Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1075 (2005) 151–158
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of T-2 and HT-2 toxins in cereal grains by immunoaffinity clean-up and liquid chromatography with fluorescence detection

Angelo Visconti *, Veronica Maria Teresa Lattanzio, Michelangelo Pascale, Miriam Haidukowski

Institute of Sciences of Food Production, National Research Council, Via G. Amendola 122/O, 70126 Bari, Italy

Received 25 February 2005; accepted 12 April 2005
Available online 28 April 2005

Abstract

A sensitive, precise and accurate method has been developed for the simultaneous determination of T-2 and HT-2 toxins in cereal grains at ppb levels using high-performance liquid chromatography (HPLC) with fluorescence detection and 1-antroylnitrile (1-AN) as labeling reagent after immunoaffinity clean-up. Cereal samples were extracted with methanol/water (90:10, v/v), and the extracts were cleaned-up through commercially available immunoaffinity columns containing monoclonal anti-T-2 antibodies (*T-2 test*™ HPLC, Vicam). T-2 and HT-2 toxins were quantified by reversed-phase HPLC with fluorometric detection (excitation wavelength 381 nm, emission wavelength 470 nm) after derivatization with 1-AN. The monoclonal antibody showed 100% cross-reactivity with both T-2 and HT-2 toxin, and the immunoaffinity column clean-up was effective up to 1.4 μg of both toxins. The method was successfully applied to the analysis of T-2 and HT-2 toxins in wheat, maize and barley. Recoveries from spiked samples with toxin levels from 25 to 500 μg/kg ranged from 70% to 100%, with relative standard deviation generally lower than 8%. The limit of detection of the method was 5 μg/kg for T-2 toxin and 3 μg/kg for HT-2 toxin, based on a signal-to-noise ratio 3:1. HT-2 toxin was detected in ten naturally contaminated wheat samples out of 14 samples analyzed, with toxin levels ranging from 10 to 71 μg/kg; three of them contained also T-2 toxin up to 12 μg/kg.

Keywords: 1-Anthroylnitrile; Cereals; Fluorescence labelling; Immunoaffinity column; Food analysis; HT-2 toxin; T-2 toxin

1. Introduction

T-2 and HT-2 toxins are type-A trichothecene mycotoxins produced by different *Fusarium* species, including *F. sporotrichioides*, *F. poae* and *F. acuminatum*, that may develop on a variety of cereal grains especially in cold climate regions or during wet storage conditions [1,2]. Several surveys have revealed the presence of these toxins in grains such as wheat, maize, oats, barley, rye, rice, beans, and soybean as well as in some cereal-based products [3,4].

While the toxic effects of trichothecenes have been extensively studied in animals, the toxicology of these mycotoxins remains largely unexplored in humans [3,5]. T-2 toxin, which is considered the most toxic trichothecene, is a potent inhibitor of protein synthesis and mitochondrial function both in vivo and in vitro, and shows immunosuppressive and cytotoxic effects [6–8]. Studies on dermal exposure have reported T-2 toxin to have extremely toxic effects on skin and mucous surfaces [9]. T-2 toxin, in vivo, is readily metabolized to HT-2 toxin. Little direct information is available on the toxicity of HT-2 toxin alone, however, the few comparative data available on T-2 and HT-2 toxins indicate that they induce adverse effects with similar potency [3].

Recently, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has evaluated the safety of certain mycotoxins in food, emphasizing the toxic effects of T-2 toxin. The Committee further concluded that the toxic effects of T-2 toxin and its metabolite HT-2 toxin could not be differentiated, and that the toxicity of T-2 toxin in vivo might be

* Corresponding author. Tel.: +39 080 5929333; fax: +39 080 5929373.
E-mail address: angelo.visconti@ispa.cnr.it (A. Visconti).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.04.009

due at least partly to toxic effects of HT-2 toxin. Hence, HT-2 toxin was included in the provisional maximum tolerable daily intake (PMTDI), fixed at 60 ng/kg body weight per day of T-2 and HT-2 toxins, alone or in combination [3].

A recent data collection on the occurrence of *Fusarium* toxins in food (incuding wheat, barley, oats, rye, maize and derivative products) in the European Union (EU), performed within an ad hoc SCOOP project, showed an incidence of positive samples of 20% and 14% out of 3490 and 3032 analyzed samples, for T-2 and HT-2 toxins, respectively [4]. Admissible levels of several *Fusarium* toxins in food are currently under discussion in the EU member states, that have agreed to establish maximum limits for T-2 and HT-2 toxins within July 2007 [10]. Intake estimates indicate clearly that the presence of T-2 and HT-2 can be of concern for public health. Therefore, the development of sensitive and accurate methods for determination of T-2 and HT-2 and collection of more occurrence data on their presence in cereal and cereal products are necessary and of high priority in order to protect consumer health from the risk of exposure to these toxins.

Different methods for the analysis of type-A trichothecenes have been reported. Various combinations of solvents, usually acetonitrile and water, or methanol and water, have been used to extract type-A trichothecenes from grains. Extract clean-up is usually performed on multifunctional MycoSep^{TM} columns, which contain activated charcoal and alumina [11]. Immunoassay tests are, along with TLC, the only screening tests for T-2 and HT-2 toxins that are applicable to routine analysis in cereals. Different immunochemical methods, especially enzyme-linked immunosorbent assay systems (ELISA), have been developed for determination of T-2 toxin alone or in combination with other trichothecenes [12–14]. Gas chromatographic (GC) methods based on electron-capture (ECD), flame ionisation (FID) and mass spectrometric detection (MS) are the most widely used methods for quantitative determination of type-A trichothecenes [11,12,15–19]. Recently, a comparative inter-laboratory study on method performances for trichothecene analysis (including T-2 and HT-2) using gas chromatographic methods clearly showed that method improvements are needed with respect to recovery, accuracy, and precision of the measurements. The main problems derived from matrix interferences inducing enhancement of the trichothecenes response (up to 120%) [20].

HPLC with atmospheric pressure chemical ionisation MS (APCI/MS) has also been used for the determination of T-2 and HT-2 toxins [21–23]. HPLC with UV detection is generally not applicable to type-A trichothecenes lacking of appropriate chromophores in their structure. On the other hand, HPLC methods for T-2 toxin and HT-2 toxin are being developed in which a variety of derivatization reagents are used to allow detection by fluorescence [24–27]. Recently, a method for the determination of T-2 and HT-2 toxins by HPLC with fluorescence detection after derivatization with coumarin-3-carbonyl chloride has been reported [26,27]. Although the method on itself had good sensitivity, when applied to cereal samples showed low toxin recoveries [26].

A new method for the determination of T-2 toxin in cereals by HPLC with fluorescence detection has been recently described [28]. The analytical procedure used immunoaffinity columns containing monoclonal antibodies specific for T-2 toxin for extract clean-up, pre-column derivatization with 1-AN, a commercially available reagent, and HPLC with fluorescence detection for toxin determination. The method allowed the determination of T-2 at ng/g (ppb) levels in various cereals with good accuracy and precision, enabling to quantify the toxin at levels that can occur in naturally contaminated cereal samples.

The aim of this work was to develop a sensitive, reproducible and accurate method for the simultaneous determination of T-2 and HT-2 toxins in cereal grains using immunoaffinity column clean-up and HPLC with fluorescence detection after derivatization with 1-AN. Performances of the immunoaffinity columns with respect to both T-2 and HT-2 toxins, laboratory method validation and its application to naturally contaminated cereals samples are also reported.

2. Experimental

2.1. Chemicals and materials

Acetonitrile, methanol (both HPLC grade) and toluene (for organic residue analysis) were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). T-2 toxin (T-2), HT-2 toxin (HT-2), deoxynivalenol (DON), nivalenol (NIV), T-2 triol, T-2 tetraol, acetyl T-2 toxin, 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (Milan, Italy) and 1-anthroyl cyanide (1-anthroylnitrile, 1-AN) from Wako (Neuss, Germany). Immunoaffinity columns (*T-2 test*^{TM} HPLC) were obtained from Vicam (Watertown, MA, USA). Glass microfiber filters (Whatman GF/A) and paper filters (Whatman no. 4) were obtained from Whatman (Maidstone, UK). HPTLC plates silica gel 60 F 254 and HPTLC plates silica gel 60 without fluorescence indicator (10 cm × 10 cm, 200 μm) were purchased from Merck (Darmstad, Germany).

2.2. Preparation of standard and reagent solutions

Trichothecene stock solutions (T-2, HT-2, DON, NIV, T-2 triol, T-2 tetraol and acetyl T-2 toxin) were prepared by dissolving the solid commercial toxin in acetonitrile (1 μg/μl). T-2 and HT-2 standard solutions for HPLC calibration or spiking purposes were prepared by dissolving adequate amounts of the stock solution, previously evaporated to dryness under nitrogen stream, in acetonitrile.

DMAP and 1-AN solutions were prepared in toluene at concentrations of 0.325 and 0.3 μg/μl, respectively. Tri-

chothecene and reagent solutions were stored at −20 °C and warmed to room temperature before use.

2.3. Apparatus

The HPLC apparatus consisted of a Perkin-Elmer (Norwalk, CT, USA) Series 200 LC binary pump equipped with a Rheodyne (Cotati, CA, USA) model 7125 injection valve, a Jasco (Tokyo, Japan) FP-1520 fluorometric detector and a Turbochrom 4.0 data system (Perkin-Elmer). The analytical column was a Phenyl-Hexyl Luna® (150 mm × 4.6 mm, 5 μm particles) (Phenomenex, USA), preceded by a Supelco guard filter (0.5 μm, Supelco, Bellefonte, PA, USA). The flow rate of the mobile phase was 1.0 ml/min. A binary gradient was applied as follows: the initial composition of the mobile phase, 70% acetonitrile/30% water, was kept constant for 5 min, then the acetonitrile content was linearly increased to 85% in 10 min, and kept constant for 10 min. Finally, to clean the column the amount of acetonitrile was increased to 100% in 2 min and kept constant for 5 min.

The excitation and emission wavelengths of the fluorometric detector were set at 381 and 470 nm, respectively.

2.4. Sample preparation and immunoaffinity clean-up

Fifty grams of cereal samples, finely ground by a Model MLI-204 Bühler (Milan, Italy), were weighed into a blender jar, added with 1 g NaCl, and extracted with 100 ml methanol/water (90:10, v/v) by blending at high speed for 2 min with a Sorvall Omnimixer (Dupont Instruments, Newtown, CT, USA). The mixture extract was filtered through filter paper. Ten millilitres of filtrate were collected and mixed with 40 ml of distilled water. The diluted extract was filtered through a glass microfibre filter and the filtrate collected. Ten millilitres of filtrate (equivalent to 1.0 g sample) were passed through the T-2 immunoaffinity column at a flow rate of about one drop per second, followed by 10 ml distilled water at one to two drops per second. T-2 and HT-2 were then eluted with 1.5 ml methanol and collected in a 4-ml screw-cap amber vial. The eluted extract was evaporated under a stream of air at ca. 50 °C in a heating block and the dried residue derivatized with 1-AN as reported below.

2.5. Derivatization procedure and HPLC determination

The derivatization procedure was performed as previously described for T-2 toxin by Pascale et al. [28]. Fifty microliters of DMAP solution followed by 50 μl of 1-AN reagent were added to the dried residue. The vial was closed 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 in ice for 10 min. The reaction mixture was dried under a stream of air at ca. 50 °C and reconstituted with 1000 μl mobile phase (acetonitrile/water, 70:30). Twenty microliters of the solution were injected into the chromatographic apparatus by a full loop injection system.

Quantification of T-2 and HT-2 toxins was performed by measuring peak areas at the retention times of T-2 and HT-2 derivatives, respectively, and comparing them with the relevant calibration curve in the range 10–500 μg/kg of each toxin.

For calibration curve and linearity response of the T-2 and HT-2 derivative-peak areas, equivalent aliquots of T-2 and HT-2 standard solutions (corresponding to 0.01–5 μg of each toxin) were placed in screw-cap amber vials, and the solvent was evaporated to dryness at about 50 °C under a stream of air before derivatization.

To test the stability of the T-2 and HT-2 derivatives, different amounts of T-2 and HT-2 toxins (from 0.1 to 1.5 μg) were derivatized with 1-AN, and the reaction mixture was reconstituted in acetonitrile/water, 70:30 (v/v), and stored at room temperature up to 7 days, and at 4 °C up to one month. At selected time intervals, 20 μl of the reaction mixture was injected into the HPLC apparatus.

2.6. Confirmation of T-2 and HT-2 toxins in naturally contaminated samples

Confirmation of T-2 and HT-2 toxins was performed in two samples of naturally contaminated wheat samples by GC with ECD and MS detection. In particular, the dried extracts from the immunoaffinity column were derivatized with Tri-Sil TBT and analyzed by GC–ECD and GC–MS according to Rizzo et al. [18] and Visconti et al. [19], respectively.

2.7. Specificity of the anti-T-2 antibody (cross-reactivity)

To assess the specificity of the anti-T-2 antibody, 1.0 μg of T-2, HT-2, T-2 triol, T-2 tetraol, acetyl T-2 toxins, DON or NIV were dissolved in 10 ml of a mixture methanol/water, 18:82 (v/v) and eluted through the immunoaffinity columns at a flow rate of one drop per second. Columns were washed with 10 ml of distilled water at a flow rate of one to two drops per second and the toxins were eluted with 1.5 ml of methanol. The eluate was dried under a stream of air at ca. 50 °C, and analyzed by HPTLC. Dried eluates were reconstituted with 100 μl of acetonitrile, and 20 μl of each toxin solution were spotted on HPTLC plate as compared to the corresponding standards. HPTLC plates without fluorescence indicator were used for the determination of T-2, HT-2, T-2 triol, T-2 tetraol, and acetyl T-2 toxins. The display of the spots was carried out on plates sprayed with a 20% H_2SO_4 solution after heating at 120 °C for 15 min. HPTLC plates with fluorescence indicator were used for the determination of DON and NIV. The display of the spots was carried out by UV light (254 nm). The elution solvent was a mixture of toluene–ethyl acetate–formic acid in ratio of 6:3:1 (v/v).

2.8. Immunoaffinity column capacity

The capacity of the immunoaffinity column was determined for both T-2 and HT-2 toxin by comparing

(duplicate measurements) the amount of toxin added to the immunoaffinity column with the respective bound amount. Different amounts of T-2 and HT-2 toxin, each from 0.1 to 5 μg, were added together to the immunoaffinity column by loading 10 ml (equivalent to 1.0 g matrix) of diluted extract of blank wheat spiked with the corresponding amount of T-2 and HT-2 toxin.

2.9. Identification of T-2 and HT-2 anthroyl esters

The identity of fluorescent derivatives [T-2-(1-AN), HT-2-(1-AN)] was confirmed by HPLC–MS (positive chemical ionisation mode) using a Varian 9012 chromatographic system interfaced to a QqTOF/mass spectrometry QSTAR® (Applied Biosystem/MSD Sciex, Concord, ON, Canada) equipped by a turbo-ionspray interface. After derivatization of 2 μg of each toxin with 1-AN, 20 μl of the reaction mixture were injected into the HPLC apparatus. Interface conditions were as follows: nebulizer gas (air), 1.3 l/min; curtain gas (nitrogen), 1.2 l/min; heater gas (air, 300 °C), 6 l/min; mass range, 100–1000 amu; scan time, 1 s; needle voltage, 5500 V; focusing potential, 120 V; declustering potential, 40 V. Accurate mass measurements (four decimal figures) were carried out by obtaining averaged spectra of each peak and then by calibrating them with two ions of known chemical structure present in same spectra. Errors associated with such determinations were usually within 5 ppm.

2.10. Recovery experiments

Recovery experiments were performed in quadruplicate by spiking blank wheat samples with T-2 and HT-2 toxin at levels of 25, 50, 125, 250 and 500 μg/kg. Spiked samples were left overnight at room temperature to allow solvent evaporation prior to extraction with methanol/water.

3. Results and discussion

3.1. Fluorescence labelling of T-2 and HT-2 toxins for HPLC analysis

T-2 and HT-2 fluorescent derivatives T-2-(1-AN) and HT-2-(1-AN) were synthesized by reaction with 1-AN in toluene, in the presence of DMAP as catalyst, following the experimental procedure previously developed for the determination of T-2 toxin by HPLC/fluorometric detection [28]. The identity of fluorescent derivatives was confirmed by HPLC–MS analysis of the reaction mixture. In order to increase the sensitivity, 2 mM ammonium acetate was added to the mobile phase, allowing the detection of molecular ions as ammonium adducts $[M + NH_4]^+$. Total ion chromatogram (TIC) of the derivatization products from 2 μg of T-2 toxin and 2 μg of HT-2 toxin revealed the presence of a molecular ion of *m/z* 688.3111 (calculated *m/z*, 688.3116; error, −0.8 ppm), at the T-2 derivative retention time, corresponding to the adduct $[\text{T-2-(1-AN)-NH}_4]^+$, and of a molecular ion of *m/z* 850.3585 (calculated *m/z*, 850.3631; error, 5.3 ppm), at the HT-2 derivative retention time, corresponding to the adduct $[\text{HT-2-(1-AN)}_2\text{-NH}_4]^+$. High resolution ESI-MS–MS spectra of both ions of nominal *m/z* 688 and 850 showed an intense peak at *m/z* 205 due to declastering of 1-AN. The derivatization scheme proposed for T-2 and HT-2 toxins on the basis of the above data is reported in Fig. 1.

Under the chromatographic conditions reported for T-2 detection by Pascale et al. [28], the HT-2 derivative was not eluted within a reasonable time. Also when a gradient elution was used increasing acetonitrile in the mobile phase up to 90%, the HT-2 derivative eluted after 30 min with a broad peak shape. The use of a phenyl-hexyl column instead of the C_{18} column improved considerably the HPLC analysis of T-2 and HT-2 fluorescent derivatives, which eluted after approximately 10 and 20 min, respectively, with a better sensitivity especially for HT-2 toxin.

Fig. 2 shows chromatograms relevant to a "blank" reagent solution (containing only 1-AN and DMAP) and a solution of the derivatization products from a reaction with 0.25 μg of T-2 and 0.25 μg of HT-2 toxin. Peaks corresponding to T-2-(1-AN) and HT-2-(1-AN) derivatives were well resolved from those of unreacted reagents or impurities derived from the labelling reaction.

The linearity range of the derivatization reaction of T-2 and HT-2 toxins was evaluated. A good linearity of the response (peak area vs. injected amount) was observed in the range 0.025–4.00 μg for either T-2 or HT-2 toxin; coefficient of correlation values (*r*) were 0.9993 for T-2 toxin and 0.9970 for HT-2 toxin. The repeatability (relative standard deviation) of the reaction in the same range of T-2 and HT-2 toxin concentrations was in general within 8%.

Experiments to test the stability of fluorescent derivatives showed no statistically significant decrease in fluorescence intensity of T-2 and HT-2 anthroylnitrile esters, after storage up to 7 days at room temperature, or up to 30 days at 4 °C. The high stability of the derivatives should allow the use of automatic sampler coupled with HPLC apparatus for the analysis of a large number of samples.

3.2. Performance of T-2 test™ HPLC immunoaffinity columns

In order to evaluate the specificity of the monoclonal anti-T-2 antibody, different solutions of structurally related trichothecene mycotoxins were used: DON, NIV, T-2, T-2 triol, T-2 tetraol, HT-2 and acetyl T-2 toxin. Each toxin was eluted through the immunoaffinity column and the eluate was analyzed by HPTLC. No spots were observed for DON, NIV and T-2 tetraol in correspondence of the R_f values of the relevant standards, indicating the absence of affinity of the antibody towards these toxins. On the contrary, well-defined spots were observed for T-2, T-2 triol, HT-2 and acetyl T-2 in correspondence of the relevant standards. In particular, based on the spot intensity as compared with the relevant standards, the

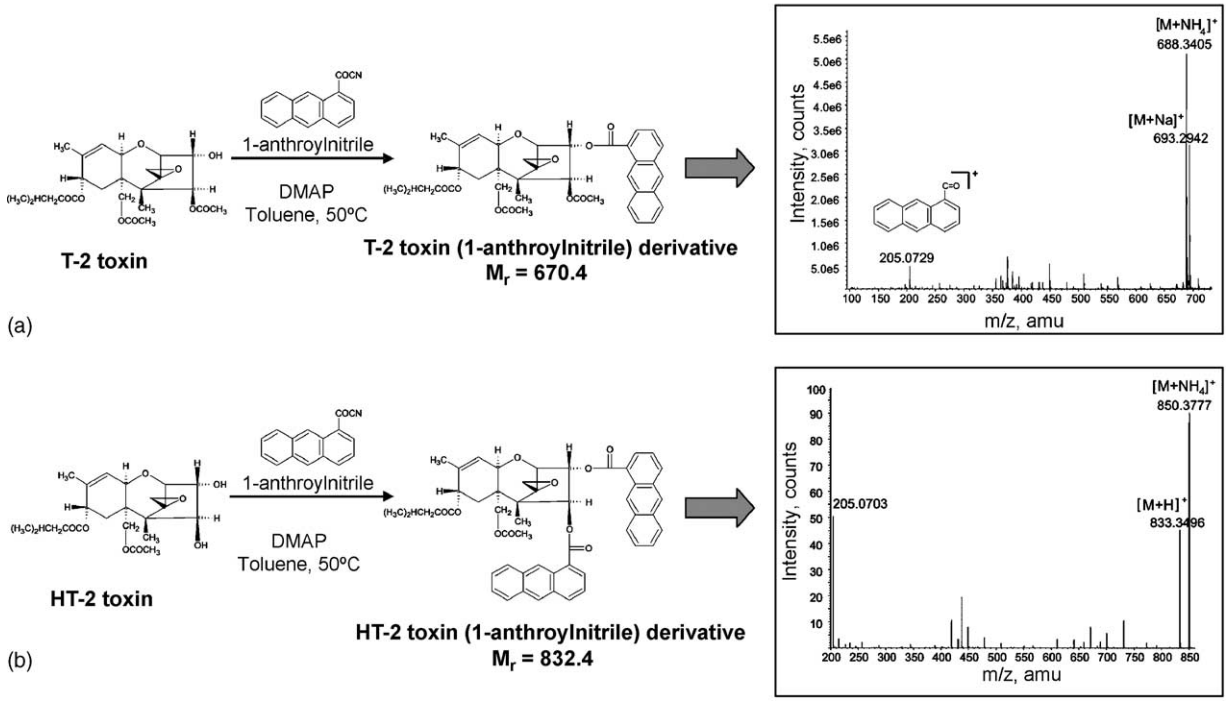

Fig. 1. Scheme of the derivatization reaction of (a) T-2 and (b) HT-2 toxins with 1-anthroylnitrile (1-AN), and characterization of the fluorescent derivatives by HPLC–MS. Figure shows also (a) ESI-MS spectrum of the T-2-(1-AN) derivative, and (b) ESI-MS–MS spectrum of the molecular ion of *m/z* 850.4 relevant to the adduct $[HT\text{-}2\text{-}(1\text{-}AN)\text{-}NH_4]^+$. The peak at *m/z* 205.1, due to declastering of 1-AN, is present in both spectra.

cross-reactivity of the antibody was 100% for T-2 and HT-2 toxins, and 90% for T-2 triol and acetyl T-2. Therefore, it can be assumed that the specificity of the antibody is strictly related to the presence of the isovaleroxy group at the C_8 position, which occurs in T-2 toxin, T-2 triol, HT-2 toxin and acetyl T-2, but not in DON, NIV and T-2 tetraol. This is in agreement with the results obtained with similar antibodies by Hunter et al. [29]. The high cross-reactivity of the anti-T-2 antibody with T-2 triol and acetyl T-2 should not be a problem for the HPLC determination of T-2 and HT-2 toxins after pre-column derivatization whit 1-AN. In particular acetyl T-2, lacking of free hydroxyl groups, should not react with 1-AN to give fluorescent derivatives, while T-2 triol, carrying three hydroxyl groups, should react with 1-AN to form derivatives with polarity quite different from T-2 and HT-2 anthroylnitrile derivatives. The derivatization of T-2 triol with 1-AN led to the formation of a major compound with retention time at 28.5 min and two minor compounds giving peaks at 16.0 and 17.5 min, respectively, that did not interfere with peaks relevant to T-2 and HT-2 derivatives.

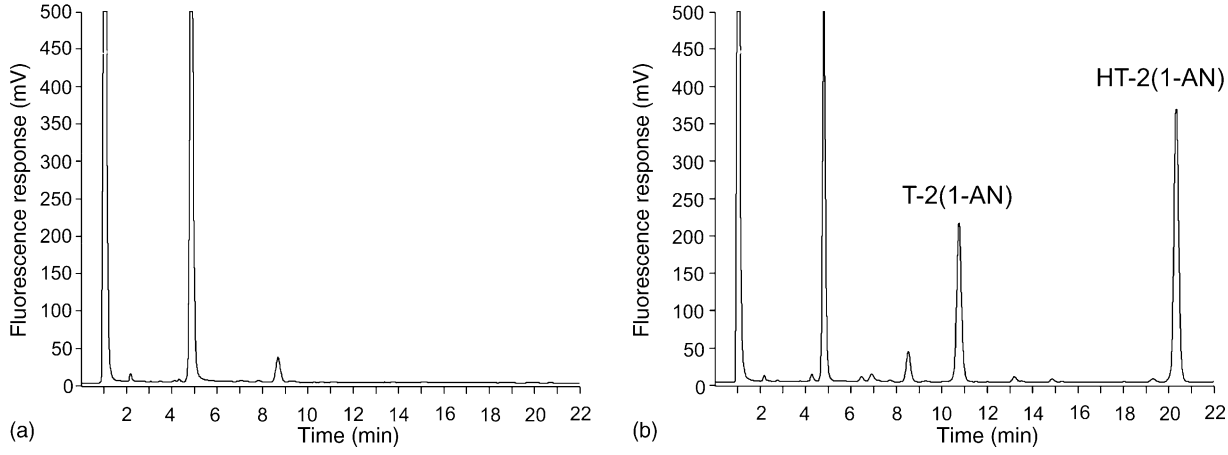

Fig. 2. Chromatograms relevant to (a) the "blank reaction mixture" (1-AN + DMAP) and (b) the derivatization products of 0.25 μg each of T-2 and HT-2 toxin. Chromatographic conditions are reported in Section 2.3.

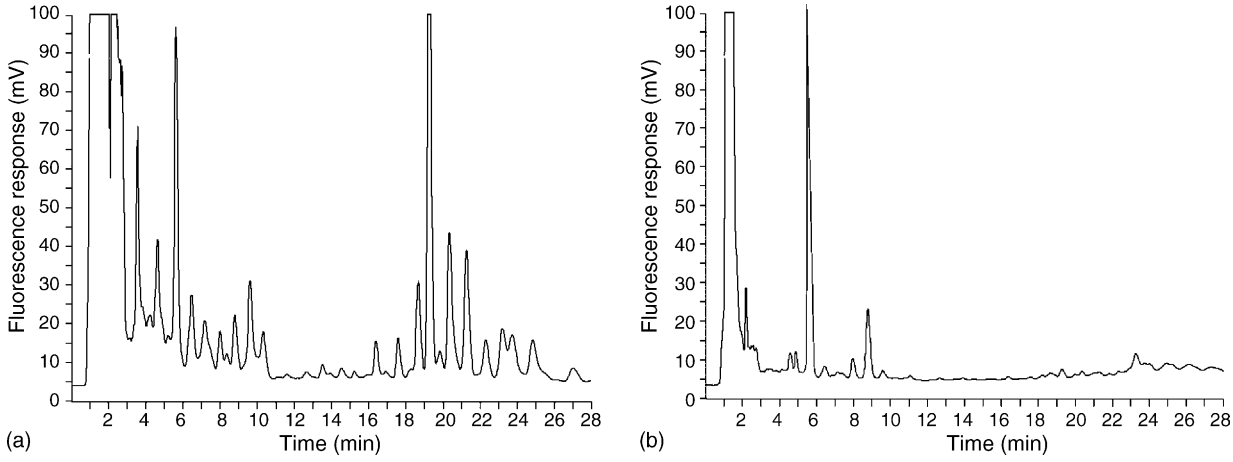

Fig. 3. Chromatograms of extracts from blank wheat samples, after derivatization with 1-AN, obtained (a) in absence and (b) in presence of NaCl (1 g/50 g of wheat). Chromatographic conditions are reported in Section 2.3.

The immunoaffinity column capacity was found to be about 1.4 μg for the mixture of T-2 and HT-2 toxins. Above this level no increase of fluorescence was observed, indicating the saturation of the T-2/HT-2 binding sites. No preferential binding for one of the two toxins was observed near the saturation level. Recoveries of both T-2 and HT-2 toxin (below the saturation level) were higher than 90%.

3.3. Analysis of T-2 and HT-2 toxins in cereals

T-2 test™ HPLC immunoaffinity columns were tested on different cereal extracts (wheat, corn, barley, and oats) prior to the HPLC determination of T-2 and HT-2 toxins. After testing different methanol–water ratios (60:40, 70:30, 80:20 and 90:10, v/v), methanol–water (90:10, v/v) was selected as the best extraction solvent for cereal matrices, based on recovery values and absence of interfering peaks in the chromatograms. In a preliminary experiment, acetonitrile–water (84:16, v/v) was also tested, with no significant improvement. Moreover, taking into account that the presence of low levels of acetonitrile (3–5%) in the extraction solvent could cause antibody denaturation, the mixture methanol–water was chosen as the extraction solvent, also due its reduced toxicity.

The addition of NaCl to the extraction solvent provided cleaner extracts, reducing significantly both noise and number of peaks in the chromatogram, as shown in Fig. 3. The effect of the NaCl concentration was investigated by adding different amounts of salt (from 0.1 to 5.0 g) to 50 g of wheat samples spiked at 250 μg/kg each of T-2 and HT-2 toxin, and by comparing the obtained recovery values. A good compromise between the clearness of the extract and the effect of salt on possible denaturation of the antibody was found by adding 1 g NaCl to 50 g of cereal sample.

Results of recovery experiments (quadruplicate measurements) for the full analytical procedure carried out on different cereal grains spiked with T-2 and HT-2 at different levels are reported in Table 1. Recoveries from wheat, maize and barley spiked with T-2 and HT-2 toxins at levels from 25 to 500 μg/kg of each toxin ranged from 70 to 100%, with relative standard deviation generally lower than 8%. The limit of detection of the method was 5 μg/kg of T-2 toxin and 3 μg/kg of HT-2 toxin, based on a signal-to-noise ratio 3:1.

The proposed analytical method did not allow the determination of T-2 and HT-2 toxins in oats because of interfering chromatographic peaks occurring at the retention time of HT-2-(1-AN) derivative. No problem was observed for the determination of T-2 toxin, thus confirming previously reported results [28].

Recovery and repeatability values of the method fulfil the criteria established by CEN (European Committee for Standardization) for the acceptance of an analytical method for T-2 and HT-2 toxin, i.e. recoveries between 60% and 120% and RSD_r <40% for T-2 and HT-2 concentrations in the range 50–250 μg/kg, and recoveries between 60% and 110% and RSD_r <30% for concentration >250 μg/kg [30].

Chromatograms relevant to a blank wheat sample, to the same sample spiked with T-2 and HT-2 toxins, and to a sample of naturally contaminated wheat are shown in Fig. 4. Similar chromatogram profiles were obtained for blank and spiked samples of corn and barley.

The method has been applied to 26 cereal samples (14 wheat samples and 12 maize samples). HT-2 toxin was detected in ten wheat samples at levels ranging from 10 to 71 μg/kg (mean value of positives, 23 μg/kg), while T-2 toxin was detected in three of these samples at lower levels (up to 12 μg/kg). No toxin contamination was observed in maize samples. The contamination levels of wheat samples were in agreement with those found in previous investigations, while the incidence of HT-2 contamination (71%) was higher (about 10%), probably due the low detection limit (3 μg/kg) of the present method for HT-2 toxin [3,4]. Chromatogram relevant to a naturally contaminated sample with T-2 toxin at 9 μg/kg and HT-2 toxin at 35 μg/kg is shown in Fig. 4(d). The identity

Table 1
Recoveries of T-2 and HT-2 toxin from blank cereals spiked at different toxin levels

Spiking level (μg/kg)	Recovery, % (RSD_r, %)					
	Wheat		Corn		Barley	
	T-2	HT-2	T-2	HT-2	T-2	HT-2
25	69.5 (5.9)	82.3 (11.6)	83.9 (0.4)	91.4 (4.6)	96.4 (7.7)	96.4 (3.0)
50	80.3 (3.2)	101.5 (5.5)	71.6 (6.2)	92.2 (4.5)	99.4 (2.5)	96.0 (0.9)
125	84.3 (5.7)	82.7 (6.6)	81.8 (3.9)	82.4 (2.5)	100.5 (3.7)	96.8 (2.1)
250	95.6 (2.3)	97.7 (1.3)	73.4 (1.6)	72.0 (2.0)	103.0 (1.2)	98.3 (3.6)
500	100.1 (7.9)	92.7 (7.7)	81.3 (8.6)	70.1 (7.7)	93.3 (5.9)	97.0 (5.1)

RSD, relative standard deviation ($n = 4$).

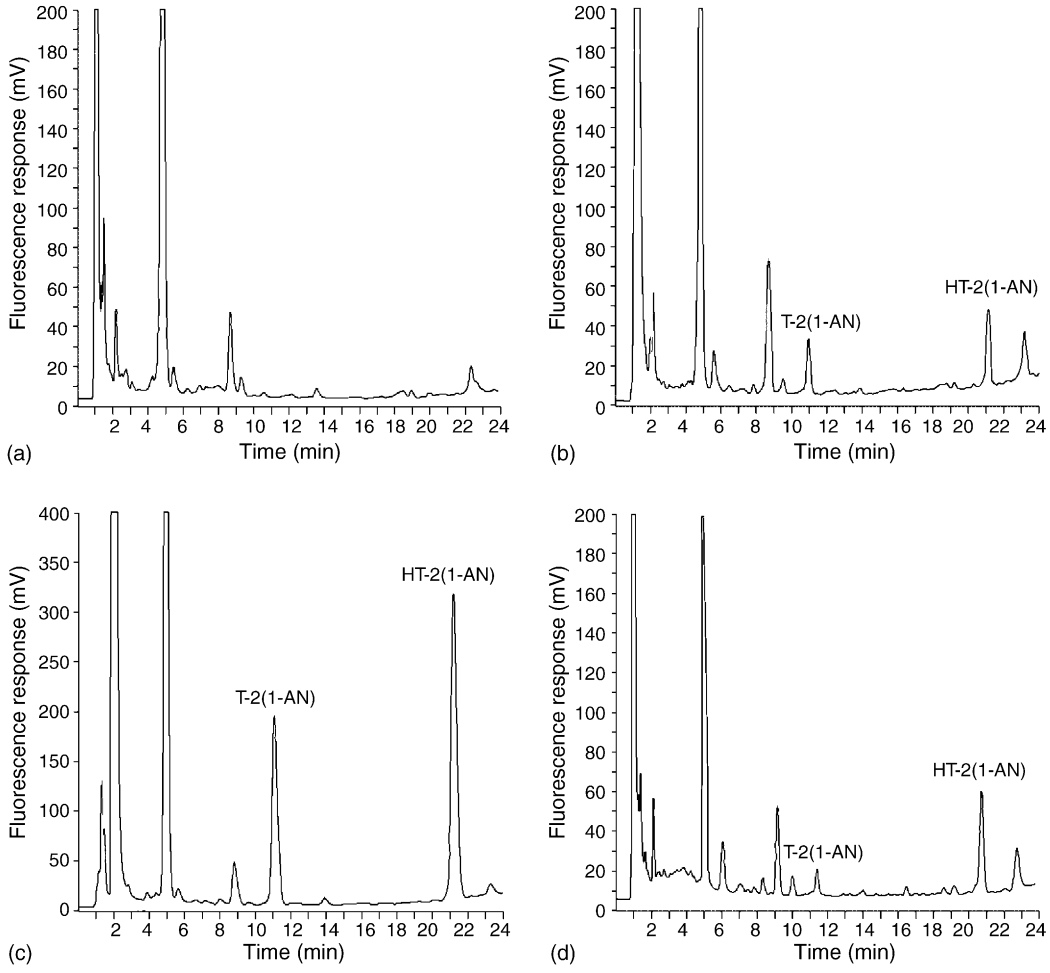

Fig. 4. Chromatograms of wheat samples: (a) blank (<5 μg/kg T-2, <3 μg/kg HT-2); (b) blank sample spiked with T-2 and HT-2 toxins at 25 μg/kg (T-2 found 17 μg/kg, HT-2 found 20 μg/kg); (c) blank sample spiked with T-2 and HT-2 toxins at 250 μg/kg (T-2 found 239 μg/kg, HT-2 found 244 μg/kg); (d) naturally contaminated sample (T-2 found 9 μg/kg, HT-2 found 35 μg/kg). Chromatographic conditions are reported in Section 2.3.

of the two toxins was confirmed by GC–MS and GC–ECD after derivatization with Tri-Sil-TBT reagent of extracts purified by immunoaffinity columns [18,19].

4. Conclusions

The method proposed herein for the simultaneous determination of T-2 and HT-2 toxins in contaminated cereal grains combines the selectivity of the immunoaffinity clean-up, due to the specificity of the antibody, with the sensitivity of the HPLC with fluorescence detection, thus allowing the determination of both toxins at levels that can occur in naturally contaminated samples.

By applying the derivatization procedure with 1-AN previously reported for T-2 toxin [28], a stable fluorescent derivative was obtained also with HT-2 toxin. The cross-reactivity with HT-2 toxin of the anti-T-2 antibody used in commercial

immunoaffinity columns (*T*-2 *test*™ HPLC, Vicam) allowed the effective clean-up of both T-2 and HT-2 toxins from extracts of different cereal grains, including wheat, corn and barley.

The proposed HPLC method is simple to use and shows good laboratory performances in terms of accuracy, sensitivity and precision.

Acknowledgement

The authors would like to thank Dr. G. Mascolo of Water Research Institute, National Research Council (Bari, Italy), for HPLC–MS analysis.

References

[1] A. Bottalico, J. Plant Pathol. 80 (1998) 85.
[2] A. Visconti, C.J. Mirocha, A. Bottalico, J. Chelkowski, Mycotoxin Res. 1 (1985) 3.
[3] WHO Food Additives Series 47, FAO Food and Nutrition Paper 74, Presented at the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), WHO, Geneva, 2001, p. 557.
[4] R.C. Schothorst, H.P. van Egmond, Toxicol. Lett. 153 (2004) 133.
[5] G.S. Eriksen, H. Pettersson, Anim. Feed Sci. Technol. 114 (2004) 205.
[6] A. Visconti, F. Minervini, G. Lucifero, V. Gambatesa, Mycopathologia 113 (1991) 181.
[7] D.B. Prelusky, B.A. Rotter, R.G. Rotter, in: J.D. Millet, H.L. Trenholm (Eds.), Mycotoxins in Grain. Compounds other than Aflatoxins, Eagan Press, Pt. Paul (MN), USA, 1994, p. 359.
[8] A. Visconti, Bull. Inst. Compr. Agr. Sci. Kinki Univ. 9 (2001) 39.
[9] D.L. Sudakin, Toxicol. Lett. 143 (2003) 97.
[10] DG-SANCO, Draft of Commission Regulation (EC) Amending Regulation (EC) No. 466/2001 as Regards Fusarium-toxins, SANCO/0006/2004-rev 5, p. 10.
[11] W. Langseth, T. Rundberget, J. Chromatogr. A 815 (1998) 103.
[12] R. Krska, S. Baumgartner, R. Josephs, Fresenius J. Anal. Chem. 371 (2001) 285.
[13] J. Park, F. Chu, J. AOAC Int. 79 (1996) 465.
[14] L. Sibanda, S. De Saeger, C. van Peteghem, J. Grabarkiewicz-Szczesna, M. Tomczak, J. Agric. Food Chem. 48 (2000) 5864.
[15] R.C. Schothorst, A.A. Jekel, Food Chem. 73 (2001) 111.
[16] M. Schollenberger, U. Lauber, H.T. Jara, S. Suchy, W. Drochner, H.M. Muller, J. Chromatogr. A 815 (1998) 123.
[17] M.W. Trucksess, in: A.E. Pohland (Ed.), Mycotoxin Protocols, Methods in Molecular Biology, vol. 157, Humana Press, Totowa, NJ, 2001, p. 115.
[18] A.F. Rizzo, L. Saari, E. Lindfords, J. Chromatogr. 368 (1986) 381.
[19] A. Visconti, M.L. Treeful, C.J. Mirocha, Biomed. Mass Spectrom. 12 (1985) 689.
[20] H. Petterson, W. Langseth, BCR Information, Project Reports EUR 20285/1 EN (Part I) and EUR 20285/2 EN (Part II), European Commission, Brussels, 2002.
[21] U. Berger, M. Oehme, F. Kuhn, J. Agric. Food Chem. 47 (1999) 4240.
[22] E. Razzazi-Fazeli, B. Rabus, B. Cecon, J. Böhm, J. Chromatogr. A 968 (2002) 129.
[23] F. Berthiller, R. Schumacher, G. Buttinger, R. Krska, J. Chromatogr. A 1062 (2005) 209.
[24] H. Cohen, B. Boutin-Muma, J. Chromatogr. 595 (1992) 143.
[25] M. Jiménez, J.J. Mateo, R. Mateo, J. Chromatogr. A 870 (2000) 473.
[26] J.J. Mateo, R. Mateo, M. Jiménez, Int. Food Microbiol. 72 (2002) 115.
[27] C. Dall'Asta, G. Galaverna, A. Biancardi, M. Gasparini, S. Sforza, A. Dossena, R. Marchelli, J. Chromatogr. A 1047 (2004) 241.
[28] M. Pascale, M. Haidukowski, A. Visconti, J. Chromatogr. A 989 (2003) 257.
[29] K.W. Hunter, A.A. Brimfiled, M. Miller, F.D. Finkelman, S.F. Chu, Appl. Environ. Microbiol. 49 (1985) 168.
[30] CEN (European Committee for Standardization), TC275-WG5, Biotoxin, Doc. No. 195, CEN Report CR 13505, Brussels, 1999.