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# Determination of HT-2 and T-2 toxins in oats and wheat by ultra-performance liquid chromatography with photodiode array detection

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ABSTRACT

European intake estimates indicate that the presence of HT-2 and T-2 toxins in cereals, mainly in oats, can be of concern for human health. Therefore, the development of sensitive, rapid and reliable methods for determining these mycotoxins in cereals, in particular oats, has high priority. A rapid ultra-performance liquid chromatographic (UPLC) method has been developed for the simultaneous determination of HT-2 and T-2 toxins in oats and wheat at $\mu g\,kg^{-1}$ level. Ground samples were extracted with methanol/water (90:10, v/v) and the diluted extracts were cleaned up through immunoaffinity columns. HT-2 and T-2 toxins were separated and quantified by UPLC with photodiode array (PDA) detector ($\lambda = 202$ nm) in less than 5 min. Mean recoveries from blank oats samples spiked with HT-2 and T-2 toxins at levels of 50–1000 $\mu g\,kg^{-1}$ ranged from 87 to 96%, with relative standard deviations (RSDs) lower than 7%; mean recoveries from wheat spiked with HT-2 and T-2 toxins at levels of 25–100 $\mu g\,kg^{-1}$ ranged from 91 to 103%, with RSDs lower than 5%. The limit of detection of the method was 8 $\mu g\,kg^{-1}$ for both toxins (signal-to-noise ratio 3:1). The method was successfully applied to the analysis of HT-2 and T-2 toxins in naturally contaminated oats and wheat samples. A good correlation was found by comparative analysis of naturally contaminated samples of oats ($r = 0.9985$) and wheat ($r = 0.9058$) using the proposed method or a reliable HPLC method with fluorescence detection after pre-column derivatization with 1-anthroylnitrile.



## 1. Introduction

HT-2 toxin (HT-2) and T-2 toxin (T-2) are two of the most toxic type A trichothecenes produced mainly by *Fusarium sporotrichioides*, *F. poae* and *F. langsethiae* that may develop on a variety of cereal grains, especially in cold climate regions or during wet storage conditions [1–4]. A detailed report on toxicity data of HT-2 and T-2 has been recently produced on behalf of EFSA (the European Food Safety Authority) [5]. T-2 is a potent inhibitor of protein synthesis and, at higher concentrations, of DNA and RNA synthesis. Acute toxicity of T-2 is quite high, with LD50 values for rodents in the range 5–10 $mg\,kg^{-1}$ body weight. The hematologic/immune system is the main target of T-2 toxicity, both *in vitro* and *in vivo* [5]. Long-term studies on poultry showed that T-2 causes mouth and intestine lesions [5]. The toxicity of HT-2 has been less investigated, nevertheless due to the fact that T-2 is rapidly metabolized to HT-2 *in vivo*, it is widely accepted that the toxicity of T-2 *in vivo* includes that of HT-2 [1,5,6]. Therefore, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has proposed a common provisional maximum tolerable daily intake (PMTDI) of 0.06 $\mu g\,kg^{-1}$ body weight per day for T-2 and HT-2, alone or in combination [1].

Data on the occurrence of T-2 and HT-2 in cereals are mainly restricted to Europe [2–4,7–14]. Results from the European project "SCOOP" (task 3.2.10) showed a low incidence of contamination by T-2 (20% out of 3490 analyzed samples) and HT-2 (14% out of 3032 analyzed samples) in cereals, including oats, maize, wheat, barley and rye [2]. More recent data have shown oats to be the cereal most susceptible to T-2 and HT-2 contamination with incidence and concentration of toxins depending on the crop year. Incidence over 90% and levels up to 9990 $\mu g\,kg^{-1}$ were found in oats samples from UK in the period 2002–2005. Much lower levels of T-2 and HT-2 occurred in wheat, maize and barley, with levels of contamination up to 214 $\mu g\,kg^{-1}$ in wheat [3,4,7,8]. Regulatory limits are currently under discussion by the European Commission, considering the sum of T-2 and HT-2 in cereals and cereal products. The latest proposal at this regard is 100 $\mu g\,kg^{-1}$ for unprocessed cereals and cereals products, 500 $\mu g\,kg^{-1}$ for unprocessed oats and 200 $\mu g\,kg^{-1}$ for oats products [9,15].

Different methods have been proposed for the determination of T-2 and HT-2 in cereals. Screening or rapid methods, including immunochemical assays such as enzyme-linked immunosorbent

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assays (ELISAs), lateral flow devices (LFDs) or surface plasmon resonance (SPR) based biosensor assays have been developed for determination of T-2 alone or in combination with HT-2 [16–20]. Most ELISA and LFD methods may be limited by the specificity of the antibody that become a problem when the sum of T-2 and HT-2 has to be measured according to the current European Commission opinion on future regulation [18]. With respect to traditional methods, chromatographic methods (GC and HPLC) are more reliable for the simultaneous determination of T-2 and HT-2. GC methods based on electron-capture (ECD) and mass spectrometric (MS) detection have been the most widely used for quantitative determination of type-A trichothecenes after derivatization to increase volatility and sensitivity. In addition, HPLC methods with fluorescence detection (FLD) have been developed for the determination of these mycotoxins at low levels in several cereals, including oats, after derivatization with fluorescent labeling reagents [16,17,21–24]. LC–MS/MS is being widely used for the simultaneous determination of mycotoxins, including T-2 and HT-2, in cereals and derived products, due to its sensitivity and due to the fact that extracts do not require derivatization. On the other hand, LC–MS equipments are very expensive and require skilled personnel for their use [17,25–28]. UV detection coupled to HPLC is not sensitive enough to allow the determination of these toxins at levels commonly found in naturally contaminated samples [16,17]. Recently separation science has been revolutionized with the introduction of ultra-performance liquid chromatography (UPLC) based on the use of innovative instrumentation and column technology (particle size < 2 μm). The UPLC allows to minimize extra column volume and column band spreading leading much greater sensitivity and effective chromatographic separation [29].

The aim of this work was to develop a sensitive and accurate method for the simultaneous determination of HT-2 and T-2 in unprocessed cereals (in particular oats and wheat) using immunoaffinity column for clean up of extracts and UPLC-photodiode array (PDA) for toxin detection, avoiding the pre-column derivatization step. Performances and advantages of the UPLC-PDA method with respect to HPLC with fluorescence detection are discussed. The application of UPLC-PDA to the analysis of naturally contaminated oats and wheat samples is also reported.

## 2. Experimental

### 2.1. Materials and chemicals

Acetonitrile, "gold" for HPLC (ultragradient grade), and methanol (HPLC grade) were purchased from Carlo Erba Reagents (Milan, Italy). Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, MA, USA). T-2 toxin (lot #080M4083, purity 99.0%) and HT-2 toxin (lot #120M4010V, purity 98.5%), sodium chloride (NaCl) and Tween 20 for molecular biology were purchased from Sigma–Aldrich (Milan, Italy). 1-Anthroyl cyanide (1-anthroylnitrile, 1-AN) was purchased from Wako Chemicals GmbH (Neuss, Germany). Easi-Extract® T-2 & HT-2 immunoaffinity columns were purchased from r-Biopharm AG (Darmstadt, Germany); glass microfibre filters (Whatman GF/A) and paper filters (Whatman No. 4) from Whatman (Maidstone, UK). FAPAS® oats test material (T-2261) was purchased from The Food and Environment Research Agency (Sand Hutton, York, UK).

### 2.2. Preparation of standard solutions

T-2 and HT-2 stock solutions (1 mg mL$^{-1}$ each) were prepared by dissolving T-2 and HT-2 solid commercial toxins in acetonitrile (HPLC grade). Mixed T-2 and HT-2 standard solutions (100 μg mL$^{-1}$ and 20 μg mL$^{-1}$ each) for spiking purposes were prepared by diluting adequate amounts of the stock solutions in acetonitrile. T-2 and HT-2 standard solutions for UPLC calibration curve were prepared by redissolving aliquots of the 20 μg mL$^{-1}$ solution in acetonitrile, previously evaporated to dryness under nitrogen stream, with water:acetonitrile (80:20, v/v).

### 2.3. Apparatus

The UPLC apparatus consisted of a Waters Acquity UPLC® system (Milford, MA, USA) equipped with a binary solvent manager, a sample manager, a column heater and a PDA detector. The analytical column was an Acquity UPLC® BEH C18 (2.1 mm × 50 mm, 1.7 μm) preceded by an Acquity UPLC™ column in-line filter (0.2 μm). The chromatographic separation was performed by a gradient elution (solvent A: $H_2O$, solvent B: $CH_3CN$) as follows: the initial composition of the mobile phase (80% solvent A, 20% solvent B) was kept constant for 2 min, then solvent B was linearly increased to 50% in 3 min, and kept constant for 1 min; solvent B was increased again to 90% in 1 min and kept constant for 1 min to clean the column, then returned to the initial conditions in 1.0 min. The column was equilibrated for 2 min prior to the successive sample injection. The flow rate of the mobile phase was 0.7 mL min$^{-1}$. The column was kept at a temperature of 50 °C; the detector was set at 202 nm wavelength. Data acquisition and instrument control were performed by Empower™ 2 Software (Waters).

The HPLC-FLD apparatus was an Agilent 1100 series (Agilent, Waldbronn, Germany) equipped with a binary pump, autosampler, column thermostat set at 25 °C and a spectrofluorometric detector with excitation and emission wavelengths set at 381 nm and 470 nm, respectively. The analytical column was a Phenyl-Hexyl Luna® (150 mm × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA), preceded by a SecurityGuardTM C18 cartridge (4 mm × 3 mm i.d., 5 μm) (Phenomenex).

### 2.4. Sample extraction and clean up

Sample extraction and clean up were performed according to the method originally developed by Visconti et al. [21] for the analysis of T-2 and HT-2 in cereals, and subsequently optimized by Trebstein et al. [23] for oats and cereal products, with minor modifications. In particular, 25 g of oats or wheat samples finely ground (particle size ≤ 1.0 mm) by a Cyclone sample mill (PBI International, Milan, Italy) after addition of 2.5 g of NaCl and 100 mL of methanol–water (90:10, v/v) were extracted by shaking at 250 rpm for 60 min (KS 4000i, IKA Werke GmbH & Co. KG., Staufen, Germany). The extracts were filtered through filter paper (Whatman n. 4) and 7.5 mL were diluted with 30 mL of a 4% NaCl solution. To let precipitation of proteins and matrix insoluble compounds after dilution, extracts were left to rest for 5 min, then mixed by stirring for 3 min and left again to rest for additional 5 min. The diluted extracts were filtered through a glass microfiber filter (Whatman GF/A), and 25 mL passed through immunoaffinity columns at a flow rate of about one drop per second. To avoid saturation of the antibody binding sites, for samples contaminated with T-2 and HT-2 at levels equal to or higher than 1000 μg kg$^{-1}$ (sum of toxins), 5 mL of diluted extracts were loaded on immunoaffinity columns. Columns were washed with 10 mL of a 0.01% Tween 20 aqueous solution followed by 10 mL distilled water at a flow rate of 1–2 drops/s. T-2 and HT-2 were eluted from the column with methanol (2× 1.0 mL) at a flow rate of 1 drop/s. Cleaned up extracts were collected in a 4 mL screw cap vial and dried

under air stream at 50 °C in a heating block. Dried residues were reconstituted with 200 μL of water:acetonitrile (80:20, v/v) and 10 μL were injected into the UPLC apparatus by full loop injection system.

### 2.5. In house method validation

Recovery experiments were performed in quadruplicate by spiking blank oats samples with T-2 and HT-2 at levels of 50, 100, 250, 500 and 1000 μg kg$^{-1}$, and blank wheat samples at levels of 25, 50 and 100 μg kg$^{-1}$. Spiked samples were left 1 h at room temperature to allow solvent evaporation prior to extraction with methanol/water.

The trueness of the method was determined by analyzing a FAPAS® oats test material (T2261) containing 164 μg kg$^{-1}$ T-2 (satisfactory range: 95–233 μg kg$^{-1}$) and 257 μg kg$^{-1}$ HT-2 (satisfactory range: 156–358 μg kg$^{-1}$).

The UPLC-PDA method was applied to the analysis of 28 naturally contaminated oats samples and 19 naturally contaminated wheat samples and compared with published and reliable methods for the determination of T-2 and HT-2 in oats [23] and wheat [21], respectively.

### 2.6. Statistical analysis

Data of toxin contamination were processed by one-way analysis of variance (ANOVA) at $P = 0.001$ to indicate statistically significant differences between means (Student–Newman–Keuls test). Data were processed using the Sigma Plot® 11 statistical software (Systat Software Inc, London, UK).

## 3. Results and discussion

### 3.1. Optimization of the UPLC-PDA method

HPLC with UV detection is not applicable to the determination of type A trichothecenes, including T-2 and HT-2, at levels occurring naturally in cereals due to the weak UV absorption of these mycotoxins [16,17]. Therefore, methods based on HPLC coupled with fluorescence or tandem mass spectrometry detectors have been developed for sensitive determination of these mycotoxins in cereals and cereal based products, including oats and derived products [17,18]. Both methodologies have some disadvantages. The first requires tedious and time-consuming pre-column derivatization reactions, the latter is quite expensive and requires specialist expertise.

Recently a LC-diode array detector method has been proposed for the determination of T-2 and HT-2 toxins in cultures of *F. langhsethiae* in oats-based media with limit of detection of about 160 μg kg$^{-1}$ [30]. In addition, a questionable paper on the simultaneous detection of 12 mycotoxins, including T-2 and HT-2, in cereals using HPLC-PDA-FLD and multifunctional immunoaffinity column clean up, reported limits of detection of 9.3 and 6.2 μg kg$^{-1}$ for T-2 and HT-2, respectively, by PDA detector [31]. A clear mistake could be ascertained in the paper due to the fact that these limits (for type A trichothecenes) were lower than the one reported therein for deoxynivalenol (i.e. 18.7 μg kg$^{-1}$), a type B trichothecene commonly determined by HPLC-UV due to the presence in the molecule of a conjugated carbonyl group chromophore (absent in type A trichothecenes) that allows UV absorption. Experiments carried out in our laboratory under the same experimental procedure and chromatographic conditions resulted in detection limits of about 1000 μg kg$^{-1}$ for both T-2 and HT-2.

The significant improvements in terms of sensitivity, resolution and speed that can be achieved by UPLC system with respect to HPLC led us to investigate the use of an UPLC-PDA system for developing a rapid and sensitive method for the determination of T-2 and HT-2 in oats without the need of derivatization. Recently, Trebstein et al. optimized extraction and clean up procedures for developing a reliable method for the determination of T-2 and HT-2 in oats by HPLC with fluorescence detection after derivatization of extract with 1-anthroylnitrile [23]. In our study we used a similar extraction procedure and immunoaffinity clean up, although a washing of the immunoaffinity column with 10 mL of a 0.01% Tween 20 aqueous solution instead of water was necessary due to the presence of interfering peaks at the retention time of T-2 in the chromatogram. Experiments carried out with a naturally contaminated oats sample to verify the need to perform two extractions to ensure the complete extraction of the toxins showed no statistical difference in toxins concentrations ($P < 0.001$) when the extraction was carried out by shaking for 60 min (our method) or by consecutive extraction by blending for 3 min and shaking for 30 min (Trebstein et al. method). Extraction by blending for 3 min was not sufficient to extract quantitatively T-2 and HT-2 toxins from naturally contaminated oats (Table 1).

**Table 1**
Statistical comparison between different extraction modes for HT-2 and T-2 toxins from a naturally contaminated oats sample.

| Extraction mode | HT-2 (μg kg$^{-1}$) | T-2 (μg kg$^{-1}$) | Reference |
|---|---|---|---|
| Blending (3 min) + shaking (30 min) | 532.8 a* | 126.6 a | [23] |
| Shaking (60 min) | 494.0 a | 119.6 a | This work |
| Blending (3 min) | 399.5 b | 95.8 b | This work |

* Mean values of 6 replicate analyses; values followed by the same letter in the same column are not significantly different at $P < 0.001$ according to Student–Newman–Keuls test.

Fig. 1 shows the chromatogram of a standard solution of T-2 and HT-2, obtained with the gradient profile described in Section 2.3. The use of acetonitrile "gold" for HPLC in the mobile phase was necessary to reduce baseline drift of chromatograms at the wavelength of 202 nm (PDA detector). Although UV spectra of T-2 and HT-2 showed maximum absorption at wavelengths of 191 nm and 192 nm, respectively, the wavelength of 202 nm was chosen as a good compromise between baseline drift and toxin's sensitivity. Elution gradient was optimized to reach good separation between toxins and interfering compounds present in matrix extract with a good sensitivity for both T-2 and HT-2. In the optimized conditions, the limits of detection of the method, based on a signal to noise ratio of 3, for oats and wheat were 8 μg kg$^{-1}$ for both T-2 and HT-2. These limits are quite similar to those reported for HPLC methods using fluorescence detection that require pre-column derivatization reactions with labeling reagents. Chromatograms of blank oats sample (<8 μg kg$^{-1}$ HT-2 and T-2), and oats samples artificially or naturally contaminated with T-2 and HT-2 are shown in Fig. 2. Similar chromatograms were observed for wheat samples.

### 3.2. Method validation and application to naturally contaminated samples

Results of recovery experiments of the full analytical procedure carried out with oats and wheat samples spiked with T-2 and HT-2 at different levels are reported in Table 2. Within the spiking range 50–1000 μg kg$^{-1}$ mean recoveries for oats ranged from 92% to 96% (average value 94.3%) for HT-2, with relative standard deviations (RSDs) less than 6% and from 87% to 93% (average value 90.7%) for T-2, with RSDs less than 7%. Mean recoveries from wheat spiked at levels ranging from 25 to 100 μg kg$^{-1}$ ranged from 100% to 103% (average value 101.9%) for HT-2, with RSDs less than 1.2% and from 91% to 100% (average value of 96.2%) with RSDs less than

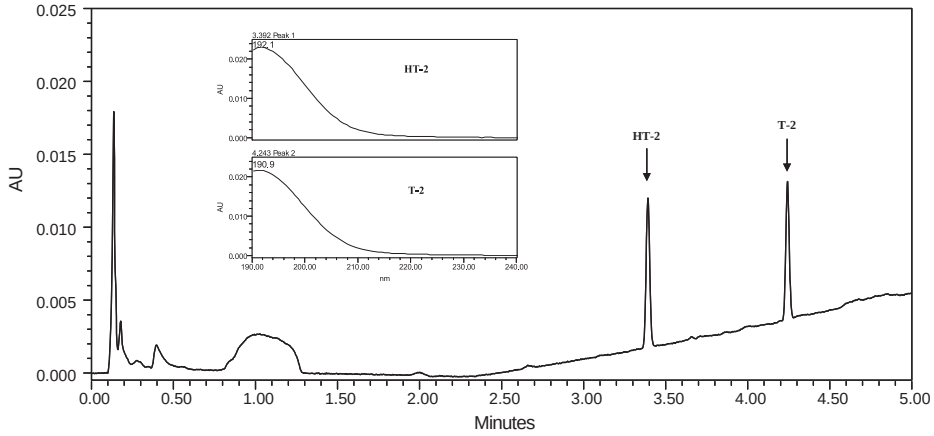


**Fig. 1.** UPLC-PDA chromatogram of standard solution of T-2 toxin (10 ng) and HT-2 toxin (10 ng). In boxes are reported the UV spectra (190–240 nm) of the toxins. Chromatographic conditions are reported in Section 2.3.

5%. Recovery and repeatability values fulfill the performance criteria established by the European Union for the acceptance of an analytical method for T-2 and HT-2 for the official control of mycotoxin levels in foodstuffs, i.e. recoveries between 60% and 130% and RSDr ≤ 40% (or RSDr ≤ 30%) for T-2 and HT-2 concentrations in the range 50–250 μg kg$^{-1}$ (or >250 μg kg$^{-1}$) and 100–200 μg kg$^{-1}$ (or >200 μg kg$^{-1}$), respectively [32].

The Easi-Extract® T-2 & HT-2 immunoaffinity columns showed saturation of T-2/HT-2 binding sites at levels higher than 1000 μg kg$^{-1}$, as sum of T-2 and HT-2 in naturally contaminated oats samples (data not shown). Considering the column capacity, a lower volume of diluted extract was loaded on the immunoaffinity columns when the sum of T-2 and HT-2 content exceeded 1000 μg kg$^{-1}$. The range of applicability of the method, as sum of T-2 and HT-2 in oats and wheat, was from 8 to 1000 μg kg$^{-1}$ when 25 mL of diluted extract were loaded on the immunoaffinity column and from 40 to 5000 μg kg$^{-1}$ when 5 mL of diluted extract were loaded on the immunoaffinity column.

Inter-day repeatability of results obtained with the UPLC-PDA method was shown by analyzing a certified FAPAS® oats test material (T2261) in five consecutive days. Results obtained with the

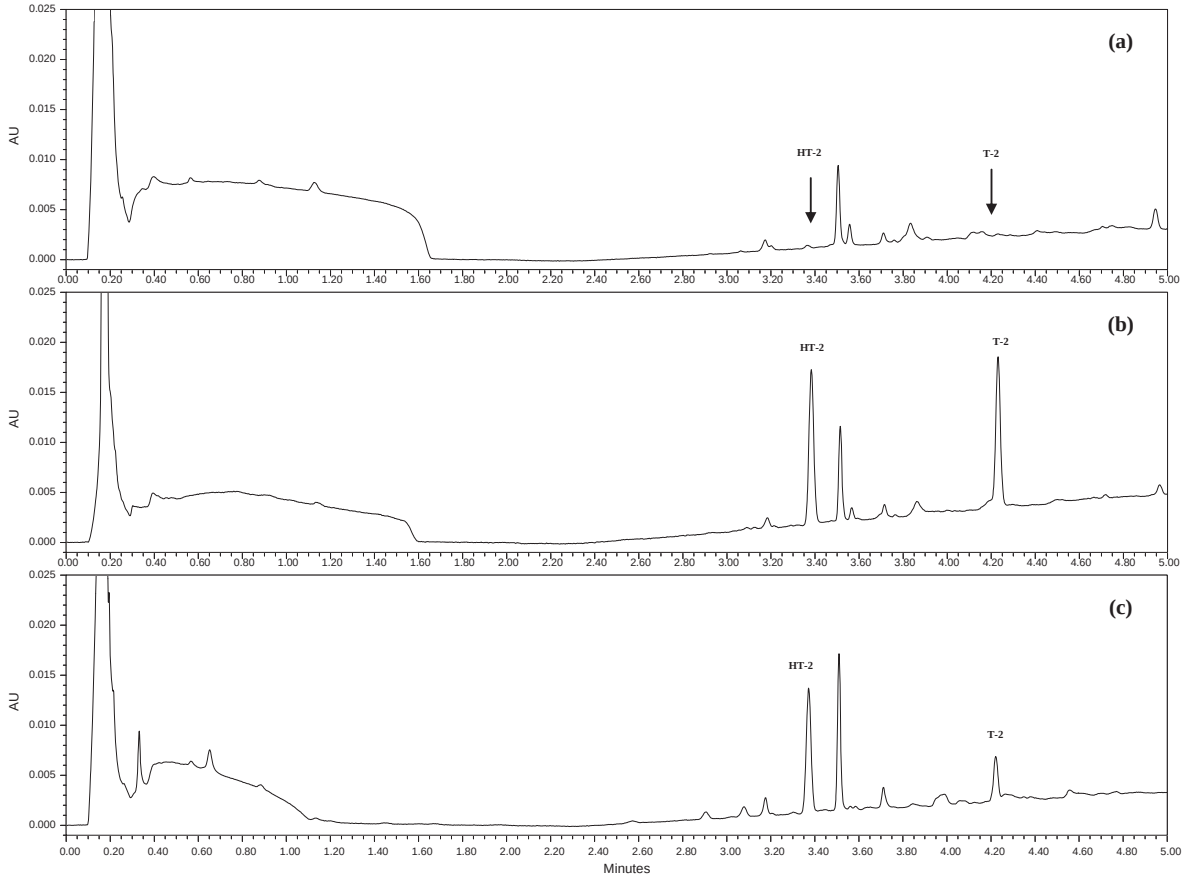


**Fig. 2.** UPLC-PDA chromatograms of oats samples: (a) blank sample (<8 μg kg$^{-1}$ HT-2 and T-2), (b) sample spiked with 250 μg kg$^{-1}$ of HT-2 and 250 μg kg$^{-1}$ of HT-2, (c) naturally contaminated sample (HT-2 found 1030 μg kg$^{-1}$, T-2 found 497 μg kg$^{-1}$). Chromatographic conditions are reported in Section 2.3.

**Table 2**
Recovery data of HT-2 and T-2 toxins and relative standard deviations.

| Spiking levels ($\mu g\,kg^{-1}$) | Oats | | Wheat | |
|---|---|---|---|---|
| | Recovery, % (RSD, %)[a] | | Recovery, % (RSD, %)[a] | |
| | HT-2 | T-2 | HT-2 | T-2 |
| 25 | – | – | 103.5 (1.2) | 91.3 (4.9) |
| 50 | 91.9 (4.3) | 86.9 (6.8) | 100.2 (1.0) | 97.4 (3.4) |
| 100 | 96.2 (0.3) | 90.4 (3.7) | 101.9 (1.1) | 99.9 (2.1) |
| 250 | 91.7 (5.9) | 92.1 (1.1) | – | – |
| 500 | 96.1 (5.3) | 92.7 (4.4) | – | – |
| 1000 | 95.5 (0.8) | 91.3 (1.0) | – | – |
| Mean of means | 94.3 (2.4) | 90.7 (2.5) | 101.9 (1.7) | 96.2 (4.6) |

–, not determined.
[a] RSD, relative standard deviation ($n = 4$).

validated UPLC method were always within the FAPAS® satisfactory range (156–358 $\mu g\,kg^{-1}$ per HT-2 and 95–233 $\mu g\,kg^{-1}$ for T-2). In particular, found values (after correction for recovery) of 247 $\mu g\,kg^{-1}$ (RSDs of 4.8%, $n = 5$) and 159 $\mu g\,kg^{-1}$ (RSDs of 1.7%, $n = 5$) for HT-2 and T-2, respectively, were very close to the FAPAS® assigned values of 257 $\mu g\,kg^{-1}$ for HT-2 and 164 $\mu g\,kg^{-1}$ for T-2, demonstrating good accuracy and precision of the UPLC-PDA method. A similar study could not be carried out with wheat due to the lack of wheat certified materials.

The UPLC-PDA method was compared with the method described by Trebstein et al. for the determination of T-2 and HT-2 in oats [23] and the method of Visconti et al. for the determination of T-2 and HT-2 in wheat [21], both based on immunoaffinity column clean up and HPLC-FLD analysis after pre-column derivatization with 1-anthroylnitrile. The regression curves comparing T-2 and HT-2 levels determined with the two methods (UPLC-PDA and HPLC-FLD) in 28 oats samples naturally contaminated with T-2 and HT-2 in the range 70–2330 $\mu g\,kg^{-1}$ and in 22 wheat samples naturally contaminated with T-2 and HT-2 in the range 30–120 $\mu g\,kg^{-1}$ (single analysis) are shown in Fig. 3. A good correlation was observed for both matrices when the sum of toxins was considered, with coefficients of correlation of 0.9985 for oats and 0.9058 for wheat. Similar correlation values were observed also when the individual toxins were evaluated separately (data not shown).

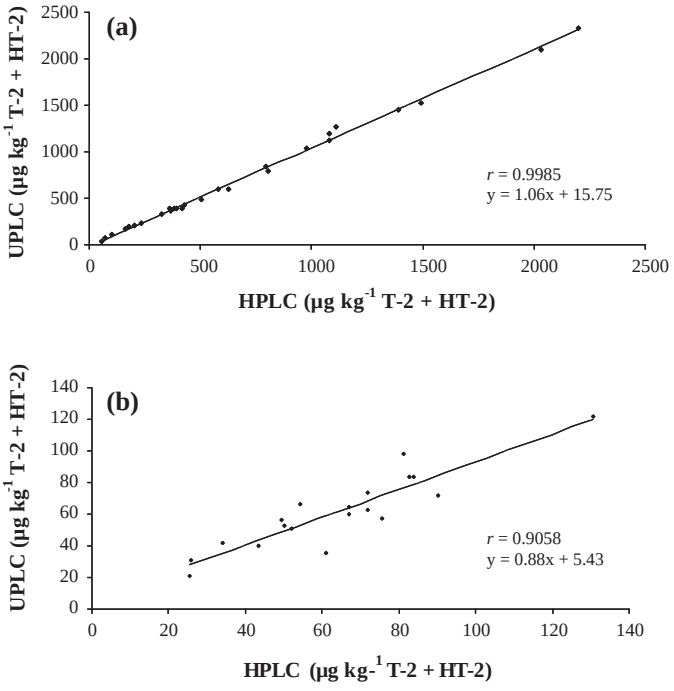


**Fig. 3.** Comparison of T-2 and HT-2 contents in (a) naturally contaminated oats samples and (b) naturally contaminated wheat samples analyzed by UPLC-PDA and HPLC-FLD after derivatization with 1-anthroylnitrile.

## 4. Conclusions

The use of UPLC allows rapid chromatographic runs leading to time-saving analysis and higher sample throughput, reducing at the same time the consumption of hazardous solvents. In addition to the drastic reduction of the chromatographic run time (5 min vs. 30 min), a major advantage of the UPLC-PDA method with respect to the HPLC-FLD methods consists in avoiding the tedious derivatization step. The sensitivity of the UPLC-PDA method, although 1.5–2.5 times lower than the HPLC-FLD methods, is suitable for quantitative determination of T-2 and HT-2 below the maximum admissible levels under discussion at the European Commission for oats and wheat. The proposed method provides a rapid, accurate and sensitive tool for generating reliable surveys on the occurrence data of these highly toxic trichothecenes in cereals, particularly oats and wheat, that are relevant for risk assessment and high priority within the European Union.

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