); ultraviolet (UV) detector; mass spectrometric (MS) detector; interlaboratory study; wheat

* kouji_aoyama@nm.famic.go.jp

Introduction

Deoxynivalenol (DON) and nivalenol (NIV), which are trichothecene mycotoxins produced by plant pathogenic *Fusarium* fungi, cause worldwide health and economic damage. These mycotoxins contaminate grains in temperate and sub-frigid regions and ingestion of contaminated grains can cause growth suppression, immunotoxicity, and hematotoxicity[1].

JECFA set the provisional maximum tolerable daily intake (PMTDI) of DON at 1.0 μg/kg of body weight per day in 2001[2]. This PMTDI was established based on the "no observed effect level" (NOEL, 100 μg/kg of body weight per day) obtained from a feeding study in mice for 2 years and on a safety factor of 100. The Scientific Committee on Food (SCF) set the temporary TDI (t-TDI) of NIV at 0.7 μg/kg of body weight per day in 2000[*1]. This t-TDI was established based on the "lowest observed adverse effect level" (LOAEL, 0.7 mg/kg of body weight per day) obtained from feeding studies in mice for 1 and 2 years and on a safety factor of 1,000. However, the LOAEL of NIV has also been reported to be 0.4 mg/kg of body weight per day based on the results of feeding studies in rats for 90 days[3),4)].

Both DON and NIV are frequently detected in the same sample, and the naturally occurring level of DON is generally higher than that of NIV[5]. However, cases in which the level of NIV is higher than that of DON are often observed in Japanese wheat. A provisional tolerable level of DON in unpolished wheat has been set at 1.1 mg/kg in Japan. A tolerable level of NIV may also be set in Japan. This is because the LOAEL of NIV is lower than that of DON, and the actual level of NIV is often higher than that of DON in Japan. Therefore, to quantify these mycotoxins reliably and rapidly, an analytical method for simultaneous measurement of the concentrations of DON and NIV is required.

Many methods for simultaneous analysis of DON and NIV have been reported. The analytical instrument most frequently used to determine concentrations of these trichothecenes is GC with electron capture detection (ECD) or with MS detection[6)-9)]. However, the GC method requires a derivatization procedure. This procedure is generally time-consuming and leads to poor recovery. On the other hand, LC methods using an UV or MS detector, which requires no derivatization procedure, have also been reported[9)-12)]. In recent years, many LC-MS and LC-MS/MS methods have been reported for the simultaneous analysis of trichothecene mycotoxins, including DON and NIV. These methods have been applied to hygiene control and the surveillance of these mycotoxins. However, these LC-MS methods require the use of expensive internal standards such as isotopically substituted compounds. Meanwhile, the LC-UV method offers high precision, although the sensitivity of the method is lower than that of the LC-MS method.

We have previously reported the results of an interlaboratory study of an analytical method for measuring the concentration of DON in wheat by LC-UV coupled with a multifunctional column for cleanup[13)]. In the present study, based on the analytical method evaluated in the previous study, we evaluated the LC-UV and LC-MS(/MS) methods for simultaneous analysis of DON and NIV concentrations in wheat through an interlaboratory study.

Materials and Methods

Standard and reagents

DON and NIV standards were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A 5-mg portion of DON or NIV was accurately weighed and placed in a 50-mL volumetric flask, dissolved in acetonitrile as a stock solution (*ca.* 100 μg/mL), and stored at −20℃. Portions of the two stock solutions were mixed and diluted with acetonitrile to make a mixed standard solution (20 μg/mL each of DON and NIV) and three concentrations of mixed spiking solutions (5, 25, and 50 μg/mL of DON and NIV). Acetonitrile and methanol for LC were LC grade, and acetonitrile for extraction was reagent grade. The other reagents were of the highest analytical grade available. The Autoprep MF-T 1500 (Showa Denko K. K., Tokyo, Japan) was used as a multifunctional column.

Preparation of samples

Two samples of wheat, one of which was naturally contaminated with DON and NIV and the other of which was not contaminated, were donated by Dr. T. Nakajima. These wheat samples were ground until all particles could pass through a mesh of 1 mm. After mixing and homogenizing in a V-style mixer (Ikemoto Scientific Technology Co., Ltd., Tokyo, Japan), these wheat samples were packed into Stomacher bags containing approximately 30 g each. Six bags each out of 40 bags of naturally contaminated wheat and 200 bags of non-contaminated wheat were chosen at random, and the homogeneity was assayed by the LC-MS/MS method detailed in this report. Each laboratory in the interlaboratory study received the following: (a) six non-contaminated samples for the spike test of wheat found to be free of DON and NIV (<0.005 mg/kg); (b) four random-numbered samples, two of which were naturally contaminated and the others of which were the same as the samples for the spike test; (c) a mixed standard solution; and (d) three mixed spiking solutions, regarding which the laboratory, had no knowledge of the DON and NIV concentrations.

Fortification procedure

For evaluating recoveries, 500 μL of mixed spiking solution was added to 25.0 g of non-contaminated sample in a 200-mL flask (final concentration, 0.1, 0.5, or 1 mg/kg) and kept at room temperature in the dark. After 1 h,

*1 Scientific Committee on Food, Opinion of the Scientific Committee on Food on Fusarium toxins, Part 4: Nivalenol (SCF/CS/CNTM/MYC/26), 2000. http://ec.europa.eu/food/fs/sc/scf/out74_en.pdf

DON and NIV were extracted from each spiked sample and quantified according to the protocol.

Protocol used

The method used in this study was based on the method described in our previous report[13]. Briefly, 25.0 g of sample was extracted with 100 mL of acetonitrile–water (85 : 15) without shaking for 10 min, followed by shaking for 30 min. The extract was transferred to a 50-mL centrifuge tube and centrifuged at 1,500×*g* for 5 min. Aliquots of 10 to 20 mL of the supernatant solution were applied to a multifunctional cleanup column (Autoprep MF-T 1500) without pre-conditioning. The first 4 mL of eluate was discarded, and the next 4 mL was collected. The collected eluate was divided into two vials; 2.0 mL for LC-UV and 1.0 mL for LC-MS or LC-MS/MS. The divided eluates were dried under nitrogen at approximately 45℃. Each residue was re-dissolved in 1.0 mL of water–methanol–acetonitrile (90 : 5 : 5) for LC-UV or 10 mmol/L aqueous ammonium acetate solution–methanol (90 : 10) for LC-MS or LC-MS/MS. Each solution was filtered through a membrane filter (0.45 μm) and the filtrate was analyzed by LC-UV and LC-MS (/MS).

LC-UV conditions

The final solution for LC-UV was loaded onto an octadecylsilane (ODS) column (250 by 4.6 mm i.d., 3–5 μm) at 40℃. The mobile phase was water–methanol–acetonitrile (90 : 5 : 5), and the flow rate was set at 0.6–1.0 mL/min. The UV detector was set at a wavelength of 220 nm. The injection volume was set by each laboratory so that the height of the signal peak of 0.05 μg/mL standard solution (equivalent to 0.1 mg/kg in sample) was more than 10 times larger than that of the background noise.

LC-MS or LC-MS/MS conditions

Each laboratory determined the DON and NIV concentrations by LC-MS or LC-MS/MS. Five to twenty microliters of the final solution for LC-MS or LC-MS/MS was loaded onto an ODS column (150 by 2.1 mm i.d., 3–5 μm) at 40℃. The mobile phase was a binary gradient of 10 mmol/L aqueous ammonium acetate solution and methanol, and the flow rate was set at 0.2 mL/min. Electrospray ionization in the negative mode was used for ionization in the MS detector. The selected ion monitoring mode of LC-MS or the selected reaction monitoring mode of LC-MS/MS was selected by each laboratory. All other conditions were set by each laboratory so that the height of the signal peak of 0.025 μg/mL standard solution (equivalent to 0.1 mg/kg in sample) was more than 10 times larger than that of the background noise.

Calibration curve

To prepare standard solutions for calibration, mixed standard solution was diluted with water–methanol–acetonitrile (90 : 5 : 5) for LC-UV or with 10 mmol/L aqueous ammonium acetate solution–methanol (90 : 10) for LC-MS or LC-MS/MS. The concentration of DON or NIV and the peak signal (area or height) were plotted for five standard solutions with different concentrations. The concentration of DON or NIV in the sample solution was calculated from this calibration curve.

Interlaboratory study design

For evaluation of the methods, an interlaboratory study was carried out using pairs of five materials (three spiked samples of wheat, a naturally contaminated sample, and a blank sample). The interlaboratory study involved 10 laboratories in Japan and one laboratory in Korea.

Statistics

The homogeneities of DON and NIV in the naturally contaminated material supplied in this study were tested using one-way analysis of variance (ANOVA) and *F*-test. The results from laboratories have previously been examined for evidence of outliers using statistical Cochran (between duplicates) and Grubbs single and Grubbs pair value tests (between laboratory means)[14]. The relative standard deviations for repeatability (RSDr) and reproducibility (RSD$_R$), and the HorRat value, which is the ratio of the RSD$_R$ to the predicted RSD$_R$, were obtained using one-way ANOVA according to the AOAC guidelines[15]. However, the predicted RSD$_R$ of the HorRat value was calculated according to the report of Thompson[16].

Results and Discussion

The *F*-test at the 95% confidence level showed that each of the naturally contaminated samples could be regarded as homogeneous, because the calculated *F*-value was less than the critical *F*-value. No laboratories detected any DON or NIV in the blank. For both LC-UV and LC-MS(/MS) methods, 11 laboratories reported results, and there were no outliers. Laboratory B reported the result of 0.1 mg/kg spiked wheat as "trace" because the ratio of the signal peak obtained from the spiked test to the background noise was less than 10.

The results of the interlaboratory study are shown in Table 1 (DON) and Table 2 (NIV). The Commission of European Communities has laid down criteria for analytical methods used for the official control of the levels of mycotoxins in foodstuffs[17]. According to the criteria, the recovery, RSDr and RSD$_R$ for 0.1–0.5 mg/kg of DON level are required to be in the ranges of 60–110%, ≤ 20% and ≤ 40%, respectively; for >0.5 mg/kg, they are required to be in the ranges of 70–120%, ≤ 20% and ≤ 40%, respectively. All the results for DON in this study were acceptable. Although criteria for NIV were not given, all results for NIV were also acceptable according to the DON criteria. As for the HorRat value, a range of 0.5–1.5 was acceptable according to AOAC International[15]. Although some HorRat values were less than 0.5, no problems were recognized throughout this interlaboratory study. Therefore, the results obtained from both LC-UV and LC-MS(/MS) methods satisfied

Table 1. Results of the interlaboratory study on the determination of deoxynivalenol by LC-UV and LC-MS(/MS) in wheat

Laboratory	Spiked (mg/kg)												Naturally contaminated (mg/kg)			
	0.1				0.5				1							
	UV		MS		UV		MS		UV		MS		UV		MS	
A	0.10	0.09	0.11	0.10	0.49	0.48	0.49	0.48	0.97	0.96	0.96	0.98	0.97	0.99	0.93	0.97
B	trace	trace	trace	trace	0.47	0.45	0.54	0.57	0.89	0.89	0.91	0.93	1.12	1.07	1.35	1.26
C	0.10	0.12	0.09	0.09	0.39	0.51	0.40	0.39	0.80	0.81	0.76	0.78	1.00	0.93	0.85	0.86
D	0.05	0.05	0.12	0.11	0.46	0.47	0.53	0.59	0.97	0.93	1.08	1.04	1.13	1.15	1.31	1.29
E	0.09	0.08	0.08	0.07	0.53	0.53	0.51	0.51	1.08	1.09	1.02	1.00	1.09	1.08	1.09	1.02
F	0.076	0.080	0.105	0.097	0.446	0.459	0.537	0.584	0.915	0.910	1.030	1.010	0.891	0.961	1.028	1.092
G	0.08	0.09	0.13	0.12	0.47	0.47	0.50	0.50	0.92	0.92	0.91	0.91	0.96	0.98	0.96	0.97
H	0.09	0.08	0.09	0.09	0.45	0.45	0.45	0.46	0.91	0.91	0.91	0.92	0.77	0.93	0.77	0.94
I	0.13	0.14	0.12	0.10	0.53	0.51	0.47	0.47	0.94	0.93	0.89	0.93	1.00	1.01	0.96	0.98
J	0.09	0.09	0.11	0.10	0.46	0.47	0.45	0.42	0.95	0.94	0.89	0.86	0.90	0.86	0.89	0.86
K	0.08	0.08	0.14	0.13	0.43	0.43	0.64	0.63	0.95	0.95	1.10	1.38	1.01	1.03	0.90	1.09
No. of labs	10		10		11		11		11		11		11		11	
Mean (mg/kg)	0.09		0.11		0.47		0.51		0.93		0.96		0.99		1.02	
Recovery (%)	90		110		94		101		93		96		—		—	
RSDr (%)	7.6		6.9		5.7		3.8		1.1		6.4		4.4		6.1	
RSDR (%)	25.2		17.5		7.6		10.8		7.2		13.4		9.6		16.1	
HorRat	1.1		0.8		0.4		0.6		0.4		0.8		0.6		1.0	

trace: The ratio of the signal peak obtained from the spiked sample to the background noise was less than 10.
No. of labs: Number of laboratories included in statistical analyses
RSDr: Relative standard deviations for repeatability
RSDR: Relative standard deviations for reproducibility

Table 2. Results of the interlaboratory study on the determination of nivalenol by LC-UV and LC-MS(/MS) in wheat

Laboratory	Spiked (mg/kg)												Naturally contaminated (mg/kg)			
	0.1				0.5				1							
	UV		MS		UV		MS		UV		MS		UV		MS	
A	0.06	0.07	0.09	0.08	0.45	0.40	0.43	0.41	0.76	0.76	0.83	0.83	1.13	1.13	1.11	1.17
B	trace	trace	trace	trace	0.44	0.42	0.48	0.49	0.82	0.73	0.82	0.80	1.31	1.33	1.50	1.42
C	0.08	0.09	0.10	0.10	0.34	0.33	0.34	0.33	0.64	0.68	0.62	0.63	1.01	1.07	0.94	0.98
D	0.03	0.03	0.09	0.09	0.34	0.34	0.46	0.48	0.75	0.65	0.89	0.88	1.07	1.13	1.43	1.48
E	0.08	0.08	0.07	0.08	0.42	0.41	0.43	0.42	0.81	0.83	0.84	0.83	1.19	1.19	1.21	1.22
F	0.067	0.073	0.072	0.084	0.353	0.356	0.402	0.408	0.746	0.735	0.821	0.849	1.031	1.220	1.132	1.186
G	0.07	0.08	0.08	0.08	0.38	0.38	0.40	0.41	0.75	0.77	0.79	0.79	0.95	1.15	1.13	1.14
H	0.06	0.05	0.07	0.08	0.38	0.37	0.38	0.39	0.75	0.75	0.77	0.77	1.14	1.11	0.94	1.13
I	0.09	0.10	0.07	0.06	0.39	0.40	0.40	0.44	0.80	0.80	0.80	0.82	1.12	1.04	1.15	1.15
J	0.12	0.10	0.12	0.11	0.42	0.45	0.37	0.39	0.76	0.83	0.84	0.78	1.16	1.09	1.10	1.00
K	0.09	0.11	0.07	0.07	0.42	0.41	0.40	0.43	0.81	0.80	0.82	0.82	1.24	1.23	0.98	1.07
No. of labs	10		10		11		11		11		11		11		11	
Mean (mg/kg)	0.08		0.08		0.39		0.41		0.76		0.80		1.14		1.16	
Recovery (%)	80		80		78		83		76		80		—		—	
RSDr (%)	10.7		6.8		3.6		3.4		4.5		2.0		5.8		4.9	
RSDr (%)	31.4		18.6		9.6		10.2		7.0		8.3		8.3		14.5	
HorRat	1.4		0.8		0.5		0.6		0.4		0.5		0.5		0.9	

trace: The ratio of the signal peak obtained from the spiked sample to the background noise was less than 10.
No. of labs: Number of laboratories included in statistical analyses
RSDr: Relative standard deviations for repeatability
RSDR: Relative standard deviations for reproducibility

the requirements for use as an official method. For determinations using MS, internal standards are frequently used to correct for purification losses and ionization efficiency. In this study, MS detection gave acceptable results for both DON and NIV even without the use of internal standards.

We have previously reported the results of an interlaboratory study of the determination of DON concentrations in wheat by LC-UV coupled with a multifunctional column[13]. In that study, the RSDr ranged from 5.8% to 11.3%, and the RSDR ranged from 12.0% to 20.7%. The HorRat value was below 1.0, and the recovery was

100%. Trucksess et al. have reported the results of an interlaboratory study for the determination of DON concentrations in white flour, wheat flour and bran by LC-UV coupled with a multifunctional column[18]. In their study, the RSDr ranged from 3.1% to 21.7%, and the RSDR ranged from 10.8% to 38.7%. The recovery ranged from 80% to 115%. McDonald et al. have reported the results of an interlaboratory study of the determination of DON concentrations in cereals and cereal products by LC-UV with an immunoaffinity column cleanup step[19]. In that study, the RSDr ranged from 3.1% to 14.1%, and the RSDR ranged from 11.5% to 26.3%. The HorRat value was below 1.3, and the recovery ranged from 74% to 87%. As compared with those results, the results of the UV and MS detection methods presented in this report are similar for both DON and NIV.

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Comparison of murine anorectic responses to the 8-ketotrichothecenes 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X and nivalenol

Wenda Wu [a,b], Brenna M. Flannery [b,c], Yoshiko Sugita-Konishi [e], Maiko Watanabe [e], Haibin Zhang [a], James J. Pestka [b,c,d,*]

[a] College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, PR China
[b] Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824-1224, USA
[c] Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824-1224, USA
[d] Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824-1224, USA
[e] Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

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Nivalenol

ABSTRACT

While induction of food refusal by the trichothecene mycotoxin deoxynivalenol (DON) has been described in several animal models, much less is known about the anorectic effects of structurally related 8-ketotrichothecenes, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenon X (FX) and nivalenol (NIV). Here, we compared the capacities of these congeners to induce anorexia in the mouse. As previously observed for DON, anorectic responses to 3-ADONand 15-ADON in the B6C3F1 female mouse following both intraperitoneal (IP) and oral exposure were transient, lasting only a few hours, with food intake recovering to control levels within 16 h. For both ADONs, the no observed adverse effect levels (NOAEL) and lowest observed adverse effect levels (LOAEL) were 0.5 and 1 mg/kg bw following IP exposure, respectively, and 1 and 2.5 mg/kg bw after oral exposure, respectively. In contrast, food refusal persisted from 48 to 96 h following IP and oral exposure to FX and NIV. For both IP and oral FX exposure, the NOAEL was 0.025 mg/kg bw and LOAEL was 0.25 mg/kg bw, whereas the NOAELs and LOAELs for NIV were 0.01 and 0.1 mg/kg bw, respectively, after IP exposure and 0.1 and 1 mg/kg bw, respectively, following oral exposure. Both these data and a prior DON study suggest that anorectic responses to 8-ketotrichothecenes were always greater when administered IP as compared to oral exposure and follow an approximate rank order of NIV > FX > DON ≈ 3-ADON ≈ 15-ADON for IP exposure and FX > NIV > DON ≈ 3-ADON ≈ 15-ADON for oral exposure. Toxic potency data such as is described here will be applicable to future comparative risk assessments for this important group of trichothecene mycotoxins.

1. Introduction

The 8-ketotrichothecenes, toxic sesquiterpenoid mycotoxins produced by *Fusarium graminearum* and *Fusarium culmorum*, frequently contaminate cereal staples such as wheat, barley and corn (Desjardins et al., 2000; Su, 1998; Szecsi et al., 2005). Sometimes referred to as Type B trichothecenes, these mycotoxins have been associated with a spectrum of toxic effects in experimental animals that include anorexia, growth retardation, nausea, emesis, neuroendocrine changes and immunosuppression (Pestka, 2010b). Three 8-ketotrichothecene chemotypes have been proposed for *Fusarium* sp. based on the primary production of: (1) deoxynivalenol (DON) and its acetylated precursor, 3-acetyldeoxynivalenol (3-ADON), (2) DON and 15-acetyldeoxynivalenol (15-ADON), and (3) nivalenol (NIV) and its acetylated precursor, 4-acetylnivalenol (fusarenon X; FX) (Miller et al., 1991; Pestka, 2010b; Ward et al., 2002) (Fig. 1). Regional studies have demonstrated that clear geographic differences exist among these *Fusarium* chemotypes (Starkey et al., 2007; Sugita-Konishi and Nakajima, 2010).

Incidence of *Fusarium* head blight (FHB) in cereal grains has increased throughout the world, possibly because of changes in climate and agricultural practices and this has corresponded with elevated contamination by DON and other 8-ketotrichothecenes (Pestka, 2010b). Since these trichothecenes survive milling and processing (Jackson and Bullerman, 1999), they can enter into foods and potentially adversely affect human and animal health. Regulatory standards have been established worldwide for DON,

Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; FX, fusarenon X; NIV, nivalenol.

* Corresponding author at: Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824-1224, USA. Tel.: +1 517 353 1709; fax: +1 517 353 8963.
E-mail address: pestka@anr.msu.edu (J.J. Pestka).

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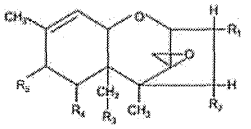

Trichothecene	Abbreviation	R1	R2	R3	R4	R5
Deoxynivalenol	DON	OH	H	OH	OH	=O
3-Acetyldeoxynivalenol	3-ADON	OAc	H	OH	OH	=O
15-Acetyldeoxynivalenol	15-ADON	OH	H	OAc	OH	=O
Nivalenol	NIV	OH	OH	OH	OH	=O
Fusarenon X	FX	OH	OAc	OH	OH	=O

Fig. 1. Structures of 8-ketotrichothecenes.

but not for other 8-ketotrichothecenes. Induction of food refusal and growth retardation by DON has been of particular concern from the perspective of children's health and has been a specific focus for establishing regulations in many countries. The mechanisms for DON-induced anorexia are not fully understood but likely result from induction of neuroendocrine hormones and pro-inflammatory cytokines that impact appetite, food intake and energy balance (Amuzie et al., 2009; Flannery et al., 2011; Girardet et al., 2011).

Our lab recently developed a simple mouse bioassay for anorexia induction in an effort to better understand the mechanistic basis for toxic effects resulting from acute and chronic trichothecene exposure (Flannery et al., 2011). In this model, DON was observed to dose-dependently induce food refusal within 2 h of exposure when administered either by intraperitoneal injection (IP) or by oral gavage with doses as low as 1 and 2.5 mg/kg bw for inducing food refusal, respectively. Given that 3-ADON, 15-ADON, FX and NIV also contaminate cereal-based foods, knowledge of their anorectic potencies relative to DON is an important consideration for assessing the hazards and risks of consuming *Fusarium*-infected grains.

The objective of this investigation was to compare effects of these 8-ketotrichothecenes on food intake using the above-described mouse bioassay. Two methods of exposure, intraperitoneal (IP) and oral gavage, were used to facilitate comparisons with prior acute studies of these toxins which have employed a variety of exposure methods. The results presented herein indicate that (1) similar to DON, the anorectic effects of 3-ADON and 15-ADON were transient, with mice refusing food within 2 h of exposure but then exhibiting a compensatory food intake increase over the next 14 h, (2) FX and NIV were more toxic, inducing much longer, persistent anorectic effects as compared to DON or the ADONs, and (3) IP exposures, in general, provoked stronger anorectic responses than did oral exposures.

2. Materials and methods

2.1. Animals and toxins

Female B6C3F1 mice were obtained from Charles River Breeding (Portage, MI) at age of 10–12 weeks and housed individually in polycarbonate cages in a room maintained at 21–24 °C and 40–55% relative humidity under a 12 h light (6:00–18:00 h)/dark (18:00–6:00 h) cycle. All guidelines for animal treatment were followed according to National Institutes of Health as supervised by the University Committee on Animal Use and Care at Michigan State University (Approval No. 01/08-007-00). High fat diet (Research Diets Inc., New Brunswick, NJ) put in 2" high glass jars was employed for feeding bioassay and sifted aspen chips used for bedding as described previously (Flannery et al., 2011). 3-ADON was purified, identified by NMR and supplied by Dr. Tony Durst (University of Ottawa, Canada) and 15-ADON was produced from culture and identified by high performance liquid chromatography as described previously (Pestka et al., 1986). Purities of 3-ADON and 15-ADON were verified to be >99% by elemental analysis. FX and NIV were isolated from cultures and purities (>98%) validated by LC–MS as previously described (Takahashi et al., 2008).

2.2. Experimental design

The general experimental design for this study, based on preliminary experiments, is summarized in Fig. 2. All procedures were consistently and rapidly performed to minimize stress to the animals. To reduce the number of animals required for this study, mice treated IP with an individual toxin, were held for a minimum 1 week washout period after cessation of the experiment, randomized and then used for the subsequent oral challenge study for the same toxin. The validity of this approach was based on our prior study that mice given a 1 week washout period responded identically to DON with no evident attenuation or potentiation of anorectic response (Flannery et al., 2011). Briefly, 1 d prior to the experiment, mice were randomly divided into different groups according to body weight and fresh sifted aspen bedding included in cages. On the day of experiment, mice were fasted from 10:00 h to 18:00 h and water provided ad lib. Toxins were dissolved in phosphate buffered saline (PBS) and administered to mice in 100 μl volumes by IP injection with a sterile 27 G, 0.5 in. needle or by oral gavage using a sterile 22 G, 1.5 in. disposable feeding tube (Instech Solomon; Plymouth Meeting, PA). Dose selection was based on our previously published mouse anorexia bioassay for DON (Flannery et al., 2011) as well as preliminary range finding studies for the various 8-ketotrichothecenes. Food intake was monitored for varying lengths of time depending on the duration of anorexia observed in preliminary experiments.

For 3-ADON, groups of mice ($n = 8$) were dosed with 0, 0.1, 0.5, 1 and 2.5 mg/kg bw 3-ADON in 100 μl PBS by IP injection or 0, 0.25, 1, 2.5 and 5 mg/kg bw 3-ADON in 100 μl PBS by gavage. Mice were then immediately provided two pre-weighed food pellets (≈7 g) and allowed to eat for 2 h in the dark. After 2 h, food pellets and shredded food were removed, weighed and then food was put back to the food jar immediately. On the second day at 10:00 h (16 h after first exposure), remaining food was measured again. The approach for 15-ADON was identical to 3-ADON, except that mice were given 0, 0.1, 0.5, 1 and 2.5 mg/kg bw by IP injection and 0, 0.5, 1, 2.5 and 5 mg/kg bw of toxin by gavage.

For FX, groups of mice ($n = 8$) were given 0, 0.025, 0.25, 1 and 2.5 mg/kg bw of the toxin in 100 μl PBS by IP or oral exposure. Mice were then provided four pre-weighed food pellets (≈14 g), and food intakes were measured up to 48 h following IP injection and up to 60 h after gavage (Fig. 2). For NIV, groups of mice ($n = 8$) were given 0, 0.01, 0.1, 0.5 and 2.5 mg/kg bw NIV in 100 μl PBS by IP injection or 0, 0.01, 0.1, 1 and 5 mg/kg bw of the toxin in 100 μl PBS by oral exposure. Mice were fed six preweighed food pellets (≈21 g) and food intake measured over 96 h.

2.3. Statistics

Data were analyzed using SigmaPlot 11 for Windows (Jandel Scientific; San Rafael, CA). Statistical methods were implemented as described in figure legends. Significant differences were established at $p < 0.05$.

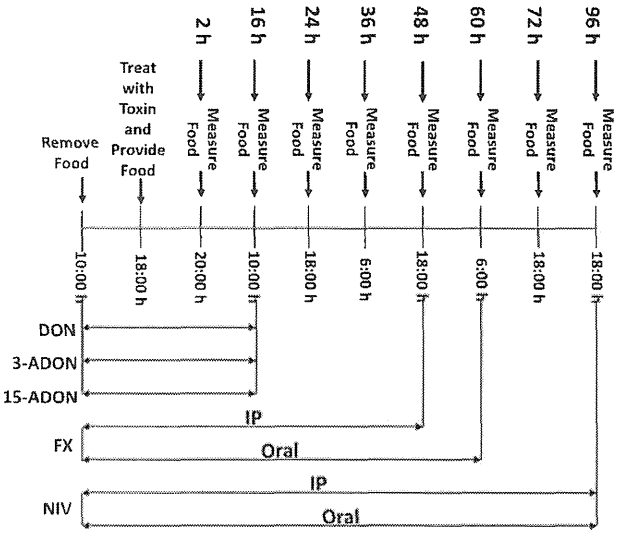

Fig. 2. Experimental design for mouse feed refusal assay. Mice were fasted from 10:00 to 18:00 h on day 1. At 18:00 h mice were treated with toxin or vehicle and then immediately provided with pre-weighed food. For 3-ADON and 15-ADON, food was measured at 20:00 h and again at 10:00 h on day 2 as described previously for DON (Flannery et al., 2011). For FX, food was measured at 20:00 h on day 1, 10:00 and 18:00 h on day 2, 6:00 and 18:00 h on day 3, and 6:00 h on day 4. For NIV, food was measured at 20:00 h on day 1, 10:00 and 18:00 h on day 2, 6:00 and 18:00 h on day 3, 6:00 and 18:00 h on day 4, and 18:00 h on day 5.

3. Results

3.1. 3-ADON exposure causes rapid and transient feed refusal

Following IP administration with 3-ADON, marked reductions in food intake were observed after 2 h at 0.5 (26%), 1 (38%) and 2.5 (93%) mg/kg bw whereas 0.1 mg/kg bw had no effect (Fig. 3). Between 2 and 16 h, mice dosed with 1 and 2.5 mg/kg bw 3-ADON doses consumed 6% and 16% more than the controls, respectively. When cumulative food intakes were compared over 16 h, no differences were observed between control and 3-ADON exposure groups, indicating that the mice compensated for the initial feed refusal by subsequently consuming more food.

Following oral administration with 3-ADON, reductions in food intake were observed at 2 h for mice dosed with 1.0 (18%), 2.5 (66%) and 5 (83%) mg/kg bw with a comparable trend at 0.5 mg/kg bw, while 0.25 mg/kg bw was without effect (Fig. 3). Conversely, in the 14 h following the initial food intake measurement, the 2.5 and 5 mg/kg bw 3-ADON groups exhibited significant increases in food ingestion with both groups, consuming 30% more than control mice. Again, when food intakes were compared over the entire 16 h period, no differences were observed between control and 3-ADON groups.

3.2. 15-ADON exposure also induces rapid and transient feed refusal

When the effects of IP exposure to 15-ADON were assessed at 2 h, treatment with 0.5, 1 and 2.5 mg/kg bw 15-ADON were observed to reduce food intake in 2 h by 15%, 43% and 90% compared to control, respectively, while the 0.1 mg/kg bw doses had no effect (Fig. 4). However, the 1 and 2.5 mg/kg bw treatment groups ate 8% and 16% more than controls, respectively, in the subsequent 14 h. After 16 h exposure to 15-ADON, differences in cumulative food intakes were not detectable among the groups.

Upon oral dosing with 2.5 and 5 mg/kg bw 15-ADON, food intakes were reduced at 2 h by 38% and 60%, respectively, compared to the control, but were not affected by the 0.5 and 1 mg/kg bw of the toxin (Fig. 4). Between 2 and 16 h, the 2.5 and 5 mg/kg bw 15-ADON dose groups consumed 4% and 12% more than the control

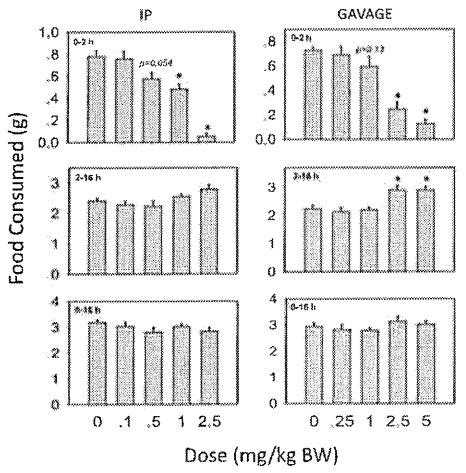

Fig. 3. Exposure to 3-ADON rapidly induces an anorectic response followed by a compensatory food intake increase in the mouse. Mice were given an IP injection or oral gavage of 3-ADON immediately before the dark cycle. Food intake was measured at 2 h and 16 h post administration time and graphically portrayed. Data are mean ± SEM (n = 8/gp).One-way ANOVA using Dunnett's Test was used to assess significant differences in food intake between doses and the control. Asterisks indicate statistically significant differences in food consumption as compared to the control ($p < 0.05$).

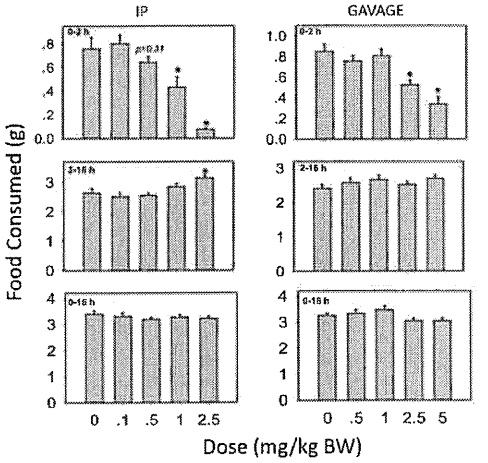

Fig. 4. Exposure to 15-ADON rapidly induces an anorectic response followed by a compensatory food intake increase in the mouse. Mice were given an IP injection or oral gavage of 15-ADON immediately before the dark cycle. Food intake was measured at 2 and 16 h post administration time and graphically shown. Data are mean ± SEM (n = 8/gp) and were analyzed as described in Fig. 3 legend.

mice, respectively. As with the IP study, no differences between control and 15-ADON exposure groups were found in cumulative food intakes after 16 h.

3.3. FX exposure causes prolonged feed refusal

Food intake measurements in mice exposed IP to FX indicated that the 0.25, 1 and 2.5 mg/kg bw doses evoked 28%, 46% and 55% reductions in food intake at 2 h compared to controls, respectively, while the 0.025 mg/kg bw dose had no effect (Fig. 5A). Between 2 and 16 h, the 0.25 and 1 mg/kg bw dose group mice consumed 20% and 19% more than the controls, suggesting that like the ADONs, compensation occurred following the initial suppression of food consumption. In contrast, the cumulative food intakes in the 2.5 mg/kg bw group at 16, 24 and 36 h were significantly less than control (Fig. 5B). The rate of food consumption from 16 to 48 h was generally higher than controls with total food intake recovering by 48 h, thus suggesting compensation eventually occurred at this higher dose.

Following oral FX exposure, 0.025, 0.25, 1 and 2.5 mg/kg bw doses caused 13%, 27%, 40% and 53% reduction in food intake at 2 h, respectively, compared to the control, (Fig. 5A). In the subsequent 14 h, increased food intake at 0.25 and 1 mg/kg bw doses compensated for the initial feed refusal, whereas the 2.5 mg/kg bw group still consumed 34% less than the control. Consumption rates at 16–24 h, 24–36 h, and 36–48 h by the 2.5 mg/kg bw group were similar to that of the control; however, cumulative food intakes at 24, 36 and 48 h for these groups were still significantly less than the control, respectively (Fig. 5C). By 60 h, the 2.5 mg/kg bw group compensated for initial feed refusal, and cumulative food consumption was the same as the control group.

3.4. NIV exposure also evokes prolonged feed refusal

IP exposure of mice to 0.1, 0.5 and 2.5 mg/kg bw doses NIV caused a 13%, 55% and 72% reduction in food intake at 2 h compared to the control, respectively, while treatment with 0.01 mg/kg bw had no effect (Fig. 6A). In the following 14 h, the 0.5 and 2.5 mg/kg bw dose groups did not compensate for the initial feed refusal, consuming 21% and 45% less than control, respectively. From 16 to 72 h, there was a general trend toward reduced food intake exposed in NIV groups (Fig. 6B). Although food consumption

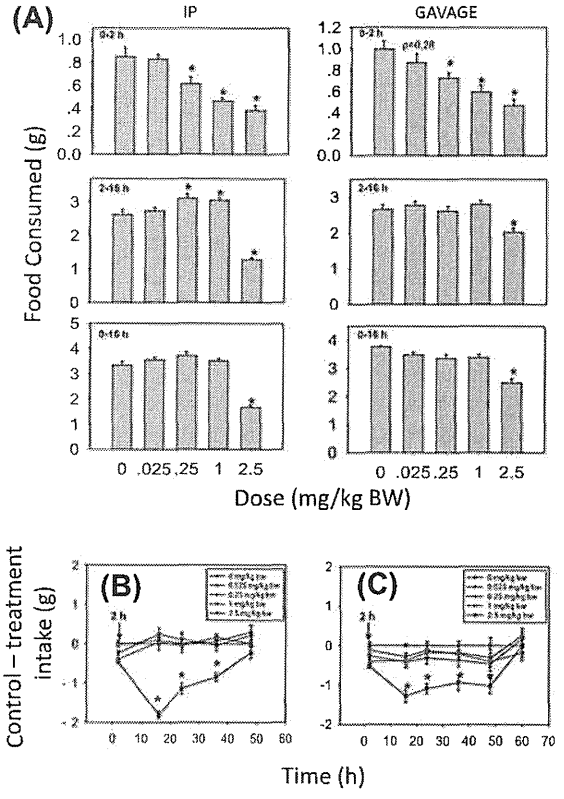

Fig. 5. FX induces prolonged anorectic responses. (A) Short term (0–16 h) feed refusal induced by intraperitoneal and oral FX exposure. Data are mean ± SEM (n = 8/gp) and were analyzed as described in Fig. 3 legend. (B) Intraperitoneal FX exposure induced a 48 h anorectic response followed by a compensatory food intake increase. Mice were given an IP injection of FX immediately before the dark cycle and food intake was measured over 48 h post and graphically depicted. Data are mean ± SEM (n = 8/gp). Two-way repeated measures ANOVA (one factor) using Bonferroni t-test was used to assess significant differences in food intake between doses and the control. (C) Oral FX exposure induces a 60 h anorectic response followed by a delayed compensatory food intake increase. Mice were orally gavaged with FX immediately before the dark cycle. Food intake was measured at 2, 16, 24, 36, 48 and 60 h post gavage time and graphically depicted. Data are mean ± SEM (n = 8/gp) and were analyzed as described in Fig. 5B legend.

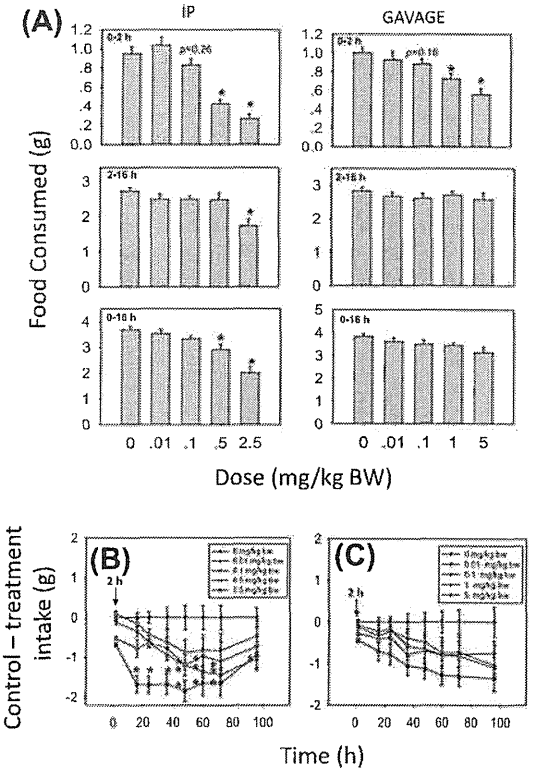

Fig. 6. NIV induces prolonged anorectic responses. (A) Short term (0–16 h) feed refusal effect induced by intraperitoneal and oral NIV exposure. Data are mean ± SEM (n = 8/gp) and were analyzed as described in Fig. 3 legend. (B) Intraperitoneal NIV exposure induced a prolonged anorectic response. Mice were given an IP injection of NIV immediately before the dark cycle. Food intake was measured at indicated time post injection time and graphically depicted. Data are mean ± SEM (n = 8/gp) and were analyzed as described in Fig. 5B legend. (C) Oral NIV exposure in induced a prolonged response. Mice were orally gavaged with NIV immediately before the dark cycle. Food intake was measured at intervals indicated time post gavage time and graphically depicted. Data are mean ± SEM (n = 8/gp) and were analyzed as described in Fig. 5B legend. Though a marked trend toward reduced food intake was seen with NIV treatment, at no point was any dose found to be statistically significant.

rates in mice administered 2.5 mg/kg bw dose at 16–24, 24–36, 36–48, 48–60 and 60–72 h were similar to that of the control, total food intake at 24, 36, 48, 60 and 72 h were 36%, 23%, 21%, 15% and 12% less than the control, respectively. A trend toward increasing food intake was observed in NIV exposure groups from 72 to 96 h; however, by 96 h these groups still did not fully compensate for initial feed refusal.

Mice orally exposed to NIV at 0.1, 1 and 5 mg/kg bw consumed 12%, 28% and 44% less food after 2 h compared to the control, respectively, while 0.01 mg/kg bw had no effect (Fig. 6A). From 16 to 96 h, there was a general trend toward reduced food consumption in NIV exposure groups, but these effects were not significant (Fig. 6C).

4. Discussion

Like DON, other members of the 8-ketotrichothecene mycotoxin family have the potential to cause toxicity in humans and animals, and therefore require scrutiny when considering the risks of consuming *Fusarium*-contaminated foods. Since DON's ability to induce anorexia and growth retardation is considered to be critical toxic effect, a mouse-based feed refusal bioassay was employed here to compare anorectic responses of the 8-ketotrichothecene family members following IP and oral administration. In a prior study, we demonstrated that DON dose-dependently induces anorexia within 2 h following IP injection or oral gavage, and that this was followed by a dose-dependent compensatory food intake increase (Flannery et al., 2011). Likewise, mice exposed here to 3-ADON and 15-ADON exhibited short-term anorexia followed by an increase in the rate of food intake. While comparable effects were observed in mice exposed to low doses of FX (0.25–1 mg/kg bw) and NIV (0.5–1 mg/kg bw), exposure to ⩾2.5 mg/kg bw of these toxins caused a much more persistent anorexia with a markedly delayed recovery.

The ADONs are identical to DON with the exception of being acetylated at the 3- or 15-positions (Fig. 1). As described here, both ADONs induced feed refusal comparable to DON, as evidenced by immediate anorexia in 2 h and a subsequent increased rate of food intake during the next 16 h. The results presented here are consistent with previous studies demonstrating that, like DON, the ADONs induce anorexia. For example, 3-ADON at 0.15 mg/kg bw via diet induces 50% feed refusal in rats (Abbas et al., 1986; Yoshizawa et al., 1978), which equates approximately with DON's feed refusal effects in this species (Yoshizawa et al., 1977). Both DON and conjugated DON metabolites are detectable in plasma