Natural occurrence of type B trichothecenes in formula feeds and feedstuffs in Japan

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Summary

The natural occurrence of type B trichothecenes (deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenone-X (F-X)) was investigated in formula feeds and feedstuffs. Twenty-one formula feed samples and 44 feedstuff samples (maize, milo, barley, wheat, rye, wheat bran, soybean meal, rapeseed meal, and cottonseed) were collected from the Japanese Feed Industry. These samples were analyzed by gas chromatography after purification with a multifunctional cleanup (MFC) column and trimethyl silyl derivatization. DON was detected in formula feed, cereals and bran samples. The concentrations in positive samples were 16–190 µg/kg in formula feeds and 14–2,200 µg/kg in cereals and wheat bran. 15-ADON was detected in formula feed, maize, wheat and wheat bran samples at concentrations of 10–94 µg/kg in positive samples. NIV was detected in the samples of formula feed, milo, barley, rye, wheat and wheat bran samples at concentrations of 26–140 µg/kg in positive samples. 3-ADON and F-X were not detected in any sample. None of the samples of cottonseed, soybean meal and rapeseed meal contained these trichothecenes.

Key words: trichothecene, deoxynivalenol (DON), nivalenol (NIV), multifunctional cleanup (MFC) column, feed

Introduction

Trichothecenes produced by Fusarium fungi are toxicologically significant mycotoxins in food and feed production. They are characterized by the 12,13-epoxy-thichothec-9-ene ring system. They differ from one another in the number and type of functional groups attached to the ring. The type B trichothecenes, such as deoxynivalenol (DON, vomitoxin), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenone-X (F-X, 4-acetylNivalenol), are characterized by a carbonyl function at C-8.

The natural occurrence of DON\textsuperscript{1-10} and NIV\textsuperscript{17,18} has been reported in several countries, and they have been found in cereal around the world. However, there are only a few reports of natural occurrence survey of these metabolites, such as 3-ADON\textsuperscript{13,15,16}, 15-ADON\textsuperscript{15} and F-X\textsuperscript{13,15,16}, and trichothecenes in feed\textsuperscript{9}. Furthermore, there has been no report of natural occurrence survey of these analogues in Japanese feeds.

This paper describes the analytical results of the trichothecenes in 21 formula feed samples and 44 feedstuff samples that circulate in Japan. It was analyzed using the gas chromatographic method\textsuperscript{11}.
Materials and Methods

Survey samples  In 2001~2002, 21 samples of formula feed and 44 samples of feedstuff, each of 500~1000 g, were randomly taken from the Japanese feed industry according to Establishment of Enforcement Outline for Inspection of Feed. Each sample was ground to pass through a 1 mm mesh and thoroughly mixed. The ground samples were stored in closed polyethylene bags in refrigerators until analyzed.

Apparatus  A gas chromatograph (GC) system was composed of a GC model Agilent 6890 (Agilent, Palo Alto, CA, USA) equipped with an electron capture detector (ECD) and an auto-injector. A GC/MS system was composed of a GC model GC-17A and an MS model GCMS QP5050A (Shimadzu, Kyoto, Japan) equipped with an auto-injector. A DB-35 column (0.25 mm I.D. x 30 m, 0.25 µm film thickness, J & W Scientific, Folsom, CA, USA) was used for separation of the trichothecenes.

Chemicals and reagents  The standards of DON, 3-ADON, 15-ADON, NIV and F-X were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N-Trimethylsilylimidazole (TMSI), N,O-bis(trimethylsilyl)acetamide (BSA) and trimethylchlorosilane (TMCS) were purchased from GL Sciences Inc. (Tokyo, Japan). The multifunctional cleanup (MFC) column, which was Autoprep M F-T 1500 purchased from Showa Denko K.K. (Tokyo, Japan) or MultiSep #228 purchased from Romer Labs, Inc. (Union, MO, USA), was used for sample cleanup. All organic solvents were of analytical reagent grades. Water was passed through a Millipore Milli-Q (Bedford, MA, USA) water purifier.

Preparation of standards solution  Each stock standard solution of DON, 3-ADON, 15-ADON, NIV and F-X at 200 µg/ml was prepared with acetonitrile and stored at -20°C. The stock standard solutions of 10 µg equivalency as each trichothecene were added to identical 50 ml round bottom flask, and evaporated to dryness under a stream of nitrogen. The trichothecenes were derivatized in same manner. To prepare the mixed derivatized standard solution at concentrations of 0.01~2 µg/ml as each trichothecene for GC, the upper layer was diluted in accuracy with 2,2,4-trimethylpentane.

Analysis of trichothecenes  The trichothecenes were analyzed according to the method reported in the previous paper and showed the following validation data. The analytical mean recoveries of the trichothecenes from milo, barley and two kinds of formula feed spiked at 0.1 and 1 µg/kg ranged from 85.8 to 105.2 %. In the collaborative study using the trichothecenes spiked milo and naturally contaminated formula feed in eight laboratories by this method, the relative standard deviations (RSD) were within 17.3 %in positive trichothecene. Limits of detection for each trichothecene were 10 µg/kg. This method is briefly explained as follows.

1) Extraction and clean-up  Twenty-five grams of the ground sample was weighed into a 200 ml Erlenmeyer flask and extracted with 100 ml of acetonitrile-water (84:16, v/v) for 60 min on a shaker at 300 per min. Meanwhile, in case of samples as bran, 25 g of the ground samples was weighed into a 300 ml Erlenmeyer flask and extracted with 150 ml of acetonitrile-water (84:16, v/v) for 60 min on a shaker. The extract was centrifuged for 5 min at 760 X g. The supernatant was
cleaned through to a MFC column\textsuperscript{12,13}. After 3 ml of the first elute was removed, 3 ml of the second elute was collected. A 2 ml portion of the collected elute was transferred to a 50 ml round bottom flask, and was evaporated with rotary evaporator on a water bath of 50 °C to exsiccation almost and under a stream of nitrogen gas to dryness. The dry residue was subjected to derivatization.

2) Derivatization \  A 0.1 ml portion of derivatization agent (TMSI-BSA-TMCS (3:3:2, v/v/v))\textsuperscript{13,16} was added to the dry residue. The mixture was shaken for 10 sec on a tube shaker and allowed to react for 15 min at room temperature. To remove derivatization agent, 1 ml of 2,2,4-trimethylpentane and 1 ml of water were added. The mixture was shaken for 5 min on shaker and transferred to 10 ml of test tube. After shaking, layers were allowed to separate. The upper layer was transferred to a vial for GC.

3) GC-ECD analysis \  A 1 µl portion of the solution was injected into GC-ECD in splitless mode under the following conditions: Helium was used as a carrier gas with a flow-rate of 1 ml/min and nitrogen as makeup gas with a flow-rate of 40 ml/min. The temperatures of an injection port and a detector were 250 °C and 300 °C, respectively. The column oven temperature program was: 80 °C held for 1 min, 20 °C/min to 180 °C, 5 °C/min to 300 °C.

4) GC/MS confirmation \  A 1 µl portion of the solution, in which the trichothecene was detected, was injected into GC/MS in splitless mode under the following conditions: The carrier gas, the injection port and the column oven conditions were identical with GC-ECD analysis conditions. The interface temperature was 300 °C. The MS was operated in the electron impact - selected ion monitoring mode with the ionizing voltage at 70 eV. The monitored ions were 422 and 512 m/z for DON, 392 and 467 m/z for 3-ADON, 392 and 407 m/z for 15-ADON, 379 and 510 m/z for 3-NIV and 480 and 450 m/z for F-X.

Results and Discussion

Natural occurrence of trichothecenes in feedstuffs \  The analytical results on the contamination with the trichothecenes in feedstuffs of different origin and in formula feeds for chicken, swine and cattle are summarized in Table 1. DON was detected in cereals and wheat bran samples except two milo samples. DON in wheat and wheat bran was detected at high concentrations, and the concentrations in the three samples exceeded 1000 µg/kg. The concentrations in maize and barley were 110~370 and 24~200 µg/kg, respectively, and the concentrations in rye and milo were at low levels of less than 100 µg/kg. These results were consistent with those reported by Rafai et al.\textsuperscript{7).} DON can be considered as an indicator of other trichothecenes\textsuperscript{11} because the samples negative for DON were confirmed to contain no other trichothecenes.

15-ADON was detected in all of seven maize samples, three of four wheat samples and two of five wheat bran samples. However the concentrations of 15-ADON in cereals were at low levels of less than 100 µg/kg.

NIV was detected in two of five milo samples, two of six barley samples, and one of six rye samples. The concentrations of NIV in milo were 130 and 140 µg/kg in positive samples, and the concentrations in barley, rye, wheat and wheat bran were at low levels of less than 100 µg/kg. The ratios of concentration of NIV to the concentration of DON were 4.4~10 with milo, 0.02~0.15 with
wheat and 0.02 with wheat bran. The results may be considered higher than the incidence and
concentration reported by Rafai et al. 7).

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3-ADON and F-X were not detected in any cereal samples analyzed. These results were
consistent with those reported by Li et al. 5) and Ryu et al. 9).

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None of the samples of cottonseed, soybean meal and rapeseed meal contained any trichothecenes.
These results were different from those reported by Rafai et al. 7).

Natural occurrence of trichothecenes in feeds

Maize, milo and oil seed meal are used
as main feedstuffs of formula feed for chicken and swine. Mean ratios of maize in formula feed for
corn and swine are 52 % and 55 % respectively. Maize, barley, rye, cereal brans and oil seed meals
are used as main feedstuffs of formula feed for cattle. Mean ratios of maize, cereal brans and barley in
formula feed for cattle are 42 %, 17 % and 10 % respectively. Japan has notified a provisional standard
for DON with tolerance limits of 4 mg/kg in formula feeds for cattle of 3 months or older and 1
mg/kg in formula feed for other livestock.20)

DON was detected in all of 21 formula feed samples, and is considered to be of corn, wheat and
wheat bran origin. However the concentrations of DON were less than 200 µg/kg and below the
tolerance limits. 15-ADON was detected in 13 of 21 formula feed samples, and is considered to be of
corn origin. The concentrations of 15-ADON in formula feed were at low levels of less than 100
µg/kg. NIV was detected in five of six formula feed samples for cattle and one of eight formula feed
samples for swine. NIV in the former samples is considered to be of cereal origin except maize. NIV
in the latter samples is considered to be of milo origin because this sample is formula feed for
breeding swine consisting mainly of milo. The concentrations of NIV in formula feed were at low
levels of less than 100 µg/kg. 3-ADON and F-X were not detected in any formula feeds analyzed.

### Table 1 Natural occurrence of trichothecenes in feeds

<table>
<thead>
<tr>
<th>Kinds of feed</th>
<th>Number</th>
<th>Mean (n)</th>
<th>Range</th>
<th>Mean (n)</th>
<th>Range</th>
<th>Mean (n)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>7</td>
<td>200 (7)</td>
<td>110 –</td>
<td>38 (7)</td>
<td>12 – 94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milo</td>
<td>5</td>
<td>27 (3)</td>
<td>14 – 39</td>
<td>–</td>
<td>–</td>
<td>135 (2)</td>
<td>130 – 140</td>
</tr>
<tr>
<td>Barley</td>
<td>6</td>
<td>83 (6)</td>
<td>24 – 200</td>
<td>–</td>
<td>–</td>
<td>34 (2)</td>
<td>21 – 48</td>
</tr>
<tr>
<td>Rye</td>
<td>6</td>
<td>38 (6)</td>
<td>16 – 75</td>
<td>–</td>
<td>–</td>
<td>20 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Wheat</td>
<td>4</td>
<td>944 (4)</td>
<td>75 – 2,200</td>
<td>23 (3)</td>
<td>12 – 41</td>
<td>17 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>5</td>
<td>707 (5)</td>
<td>16 – 1,300</td>
<td>23 (2)</td>
<td>21 – 26</td>
<td>26 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cottonseed</td>
<td>4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rapeseed meal</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Formula feed for chicken</td>
<td>7</td>
<td>65 (7)</td>
<td>34 – 130</td>
<td>18 (3)</td>
<td>15 – 23</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Formula feed for swine</td>
<td>8</td>
<td>73 (8)</td>
<td>16 – 190</td>
<td>20 (5)</td>
<td>13 – 47</td>
<td>23 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Formula feed for cattle</td>
<td>6</td>
<td>106 (6)</td>
<td>78 – 140</td>
<td>14 (5)</td>
<td>10 – 26</td>
<td>61 (5)</td>
<td>34 – 92</td>
</tr>
</tbody>
</table>

*1 3-ADON and F-X were not detected in any sample.
*2 Not detected. Limits of detection for each trichothecene were 10 µg/kg.
References

21) Notification 14 Sei-chiku No.2267 (July 5, 2002), “Deoxynivalenol in Feeds”, Manager of Division of Feed, Department of Livestock and Industry Department, Bureau of Agricultural Production, Ministry of Agriculture, Forestry and Fisheries, Japan.
配合飼料及び飼料原料中のトリコテセン系マイコトキシン（B タイプ）の汚染実態

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ガスクロマトグラフィーによる飼料中の 5 種類の B タイプのトリコテセン系マイコトキシン（デオキシニバレノール（DON）, 3-アセチルデオキシニバレノール（3-ADON）, 15-アセチルデオキシニバレノール（15-ADON）, ニバレノール（NIV）及びフザレノンX（F-X））の同時分析法を開発し, 鶏, 豚及び牛用配合飼料計 21 検体及び飼料原料計 44 検体（とうもろこし, マイロ, 大麦, 小麦, ライ麦, ふすま, 大豆油かす, 菜種油かす及び綿実）についてこれらの汚染状態を調査した。DON は配合飼料及び穀物等（ふすまを含む）から検出され, 検出された DON の濃度はそれぞれ 16-190 及び 14-2,200 µg/kg であった。15-ADON は配合飼料, とうもろこし, 小麦及びふすまから検出されたが, 検出された 15-ADON の濃度は 10-92 µg/kg であった。NIV は配合飼料, マイロ, 大麦, 小麦, ライ麦及びふすまから検出されたが, 検出された NIV の濃度は 21-140 µg/kg であった。3-ADON 及び F-X はいずれの試料からも検出されなかった。また, 大豆油かす, 菜種油かす及び綿実についてはいずれの B タイプのトリコテセン系マイコトキシンも検出されなかった。

キーワード：トリコテセン, デオキシニバレノール（DON）, ニバレノール（NIV）, 多機能クリーンナップ（MFC）カラム, 飼料