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REVIEW

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# T-2 Toxin, a Trichothecene Mycotoxin: Review of Toxicity, Metabolism, and Analytical Methods

Yanshen Li,[†] Zhanhui Wang,[†] Ross C. Beier,[‡] Jianzhong Shen,[†] David De Smet,[§] Sarah De Saeger,[§] and Suxia Zhang*[,†]

[†]Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, China Agricultural University, Beijing 100094, People's Republic of China

[‡]Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, Texas 77845-4988, United States

[§]Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium

**ABSTRACT:** This review focuses on the toxicity and metabolism of T-2 toxin and analytical methods used for the determination of T-2 toxin. Among the naturally occurring trichothecenes in food and feed, T-2 toxin is a cytotoxic fungal secondary metabolite produced by various species of *Fusarium*. Following ingestion, T-2 toxin causes acute and chronic toxicity and induces apoptosis in the immune system and fetal tissues. T-2 toxin is usually metabolized and eliminated after ingestion, yielding more than 20 metabolites. Consequently, there is a possibility of human consumption of animal products contaminated with T-2 toxin and its metabolites. Several methods for the determination of T-2 toxin based on traditional chromatographic, immunoassay, or mass spectroscopy techniques are described. This review will contribute to a better understanding of T-2 toxin exposure in animals and humans and T-2 toxin metabolism, toxicity, and analytical methods, which may be useful in risk assessment and control of T-2 toxin exposure.

**KEYWORDS:** trichothecenes, T-2 toxin, toxicity, apoptosis, metabolism, analysis

## INTRODUCTION

Trichothecenes are agriculturally important mycotoxins that present a potential threat to animal health throughout the world. Trichothecenes are a large family of chemically related toxins produced by fungi in taxonomically unrelated genera, such as *Fusarium*, *Myrothecium*, and *Stachybotrys*. Trichothecenes have a tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring in common (Figure 1), and the 12,13-epoxy ring is responsible for the toxicological activity.[1] On the basis of the absence or presence of characteristic functional groups, the trichothecenes can be classified into four types. Type A trichothecenes are mainly represented by T-2 toxin (T-2) and HT-2 toxin (HT-2) and do not contain a carbonyl group at the C-8 position (Figure 1). In type B trichothecenes, a carbonyl group is present at the C-8 position. The main representatives of type B trichothecenes are deoxynivalenol and nivalenol (Figure 1). Trichothecenes of type C (e.g., crotocin and baccharin) have a second epoxy ring between C-7 and C-8 or between C-9 and C-10. Trichothecenes of type D, such as satratoxin and roridin, contain a macrocyclic ring between C-4 and C-15. The chemical structures of the four different types of trichothecenes are shown in Figure 1.

Trichothecenes are toxic to animals, and currently approximately 190 members of the trichothecene family have been identified. However, T-2, which belongs to the type A trichothecenes, has received much attention because it has the highest toxicity of all the trichothecenes, although it is less frequently detected compared to the other trichothecenes.[1,12,13] T-2 is often produced by different *Fusarium* species, including *F. soprotrichioides*, *F. poae*, and *F. acuinatum*, which may grow on a variety of cereal grains, especially in cold climate regions or during wet storage conditions.[12,14] In addition to fungal genetics, T-2 production can be significantly influenced by environmental conditions, such as temperature, humidity, and type of substrate.[12] A recent report from the European Union (EU) stated that T-2 toxin was a common contaminant in cereal samples from EU member states, and therefore EU members were at risk for dietary exposure to *Fusarium* toxins.[15] In particular, the most frequently contaminated cereal samples were maize (41%), wheat (21%), and oats (21%).[15]

T-2 is nonvolatile and resistant to degradation in different environments, such as light and temperature, but it is effectively deactivated by strong acid or alkaline conditions. T-2 may also be affected by the presence of coexisting bacteria or fungi that can detoxify it by altering its chemical structure.[16−18]

T-2 toxin causes a large range of toxic effects in animals, such as weight loss, decreases in blood cell and leukocyte count, reduction in plasma glucose, and pathological changes in the liver and stomach. Furthermore, T-2 is associated with an increased infection rate, alimentary toxic aleukia (ATA), deoxyribonucleic acid (DNA) damage, and induction of apoptosis.[19−23] Over the years, there have been many reports from different regions of the world describing the association of T-2 with damage to agriculture and its toxic effects in animals.[24−28] The subject of this review focuses on T-2 toxin toxicity, metabolism, and methods of analytical determination.

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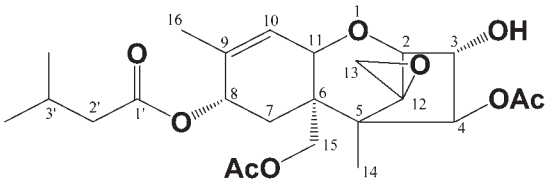
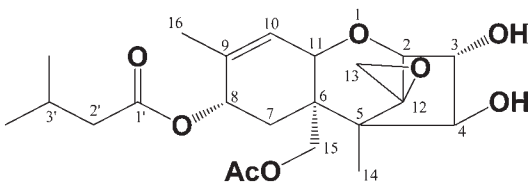
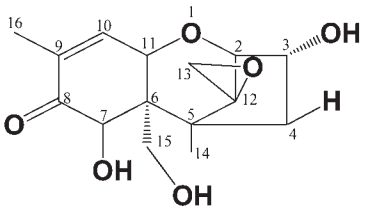


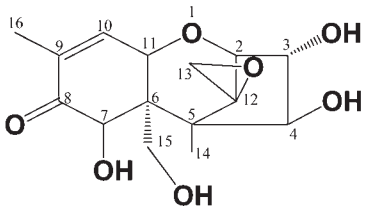
Type A: T-2 toxin



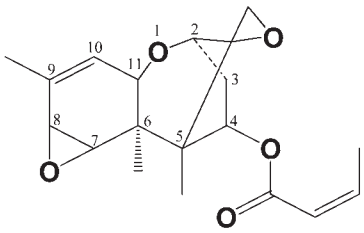
Type A: HT-2 toxin



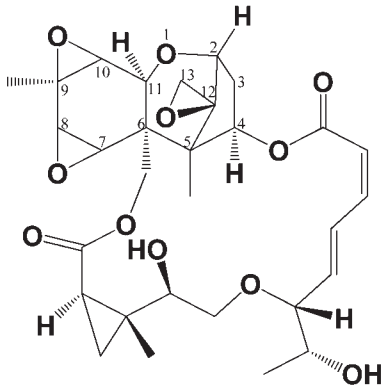
Type B: Deoxynivalenol



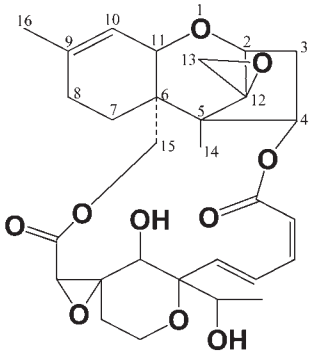
Type B: Nivalenol



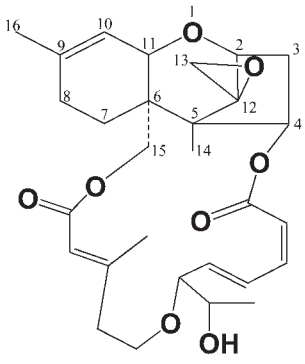
Type C: Crotocin



Type C: Baccharin



Type D: Satratoxin



Type D: Roridin

**Figure 1.** Structures of T-2 and HT-2 toxins (type A) and other trichothecenes (types B, C, and D).

## TOXICITY OF T-2

T-2 is one of the most acutely toxic members of the trichothecene family. It can cause emesis, diarrhea, lethargy, weight lose, hemorrhage, inhibition of immunity, necrosis, damage of cartilaginous tissues, apoptosis, and death.[6,29] Recently, T-2 was considered as the cause of ATA and Kashin−Beck disease (KBD).[30]

**Acute Toxicity.** Animals can be infected with T-2 through different routes of exposure. The characteristic signs of T-2 toxicity are emesis, vomiting, feed refusal, and weight loss. The level of T-2 toxicity is related to the animal species, age, exposure route, and dosage, and in particular, infants are more sensitive than adults. $LD_{50}$ values of T-2 for different animals and exposure routes are shown in Table 1.

**Table 1. $LD_{50}$ Values of T-2 Toxin in Different Animals with Different Administration Pathways**

| species | administration pathway | $LD_{50}$ (mg/kg BW[a]) | ref |
|---|---|---|---|
| mice | oral | 10 | 2 |
| mice | intraperitoneal | 5.2 | 2 |
| mice | subcutaneous | 2.1 | 2 |
| mice | intravenous | 4.2 | 2 |
| rats | intraperitoneal | 1.5 | 3 |
| rats | subcutaneous | 1.0 | 4 |
| rats | intramuscular | 0.85 | 5 |
| rats | intravenous | 0.9 | 6 |
| rats | inhalation | 0.05 | 3 |
| guinea pigs | intraperitoneal | 1.2 | 3 |
| guinea pigs | intravenous | 1−2 | 6 |
| guinea pigs | inhalation | 0.4 | 3 |
| rabbits | intramuscular | 1.1 | 5 |
| 7-day-old broilers | oral | 4 | 7 |
| laying hens | oral | 6.3 | 8 |
| day-old cockerels | oral | 1.84 | 9 |
| day-old chicks | oral | 5 | 10 |
| pigs | intravenous | 1.21 | 11 |

[a] BW, body weight.

Acute toxicity of T-2 was studied in rats, mice, guinea pigs, and pigeons resulting from different routes of exposure, including intravenous, intragastric, subcutaneous, intraperitoneal, and intratracheal.[6] At doses equal to one-fifth the $LD_{50}$ or less, T-2 caused vomiting in pigeons. Rats exposed to doses of 3.0 and 5.0 mg/kg exhibited signs of lymphopenia and reticulocytosis. In addition, changes in alkaline phosphatase and aspartate aminotransferase activities were observed. However, the most severe signs were observed in the thymus, lymph nodes, and Peyer's patches,[6] but acute toxicity of T-2 does not interfere with iron absorption or transport. The risk of erythropoietic injury due to dietary exposure to T-2 may be evaluated by studying the damage on reticulocytes and/or erythroblasts, because T-2 exposure causes inhibition of the immune system.[31]

Administering T-2 to rats resulted in increased brain concentrations of tryptophan and serotonin, followed by an increase in dopamine and a decrease in 3,4-dihydroxyphenylacetic acid (DOPAC) levels. In adrenal glands, concentrations of dopamine were increased, whereas epinephrine levels decreased. This indicated that an increase of the indoleamine levels in the brain, induced by T-2, may contribute to feed refusal.[22] Acute toxicity of T-2 in rabbits revealed pathological and histopathological changes in the digestive tract, lymphocytes, and bone marrow, whereas subacute toxicity revealed catarrhal gastritis (inflammation of the lining of the stomach), emaciation, and hypertrophy of the adrenal cortex.[29]

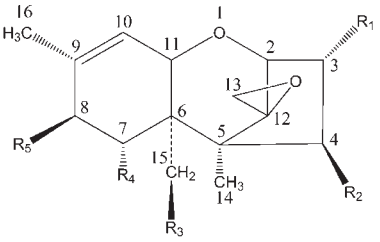
**Chronic Toxicity.** Chronic T-2 administration to female rats was characterized by an increase in cerebellar tyrosine and serotonin concentrations. Moreover, cortical tryptophan concentrations were also increased, showing a differential effect in comparison to acute administration.[32] Acute systemic T-2 toxin administration increased cerebellar and brainstem tryptophan, whereas the serotonin, a tryptophan metabolite, level was correspondingly decreased in these same brain regions.[32] Other toxic effects involved emaciation, subacute catarrhal gastritis, and necrosis of the lymphoid in rabbits.[33] In the case of chickens, intake of T-2-contaminated feed markedly decreased weight gain and egg production and also impaired egg hatch. Meanwhile, a significant reduction in serum total protein and cholesterol levels and a rise in serum uric acid and lactate dehydrogenase (LDH) levels were observed.[34−37] T-2 can also cause feather alteration in chickens.[38] In white Pekin ducks, T-2 can significantly decrease weight gain as the dosage increases and distinctly impair the blastogenic response of lymphocytes to nonspecific and specific mitogens.[39] Also, a long-term application of low-dosage T-2 toxin to BALB/c mice resulted in the production of papillomas and carcinomas.[40]

## APOPTOSIS AND T-2

T-2 is a well-known inhibitor of protein synthesis resulting from binding peptidyltransferase, which is an integral part of the 60s ribosomal subunit.[41,42] Furthermore, T-2 inhibits the synthesis of DNA and RNA, interferes with the metabolism of membrane phospholipids, and increases the level of liver lipid peroxides.[43,44] Currently, apoptosis is confirmed by histology, in situ detection of fragmented DNA, DNA agarose gel electrophoresis, or flow cytometry. There are many studies concerned with T-2-induced apoptosis of the immune system, gastrointestinal tissues, and fetal tissues.[23,42,45]

T-2 toxin-induced bone marrow and splenic red pulp in female ICR:CD-1 mice orally treated with T-2 showed significant hypocellularity, and the cell nuclei showed pyknosis or karyorrhexis. Also, DNA ladders in bone marrow were clearly detected with agarose gel electrophoresis, indicating that the T-2 toxin-induced lesions in hematopoietic tissues and in lymphoid tissues were caused by apoptosis of component cells.[23] Other studies revealed inhibition of DNA and protein synthesis in the spleen, thymus, and bone marrow after exposure to T-2, and T-2 had species-specific hemolytic effects on erythrocytes.[30,46] HL-60 human promyelocytic leukemia cells were used to characterize the apoptotic effects of T-2. Apoptotic cells were identified microscopically by chromatin condensation and nuclear fragmentation via flow cytometric analysis and DNA gel electrophorsis. Furthermore, the $Ca^{2+}/Mg^{2+}$ ratio was markedly increased after intoxication with T-2, and apoptosis could be inhibited by using chelators of intracellular $Ca^{2+}$.[47,48]

Mice orally inoculated with T-2 exhibited enteric absorption with initial effects seen in the Peyer's patches, followed by effects in the mesenteric lymph nodes and finally the thymus. The degree of lymphocyte apoptosis differed among different lymphoid tissues. The most severe effects of T-2 were observed in the thymus, then in the Peyer's patches, and finally in the lymph nodes, indicating variable susceptibility to T-2 by the lymphocyte population.[49,50] Different mouse B lymphocyte subsets were different highly sensitive targets to T-2 exposure. $CD4^+CD8^+$ T cells were the most sensitive to T-2 and markedly decreased, whereas $CD4^+CD8^-$ T cells were more depressed than $CD4^-CD8^+$ T cells in the thymus. In the mesenteric lymph nodes, $CD3^+$ cells were clearly more affected than $CD19^+$ cells, and the number of $CD4^+$ and $CD8^+$ cells decreased similarly. On the other hand, in the Peyer's patches, the numbers of $CD3^+$, $CD19^+$, $CD4^+$, and $CD8^+$ cells were only slightly decreased.[49,51−53] T-2 treatment of broiler chickens caused induction of apoptosis in the thymus, with peak induction at 24 h post treatment.[54] The thymocytes were shrunken, characterized by a condensed nucleus, and exhibited crescent



| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| T-2 toxin | OH | OAc | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| HT-2 toxin | OH | OH | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| T-2 triol | OH | OH | OH | H | $OCOCH_2CH(CH_3)_2$ |
| T-2 tetraol | OH | OH | OH | H | OH |
| 3′-hydroxy T-2 triol | OH | OH | OH | H | $OCOCH_2C(OH)(CH_3)_2$ |
| 3′-hydroxy T-2 | OH | OAc | OAc | H | $OCOCH_2C(OH)(CH_3)_2$ |
| 3′-hydroxy HT-2 | OH | OH | OAc | H | $OCOCH_2C(OH)(CH_3)_2$ |
| 4′-hydroxy HT-2 | OH | OH | OAc | H | $OCOCH_2CHCH_3CH_2OH$ |
| 3-acetyl T-2 | OAc | OAc | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| 3-acetyl HT-2 | OAc | OH | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| 3′,7-di-hydroxy HT-2 | OH | OH | OAc | OH | $OCOCH_2CH(CH_3)_2$ |
| 3-acetyl-3′-hydroxy HT-2 | OAc | OH | OAc | H | $OCOCH_2C(OH)(CH_3)_2$ |
| 8-acetyl T-2 tetraol | OH | OH | OH | H | OAc |
| 15-acetyl T-2 tetraol | OH | OH | OAc | H | OH |
| Neosolaniol | OH | OAc | OAc | H | OH |
| 4-deacetylneosolaniol | OH | OH | OAc | H | OH |
| 15-deacetylneosolaniol | OH | OAc | OH | H | OH |

**Figure 2.** Structures of T-2 toxin and its metabolites.

margination of chromatin against the nuclear envelope without any surrounding inflammation. This study points out that one route of induced cell death by T-2 in lymphoid organs of broiler chickens is by apoptosis.[54]

Following treatment with T-2 in female ICR:CD-1 mice, changes in mRNA expression of the apoptosis-related cell-surface antigen (Fas) and genes (*p53*, *bcl-2*, *c-myc*, and *c-fos*) were evaluated by reverse transcription−polymerase chain reaction (RT-PCR). Bcl-2 proteins act as anti- or pro-apoptotic regulators that are involved in cellular activities. The *c-fos* mRNA expression increased prior to the progression of apoptotic cell death in the thymus, whereas no other significant changes were observed.[55] Research to determine whether the Fas/Fas ligand (FasL) signaling pathway was involved in T-2-mediated thymocyte apoptosis used *lpr*/*lpr* (*lpr*) and *gld*/*gld* (*gld*) mice, whose Fas and FasL proteins were functionally deficient, and showed that in vivo T-2-induced thymocyte apoptosis was independent of the Fas/FasL pathway.[56]

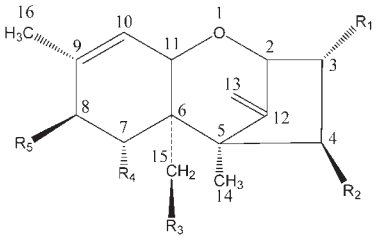
After exposure to T-2, the human chondrocyte level increased in the proteins Fas and p53, the pro-apoptotic factor Bax (Bcl-2-associated X protein), and mRNA expression, whereas the anti-apoptotic factor Bcl-xl level decreased in a dose-dependent manner. On the other hand, the expression of the anti-apoptotic factor Bcl-2 was constant. Meanwhile, T-2 could also up-regulate the expression of both pro-caspase-3 and caspase-3 in a dose-dependent manner. This up-regulation may be a possible molecular mechanism for the T-2-induced apoptosis signaling pathway in human chondrocytes.[57] Other research concerning chondrocyte apoptosis and expression of the Bcl-2/Bax proteins and mRNA induced by T-2 showed an increase of the Bax/Bcl-2 ratio, showing that Bax mRNA also increased, whereas Bcl-2 mRNA expression remained unchanged. Selenium could partially block the apoptosis induced by T-2 through decreasing the Bax/Bcl-2 ratio, and the Bax/Bcl-2 ratio may also play a critical role in governing the susceptibility to apoptosis induced by T-2 in human chondrocytes.[58] In addition, T-2-induced apoptosis was significantly correlated with levels of nitric oxide (NO) production and induced NO synthase (iNOS) and Fas protein expression in human chondrocytes. This indicated that T-2 can up-regulate NO production and enhance the expression of iNOS and Fas proteins. Both NO and Fas signaling pathways were involved in T-2-induced apoptosis.[59] T-2 induced a caspase-dependent mitochondrial apoptotic pathway in the human hepatoma cell line, HepG2.[60] The mitochondrial alterations included the following: Bax relocalization into the mitochondrial outer membrane, loss of the mitochondrial transmembrane potential, and cytochrome *c* but not apoptosis-inducing factor (AIF) release. In the presence of T-2 toxin, the reactive oxygen species (ROS) level was highly increased at an early stage, even before physical mitochondrial alterations were observed, and it appeared that the ROS levels were a consequence of early mitochondrial changes. The authors suggested these results point to a central role of mitochondria in the T-2-induced apoptotic process, which may provide insights into the mechanisms by which T-2 promotes hepatotoxicity.[60]

T-2 can induce apoptosis in basal keratinocytes when topically applied to the dorsal skin of rats.[61] The expression of apoptosis-related genes (c-*fos* and c-*jun*) and cytokine (TNF-α and IL-1$\beta$) mRNAs markedly increased before the initiation of apoptosis, indicating that c-*fos* and c-*jun* oncogenes and TNF-α and IL-1$\beta$ play an important role in the T-2-induced apoptosis in keratinocytes.[61]

T-2 can cause toxicity not only to the pregnant female, but it can easily pass through the placental barrier of the rat and cause toxicity to the embryo.[62] T-2 toxin feeding of pregnant rats at levels similar to those found in naturally contaminated foods impaired the immune systems of newborns.[62] Maternal mortality, in combination with placental hemorrhage, was observed in pregnant mice after dosing with T-2. Also, fetal loss was greater in T-2-treated groups than in controls.[63,64] T-2 can also induce a significant reduction in the level of fetal liver B lymphocytic cells, cause significant fetal thymus atrophy, and decrease $CD44^-$ and $CD45^-$ fetal liver pro-lymphoid cell subpopulations, suggesting that lymphocyte progenitors represent highly sensitive targets of T-2 toxin exposure.[65] In addition, oral administration of T-2 to pregnant rats increased histopathological apoptosis in the liver and placenta and also in the fetal liver. The mechanism of T-2-induced toxicity in pregnant rats was related to the expression of apoptosis genes, including oxidative stress-related genes detected in the tissues, suggesting that the c-*jun* gene may play an important role in T-2-induced apoptosis.[20,66,67]

## METABOLISM OF T-2

T-2 is usually metabolized and eliminated after ingestion.[68] In general, T-2 is soluble in water, and the major metabolic reactions are usually hydrolysis, hydroxylation, de-epoxidation, and conjugation. The most typical metabolites of T-2 are HT-2 toxin (hydrolysis), T-2 triol, T-2 tetraol, neosolaniol (NEO), 3′-hydroxy HT-2, 3′-hydroxy T-2, 3′-hydroxy T-2 triol, and dihydroxy HT-2 (Figure 2) and de-epoxy-3′-hydroxy T-2 and de-epoxy-3′-hydroxy HT-2 (Figure 3).[68,69] Because human consumption of animal products contaminated with T-2 and its metabolites is a possibility, distribution and metabolism studies of T-2 toxin in animals could provide important information for



| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| Deepoxy HT-2 | OH | OH | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| Deepoxy T-2 triol | OH | OH | OH | H | $OCOCH_2CH(CH_3)_2$ |
| Deepoxy T-2 tetraol | OH | OH | OH | H | OH |
| Deepoxy-3'-hydroxy HT-2 | OH | OH | OAc | H | $OCOCH_2C(OH)(CH_3)_2$ |
| Deepoxy-3'-hydroxy T-2 triol | OH | OH | OH | H | $OCOCH_2C(OH)(CH_3)_2$ |
| Deepoxy-15-acetyl T-2 tetraol | OH | OH | OAc | H | OH |

**Figure 3.** Structures of de-epoxy metabolites of T-2 toxin.

both evaluating and controlling human exposure to residual T-2 metabolites in foods of animal origin.

**In Vivo Metabolism.** In vivo studies in different animal species confirmed that ester hydrolysis and hydroxylation of the isovaleryl group are the major metabolic pathways involved in the metabolism of T-2.[70−74] In addition, the mechanisms of de-epoxidation and glucuronide conjugation of T-2 were also determined during in vivo metabolism studies.[70,71,75] The half-life of T-2 in vivo is very short. When administered to rodents, chickens, cattle, and swine, T-2 is rapidly metabolized into various products. T-2 is rapidly metabolized and eliminated in the feces and urine at ratios of 3:1 in mice, 5:1 in rats, and 1:4 in guinea pigs.[76,77] The tissue distribution and excretion of T-2 were studied in chickens, cows, rodents, and swine by following tritium-labeled T-2 administration.[10,74,78] The metabolites of T-2 are shown in Figure 2.

After oral administration of T-2 in rats and mice, metabolites of T-2 were excreted in the feces, primarily as HT-2, neosolaniol, and three unidentified metabolites.[76] In broiler chickens, about 80% of orally administered [$^3$H]T-2 was rapidly metabolized to more polar derivatives and eliminated in the excreta within 48 h. Among the various metabolites, T-2, HT-2, neosolaniol, and T-2 tetraol were detected. Furthermore, eight unknown derivatives, named TB-1−TB-8 were quantitatively more significant compared to the previously mentioned metabolites. TB-6 was identified as 4-deacetylneosolaniol (Figure 2).[74] In vivo metabolism of daily oral administration of unlabeled T-2 in a lactating cow followed by a single oral administration of [$^3$H]T-2 on day 4 was conducted.[73] T-2 was rapidly metabolized in cow tissues and excreta, primarily yielding three major unknown metabolites, referred to as TC-1, TC-3, and TC-6. These metabolites represented approximately 30−40% of the extractable radioactivity in urine, 60−70% in milk, and 50−60% in plasma within the first 24 h. Furthermore, a very polar metabolite designated TC-8 was also detected. TC-1, TC-3, and TC-6 were then identified as 3′-hydroxy T-2, 3′-hydroxy HT-2, and 3′,7-dihydroxy HT-2 (Figure 2), respectively.[10,77] The metabolites TC-1 and TC-3 correspond to TB-1 and TB-3, respectively, which were found in the excreta of broiler chickens.[74] These metabolites may also correspond to one of the unknown metabolites found in rats.[76] This suggested that many animals were capable of metabolizing T-2 in a manner similar to that of the cow and that the metabolites TC-1, TC-3, and TC-6 could be used as appropriate indices to estimate the intake of T-2. In another study, iso-TC-1, an isomer of TC-1, was detected in cow urine.[79] The structure of iso-TC-1 has been established as 3,15-diacetoxy-4-hydroxy-8(3-methyl-3′-hydroxybutyryloxy)-12,13-epoxytrichothec-9-ene (3-acetyl-3′-hydroxy HT-2, Figure 2), in which the hydroxyl and acetyl groups are at the C-3 and C-4 positions, respectively.[79]

Trace amounts of T-2 were detected in the lungs of chickens 18 h after intravenous injection of T-2, but no trichothecenes were detected in the heart and kidneys.[72] Most of the T-2 metabolites were found in the excreta, although considerable amounts were also found in the liver. In addition to the previously identified T-2 metabolites in chicken excreta, such as HT-2 toxin, 15-acetyl T-2 tetraol, and T-2 tetraol (Figure 2), another six metabolites were also detected, including 3′-hydroxy HT-2 (the major metabolite), 3′-hydroxy T-2, 4-acetyl T-2 tetraol, and trace amounts of 8-acetyl T-2 tetraol, 3-acetyl-3′-hydroxy HT-2, and T-2 triol (Figure 2).[72]

Following intravascular administration of T-2 to female pigs, metabolic products of T-2 were found in the bile and urine.[70] These products included the unconjugated metabolites 3′-hydroxy HT-2 and T-2 triol and the glucuronide conjugated metabolites of HT-2, 3′-hydroxy HT-2, 3′-hydroxy T-2, and T-2. The glucuronide conjugates accounted for approximately 70% of the total metabolite residues, with T-2 glucuronide accounting for a much greater percentage of the metabolite residues in the bile (42%) than in the urine (11%).[70] A total of 21 metabolites were identified following intravascular administration of radiolabeled T-2 to swine.[71] The conjugation of T-2 and its metabolites occurred very rapidly. As evidenced in the blood taken only 10 min after T-2 administration, 50% of 3′-hydroxy T-2 and HT-2 (Figure 2) were already conjugated. In addition, approximately 55% of the extractable radioactivity in the tissues and gastrointestinal tract of the swine corresponded to T-2, HT-2, T-2 triol, 3′-hydroxy T-2, 3′-hydroxy HT-2, and T-2 tetraol (Figure 2) and de-epoxy HT-2, de-epoxy T-2 triol, and de-epoxy T-2 tetraol (Figure 3). The major metabolite found in tissues was PM-XV and was not identified, but it represented 27% of the extractable radioactivity from dosed swine.[71] The de-epoxidation metabolites were initially detected following orally administered T-2 metabolites, 3′-hydroxy HT-2, and T-2 tetraol (Figure 2), to male Wistar rats.[75] Four de-epoxy trichothecene metabolites were found in the excreta, and their structures were confirmed as de-epoxy-3′-hydroxy HT-2, de-epoxy-3′-hydroxy T-2 triol, de-epoxy-15-acetyl T-2 tetraol, and de-epoxy T-2 tetraol (Figure 3).[75]

T-2 did not significantly accumulate in specific organs of a lactating Jersey cow, and chromatographic analysis of the tritium residues showed that T-2 was rapidly metabolized. Also, the tritium residues in bile and liver were higher than in the blood.[73] Retention of radioactivity in these same organs was also observed in broiler chickens[10] and rats and mice,[76] indicating that a large amount of the absorbed toxin and its metabolites in the intestinal tract was eliminated through the biliary excretion system. The delayed elimination of radioactivity in cow feces, compared with urinary excretion, suggested that T-2 and its metabolites probably circulate in the enterohepatic system of the cow.

**In Vitro Metabolism.** *Liver Metabolic Systems.* Studies on the metabolism of T-2 have been carried out on liver S-9 fractions and microsomes. During initial studies, HT-2 was considered as the sole metabolite in bovine and human liver homogenates and in liver microsomes of various animals.[80−82] However, new

metabolites of T-2 were subsequently detected.[83] Upon incubation of T-2 in a rat liver S-9 fraction, T-2 was rapidly converted into HT-2, T-2 tetraol, and two unknown metabolites, which were designated TMR-1 and TMR-2. TMR-1 was characterized as 4-deacetylneosolaniol (Figure 2) by spectroscopic analysis. TMR-1, TMR-2, and T-2 tetraol were also found in the incubation mixture of HT-2, which suggested that the three compounds were converted from T-2 via HT-2 by hydrolysis at the C-4 position.[83] Along with these metabolites, four other metabolites of T-2 were detected in homogenates of mouse and monkey livers. These new metabolites included neosolaniol, 15-deacetylneosolaniol, 3′-hydroxy T-2, and 3′-hydroxy HT-2 (Figure 2). The hydroxylation reactions were enhanced by treating mice with phenobarbital (PB).[84] Liver microsomes from rat, chicken, and mouse can biotransform T-2 into a variety of metabolites including HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol, 3′-hydroxy T-2, and 3′-hydroxy HT-2 (Figure 2), and two unidentified compounds, RLM-2 and RLM-3.[85] These two additional compounds were tentatively identified as isomers of 3′-hydroxy T-2 by gas chromatography−mass spectrometry (GC-MS). The major metabolite in microsomal preparations from both control and PB-induced microsomes was HT-2. Following incubation of PB-induced chickens with T-2, 3′-hydroxy T-2 was the major metabolite. However, 30 and 79% of the added T-2 remained unchanged 60 min after incubation in PB-induced and control chickens, respectively.[85] The significant increase in hydroxylated metabolites formed by liver microsomes from PB-induced animals may be caused by cytochrome P-450 catalysis. This effect has been described in liver homogenates prepared from mice and monkeys[84] and has been used to enhance the in vitro production of 3′-hydroxy metabolites by liver S-9 fractions isolated from pigs and rats.[86−88] Treatment of a rat hepatic S-9 preparation with T-2 yielded primarily 3′-hydroxy T-2 (Figure 2) as the major metabolic product (>85%), and a new minor metabolite, RLM-3, was identified by GC-MS and $^{1}$H and $^{13}$C NMR experiments as 4′-hydroxy T-2.[89] In work by Pace,[90] [$^{3}$H]T-2 was administered to perfused rat livers and used to study the metabolism and clearance of T-2. T-2 was metabolized and eliminated as 3′-hydroxy HT-2, 3′-hydroxy T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol (Figure 2) and as glucuronide conjugates of HT-2, 3′-hydroxy HT-2, and T-2 tetraol.[90] The biochemical pathways of T-2 metabolism in perfused rat liver were the same as those observed in vivo.[74,77,91] Again, bile was observed to be the major excretion route of T-2 and its metabolites, and the perfusion model was considered to be useful as a tool for the isolation of minor metabolites for structural analysis.

*Non-Liver Metabolic Systems.* The in vitro percutaneous penetration and metabolism of [$^{3}$H]T-2 on human, rabbit, guinea pig, and rat skin was evaluated.[92] Penetration was much higher when using dimethyl sulfoxide (DMSO) as the solvent vehicle compared to methanol. Using methanol as vehicle resulted in an increase of penetration when the dose of T-2 increased. The metabolism of T-2 was extensive in the human, rabbit, and rat, resulting in HT-2 being the main metabolite. Rabbit skin provided the best approximation to human skin for studying T-2 penetration.[92] Penetration of T-2 through monkey skin was 2.9 times faster than penetration through human skin.[93] The metabolism of T-2 was extensive, with the major metabolite being HT-2, which is consistent with previous observations concerning penetration of T-2 through intact rat skin. T-2 triol and T-2 tetraol (Figure 2) were also detected[92,94] and were most likely formed via acetylcholine esterases.[86] In addition, three unknown metabolites were found, which comigrated with 3′-hydroxy T-2, 3′-hydroxy HT-2, and 3′-hydroxy T-2 triol. However, these unknown metabolites were not identified because the quantities were too small. On the other hand, metabolism of the trichothecenes was related to the type of vehicle used. Metabolism of [$^{3}$H]T-2 to [$^{3}$H]HT-2 occurred to a larger extent in human skin when DMSO was used as the vehicle (70.7% of radioactivity in receptor fluids was associated with HT-2) compared to when methanol was the vehicle (18.1% associated with HT-2).[93]

Human and rat blood hydrolyzed T-2 toxin following two different metabolic pathways and resulted in HT-2 and neosolaniol as the primary metabolites, respectively.[95] The enzymes responsible for hydrolysis of T-2 to HT-2 in white blood cells and T-2 to neosolaniol (Figure 2) in red blood cells were identified, by use of specific inhibitors, as carboxylesterases. The ratio between trichothecene hydrolysis and 4-nitrophenyl butyrate hydrolysis varied among the different cell fractions, indicating that specific isoenzymes were involved.[95]

Anaerobic incubation of the gastrointestinal tract is commonly conducted during studies of non-liver metabolic systems. T-2 was incubated with rat gastrointestinal strips to provide more information about the in vitro metabolism of T-2.[83] It was found that when T-2 was incubated with stomach strips at pH 2.2, 7.6% of the toxin was metabolized to HT-2 after 60 min, and no other metabolites were detected by gas−liquid chromatography (GLC) or by thin-layer chromatography (TLC). However, at pH 7.5, stomach strips converted T-2 to HT-2 (18%), TMR-1 (3.5%), and neosolaniol (4.4%) after 180 min of incubation. TMR-1 was confirmed as 4-deacetylneosolaniol by GC-MS. Incubation of T-2 toxin with rat intestinal strips for 60 min at pH 7.5 resulted in two major metabolites, HT-2 (45.5%) and 4-deacetylneosolaniol (12.9%), including a trace amount of neosolaniol (Figure 2). The results indicated that the metabolic pathways are different in different animal tissues, and the substrate specificity of the enzymes involved in the metabolism is affected by the pH of the incubation system.[83]

T-2 toxin was incubated in vitro under anaerobic conditions with bovine rumen microorganisms obtained from a fistulated dairy cow.[96,97] In addition to HT-2 and T-2 triol, new de-epoxy metabolites were observed. GLC and GC-MS were used to confirm the new metabolites as de-epoxy HT-2, de-epoxy T-2 triol, and de-epoxy T-2 tetraol (Figure 3).[96,97] It was demonstrated that the acute toxicity of de-epoxy metabolites of T-2 was significantly less toxic to brine shrimp than were the corresponding epoxy analogues.[97] Suspensions of microflora obtained from the feces of different animals were incubated anaerobically with T-2.[98] It was completely biotransformed by rat cecal microflora to predominantly C-4 and C-15 deacylated and de-epoxy products, including HT-2 and T-2 triol (Figure 2) and de-epoxy HT-2 and de-epoxy T-2 triol (Figure 3). The major product, de-epoxy HT-2, was the result of epoxide reduction and C-4 deacetylation. The lack of T-2 C-8 ester hydrolysis by rat cecal microflora confirms that products from ester cleavage in whole animal studies are solely the result of mammalian metabolism by carboxyesterases and not microflora in the gut.[98] T-2 incubated with crossbred fistulated steer rumen fluid in vivo can be converted to 3-acetyl T-2, 3-acetyl HT-2, and HT-2 (Figure 2) via acetylation and deacetylation.[99]

Some metabolites of T-2 are equally as toxic as T-2 itself;[100] therefore, much effort has been spent on metabolism studies of

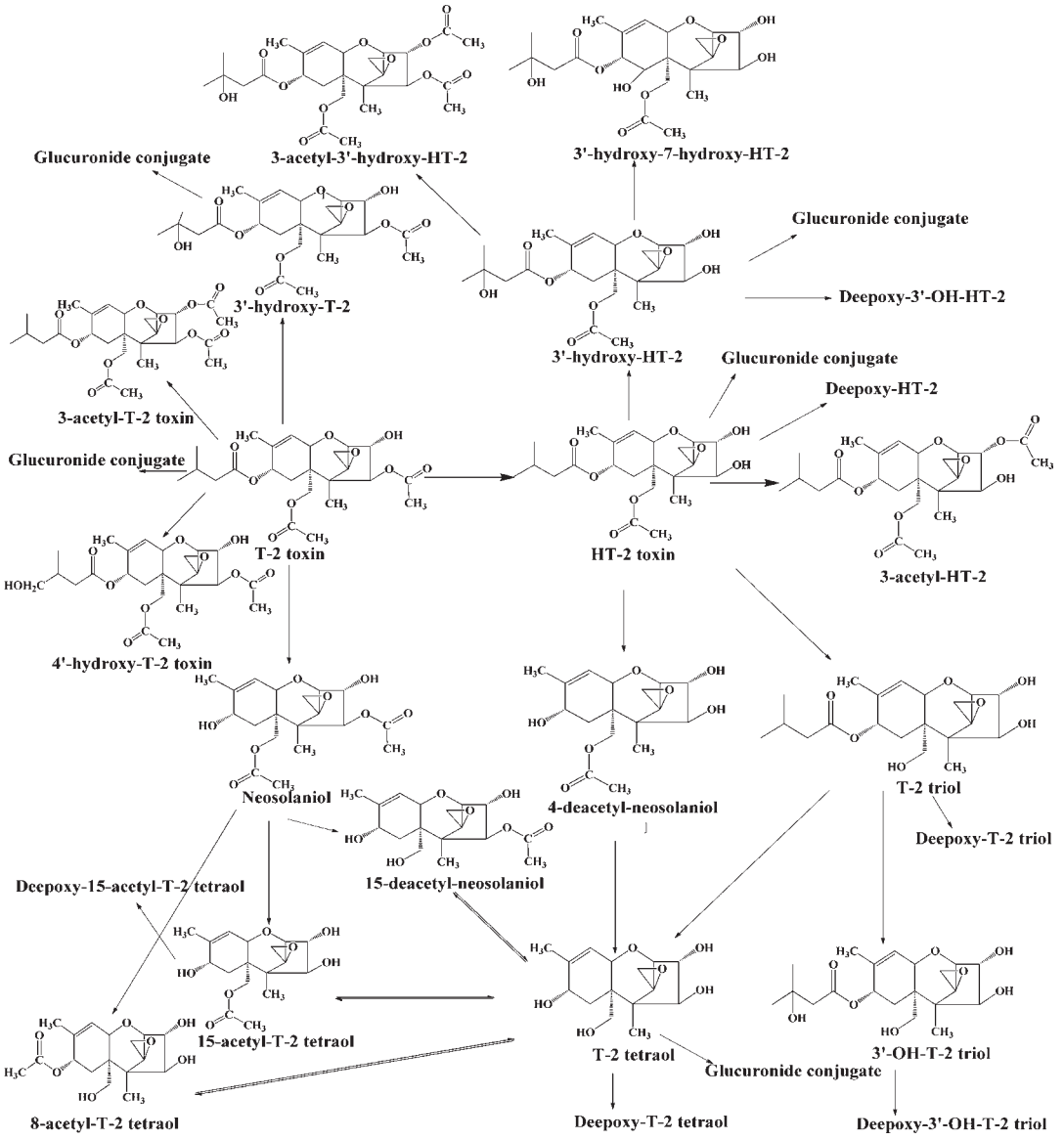



**Figure 4.** Potential metabolic pathways of T-2 toxin in vivo and in vitro.

T-2. The metabolic conversion product of T-2, 3′-hydroxy T-2 (Figure 2), had the same level of apoptosis-inducing activity and DNA fragmentation in the mouse thymus as did T-2.[100] The acetyl group at the C-4 position and the isovaleryl or 3′-hydroxyisovaleryl groups at the C-8 position of T-2 are important structural characteristics for inducing cell death through apoptosis in the thymus.[100] The known metabolites of T-2 are shown in Figure 2, and the metabolic pathways are shown in Figure 4. The metabolites of T-2 are, in general, species dependent.

## ■ ANALYTICAL SEPARATION AND DETERMINATION METHODS

As one of the most acutely toxic chemical species among the trichothecenes, T-2 and some of its metabolites are toxic to animals and to humans. Animals and humans are affected through different routes of exposure to T-2, and T-2 can cause acute and chronic toxicity. Unfortunately, there are no effective treatments for T-2 toxicity. Therefore, it is important to avoid exposure to T-2 toxin. Consequently, rapid, sensitive, and accurate analytical methods for the detection of T-2 in various matrices are needed to protect consumers from unnecessary exposure to T-2.

**Extraction and Cleanup.** Most methods used for T-2 determination rely on extraction and cleanup steps because of the presence of interfering substances from the sample matrix. However, some enzyme-linked immunosorbent assay (ELISA) methods may need only a dilution step.[101,102] Methods used to extract T-2 from a biological matrix usually depend upon the structure of the toxin. The increased polarity of mycotoxins and metabolites (e.g., HT-2) requires a higher percentage of water in the extraction solvent. Mixtures of acetonitrile/water (85:15, v/v) are often used as the extraction solvent for simultaneous extraction and determination of trichothecenes, including T-2.[103−105] Also, methanol, ethyl acetate, phosphate-buffered saline (PBS), and dichloromethane can be used as extraction solvents.[106−109]

Conventional extraction of organic analytes from tissue is usually initiated with a homogenization step, followed by tedious

liquid−liquid extraction procedures and sample cleanup followed by enrichment. However, often large amounts of sample and solvent are required. Matrix solid-phase dispersion (MSPD) is a microscale extraction technique requiring less sample and low volumes of solvents. MSPD extraction procedures for T-2 demonstrated good performance with reduction of solvent volume and extraction time.[110,111] On the other hand, when the amount of sample becomes too small, the sample is not representative of the total amount of material to be sampled, due to the variability of mycotoxin pollution. For this reason, the choices of cleanup procedure and sample size are very important.

Cleanup is an essential step in T-2 chromatographic analysis, because the purity of the sample affects the sensitivity of the results, especially at trace levels. Solid-phase extraction (SPE) is a common cleanup procedure used for T-2 purification prior to analysis by chromatography. Cleanup procedures that use SEP-PAK cartridges (reverse-phase $C_{18}$, Oasis HLB) are widely implemented in T-2 analysis and exhibit good performance traits.[110,112] MycoSep cartridges, which contain charcoal, Celite, and alumina, are multifunctional and are frequently used for T-2 purification. MycoSep 227 columns retain T-2 and its metabolites very well and resulted in high recoveries and a lower limit of detection (LOD).[103,104,109,113,114]

**High-Performance Liquid Chromatography (HPLC).** HPLC with UV detection is generally not suitable for T-2 detection because of the weak UV absorption of T-2, whereas a fluorescence detector (FLD) provides high sensitivity, selectivity, and repeatability after derivatization. Because T-2 and HT-2 are not fluorescent, derivatization is necessary to develop sensitive HPLC-FLD methods. 1-Anthroylnitrile (1-AN) is frequently used as an efficient fluorescence labeling reagent for the determination of T-2.[115−117] Some studies have indicated that the LOD for T-2 in grains reached 5 $\mu$g/kg after derivatization with 1-AN.[116,117] HPLC-FLD analysis following derivatization of T-2 with 1-AN was suitable for the detection of T-2 in eggs with an LOD as low as 1 $\mu$g/L,[118] whereas analysis in cereal resulted in an LOD of 8 $\mu$g/kg.[115] In addition, coumarin-3-carbonyl chloride, a highly fluorescent fluorophore, has also been used as a labeling reagent.[109,119] A method for the simultaneous determination of T-2, HT-2, and some other trichothecenes in wheat using coumarin-3-carbonyl chloride as derivatization reagent showed good recovery and high accuracy, with LOD values as low as 0.6 and 0.4 $\mu$g/kg for T-2 and HT-2, respectively.[109] The LOD of that study was much lower than the LOD (10 $\mu$g/kg) observed during a previous analysis of T-2 in cereals using coumarin-3-carbonyl chloride.[119]

Three fluorescent labeling reagents, 1-naphthoyl chloride (1-NC), 2-naphthoyl chloride (2-NC), and pyrene-1-carbonyl cyanide (PCC), were used to detect T-2 and HT-2 with 4-dimethylaminopyridine (DMAP) used as catalyst under mild reaction conditions in toluene.[108] The result showed a wide linear range, good stability of the derivatives, and good repeatability of the reaction. Derivatization with 1-NC, 2-NC, and PCC resulted in LODs for T-2 in cereals of 10.0, 6.3, and 2.0 $\mu$g/kg, respectively, whereas the LODs for HT-2 were 6.3, 2.3, and 2.8 ng/g, respectively. The labeling reagents PCC and 2-NC demonstrated better sensitivity and repeatability than did 1-AN. Preliminary studies showed the applicability of 2-NC and PCC as fluorescent labeling reagents for the simultaneous determination of T-2 and HT-2 in cereal grains by HPLC-FLD.[108]

Liquid chromatography−tandem mass spectrometry (LC-MS/MS) is a widely used technique for the simultaneous determination of T-2 and HT-2 because of its high sensitivity and selectivity. Also, MS/MS provides the highest reliability in analyte identification and satisfies the European Union's technical criteria for analysis.[120] In addition, the high selectivity of MS/MS theoretically leads to less sample purification. However, matrix effects can influence the analyte ionization by means of signal suppression or enhancement, which can affect the overall accuracy of the determination. Sometimes purification of samples remains a key step for a reliable quantitative analysis.[121] Various approaches have been proposed to overcome matrix effects observed during T-2 analysis by LC-MS/MS, such as matrix-matched calibration, using isotope-labeled standards, SPE purification, and extract dilution.[103,122−125] An LC-APCI-MS/MS (LC−atmospheric pressure chemical ionization-MS/MS) method for the simultaneous determination of T-2 and HT-2 in maize was reported using MycoSep 227 for sample cleanup.[113] This method resulted in a wide range of detection with LODs for T-2 and HT-2 of 0.2 and 0.1 $\mu$g/kg, respectively. In another LC-APCI-MS/MS method used for analysis of T-2 and HT-2 in cereals and cereal-based foods (e.g., wheat, barley, maize, infant semolina, and infant biscuits), Oasis HLB cartridges were used for sample cleanup. Depending on the type of sample, the LODs ranged from 0.4 to 1 $\mu$g/kg and from 0.4 to 1.7 $\mu$g/kg for T-2 and HT-2, respectively.[121] LC-ESI-MS/MS (LC−electrospray ionization−MS/MS) methods used for simultaneous determination of T-2 and HT-2 in cereals have been successfully used in recent years.[103,121,126] A reliable, sensitive, and selective LC-ESI-MS/MS method was developed to determine T-2 and HT-2 in wheat flour.[121] This analytical method was characterized by good linearity ($R^2 > 0.991$), a wide range of detection (1−200 $\mu$g/L), and an LOD of 10 $\mu$g/kg for T-2 and HT-2.[121]

In contrast to the quadrupole or ion trap instruments, time-of-flight mass spectrometry (TOF-MS) instruments provide enhanced sensitivity and accuracy in the full mass range due to higher mass resolution.[127] This results in increased reliability in the identification of analytes by estimating the elemental composition of each ion.[128−130] In addition, TOF-MS analysis has the advantage that quantitation can be performed on any observed ion in the acquired mass range.[131,132] All of the properties of LC-TOF-MS analysis showed great advantages in the screening for mycotoxins with different molecular weights. An LC-APCI-TOF-MS method, with real-time reference mass correction, was developed for the simultaneous determination of T-2, HT-2, and other trichothecenes in corn, wheat, cornflakes, and biscuits.[114] The samples were extracted with acetonitrile/water (85:15, v/v) and then purified by MycoSep 226 before determination. The LODs for T-2 toxin in wheat, biscuit, corn, and cornflake were 0.2 $\mu$g/kg, whereas the LODs for HT-2 were 0.6, 0.5, 0.9, and 0.6 $\mu$g/kg, respectively.[114]

**Gas Chromatography (GC).** GC methods are also generally used for trichothecene separation, identification, and quantification. Detection is generally performed using MS, a flame ionization detector (FID), or an FLD.[111,133,134] *N*,*N*-Dimethyltrimethylsilylcarbamate is a common derivatization reagent used to produce trimethylsilyl (TMS) derivatives of trichothecenes.[135] A GC method for T-2 analysis was developed consisting of an MSPD cleanup step, followed by analyte derivatization with TMS, and the method used either FID or MS detection.[111] The LODs for T-2 using FID detection were 0.3 and 0.47 $\mu$g/kg for semolina and corn grits, respectively. The LODs using MS detection were 0.05 $\mu$g/kg for both semolina and corn grits.[111] A gas chromatography−selected ion monitoring−mass spectrometry method (GC-MS-SIM) was

used to determine seven trichothecenes in cereal samples, including T-2, and the LOD for T-2 was 5 $\mu$g/kg.[136] A comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOF-MS) method was used for the analysis of trichothecenes in wheat grain.[137] T-2 and HT-2 were quantified using trifluoroacetic acid anhydride (TFAA) and TMS derivatization after extraction without further cleanup steps. The separation of trichothecenes from matrix constituents was possible due to the vast peak capacity of two-dimensional gas chromatography. LODs for T-2 and HT-2 were 20 and 10 $\mu$g/kg, respectively.[137]

**Immunoassays.** Immunoaffinity column chromatography (IAC) uses analyte-specific antibodies similar to immunoassays, is based upon molecular recognition, and can provide an alternative method to isolate, purify, and concentrate target analytes from complex sample matrices.[138] IACs use antibodies that are chemically bound to the column material, which can then selectively bind the analyte of interest from crude extracts. During the cleanup step, interferences can be washed from the column while the analyte is immobilized on the antibody. Finally, the analyte can be eluted from the column for determination. IACs can result in fewer matrix effects and lower LODs.[115−117] T-2 and HT-2 were determined in cereals, including oats.[115] The limit of quantitation (LOQ) was determined to be 8 $\mu$g/kg.[115] In other studies, T-2 and HT-2 were determined in cereal grains with an LOD of 5 $\mu$g/kg [116,117] and 3 $\mu$g/kg,[116] respectively. However, the overall effectiveness of IACs is based on the quality of the antibody used. Therefore, the antibody plays an important role in the performance of IACs used for cleanup procedures.

The ELISA is widely used for T-2 monitoring due to the relatively low cost and ease of application.[133] ELISAs can provide fast and inexpensive screening of trichothecenes and are generally based on a competitive assay format using an antibody specific for the target compounds and conjugates of an enzyme and the target molecule. As an immunology-based assay system, ELISAs can allow specific recognition of trichothecenes without complicated sample preparation.[139] An ELISA system for the detection of T-2 and HT-2 in wheat kernels using monoclonal antibodies specific for acetyl T-2 was developed. After extraction with acetonitrile/water (85:15, v/v), the samples were centrifuged for 5 min, and the supernatants were stored at 4 °C until used. The stored solutions were completely dried, acetylated, evaporated to dryness again, and dissolved in 100 $\mu$L of ethanol and 900 $\mu$L of PBS. Sample recovery using the ELISA ranged from 93.8 to 112.0%, and the LOD for the sum of T-2 and HT-2 was 30 $\mu$g/kg.[139]

## CONCLUSIONS

In summary, current knowledge of T-2 toxicity, metabolism, and analytical methods used for the analysis of T-2 is described here. Although the metabolites of T-2 toxin and their associated structures are well-known, the toxicity of these compounds to animals and humans is not clearly understood because of the limited scientific investigations describing their toxicity in animals. The mechanism(s) responsible for T-2 and its metabolites' apoptosis is still unknown. Also, some of the same T-2 and HT-2 toxic effects observed in animals may affect humans. Consequently, further studies on the toxicology of T-2 are required in both animals and humans, and improved analytical methods should be a goal for future investigations. Research studies focused on the analysis and detection of T-2 and HT-2 toxins with a large array of detection techniques have been discussed, but new methods are needed to achieve higher sensitivity and accuracy during analyses of T-2 and HT-2. The application of MS/MS conjugated with other tools such as GC and LC have decreased the LOD in recent years. In addition, the development of rapid assays and methods for simultaneous monitoring of multiple toxins are current trends. In the future, new and improved techniques will be used to investigate the toxicity of T-2 and its metabolism in animals, and advanced screening methods are needed to fully evaluate the exposure of humans to T-2 and its metabolites.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +86-10-6273-1201. Fax: +86-10-6273-1032. E-mail: suxia@cau.edu.cn.

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## ABBREVIATIONS USED

AIF apoptosis-inducing factor
1-AN 1-anthroylnitrile
ATA alimentary toxic aleukia
Bax Bcl-2-associated X protein
DMAP 4-dimethylaminopyridine
DMSO dimethyl sulfoxide
DNA DNA
DOPAC 3,4-dihydroxyphenylacetic acid
ELISA enzyme-linked immunosorbent assay
EU European Union
FasL Fas/Fas ligand
FID flame ionization detector
FLD fluorescence detector
GC×GC-TOF-MS two-dimensional gas chromatography−time-of-flight mass spectrometry
GC-MS gas chromatography−mass spectrometry
GC-MS-SIM gas chromatography−selected ion monitoring mass spectrometry
GLC gas−liquid chromatography
HPLC high-performance liquid chromatography
IAC immunoaffinity column
iNOS induced nitric oxide synthase
KBD Kashin−Beck disease
LC-APCI-MS/MS liquid chromatography−atmospheric pressure chemical ionization tandem mass spectrometry
LC-APCI-TOF-MS liquid chromatography−atmospheric pressure chemical ionization time-of-flight mass spectrometry
LC-ESI-MS/MS liquid chromatography−electrospray ionization tandem mass spectrometry
LC-MS/MS liquid chromatography−tandem mass spectrometry
LDH lactate dehydrogenase

$LD_{50}$ median lethal dose
LOD limit of detection
LOQ limit of quantitation
MSPD matrix solid phase dispersion
1-NC 1-naphthoyl chloride
2-NC 2-naphthoyl chloride
NO nitric oxide
PB phenobarbital
PBS phosphate-buffered saline
PCC pyrene-1-carbonyl cyanide
ROS reactive oxygen species
RT-PCR reverse transcription−polymerase chain reaction
SPE solid-phase extraction
TFAA trifluoroacetic acid anhydride
TLC thin-layer chromatography
TMS trimethylsilyl
TOF-MS time-of-flight mass spectrometry

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