Research Note

Measurement of T-2 and HT-2 Toxins in Eggs by High-Performance Liquid Chromatography with Fluorescence Detection†

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ABSTRACT

T-2 toxin is a mycotoxin produced by several species of common fungi capable of infesting human food and animal feeds. Lower-quality feeds given to chickens may be contaminated with T-2 toxin, which may affect their health. The literature suggests that T-2 toxin is transmitted from the hen to the eggs. This article describes the development of a liquid chromatographic assay for T-2 and the related mycotoxin HT-2 in eggs. T-2 and HT-2 toxins were isolated from spiked eggs with a tandem charcoal-alumina-Florisil column and immunoaffinity column cleanup. The isolated toxins were derivatized with the fluorophore 1-anthroyl nitrile, separated by high-performance liquid chromatography, and quantitated by fluorescence. The limit of detection of the method was 1 ng ml⁻¹ (parts per billion) of T-2 and HT-2 in whole (with shell removed) eggs. The limit of quantitation for both toxins was 5 ng ml⁻¹. Recoveries from spiked eggs over the range from 5 to 50 ng ml⁻¹ averaged 89.2% for T-2 and 100.3% for HT-2, with coefficients of variation of 3.5 and 8.2%, respectively. This method is sensitive enough to be used to check for the presence of T-2 or HT-2 toxins in eggs.

T-2 toxin is one of a group of trichothecene mycotoxins produced by various species of Fusaria, in particular F. sporotrichioides and F. poae. These fungi are routinely found on commodities such as wheat, maize, oats, barley, and rice, as are the trichothecene mycotoxins. A related toxin, HT-2 toxin (Fig. 1), is believed to be formed by the deacetylation of T-2 during metabolism or bioconversion by microflora (1). Excellent summaries of T-2 and HT-2 toxins can be found in reports by the Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (1) and the Council for Agricultural Science and Technology (7).

T-2 is acutely toxic in many species and is believed to act by inhibiting both protein and DNA synthesis. Some of the toxic effects of T-2 on poultry include oral necrosis, asthenia, inappetence, diarrhea, decreased growth rate, decreased egg production, thinner egg shells, and immune system toxicity (1, 5, 11). The body weight loss and decreased egg production return to normal within 18 days after removal from T-2 exposure (11).

The transmission of trichothecenes into eggs may also occur. This has been demonstrated recently for a related trichothecene mycotoxin, deoxynivalenol (9). A much earlier study (2) with tritium-labeled T-2 toxin demonstrated the transmission of the tritium label into eggs. In that study, the identity of the radioactivity as T-2 toxin was not confirmed.

As a prelude to determining the possible transmission of T-2 into eggs, an analytical method capable of detecting T-2 at relevant levels in eggs was needed and was the objective of this research. To this end, a method was developed to isolate T-2 and HT-2 toxins from eggs with a tandem charcoal-alumina-Florisil (CAF) column and immunoaffinity column (IAC) approach, with subsequent fluorescent derivatization of the toxins with 1-anthroyl nitrile (1-AN) and separation by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Reagents. Except where noted otherwise, deionized water (Nanopure II, Sybron/Barnstead, Boston, Mass.) was used in the preparation of all reagents. All solvents were HPLC grade. The T-2 toxin was prepared previously by Harlan Burmeister of the U.S. Department of Agriculture, National Center for Agricultural Utilization Research (Peoria, Ill.). Purity of the T-2 toxin was confirmed by liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (MS). HT-2 toxin was purchased from Sigma (St. Louis, Mo.), as were the neutral alumina and Florisil. Activated charcoal was obtained from EM Science (Cherry Hill, N.J.). 1-AN, also known as 1-anthroyl cyanide, was purchased from Wako Pure Chemicals (Osaka, Japan). IACs (T2-test) were obtained from Vicam (Watertown, Mass.). Chicken’s eggs were purchased from a local supermarket (Peoria, Ill.) and

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†Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the products, and the use of the name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.
were grade A medium. All other chemicals were reagent grade or better and were purchased from major suppliers.

Preparation of CAF columns. In a large, capped container, activated charcoal, neutral alumina (Brockmann I, standard grade, 10-mesh, 58 Å), Florisil (60 to 100 mesh), and Celite were combined in proportions of 1:40:40:25 (wt/wt/wt/wt) and mixed thoroughly. Thirty grams of the mixture was poured into empty 60-ml capacity cartridges containing a polyethylene frit (Varian, Walnut Creek, Calif.), and a second frit was added on top of the column.

Extraction of eggs and isolation of T-2 and HT-2. To determine recoveries, T-2 and HT-2 toxins were added to eggs. Eggs were removed from the shell, and the yolk and white were mixed with a magnetic stirrer. Each egg generally contained about 50 ml of contents. T-2 and HT-2 were added from stock solutions (1 to 10 μg ml⁻¹ in acetonitrile [ACN]) to obtain spiking levels from 1 to 50 ng ml⁻¹ in the egg.

Twenty-five milliliters of the mixed egg was transferred to a 250-ml shaker flask. Then, 105 ml of ACN was added, and the mixture was placed on a wrist-action shaker for 1 h. The mixture was decanted and filtered (Whatman 934-AH, Whatman International Ltd., Maidstone, UK), and the filtrate was applied to the CAF column, collecting the eluate. The remaining egg solids were reextracted with 125 ml of ACN-water (ACN-H₂O, 84 + 16, vol/vol) by shaking for 15 min, filtering, applying the filtrate to the CAF column, and collecting the eluate. The filters and CAF were reextracted with 125 ml of ACN-water (ACN-H₂O, 84 + 16, vol/vol) by shaking for 15 min, filtering, applying the filtrate to the CAF column, and collecting the eluate. The filters and CAF were rinsed with an additional 40 ml of ACN-H₂O, and the eluate was collected. The eluate from the CAF column was concentrated under reduced pressure at 40°C to approximately 2 to 4 ml. The concentrated extract was filtered through a 17-mm polyvinylidene difluoride syringe filter, with a pore size of 0.2 μm (Alltech, Deerfield, Ill.). The flask was rinsed with water, and this was used to rinse the filter and syringe and pooled with the filtrate, giving a final volume of approximately 10 ml.

The aqueous extract from the CAF column was applied to a T2-test IAC at a flow rate of 1 drop s⁻¹, without allowing the column to dry. The IAC was rinsed with 10 ml of water, and the T-2 was eluted into a silane-treated vial with 1.5 ml of ACN. The purified extract was dried under a stream of nitrogen gas at 50°C.

Derivatization and HPLC. The purified and dried extract was derivatized with 1-AN as follows. Fifty microliters of dimethylaminopyridine (3 mg ml⁻¹ in toluene) was added, followed by 50 μl of 1-AN (1 mg ml⁻¹ in toluene). This was mixed and heated at 50°C for 20 min, after which time it was dried under nitrogen at 50°C for 20 min. The dried, derivatized sample was reconstituted with 0.6 ml of ACN, mixed, and diluted with 0.3 ml of water. The T-2 and HT-2 derivatives were separated by HPLC.

The HPLC consisted of a Beckman Coulter System Gold 125 solvent module, a model 508 autosampler, and a JASCO (Tokyo, Japan) model FP-2020 Plus fluorescence detector (381 nm excitation, 470 nm emission). The column was a Phenomenex Luna C18(2) 3 μm 150 mm equilibrated with ACN-H₂O (4 + 1). Twenty microliters of derivatized extract was injected and separated, starting with isocratic ACN-H₂O (4 + 1) for 15 min and then a wash with ACN for 10 min and a reequilibration with ACN-H₂O (4 + 1) for 10 min (total HPLC run times, 35 min).

RESULTS AND DISCUSSION

The most recent Joint FAO/WHO Expert Committee on Food Additives report on T-2 and HT-2 (1) summarized the toxicological effects of these toxins in various species, including poultry. The effects on hens fed a diet containing T-2 toxin for 8 weeks included decreased feed consumption, egg production, and shell thickness, and the hatchability of fertile eggs from the hens fed a diet of 2 or 8 mg of T-2 per kg was lower than that of controls. The only report that describes the potential transmission of T-2 into eggs found by this author is that of Chi et al. (2). In that study, [³H]T-2 toxin was given by gastric intubation to laying hens. In hens that received a single dose, the maximum radioactivity was found in the egg 24 h after dosing. The yolk and the white contained 0.04 and 0.13% of the administered radioactivity, respectively. In hens that received eight consecutive daily doses, the radioactivity in the yolk increased with each dose, while the radioactivity in the white increased for the first three doses and then leveled off. With either dosing regimen, the white contained higher specific radioactivity than the yolk. From this, the amount of T-2 (based on radioactivity) transmitted into the eggs from hens given 1 mg kg⁻¹ for 8 consecutive days (equivalent to 1,600 μg of T-2 per kg in the diet) was about 0.9 μg. The indicated study was important for demonstrating the potential transmission of T-2 toxin into eggs; however, it did not directly recover either T-2 or HT-2 toxins from the eggs.

There are a wide variety of analytical techniques for detecting the "type A" trichothecenes, such as T-2 and HT-2 (3, 4, 8). The literature on trichothecene analysis before 1998 was summarized in an excellent review by Langseth and Rundberget (6). Currently, the most commonly used methods include gas chromatography with either electrochemical or MS detection, enzyme-linked immunosorbent assays, and HPLC detection of fluorescently derivatized T-2 or HT-2 (3, 10). For the chromatographic methods, T-2 has been extracted from grains with a variety of solvents, most notably mixtures of ACN-water in proportions of 84 + 16 (vol/vol). T-2 has generally been isolated from grains by using charcoal-alumina (CA), Florisil (magnesium silicate), or silica solid-phase extraction columns (12). For the CA and Florisil columns, the extract is passed through the column, and many of the impurities bind, whereas the trichothecenes pass through. Because of the widespread availability of HPLC instrumentation, a variety of HPLC-MS methods have been developed. For laboratories without access to MS instrumentation, a sensitive alternative has been the derivatization of T-2 and HT-2 with 1-AN and

FIGURE 1. Structures of T-2 toxin (R = OCOCH₃) and HT-2 toxin (R = OH).
subsequent fluorescent detection (10). Because of the general applicability of this approach and the availability of instrumentation, this technique was chosen for our efforts to adapt a T-2 method to the analysis of eggs.

Several approaches were initially tried, including the use of protein precipitation, followed by liquid partitioning cleanups, CA cleanup columns, and IACs. With these approaches, we were unable to attain the necessary sensitivity (data not shown). However, because both CA columns and Florisil have been used (separately), we decided to attempt to combine these materials into a single column, namely a CAF column. As with the CA and Florisil columns, the CAF columns allowed the T-2 and HT-2 to pass through while binding potential interferences. The CAF columns alone were not sufficient for cleaning up the samples for HPLC with fluorescence detection (data not shown). However, when the eluate from the CAF columns was concentrated and applied to an IAC, the degree of cleanup and the degree of concentration attained were sufficient to permit sensitive detection of both T-2 and HT-2. A chromatogram of eggs spiked with 5 ng ml⁻¹ of T-2 and HT-2 and isolated in this manner is shown in Figure 2.

Spiking and recovery studies were conducted to determine the limit of detection and limit of quantitation for the method. The signal-to-noise ratios for T-2 and HT-2 spiked into eggs at 1 ng ml⁻¹ were 8 and 15, respectively. The sensitivity for HT-2 is suspected to be greater than for T-2 because of the presence of two hydroxyl groups on HT-2 (as opposed to one on T-2), potentially leading to multiple labeling by the fluorophore 1-AN. The recoveries at both 1 and 2 ng ml⁻¹ were quite variable, with coefficients of variation ranging from 16 to 95% (Table 1). At 5 ng ml⁻¹, however, the coefficients of variation were much lower (1.8 and 3.2% for T-2 and HT-2, respectively), indicating that reliable quantitation can be conducted at or above this level. Recoveries over the range from 5 to 50 ng ml⁻¹ averaged 89.2% for T-2 and 100.3% for HT-2 (Table 1).

The sensitivity attained, as described above, is sufficient to be useful in transmission studies for determining the carryover of T-2 from poultry feed into eggs, which is a logical extension of the described method. The assay would also be useful for the random checking of T-2 in eggs. Furthermore, the CAF cleanup columns described in this study might be useful in the development of HPLC-MS methods for the detection of T-2 and HT-2 in eggs, which would have the advantage of avoiding the derivatization and fluorescence detection steps used in the current method.

ACKNOWLEDGMENT

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REFERENCES


### TABLE 1. Recovery of T-2 and HT-2 toxins from eggs

<table>
<thead>
<tr>
<th>Spiking level (ng ml⁻¹)</th>
<th>Avg recovery (%)</th>
<th>Coefficient of variation (%)</th>
<th>Avg recovery (%)</th>
<th>Coefficient of variation (%)</th>
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<tr>
<td>50</td>
<td>89.0</td>
<td>3.4</td>
<td>99.3</td>
<td>0.8</td>
</tr>
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</table>

Overall averages, LOQ and above

89.2 3.5 100.3 8.2

- Quadruplicate analyses of eggs spiked at the indicated level.
- Limit of detection (LOD), 1 ng ml⁻¹.
- Limit of quantitation (LOQ), 5 ng ml⁻¹.

![Chromatograms of eggs spiked with T-2 and HT-2 toxins at the limit of quantitation of the method. (A) 5 ng ml⁻¹ and (B) unspiked egg. Arrows depict the retention times of T-2 (11.0 min) and HT-2 (22.2 min).](image)


