Direct Enzyme-Linked Immunosorbent Assays for the Detection of the 8-Ketotrichothecene Mycotoxins Deoxynivalenol, 3-Acetyldeoxynivalenol, and 15-Acetyldeoxynivalenol in Buffer Solutions

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Polyclonal antisera against deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-AcDON) were obtained after rabbits were immunized with human serum albumin conjugates of 3,15-*O*-dihemisuccinyl-DON and 15-*O*-hemiglutaryl-3-AcDON, respectively. The specifity and sensitivity of these antisera were tested by using 3,15-*O*-dihemiglutaryl-DON or 15-*O*-hemiglutaryl-3-AcDON coupled to horseradish peroxidase as the enzyme-linked toxins in a competitive direct enzyme-linked immunosorbent assay (ELISA). The 50% inhibition level was used for the determination of the cross-reactivities. The anti-DON antiserum showed strong cross-reactivity (relative to DON = 1.0) with 3-AcDON (100.0) and 15-acetyldeoxynivalenol (15-AcDON, 1.1); minor cross-reactions were observed with nivalenol (0.063) and fusarenon X (0.016). The anti-3-AcDON antiserum was most specific for 3-AcDON and had only minor cross-reactions (relative to 3-AcDON = 1.0) with acetyl T-2 (0.025), 15-AcDON (0.016), and T-2 tetrol tetraacetate (0.013). When the antiserum against DON was employed in the direct ELISA, detection limits for DON, 3-AcDON, and 15-AcDON in buffer were 1.0, 0.05, and 1.5 ng/mL, respectively. The detection limit for 3-AcDON in the direct 3-AcDON ELISA was 0.1 ng/mL. After hydrolysis, 3-AcDON gave a response similar to that of DON in the DON ELISA, but levels up to 100 ng/mL were no longer detected by the 3-AcDON ELISA, thus providing a possibility to discriminate between these toxins.

INTRODUCTION

Deoxynivalenol (DON, vomitoxin) and 3-acetyldeoxynivalenol (3-AcDON) were first isolated and characterized in the early 1970s from *Fusarium*-infected cereals in Japan (Morooka et al., 1972; Yoshizawa and Morooka, 1973). The natural occurrence of 15-acetyldeoxynivalenol (15-AcDON) in corn infected with *Fusarium* spp. was first reported by Abbas et al. (1986). Chemically, these mycotoxins are characterized by their 12,13-epoxytrichothec-9-en-8-one skeleton, with R_2 = H and R_5 and R_6 = O (Figure 1; Table I).

The occurrence of trichothecene mycotoxins in food and feedstuff seems to be a worldwide problem (Tanaka et al., 1988). Adverse effects of DON, 3-AcDON, and 15-AcDON include dose-dependent induction of feed refusal, diarrhea, and emesis in lifestock, especially in swine (Vesonder et al., 1973; Yoshizawa and Morooka, 1974; Coppock et al., 1985; Abbas et al., 1986). Like all trichothecene mycotoxins, they possess significant cytotoxic activity (Ueno, 1983), which implies immunosuppressive effects (Tryphonas et al., 1986; Pestka et al., 1989).

Most analytical work has been done on DON, but 3-AcDON and 15-AcDON, being precursors of DON synthesis in the secondary metabolism of *Fusaria* (Miller et al., 1983a), are frequently isolated together with their parent alcohol (Apsimon et al., 1990) and thus may be associated when the presence of DON could not account for the entire degree of adverse effects, e.g., feed refusal (Foster et al., 1986). Physicochemical methods currently used for analysis of DON and its acetylated analogues, ranging in sophistication from thin-layer chromatography (TLC) (Miller et al., 1983b) to gas chromatography (Kamimura et al., 1981), require extensive and time-consuming sample cleanup prior to analysis. Furthermore, the efforts necessary for sufficient sample preparation increase greatly if different toxins should be detected simultaneously (Pestka, 1989).

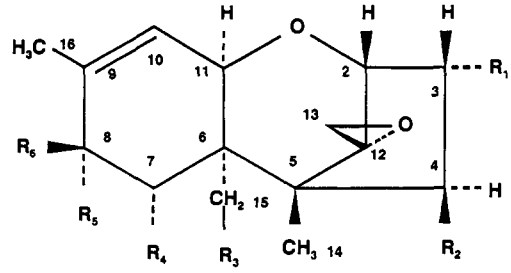

Figure 1. Structure and numbering system of the trichothecene skeleton. The side-chain residues for the trichothecenes used in this study are shown in Table I.

Immunological approaches for trichothecene analysis have proved to be good alternatives in aspects of sensitivity and performance (Chu, 1986, 1990). Few ELISA methods have been reported for DON (Casale et al., 1988; Xu et al., 1988; Mills et al., 1990) and 3-AcDON (Kemp et al., 1986) and none for 15-AcDON. This paper describes the development of direct enzyme-linked immunosorbent assays that allow the simultaneous detection of DON, 3-AcDON, and 15-AcDON as well as a preliminary differentiation between 3-AcDON and DON via a simple hydrolysis procedure.

MATERIALS AND METHODS

Materials. Nivalenol and fusarenon X were purchased from Waco Chemicals, Neuss, Federal Republic of Germany. DON, 3-AcDON, 15-AcDON, and all other trichothecenes were obtained from Sigma Chemicals, Deisenhofen, Federal Republic of Germany. Succinic anhydride, glutaric anhydride, human serum albumin (HSA), horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), and all other chemicals (highest grade available) were also obtained from Sigma. Microtitration plates (Nunc immunoplate I) were purchased from Nunc, Wiesbaden,

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Table I. Side-Chain Residues (Figure 1) of the Trichothecenes Tested

toxin	side chain[a]					
	R_1	R_2	R_3	R_4	R_5	R_6
Group A						
T-2 toxin	OH	Ac	Ac	H	IV	H
acetyl-T-2	Ac	Ac	Ac	H	IV	H
HT-2	OH	OH	Ac	H	IV	H
T-2 triol	OH	OH	OH	H	IV	H
T-2 tetrol	OH	OH	OH	H	OH	H
T-2 tetrol tetraacetate	Ac	Ac	Ac	H	Ac	H
diacetoxyscirpenol	OH	Ac	Ac	H	H	H
15-acetoxyscirpenol	OH	OH	Ac	H	H	H
3-acetyldiacetoxyscirpenol	Ac	Ac	Ac	H	H	H
neosolaniol	OH	Ac	Ac	H	OH	H
verrucarol	H	OH	OH	H	H	H
Group B						
trichothecolone	H	OH	H	H	=O	
nivalenol	OH	OH	OH	OH	=O	
fusarenon X	OH	Ac	OH	OH	=O	
nivalenol tetraacetate	Ac	Ac	Ac	Ac	=O	
deoxynivalenol	OH	H	OH	OH	=O	
3-acetyldeoxynivalenol	Ac	H	OH	OH	=O	
15-acetyldeoxynivalenol	OH	H	Ac	OH	=O	

[a] Ac, $OCOCH_3$ (acetate); IV, $OCOCH_2CH(CH_3)_2$ (isovalerate).

Table II. Thin-Layer Chromatographic Analysis of Deoxynivalenol (DON), 3-Acetyldeoxynivalenol (3-AcDON), 15-Acetyldeoxynivalenol (15-AcDON), Fusarenon X, and Their Derivatives

toxin	R_f value in solvent system[a]		detection method[b]		
	1	2	A	B	C
DON	0.43	0.62	+	+	–
3,15-*O*-dihemisuccinyl-DON	0.70	0.12	+	+	+
3,15-*O*-dihemiglutaryl-DON	0.77	0.42	+	+	+
3-AcDON	0.80	0.82	+	+	–
15-*O*-hemiglutaryl-3-AcDON	0.86	0.72	+	nd	+
15-AcDON	0.68	nd	+	nd	–
fusarenon X	0.52	nd	+	+	nd
fusarenon X oxime	0.33	nd	–	+	nd
nivalenol tetraacetate	0.89	nd	–	+	nd

[a] 1, ethyl acetate/*n*-hexane/acetic acid 75:25:5; 2, chloroform/methanol 90:10. [b] A, blue fluorescence (366 nm) after spraying with 20% aluminum chloride; B, blue fluorescence (366 nm) after derivatization with nicotinamide/acetylpyridine; C, yellow color after spraying with 0.04% bromcresol purple; nd, not determined; + and –, positive and negative reaction, respectively.

FRG. Nivalenol tetraacetate (3,4,7,15-tetraacetylnivalenol) was synthesized by reaction of fusarenon X with acetic anhydride according to the method of Tatsuno (1968). Fusarenon X oxime was prepared by reaction of fusarenon X with carboxymethoxylamine as described by Märtlbauer et al. (1989).

Derivatization of DON and 3-AcDON. 3,15-*O*-Dihemisuccinyl-DON (3,15-HS-DON), 3,15-*O*-dihemiglutaryl-DON (3,15-HG-DON), and 15-*O*-hemiglutaryl-3-AcDON (15-HG-3-AcDON) were prepared by reaction of DON or 3-AcDON with succinic anhydride or glutaric anhydride, respectively. Briefly, DON (10 mg) or 3-AcDON (20 mg) was dissolved with the respective acid anhydride (200 mg) in 1 mL of pyridine and heated at 100 °C in a steam bath for 2 (3-AcDON derivative) or 8 h (DON derivatives). Pyridine was removed under N_2 pressure and the residue dissolved in $CHCl_3$ (15 mL). After extraction with 0.1 M HCl (3 × 10 mL) the organic phase was dried under vacuum in a rotary evaporator and the remaining redissolved in 2 mL of methanol.

After TLC on silica gel, toxins and derivatives were visualized with selective spray reagents (Table II). Carboxyl groups in the toxin derivatives were detected by spraying the developed plates with a solution of 0.04% bromcresol purple in ethanol/water (1:1 by volume, pK_{ind} = 6.1). The chemical ionization mass spectrum of the DON-succinyl derivative showed m/z 497 as the protonated molecular ion and the adduct ions m/z 525 $(M + C_2H_5)^+$ and 537 $(M + C_3H_5)^+$, formed in the methane plasma. The structures of the glutaryl derivatives were characterized by 400-MHz ^1H nuclear magnetic resonance (^1H NMR) spectroscopy (see Results and Discussion).

Synthesis of Conjugates. The toxin derivatives were coupled to HSA and HRP using either an activated ester (AE) or a mixed anhydride (MA) method, described by Kitagawa et al. (1981) and Gendloff et al. (1986), respectively. The toxin derivatives were reacted with HSA in molar ratios of 50:1 and 100:1 employing AE and MA, respectively; for the HRP conjugates, molar ratios were 10:1 (AE) and 20:1 (MA). Via the AE method, 3,15-HS-DON–HSA and 15-HG-3-AcDON–HRP were synthesized; 3,-15-HG-DON–HRP and 15-HG-3-AcDON–HRP were prepared using the MA. The conjugation ratios (moles of toxin/mole of HSA) were estimated from the number of unreacted amino groups (Habeeb, 1966). The enzyme concentration of the toxin–HRP conjugates was quantified by determination of the absorbance at 403 nm.

Production of Antisera. For use as immunogens, 250 μg of 3,15-HS-DON–HSA or 15-HG-3AcDON–HSA was emulsified in 2 mL of Freund's complete adjuvant/distilled water (3:1 by volume). Rabbits (female chinchilla bastard, three each for the DON and the 3-AcDON conjugates, respectively) were immunized by using multisite intradermal injections (Nieschlag et al., 1973). Booster injections, using the same composition and amount of immunogen, were given subcutaneously 10 and 20 weeks after the primary injection.

Blood was collected from the *A. auricularis magna*. After centrifugation, the serum was precipitated with $(NH_4)_2SO_4$ (70%, in distilled water) according to the method of Hebert et al. (1973), dialyzed against 3 × 5 L of phosphate-buffered saline (PBS, 0.15 M, pH 7.2), and stored frozen at −20 °C. Antibody titer was determined as described by Märtlbauer et al. (1988).

ELISA Procedure. Appropriate dilutions of the antisera against DON (1:1000) and 3-Ac-DON (1:1400) in 0.05 M carbonate/bicarbonate buffer (pH 9.6) were dispensed to the wells (100 μL) of microtitration plates and incubated overnight at room temperature. Free protein-binding sites of the plates were blocked with 200 μL of sodium caseinate solution (2%, in PBS) for 30 min, and then the plates were washed and made semidry. To each well was added 50 μL of toxin standard in PBS containing 10% methanol (50 μL) and 50 μL of the respective enzyme conjugate, diluted in 1% sodium caseinate/PBS (3,15-HG-DON–HRP, 1.5 μg/mL; 15-HG-3-AcDON–HRP, 10 ng/mL), and the mixture was incubated overnight at 4 °C. The plate was washed, and substrate solution (1 mM TMB and 3 mM H_2O_2 per liter of potassium citrate buffer, pH 3.9) was added (100 μL/well). After 20 min, reaction was stopped with 1 M H_2SO_4 (100 μL/well), and the absorbance at 450 nm was measured by using a microplate reader (MR 5000; Dynatech GmbH, Denkendorf, Federal Republic of Germany). Using the same ELISA procedure, 16 other trichothecenes (Table I) were tested for competition with the respective enzyme conjugates in the assays for DON and 3-AcDON. The relative cross-reactivity was calculated from the concentration of toxin necessary to inhibit 50% binding of enzyme-labeled toxin (in nanomoles per milliliter).

Hydrolysis of 3-AcDON and 15-AcDON. Solutions of 3-Ac-DON in methanol/PBS (50:50, 0.8 mL) were incubated with KOH (0.1 M, 0.1 mL) for 20 min at room temperature. Then the pH was adjusted to approximately 7 with HCl (0.1 M, 0.1 mL). The efficiency of hydrolysis of 3-AcDON at concentrations ranging from 10 to 1000 μg/mL was checked by TLC. After an appropriate dilution in PBS, the mixtures were assayed using the DON ELISA and 3-AcDON ELISA, respectively. Hydrolysis of 15-AcDON was performed according to the same procedure.

RESULTS AND DISCUSSION

To introduce carboxyl moieties into the molecules of DON and 3-AcDON, DON was reacted with succinic anhydride and glutaric anhydride and 3-AcDON only with the latter. No underivatized 3-AcDON was detected by TLC after a reaction time of 2 h; conversion of DON took 8 h, as estimated by TLC. The derivatives ran as single spots with R_f values clearly different from those of the

original toxins. Using selective spray reagents, some characteristics of the derivatives could be elucidated (Table II).

Reaction with $AlCl_3$ is characteristic for trichothecenes having the 7-hydroxy-8-keto side-chain residues (Kamimura et al., 1981). Derivatives missing one of these groups, e.g., fusarenon X oxime (no carbonyl function) or nivalenol tetraacetate (C-7 esterified with acetic acid), showed no fluorescence, whereas the derivatives of DON and 3-AcDON gave a positive reaction, indicating an intact hydroxyl group at R_4. The positive reaction with nicotinamide and 2-acetylpyridine (Sano et al., 1982) confirmed the presence of the trichothecene epoxy group in the derivatives. The chemical ionization mass spectrum of the hemisuccinyl derivative of DON confirmed the expected mass for $M + H^+$ of m/z 497, i.e., 297 for DON plus 200 for two hemisuccinyl side chains. Together with the results from TLC, these data are consistent with 3,15-*O*-dihemisuccinyldeoxynivalenol (3,15-HS-DON).

The data obtained from 400-MHz ^1H nuclear magnetic resonance (^1H NMR) spectra for DON and 3-AcDON agreed with the spectra previously published by Cole and Cox (1981). The ^1H NMR spectra of the glutaryl derivatives of DON and 3-AcDON showed multiple additional chemical shifts in a range from δ 1.9 to 2.5, due to the methyl protons of the glutaryl side chains. For DON and its derivative, a comparison of the signals corresponding to the protons at C3, C15a, and C15b showed chemical shifts of δ 4.54, 3.70, and 3.88, respectively (DON), and of δ 5.19, 4.14, and 4.18 (derivative). For the 3-AcDON derivative, ^1H NMR spectra showed the expected chemical shift of the signals corresponding to the protons at C15a and C15b from δ 3.78 and 3.88 (3-AcDON) downfield to δ 4.27 and 4.29 (derivative). These results indicate esterification of the hydroxyl groups at R_1, R_3 (DON), and R_3 (3-AcDON), respectively. The signal corresponding to H7 and all other chemical shifts were unchanged in the derivatives, compared to those of the original toxins. Thus, in connection with the results obtained from TLC, these data confirmed that 3,15-*O*-dihemiglutaryl-DON and 15-*O*-hemiglutaryl-3-AcDON derivatives were formed.

Toxin–protein conjugates were prepared by coupling 3,15-HS-DON and 15-HG-3-AcDON to HSA (for use as immunogens) and 3,15-HG-DON and 15-HG-3-AcDON to HRP (for use as enzyme-linked antigens). To avoid unspecific antibody–enzyme binding due to identical epitopes in the immunogen and the enzyme conjugate, which may be introduced through the coupling reagents (Gendloff et al., 1986), toxin–HSA conjugates and toxin–HRP conjugates were produced by using different coupling methods.

On the basis of the determination of free amino groups, it was calculated that the conjugation ratios (moles of toxin derivative per mole of HSA) were approximately 15:1 for 3,15-HS-DON–HSA (AE method) and 25:1 for 15-HG-3-AcDON–HSA (the amount of toxin derivative bound to HRP was not determined).

Antibodies could be detected in the sera of all rabbits immunized with the 15-HG-3-AcDON–HSA conjugate as early as 4 weeks after the initial exposure, and the maximum serum titer (1:1 200 000) was obtained from rabbit 1 at week 26, whereas only one of the rabbits immunized with 3,15-HS-DON–HSA gave sufficient immune response after the first booster injection (maximum serum titer, 1:52 000, at week 18). These results agree with those of other authors who reported difficulties in preparing antibodies against DON (Zhang et al., 1986; Casale et al., 1988), while 3-AcDON conjugates have proved to be good immunogens (Kemp et al., 1986).

Table III. Cross-Reactivity of Deoxynivalenol (DON) and 3-Acetyldeoxynivalenol (3-AcDON) Antisera to Various Trichothecenes

	cross-reactivity[b]	
toxin[a]	DON antiserum	3-AcDON antiserum
deoxynivalenol	1	<0.001
3-acetyldeoxynivalenol	100	1
15-acetyldeoxynivalenol	1.1	0.016
nivalenol	0.063	<0.001
fusarenon X	0.014	<0.001
trichothecolone	0.007	<0.001
acetyl-T-2	<0.001	0.025
T-2 tetrol tetraacetate	<0.001	0.013

[a] Cross-reactivity with the other toxins listed in Table I was below 0.001. [b] Nanomoles of deoxynivalenol (DON antiserum) and 3-acetyldeoxynivalenol (3-AcDON antiserum), respectively, per milliliter required for 50% inhibition/nanomoles of toxin per milliliter required for 50% inhibition.

The conjugation site to the carrier protein and the choice of animal for immunization seemed to be very critical for anti-DON antibody production. Initial attempts in our laboratory, employing the 8-carbonyl function of DON for derivatization with carboxymethoxylamine (CMO), forming an oxime which was then conjugated to HSA, or reaction of DON with *p*-aminobenzoyl hydrazide and subsequent diazocoupling of the resulting hydrazone derivative to keyhole limpet hemocyanin according to the method described by Quarrie and Galfre (1985) for abscisic acid, failed to induce production of specific antibodies in rabbits, mice, and guinea pigs. Sera obtained from mice immunized with the 3,15-HS-DON–HSA immunogen reacted with a 3,15-HS-DON–HRP conjugate, but competitive inhibition of this binding by free DON could not be achieved, whereas the high specific titer in one of the rabbits provided an amount of serum that was sufficient to coat at least 10 000 microtitration plates.

Due to the modification of DON through the esterification of the hydroxyl groups at R_1 and R_3, 3-AcDON and 15-AcDON are strongly recognized by the antiserum against DON. Minor cross reactions were observed with other 8-ketotrichothecenes (Table III). The decreasing affinity of the DON antiserum for 3-AcDON, 15-AcDON, DON, nivalenol, fusarenon X, and trichothecolone suggests that the bridge region (i.e., succinyl) between DON and HSA imitated the acetyl side chaines at R_1 and R_3. Trichothecenes that lack the carbonyl group at C8 (e.g., diacetoxyscirpenol) were not recognized by the antibody. This indicates that the antiserum against DON is most specific for the trichothecene skeleton combined with the carbonyl function at C8 and acyl substituents at C3 and C15.

Derivatization of DON via the hydroxyl groups for synthesis of the immunogen has been found to be the only successful way of producing antibodies against DON. Therefore, it is unlikely that one can successfully prepare anti-DON antibodies without any cross-reaction with acetylated DON analogues. By immunizing mice with a 3-HS-DON–bovine serum albumin conjugate, Casale et al. (1988) prepared a monoclonal antibody against DON which showed higher affinity for 3-AcDON than for DON. Recently, Mills et al. (1991) described the production of antisera against DON, utilizing a 15-hemiglutaryl derivative of DON. No cross-reaction with the other trichothecenes tested has been observed by the authors; however, competitive inhibition by 15-AcDON had not been tested.

Vice versa, the antiserum against 3-AcDON prepared in this study had only weak cross-reaction with 15-Ac-

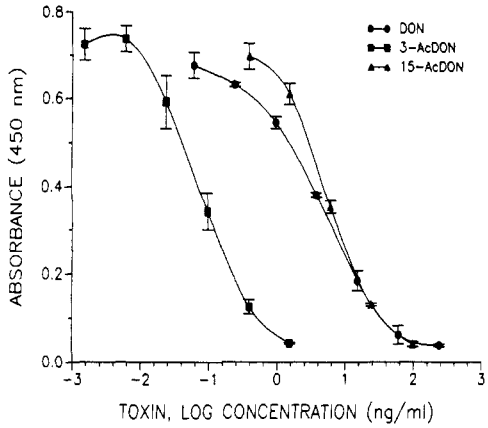

Figure 2. Standard curves of the competitive EIA for the detection of DON, 3-AcDON, and 15-AcDON, utilizing inhibition of DON–HG-HRP conjugate binding by each of these toxins to antibodies obtained after immunization with DON–HS-HSA. Each point represents the mean (± standard deviation) of four replicates.

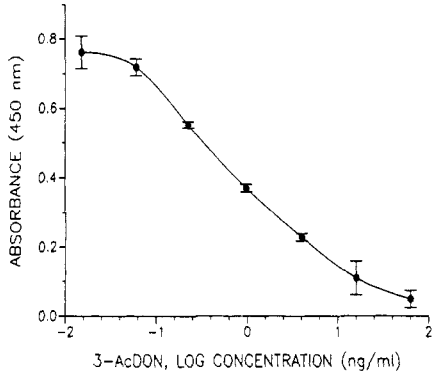

Figure 3. Standard curve of the competitive EIA for the detection of 3-AcDON, utilizing inhibition of 3-AcDON–HG-HRP conjugate binding by 3-AcDON to antibodies obtained after immunization with 3-AcDON–HG-HSA. Each point represents the mean (± standard deviation) of four replicates.

DON, which lacks the acetyl side chain at R_1, and did not react even with microgram quantities of the corresponding alcohol (DON). Only minor cross-reactions were observed with other trichothecenes (Table III). Thus, the 3-AcDON assay can be considered to be very specific for 3-AcDON.

Using toxin standards, dissolved in PBS containing 10% methanol, the detection limits (defined as the zero value minus 2 standard deviations) for DON, 3-AcDON, and 15-AcDON in the direct ELISA for DON were 1.0, 0.05, and 1.5 ng/mL, respectively (Figure 2). The detection limit for 3-AcDON in the direct ELISA for 3-AcDON was 0.1 ng/mL (Figure 3). These results suggested the possibility to discern 3-AcDON from DON and 15-AcDON, respectively, which was important for the quantitative determination of these toxins. The DON assay, with its broad range of specifity, favorably detects these three toxins simultaneously. In the presence of 3-AcDON, however, results from the DON ELISA would lead to an overestimation of the toxin concentration. This problem can be solved by utilizing selective determination of 3-AcDON by the highly specific 3-AcDON ELISA.

Because the acetyl group of 3-AcDON is very susceptible to hydrolysis (Yoshizawa and Morooka, 1975), this toxin was easily converted to DON by incubation with KOH. Up to concentrations of 1 mg/mL, no 3-AcDON was detected after hydrolysis using TLC and spraying with $AlCl_3$, whereas a new spot appeared with a R_f value identical to that of DON. From the detection limit of the TLC method (50 ng/spot), it was calculated that nearly 100% of 3-AcDON had been converted to DON. When assayed in the DON ELISA, 3-AcDON–KOH reaction mixtures gave results corresponding to the reaction product (DON), and no 3-AcDON was detected in the 3-AcDON ELISA at concentrations below 100 ng/mL. These results confirmed that, under the mild conditions used in this study, an efficient conversion of 3-AcDON into DON could be achieved. Conversion of 15-AcDON into DON using this procedure was also nearly quantitative, as estimated using TLC. However, due to the similar sensitivity of the anti-DON antiserum for both toxins, this did not significantly alter the results of the DON ELISA.

Consequently, using the DON ELISA combined with the hydrolysis procedure, the content of DON, 3-AcDON, and 15-AcDON in a solution can be expressed as DON equivalents. This is important considering that acetylated trichothecenes are deacetylated by mammalian microsomal carboxyesterase (Ohta et al., 1978). Therefore, 3-AcDON and 15-AcDON have to be estimated as approximately equivalent to DON in aspects of their toxicity in vivo (Thompson and Wannemacher, 1986). The detection of all acetylated DON analogues known to occur as natural contaminants, by the method described here, needs to be tested for the analysis of food and feed.

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