DETERMINATION OF T-2 AND HT-2 TOXINS IN FEEDSTUFFS
BY HIGH–PERFORMANCE LIQUID CHROMATOGRAPHY
WITH FLUORESCENCE DETECTOR

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Abstract

The procedure for the determination of T-2 and HT-2 toxins in feedstuffs has been developed. The test portion was extracted with methanol–water (80:20, v/v) after adding sodium chloride and cleaned-up through immunoaffinity column. Mycotoxins were quantified by a high-performance liquid chromatography coupled with fluorescence detector ($\lambda_{ex}=381$ nm, $\lambda_{em}=470$ nm) after pre-column derivatisation, which was achieved with 4-dimethylaminopyridine and 1-anthroyl cyanide solutions. The method was validated according to the Commission Regulation (EC) No 401/2006. Average recoveries for the compounds ranged from 94.0% to 106.5% for T-2 toxin and from 80.3% to 102.9% for HT-2 toxin. The limits of detection and quantification for T-2 and HT-2 toxins were 6 and 15 µg kg$^{-1}$, respectively.

Key words: T-2 toxin, HT-2 toxin, feedstuffs, high performance liquid chromatography.

Among mycotoxins, the trichothecenes are the largest group of secondary metabolites produced mainly by Fusarium species. All of them are closely-related sesquiterpenoids with a 12, 13 epoxy ring and a variable number of hydroxyl, acetoxy, or other substituents (4). The most toxic representative of the trichothecenes is T-2 toxin and its deacetylated form - HT-2 toxin. The principle fungi responsible for the production of these mycotoxins are Fusarium sporotrichioides and Fusarium poae, which occur on corn, wheat, barley, oats, rye, and other crops (2, 15, 17).

In animals, the major effect of T-2 toxin is the inhibition of protein synthesis, which is followed by a secondary disruption of DNA and RNA synthesis. The manifestations of toxicosis include signs of weight loss or poor weight gains, beak and mouth lesions, and decreased production of milk and eggs (4, 5, 15). T-2 toxin causes outbreaks of haemorrhagic disease in domestic animals and has been shown to be the causative agent of alimentary toxic aleukia (ATA), a fatal disease, which occurred in Russia in 1942-1947 in people fed overwintered cereals (3, 11, 13).

Nowadays, there are no regulatory limits for T-2 and HT-2 toxins in Europe; however, it is anticipated that these will be agreed in the near future and the legislation will refer to the sum of T-2 and HT-2 (1, 12). These incoming legislations require analytical methods (reliable and sensitive) for rapid determination of these toxins in food and feedstuff products in order to protect consumers from the risk of exposure (9).

This paper presents a reliable and sensitive HPLC-FLD method for the determination of T-2 and HT-2 toxins in feedstuffs.

Material and Methods

Reagents and chemicals. All chemicals were analytical grade. Acetonitrile, methanol (both HPLC grade), and toluene were obtained from J.T. Baker (Germany). Sodium chloride was from P.O.Ch. Gliwice.
(Poland). T-2 toxin, HT-2 toxin, and 4-
dimethylaminopyridine (DMPA) were purchased from Sigma-Aldrich Chemical Company (Germany). 1-
anthroyl cyanide (1-anthroylnitrile, 1-AN) was from WAKO (Germany). T-2 immunoaffinity columns (T-2test™HPLC, Vicam) were from Vicam Inc. (USA). Water (>14 MΩ.cm) was obtained from a Milli-Q system (Millipore, USA).

**Standard and reagent solutions.** Stock standard solutions of T-2 and HT-2 toxins were prepared by dissolving the solid commercial toxins in acetonitrile at concentrations 1,000 and 200 µg mL⁻¹, respectively. Sample spiking solutions and standard curve solutions were prepared from appropriate dilutions of the stock solutions in acetonitrile. DMAP and 1-AN derivatisation reagent solutions were prepared in toluene at concentrations 0.325 and 0.300 µg mL⁻¹, respectively. All standard and reagent solutions were stored at ≤-18°C until analysis.

**Sample preparation and clean-up procedures.** Each sample was ground in the laboratory mill (Glen Mills Inc., USA), sieved (dimension of mesh 1×1 mm), mixed for the achievement of homogeneity, and stored at ≤-18°C until analysis. A 50 g sample was extracted with 100 mL of the methanol-water mixture (80:20, v/v) by shaking on the open-air platform shaker (Barnstead/Lab-Line, USA) for 60 min after adding of 1 g of sodium chloride. Next, the extract was centrifuged (Lab-Laborfachhandel® GmbH, Germany) at 3,000 rpm at 25°C for 10 min. 10 mL of the supernatant were diluted with 40 mL of water and filtered through a glass microfibre filter. Then 10 mL of the diluted extract (=1 g sample equivalent) were passed through an immunooaffinity column (IAC) T-2test™HPLC at a flow rate of 1 drop per 4 s. The column was washed with 10 mL of water and toxins were eluted with two portions of methanol, each of 0.75 mL. The eluate was evaporated to dryness at 50°C in a heating module (Pierce Reacti- Thern III, USA) under a gentle stream of nitrogen.

**Derivatisation procedure.** For fluorescence detection, dryness residue was dissolved in 50 µL of DMAP and 50 µL of 1-AN regent solutions and mixed by vortex for 1 min. The mixture was left to react for 15 min at 50°C in a heater block and then cooled on ice for 10 min. Then the reaction mixture was dried at 50°C under a stream of nitrogen. Finally, the residue was redissolved with 1 mL of acetonitrile-water (7:3, v/v) and injected into the chromatographic column.

**LC-FLD analysis.** Instrumental analysis was performed using an HPLC Series 1100 from Agilent Technologies (Germany), equipped with quaternary pump, autosampler, column oven, and fluorescence detector (FLD, λex=381 nm, λem=470 nm). Software used to manage the chromatographic data was Agilent ver. ChemStation Rev.A.10.01. The chromatographic separation was performed on a Luna 5 µm Phenyl-Hexyl (150×4.6 mm) (Phenomenex) with the column oven temperature at 40°C. The mobile phase consisted of A-water and B-acetonitrile in gradient mode. A gradient elution was performed: 0-5 min - 30% A and 70% B; 15-25 min - 15% A and 85% B; 27-32 min - 30% A and 70% B. The flow rate was 1 mL min⁻¹ and the injection volume was 50 µL.

**Validation.** The method was validated in-house according to the Commission Regulation (EC) No. 401/2006 (1). The calibration curves for T-2 and HT-2 toxins were obtained by preparing mix-standard solutions at six concentrations: 0.015, 0.05, 0.1, 0.2, 0.4, and 0.5 µg mL⁻¹. Each standard solution was injected into chromatographic column three times and the working range was established. Limit of detection (LOD) and limit of quantification (LOQ) were calculated on the basis of signal-to-noise ratio and were: S/N=3 for LOD and S/N=10 for LOQ. The repeatability was determined for four concentrations: 15, 100, 200, and 300 µg kg⁻¹ and each group of fortified samples was analysed during one day. The reproducibility was established by analysing other two groups (six samples in each group) of samples fortified at the concentrations described above, on two different days and by two different analysts. Recoveries were calculated by comparing peak areas of spiked samples with peak areas of standard solutions.

**Results**

All results of the method validation are shown in Table 1. LOD and LOQ for both, T-2 and HT-2, toxins were 6 and 15 µg kg⁻¹, respectively. The calibration curves were linear in the ranges: 15-500 µg and the correlation coefficients were higher than 0.999. The analytes’ peaks were sharp and symmetrical. Retention time for T-2 toxin was 9 min and for HT-2 toxin ~ 18 min. Figs 1, 2, and 3 show the chromatograms of T-2 and HT-2 standard solution at concentration level of 0.2 µg mL⁻¹, blank feedstuff sample, and feedstuff sample spiked with T-2 and HT-2 toxins at concentrations ~ 200 µg kg⁻¹, respectively. The repeatability and reproducibility values for T-2 and HT-2 toxins fulfil the criteria established by Commission Regulation (EC) No. 401/2006 (1). For T-2 and HT-2 toxins in the range of 15-300 µg kg⁻¹, a relative standard deviation of repeatability (RSD) was lower than 30% and relative standard deviation of reproducibility (RSDh) was lower than 50%. Recovery values for all fortified levels, prepared using immunoaffinity columns and derivatisation reagents (DMAP and 1-AN) varied from 94.0% to 106.5% for T-2 toxin and from 80.3% to 102.9% for HT-2 toxin.

**Discussion**

A wide variety of analytical methods exist for the detection and quantitative measurement of trichothecenes in foods and feedstuffs. The majority of the used rapid methods are based on enzyme linked immunosorbent assays (ELISA). However, there are problems associated with many of these tests, namely lengthy incubation periods, lack of sensitivity, and over-estimation (13). Nowadays, high-performance liquid chromatography has become an almost universal method for the determination of trichothecenes.
Table 1
Validation results obtained for the method of determining T-2 and HT-2 toxins in feedstuffs

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycotoxin</td>
<td>T-2 toxin</td>
</tr>
<tr>
<td>LOD, µg kg⁻¹</td>
<td>6</td>
</tr>
<tr>
<td>LOQ, µg kg⁻¹</td>
<td>15</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9992</td>
</tr>
<tr>
<td>Working range, µg kg⁻¹</td>
<td>15-500</td>
</tr>
<tr>
<td>Level of spiked samples, µg kg⁻¹</td>
<td>15 100 200 300 15 100 200 300</td>
</tr>
<tr>
<td>Recovery, %</td>
<td>102.8 106.5 100.3 94.0 80.3 102.9 84.2 83.1</td>
</tr>
<tr>
<td>Repeatability, CV%</td>
<td>7.9 7.0 12.6 8.8 5.8 12.1 10.3 17.4</td>
</tr>
<tr>
<td>Reproducibility, CV%</td>
<td>6.3 10.9 8.6 11.1 5.0 12.5 10.9 12.1</td>
</tr>
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Fig. 1. Chromatogram of standard solution of T-2 and HT-2 toxins at 0.2 µg mL⁻¹.

Fig. 2. Chromatogram of blank feedstuff sample.

Fig. 3. Chromatogram of feedstuff sample spiked with T-2 and HT-2 toxins at the level of 200 µg kg⁻¹.

In different methods of type-A trichothecenes analysis (e.g. T-2 toxin, HT-2 toxin, diacetoxyscirpenol), various combinations of solvents, usually acetonitrile and water, or methanol and water have been used (20). In our study, different methanol-water ratios (80:20, 84:16, and 90:10, v/v) were tested on the basis of previously published reports (14), and the methanol-water ratio: 80:20 (v/v) was found to be the best extraction solvent, while other authors (10, 18, 20) preferred the ratio of methanol-water: 90:10, v/v. An addition of 1 g of sodium chloride to the extraction solvent provided cleaner extracts and reduced significantly interfering peaks in the chromatogram.

Trichothecenes can be extracted from ground solid matrices by shaking or blending with extraction solvents but in literature, the main extraction technique
for determining T-2 and HT-2 toxins is blending (9, 10, 14, 18, 20). During the development of the presently described method, mechanically shaking for 60 min, followed by centrifuging at 3,000 rpm at 25°C was found to be the most appropriate, and the recoveries were higher than those reported by some authors, who applied only blending as an extraction technique (10, 14, 20).

In the literature, solid phase extraction (SPE), column chromatography, immunoaffinity columns, and multifunctional clean-up columns are the most popular techniques (8, 12, 19). Furthermore, the use of immunoaffinity columns provides a number of advantages, which include provision of clean extracts due to the specificity of the antibodies for the single toxin or a group of related toxins, that can be separated by HPLC, and optimal performances in terms of precision and accuracy within the wide range of concentrations and reduction in the use of dangerous solvents (12). Among commercially available SPE columns, MycoSep columns are the most frequently used. They are multifunctional, containing charcoal, celite, and alumina. In addition, a clean-up procedure, based on polymeric reversed-phase SPE columns (Oasis HLB), was used for type-A trichothecenes (9). In our study, IAC from Vicam (T-2test), was applied only blending as an extraction technique (10, 14, 18, 20). During the development of the present study, IAC from Vicam (T-2test) has been also validated with success in the screening of methanol.

The prepared procedure is simple, selective, and reliable. It has been also validated with success in compliance with the requirements of the Commission Regulation (EC) No. 401/2006 and can be used in routine analyses of T-2 and HT-2 toxins in feedstuffs.

References