

A rapid fluorescence polarization immunoassay for the determination of T-2 and HT-2 toxins in wheat

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Abstract A rapid fluorescence polarization (FP) immunoassay has been developed for the simultaneous determination of T-2 and HT-2 toxins in naturally contaminated wheat samples. Syntheses of four fluorescein-labelled T-2 or HT-2 toxin tracers were carried out and their binding response with seven monoclonal antibodies was evaluated. The most sensitive antibody-tracer combination was obtained by using an HT-2-specific antibody and a fluorescein-HT-2 tracer. The developed competitive FP immunoassay in solution showed high cross-reactivity for T-2 toxin (CR%=100%) while a very low CR% for neosolaniol (0.12%) and no cross-reactivity with other mycotoxins frequently occurring in wheat. A rapid extraction procedure using 90% methanol was applied to wheat samples prior to FP immunoassay. The average recovery from spiked wheat samples (50 to 200 $\mu\text{g kg}^{-1}$) was 96% with relative standard deviation generally lower than 8%. A limit of detection of 8 $\mu\text{g kg}^{-1}$ for the combined toxins was determined. Comparative analyses of 45 naturally contaminated and spiked wheat samples by both the FP immunoassay and high-performance liquid chromatography/immunoaffinity clean-up showed a good correlation ($r=0.964$). These results, combined with the rapidity (10 min) and simplicity of the

assay, show that this method is suitable for high throughput screening as well as for quantitative determination of T-2 and HT-2 toxins in wheat.

Keywords T-2 toxin · HT-2 toxin · Fluorescence polarization · Immunoassay · Wheat

Introduction

T-2 toxin (T2) and HT-2 toxin (HT2) are type-A trichothecene mycotoxins produced by several *Fusarium* species, mainly *Fusarium sporotrichioides*, *Fusarium poae* and *Fusarium langsethiae*, that may grow on a variety of cereal grains in the field or after harvesting at high water activity, especially in cold regions. Several surveys have shown the presence of these toxins in grains including maize, oats, barley, wheat, rice, beans and soybean as well as in some cereal-based products [1–3].

T2, the most toxic trichothecene, is a potent inhibitor of DNA, RNA and protein synthesis, and shows immunosuppressive and cytotoxic effects both in vivo and in vitro [1, 4]. T2, in vivo, is rapidly metabolized to HT2 that also induces adverse effects similar to T2, with not remarkably difference in terms of potency [1]. Therefore, a combined provisional maximum tolerable daily intake was set at 0.06 $\mu\text{g kg}^{-1}$ body weight per day for T2 and HT2 alone or in combination [1], and the toxicity of T2 in vivo is considered to include that of HT2 assuming that the available toxicological data for T2 approximate the effects of HT2 [5].

The limited data concerning the exposure of consumer makes it difficult to set scientifically based and practicable regulatory limits for these toxins. The European Commission has established, with Regulations No. 1881/2006 and No.

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1126/2007, admissible levels of several *Fusarium* toxins in cereals and cereal-based products giving emphasis to the need of reliable and sensitive analytical methods and collection of more occurrence data [6, 7]. Within the European Union, there have been suggestions that regulatory limits of 100, 500, 200 and 50 $\mu\text{g kg}^{-1}$, intended as the sum of T2 and HT2, will be agreed for unprocessed cereals, unprocessed oats, oat products and infant food, respectively [8].

Analytical methods for the detection of T2 and HT2, alone or in combination with other trichothecenes, have been reviewed [9–11]. Several methods of analysis of trichothecenes used gas-chromatography (GC) based on electron-capture, flame ionisation or mass spectrometric detection (MS); but a tedious derivatisation was required to increase volatility and sensitivity of the toxins and problems of accuracy due to matrix effect were observed [12]. In addition, several studies have described the determination of T2 and HT2 by HPLC coupled with fluorescence detectors (FLD) after derivatisation with different fluorescent labelling reagents [13–15]. HPLC-MS/MS has become more popular than GC-MS for the analysis of T2 and HT2 due to its sensitivity and the fact that samples do not require derivatisation [10, 16, 17], combined to its applicability to simultaneous determination of mycotoxins having great chemical diversity [18].

Although these analytical methods permit sensitive and accurate determination of T2 and HT2, they require a preliminary clean-up of the extracts and are time-consuming, expensive, and unsuitable for screening purposes. Simpler, rapid, reliable and more effective screening methods are needed for T2 and HT2.

At this regard antibody-based methods such as enzyme-linked immunosorbent assays are commonly used [19, 20] due to their easy to use and high sample throughput; however, problems associated with long incubation times, lack of sensitivity and matrix effects were observed [18]. Other antibody-based methods applied to the analysis of type-A trichothecenes include dipstick enzyme immunoassay [21] and surface plasmon resonance immunoassay [22]. For accurate screening of T2 and HT2, the antibody should have a high specificity for both, based on the legislative that will cover the sum of the two toxins. Several commercial kits have low specificity for HT2 and can lead to an underestimation of the analyte [22].

Fluorescence polarization (FP) immunoassay is a homogeneous technique that is getting attention as screening tool in environmental monitoring and food-safety control due to its simplicity, rapidity, cheapness and reliability. FP immunoassay measures the rate of rotation of a toxin-fluorophore conjugate (tracer) in solution by monitoring the interaction between the tracer and a specific antibody. The technique is based on the measurement of the polarization

value (P), commonly expressed as millipolarization units (mP) and defined by the equation $P = (I_V - I_H)/(I_V + I_H)$ where I_V and I_H are the intensities of fluorescence of the tracer along the vertical axis and the horizontal axis, respectively. The polarization value is inversely proportional to free unlabelled antigen (i.e. mycotoxin) content in solution that competes with the tracer, and it increases when the binding of specific antibody to the tracer increases [23]. FP immunoassays have been reported for the determination of major mycotoxins, including aflatoxins, zearalenone, fumonisins, deoxynivalenol and ochratoxin A in different matrices (i.e. cereals, cereal-based products, peanut-based products and wine) [24–29].

The aim of this study was the development of a rapid, sensitive and quantitative FP immunoassay for the simultaneous determination of T2 and HT2 in wheat. The method has been validated with naturally contaminated and spiked wheat samples and compared to a widely used HPLC-FLD/immunoaffinity method.

Materials and methods

Reagents and chemicals

T2, HT2, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-Ac-DON), 15-acetyldeoxynivalenol (15-Ac-DON), diacetoxyscirpenol (DAS), neosolaniol (NEO), nivalenol (NIV), zearalenone (ZEN), ochratoxin A (OTA), phosphate-buffered saline (PBS), sodium azide (NaN_3), sodium chloride (NaCl), sodium bicarbonate, ovalbumin (OVA), 1,1'-carbonyldiimidazole (CDI) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (Milan, Italy). 4'-(Aminomethyl)-fluorescein hydrochloride was purchased from Molecular Probes (Eugene, OR, USA). A monoclonal antibody specific for T2 (clone G10-E11) and three monoclonal antibodies specific for HT2 (clones H10-A10, C12-G8, D7-C11) were obtained from the University of Natural Resources and Life Science, Vienna, Department for Agrobiotechnology IFA-Tulln (Tulln, Austria). Commercial monoclonal antibodies specific for T2 (AK-T2, Order No. AB-07-0X) and HT2 (AK-HT2, Order No. AB-08-0X), respectively, were purchased from Aokin AG (Berlin, Germany) and a monoclonal antibody specific for T2 (clone 8H2, Order No. 201062-8H2) was purchased from Softflow Biotechnology (Pécs, Hungary). Glass culture tubes (10 × 75 mm) were purchased by VWR International s.r.l. (Milan, Italy). Glass microfibre filters (Whatman GF/A) and paper filters (Whatman No. 4) were obtained from Whatman (Maidstone, UK). 1-Anthroyl cyanide (1-anthroylnitrile, 1-AN) was purchased from Wako (Neuss, Germany). T2 and HT2 immunoaffinity columns Easi-Extract® T-2 & HT-2 were purchased from R-Biopharm Rhône Ltd (Glasgow,

UK). All other chemicals and solvents were reagent grade or better and purchased by Carlo Erba Reagents (Milan, Italy). Ultrapure water was produced by a Waters Milli-Q system (Waters Corp., Milford, MA, USA).

Preparation of toxin-fluorescein tracers

T2 and HT2 were conjugated to aminofluorescein derivative in two different reactions according to the procedure reported by Maragos and Plattner for DON-fluorescein preparation [26], with minor modifications. In particular, an aliquot (224 μL) of T2 (16.2 mg mL^{-1}) or HT2 (14.7 mg mL^{-1}) solutions in dry acetone was added under vigorous mixing to 64 mg of CDI and kept at room temperature for 90 min. Water (20 μL) was added, and then 460 μL of 4'-(aminomethyl)-fluorescein (10 mg mL^{-1} in dimethylformamide) and 480 μL of 0.1 M sodium bicarbonate, pH=8.2, were added. The reaction was held at 4 $^{\circ}\text{C}$ and tested periodically over a period of 5 days by using an analytical HPLC. Chromatographic separation was performed by an Agilent 1100 Series system (Agilent Technologies, Palo Alto, CA, USA) equipped with a fluorescence detector ($\lambda_{\text{ex}}=488 \text{ nm}$, $\lambda_{\text{em}}=520 \text{ nm}$) and a Symmetry[®] column (5 μm , 150 \times 4.6 mm), Waters Corp. (Milford, MA, USA). A binary gradient was applied at a flow rate of 1 mL min^{-1} starting from an initial composition of 50% acetonitrile containing 1% acetic acid with a linear increase of acetonitrile content up to 70% in 20 min.

T2- and HT2-fluorescein tracers (T2-FL, HT2-FL_{1a}, HT2-FL_{1b} and HT2-FL₂; see Fig. 1 for structures) were isolated by semi-preparative HPLC with a PerkinElmer Series 200

pump (PerkinElmer, Norwalk, CT, USA), a Symmetry[®] semi-preparative column (7 μm , 150 \times 7.8 mm, Waters Corp.) and a fluorescence detector ($\lambda_{\text{ex}}=488 \text{ nm}$, $\lambda_{\text{em}}=520 \text{ nm}$) Jasco FP-1520 (Jasco Inc., Easton, MD, USA). Aliquots of the reaction mixtures (50 μL) were injected. T2-FL was isolated by using the above described HPLC binary gradient and applied at a flow rate of 4 mL min^{-1} . Fractions eluting between 8.5 and 9.7 min (containing T2-FL) were collected from the column, and the solvent was removed by a combination of vacuum evaporation and lyophilization. The tracers derived of HT2 (HT2-FL_{1a}, HT2-FL_{1b}, HT2-FL₂) were isolated at a flow rate of 4 mL min^{-1} by using the following binary gradient: the initial composition of the mobile phase (45% acetonitrile, containing 1% acetic acid) was kept constant for 12 min, and then acetonitrile content was linearly increased up to 100% in 10 min, and then kept constant for 10 min. Fractions eluting between 8.5 and 10, 10.5 and 12 and 17.5 and 18.2 min containing HT2-FL_{1a}, HT2-FL_{1b} and HT2-FL₂, respectively, were collected and dried as described above.

The identity of tracers (T2-FL, HT2-FL_{1a}, HT2-FL_{1b}, HT2-FL₂) was confirmed by HPLC-High Resolution Mass Spectrometry (HPLC-HRMS) analysis performed on a benchtop single stage mass spectrometer Exactive[™] equipped with an heated electrospray ion source (H-ESI; Thermo Fisher Scientific, Bremen, Germany), coupled to a HPLC system Accela (Thermo Fisher Scientific, San Jose, USA). HPLC-HRMS analyses were performed with H-ESI interface in positive ion mode, with the following settings: spray voltage, 3,000 V; capillary temperature, 300 $^{\circ}\text{C}$; sheath gas, 35 arbitrary units; auxiliary gas, 10 arbitrary

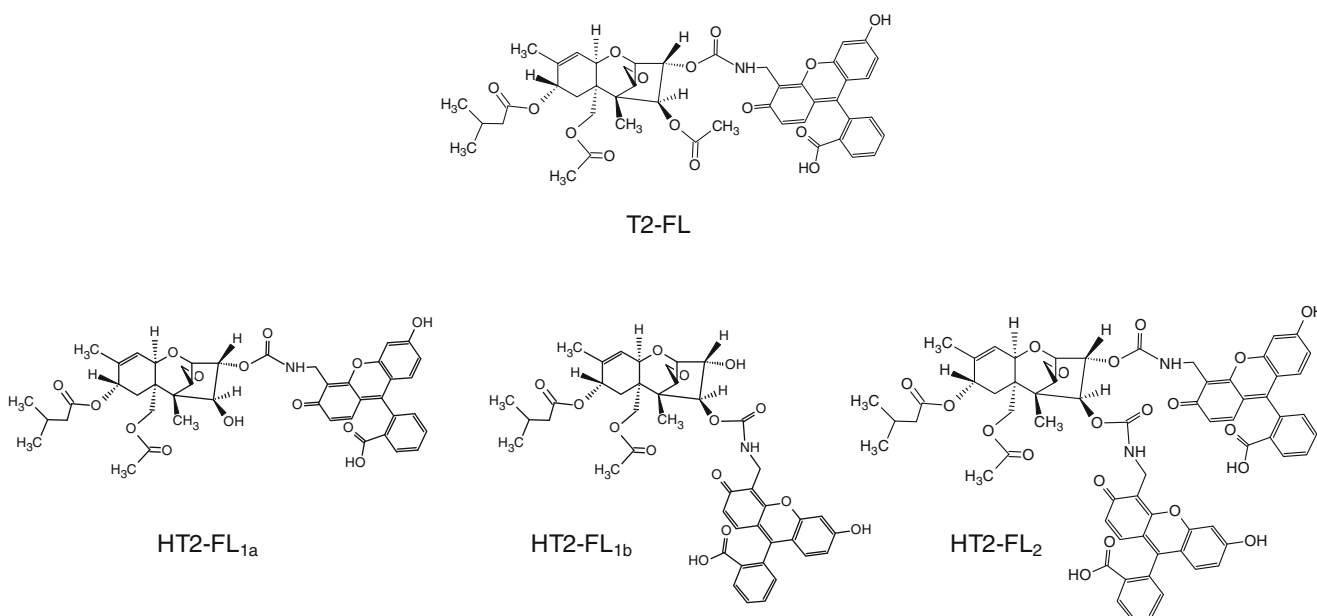


Fig. 1 Structures of fluorescein-labelled T2 toxin tracer (T2-FL) and of monosubstituted (HT2-FL_{1a} and HT2-FL_{1b}, arbitrarily ascribed to each isomeric product) and bi-substituted (HT2-FL₂) fluorescein-labelled HT2 toxin tracers

units; spare gas, 0 arbitrary units; heater temperature, 300 °C. The mass spectrometer operated in a scan range from 400 to 1,300 m/z with a resolving power of 100,000 FWHM and scan rate 1 spectra per second.

Stock solutions of the four tracers (T2-FL, HT2-FL_{1a}, HT2-FL_{1b}, HT2-FL₂) were prepared by reconstituting the isolated compounds with 2 mL of 50% acetonitrile and stored at -20 °C.

T2 and HT2 standard solutions

T2 and HT2 stock solutions were individually prepared by dissolving solid commercial toxins in acetonitrile at a concentration of 1 mg mL⁻¹. Diluted T2 and HT2 solutions in acetonitrile, at the concentration of 0.1 mg mL⁻¹, were prepared. Standard solutions of single or mixed T2 and HT2 for FP immunoassay calibration curve were prepared by dissolving, in PBS-A (PBS, sodium phosphate 10 mM, 0.85% of NaCl, pH=7.4; containing 0.1% of NaN₃), adequate amounts of the diluted solutions, previously evaporated to dryness under nitrogen stream. A mixed standard solution of T2 and HT2 was prepared in acetonitrile, at a concentration of 10 µg mL⁻¹ each toxin, for spiking purposes in recovery experiments and for the preparation of standard solutions for HPLC and FP calibrations.

Sample preparation

Durum wheat kernels belonging to different cultivars were obtained from various fields in Italy. Sample preparation was performed according to the method described by Visconti et al. [14], with minor modifications. Wheat samples were finely ground with a Tecator Cyclotec 1093 (International PBI, Milan, Italy) laboratory mill equipped with a 500 µm sieve. Ground samples (50 g) were weighed into a blender jar, added with 1 g of NaCl, and extracted with 100 mL methanol/water 90:10 (v/v) by blending at high speed for 2 min with a Steril Mixer12 blender (International PBI). The extract was filtered through filter paper and diluted with water in a ratio 1:5 (v/v). The diluted extract was then filtered through a glass microfibre filter and analysed by FP immunoassay (without further treatment) or by HPLC (after immunoaffinity column clean-up) as described below. To determine the matrix effect on the FP signal, diluted extracts of uncontaminated wheat samples were spiked at different T2/HT2 levels (ranging from 1.88 to 35 ng mL⁻¹) and analysed by the FP immunoassay.

FP immunoassay

FP analyses were performed by a Sentry® 100 portable system (Diachemix Corporation, Milwaukee, WI, USA), a

manual single-well instrument using glass culture tubes and excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 485 and 535 nm, respectively.

Tracers working solutions were prepared by diluting aliquots of the tracer stock solutions with methanol. For each tracer, the optimum tracer concentration in the test solution was determined as the dilution of the stock solution producing a total fluorescence intensity equal to about 2- to 3-fold the blank signal measured against PBS-A.

Working solutions of each monoclonal antibody (MAb) specific for T2 or HT2 were prepared by diluting aliquots of the stock solutions with PBS-OVA (PBS-A containing 0.1% of ovalbumin). The antibody binding to the tracer was assessed by measuring the maximum polarization shift ($\Delta P_{max} = mP_{MAb} - mP_{tracer}$) observed between the test solution containing the tracer (at the optimized concentration) and the test solution after adding and incubating with MAb working solutions at different concentrations (7–140 µg mL⁻¹ for clone G10-E11, 1.48–39.4 µg mL⁻¹ for clone H10-A10, 3.1–124 µg mL⁻¹ for clone C12-G8, 5.4–107 µg mL⁻¹ for clone D7-C11, 2.3–183 µg mL⁻¹ for clone AK-T2, 0.5–81 µg mL⁻¹ for clone AK-HT2 and 0.3–20 µg mL⁻¹ for clone 8H2). Optimized MAb concentrations in the test solution, corresponding to the lowest MAb concentration providing ΔP_{max} , were determined per each antibody-tracer combination.

Competitive FP immunoassays were carried out with single T2 and HT2 standard solutions by using the selected tracers (T2-FL and HT2-FL_{1a}) and antibodies (clones H10-A10, C12-G8, AK-HT2 and 8H2) at the optimized concentrations. FP analyses were performed by adding in a test tube 100 µL of antibody working solution with 50 µL of single T2/HT2 standard solution and PBS-A up to 1,000 µL. After thorough mixing by vortex, the test solution was placed in the instrument and FP signal was used as the blank. Twenty-five microlitres of tracer working solution was added and mixed by vortex. The tracer containing test solution was then placed in the instrument, and the polarization value, expressed in millipolarization units (mP), was measured. In these experiments to evaluate the kinetics of the competition, measurements were made after holding at room temperature for time ranging from 15 s and 10 min.

Optimized FP immunoassays were performed by adding 100 µL of antibody working solution (clone H10-A10) and then 200 µL of filtered extract (equivalent to 20 mg of matrix) or 50 µL of mixed T2/HT2 standard solution and PBS-A up to 1,000 µL. After mixing the test solution was placed in the instrument and the signal was used as the blank. Tracer working solution (25 µL of HT2-FL_{1a}) was added and mixed. Polarization value was measured after 5 min (incubation time).

The polarization values were normalized to fit the range 0–1, using the equation $Y_{obs} = (mP_{obs} - mP_0) / (mP_1 - mP_0)$,

where mP_{obs} , mP_0 and mP_1 are the polarization of the test solution, the polarization of an antibody-free control solution and of a toxins-free control solution, respectively, and Y_{obs} is the normalized result for the test solution [27].

Cross-reactivity of the FP immunoassay

The cross-reactivity of the optimized FP immunoassay was determined for structurally related (DAS, NEO, NIV, DON, 3-Ac-DON and 15-Ac-DON) and commonly occurring (OTA and ZEN) mycotoxins. Stock solutions of the mycotoxins were prepared either in acetonitrile, acetonitrile/water or methanol depending upon the solubility characteristics of each toxin. Once in solution, the toxins were diluted to $100 \mu\text{g mL}^{-1}$ with methanol, and $50 \mu\text{L}$ was tested as described above. NEO that showed reactivity with the immunoassay at this level was further tested over the concentration range of 0.1 to $100 \mu\text{g mL}^{-1}$. The midpoint relative response of the calibration curve was used to calculate the midpoint concentration (IC_{50}) for NEO and cross-reactivity was calculated as a percentage relative to T2/HT2 ($[IC_{50[\text{T2+HT2}]} / IC_{50[\text{NEO}]}] \times 100$, where $IC_{50[\text{T2+HT2}]}$ and $IC_{50[\text{NEO}]}$ represent midpoint concentrations for T2/HT2 and NEO, respectively).

HPLC analysis

T2 and HT2 analyses of wheat samples were performed according to the method described by Visconti et al. [14], with minor modification. Ten millilitres of the diluted filtered extract (equivalent to 1 g sample) were passed through the immunoaffinity column at a flow rate of about one drop per second, followed by 10 mL of distilled water at a flow rate of about one or two drops per second. T2 and HT2 were then eluted with 1.5 mL of methanol and collected in a 4-mL screw-cap amber vial. The eluted extract was evaporated under a stream of nitrogen at ca. $50 \text{ }^\circ\text{C}$, and the dried residue was derivatised with 1-AN. In particular, $50 \mu\text{L}$ of DMAP solution (0.325 mg mL^{-1} in toluene), followed by $50 \mu\text{L}$ of 1-AN solution (0.3 mg mL^{-1} in toluene), was 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 $50 \text{ }^\circ\text{C}$ in a heater block and then cooled at $-20 \text{ }^\circ\text{C}$ for 10 min. After the cap was removed, the mixture was allowed to dry under a gentle stream of nitrogen for 2 min at ca. $50 \text{ }^\circ\text{C}$ and reconstituted with $1,000 \mu\text{L}$ of a mixture 70% acetonitrile: 30% water (v/v). Twenty microlitres of the solution was injected into the chromatographic apparatus. HPLC analyses were carried out using a Waters Alliance HT 2795 system (Waters Corp., Milford, MA, USA) equipped with a fluorometric detector (model 2475, $\lambda_{\text{ex}}=381 \text{ nm}$, $\lambda_{\text{em}}=470 \text{ nm}$) and the Empower 2 data software (Waters Corp.).

The analytical column was a Phenyl-Hexyl Luna[®] ($150 \times 4.6 \text{ mm}$, $5 \mu\text{m}$) (Phenomenex, Torrance, CA, USA), preceded by a SecurityGuard[™] C18 cartridge ($4 \times 3 \text{ mm}$, $5 \mu\text{m}$; Phenomenex). The flow rate of the mobile phase was 1 mL min^{-1} . A binary gradient was applied as follows: the initial composition of the mobile phase, 70% acetonitrile, 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 then kept constant for 5 min. The detection limit (signal to noise ratio of 3:1) of the method was 5 and 3 mg kg^{-1} for T2 and HT2, respectively.

Recovery experiments

Recovery experiments were performed in triplicate by spiking uncontaminated wheat samples with a mixed T2 and HT2 spiking solution at levels of 50, 100 and $200 \mu\text{g kg}^{-1}$. Spiked samples were left overnight at room temperature to allow solvent evaporation prior to extraction and analysis by both FP immunoassay and HPLC method.

Statistical analysis

Linear and sigmoidal fits of the FP immunoassay data were performed by means of the unweighted least-square method by Origin version 6.0 (OriginLab Corporation, Northampton, MA, USA). In particular, sigmoidal curves used the following logistic equation: $y=A_2+[A_1-A_2/1+(x/x_0)^P]$, where A_1 and A_2 represent the initial (left horizontal asymptote) and final value (right horizontal asymptote), respectively, x_0 the centre (inflection point) and P the power. Comparison between linear regression curves were performed by parallelism and position statistical tests [30]. Limit of detection (LOD) of the FP immunoassay was calculated from the mean FP signal of representative uncontaminated wheat samples ($n=10$) minus 3 standard deviation of the mean signal [31]. For recovery experiments, homogeneities of variances and means among the three spiking levels of contamination were confirmed using Bartlett's test and one-way ANOVA ($p=0.05$), respectively.

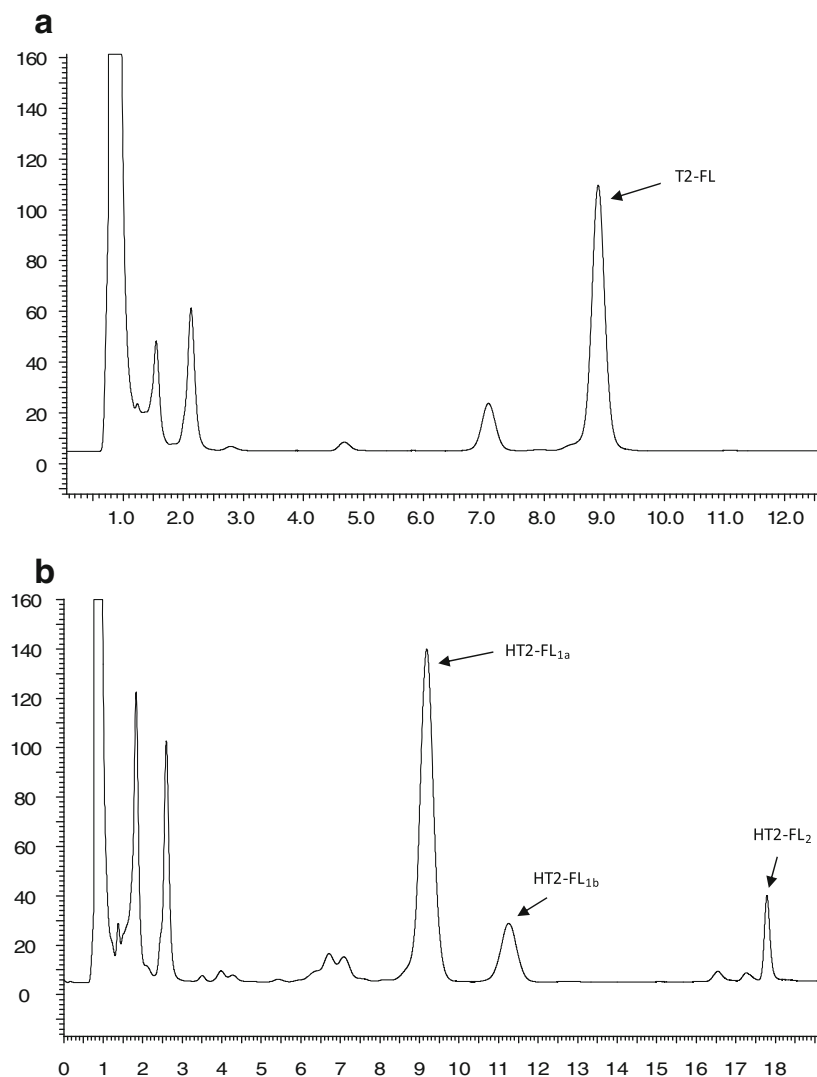
Statistical evaluation of data (means and standard deviations) relevant to recovery experiments and matrix effect was performed using Sigma-Stat for Windows version 5.0 statistical software (Graphpad Software, La Jolla, CA, USA).

Results

Synthesis of the fluorescein-toxin tracers

Fluorescein-toxin tracers (T2-FL, HT2-FL_{1a}, HT2-FL_{1b}, HT2-FL₂) were obtained from the reaction of T2 or HT2 with 4'-

Fig. 2 Semipreparative HPLC chromatograms of the reaction mixtures for the preparation of fluorescein-labelled tracers obtained, after 4 days, starting from (a) T2 and (b) HT2. Chromatographic conditions are described in the “Materials and methods” section (preparation of toxin-fluorescein tracers)



(aminomethyl)fluorescein after activation with 1,1'-carbonyldiimidazole, as previously reported for deoxynivalenol by Maragos and Plattner [26]. T2-FL was the only T2-fluorescein derivative, whereas the derivatisation reaction of HT2 led to two isomeric monosubstituted products (HT2-FL_{1a} and HT2-FL_{1b}) and a bi-substituted product (HT2-FL₂), due to the presence of two hydroxyl groups at -C3 and -C4 positions,

respectively (Fig. 1). Figure 2 shows the chromatograms of the reaction mixtures obtained after a 4-day reaction starting from T2 or HT2. In the first chromatogram, the peak at 8.9 min retention time corresponded to T2-FL (Fig. 2a); whereas in the second chromatogram, the peaks at 9.2, 11.2 and 17.8 min were ascribed to HT2-FL_{1a}, HT2-FL_{1b} and HT2-FL₂, respectively (Fig. 2b).

Table 1 Maximum values of polarization shift (ΔP_{\max}) and relevant optimized antibody concentrations obtained for each antibody-tracer combination

Mab	Clone	[Mab] ($\mu\text{g mL}^{-1}$)	ΔP_{\max}^a			
			T2-FL	HT2-FL _{1a}	HT2-FL _{1b}	HT2-FL ₂
Anti-T2	G10-E11	27.9	–	–	–	–
	AK-T2	24.4	80	45	–	–
	8H2	3.3	230	20	–	–
Anti-HT2	H10-A11	11.8	200	230	110	50
	C12-G8	24.8	100	130	110	15
	D7-C11	21.4	25	25	–	–
	AK-HT2	5.4	200	230	110	15

^a $\Delta P_{\max} = mP_{\text{Mab}} - mP_{\text{tracer}}$
 $-\Delta P_{\max} < 10$ (no significant antibody-tracer binding)

The identity of the tracers was confirmed by HPLC-HRMS analysis (positive chemical ionization mode) of the reaction mixtures. Full scan chromatogram of the T2 reaction mixture revealed the presence, at the T2-FL retention time, of a molecular ion with m/z 854.2998 ($C_{46}H_{48}O_{15}N$ calculated m/z , 854.3018; error, -2.3 ppm), corresponding to the $[(T2-FL)-H]^+$ adduct. On the other hand, full scan chromatogram of the HT2 reaction mixture showed, at the retention times of isomeric monosubstituted products HT2-FL_{1a} and HT2-FL_{1b}, the presence of molecular ions with m/z 812.2896 and 812.2893 ($C_{44}H_{46}O_{14}N$ calculated m/z , 812.2913), corresponding to the $[(HT2-FL)-H]^+$ adducts, with errors of -2.1 and -2.5 ppm, respectively. In addition, at the retention time of bi-substituted product (HT2-FL₂), a molecular ion with m/z 1,199.3634 ($C_{66}H_{59}O_{20}N_2$ calculated m/z , 1,199.3656; error, -1.8 ppm) was observed, corresponding to the $[(HT2-FL_2)-H]^+$ adduct. The tracers were isolated and purified by semi-preparative HPLC.

Study of the antibody-tracer interactions

The assessment of the antibody-tracer binding was performed by FP immunoassay for the four synthesized tracers and seven monoclonal antibodies (MAbs), three specific for T2 and four specific for HT2. Table 1 shows the maximum values of polarization shift (ΔP_{max} , maximum tracer-antibody binding) and optimized MAbs concentrations obtained for each antibody-tracer combination. Antibody clone G10-E11 exhibited no binding with all tested tracers. While antibodies clones AK-T2, 8H2 and D7-C11 showed binding only with T2-FL and HT2-FL_{1a} tracers, clones H10-A10, C12-G8 and AK-HT2 bound all tested tracers. Highest binding were observed for antibody-tracer combinations 8H2/T2-FL, H10-A10/HT2-FL_{1a} and AK-HT2/HT2-FL_{1a} ($\Delta P_{max}=230$ mP). These combinations were subsequently used in the optimization of competitive FP immunoassays.

Optimization of the FP immunoassay

The FP immunoassay was used to measure the competition between the tracer and unlabeled T2/HT2 in solution for binding the toxin-specific antibody. The effect of the antibody-tracer incubation time (from 15 s to 10 min), in the presence of T2/HT2, upon FP response was investigated. The time required for the FP signal to reach equilibrium was 2 min for competitive FP immunoassays using the antibody-tracer combinations of 8H2/T2-FL and AK-HT2/HT2-FL_{1a} and 5 min for the immunoassay with H10-A10/HT2-FL_{1a} combination.

Calibration curves obtained with single T2 and HT2 standard solutions for antibody-tracer combinations 8H2/

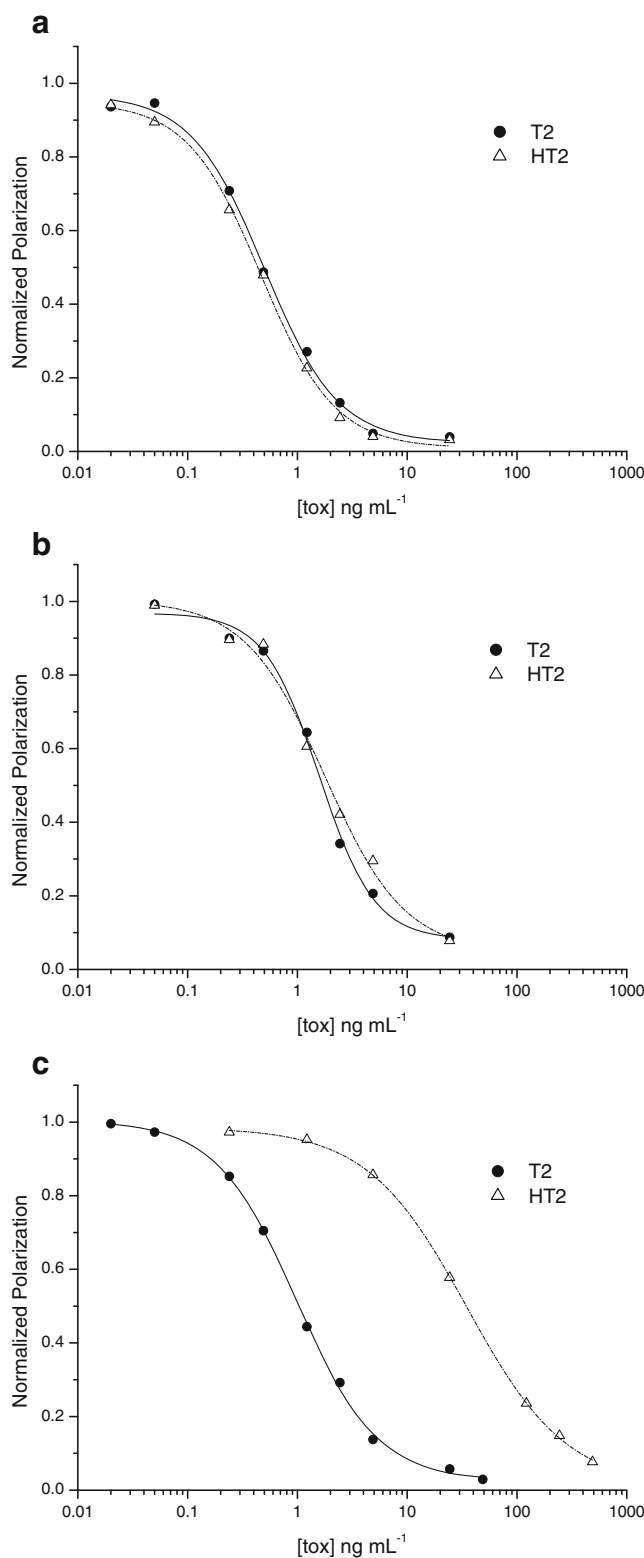
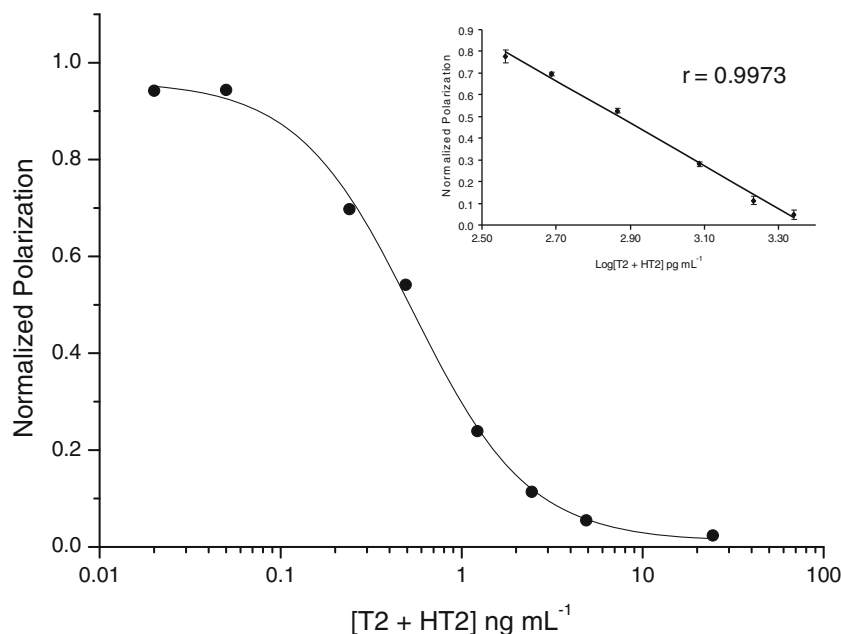


Fig. 3 Normalized calibration curves obtained with standard solution of T2 (filled circle) and HT2 (triangle) for FP immunoassay using antibody-tracer combination of: (a) H10-A10/HT2-FL_{1a}, (b) AK-HT2/HT2-FL_{1a} and (c) 8H2/T2-FL

Fig. 4 Normalized calibration curve of the FP immunoassay, using antibody-tracer combination of H10-A10/HT2-FL_{1a}, obtained with mixed standard solutions of T2 and HT2 (expressed as sum) in PBS-A solution. The FP linearity range vs log [T2+HT2] is reported in the *insert*



T2-FL, H10-A10/HT2-FL_{1a} and AK-HT2/HT2-FL_{1a} are shown in Fig. 3. Competitive FP immunoassays using the antibody-tracer combination H10-A10/HT2-FL_{1a} (Fig. 3a) and AK-HT2/HT2-FL_{1a} (Fig. 3b) showed 100% cross-reactivity for both T2 and HT2 that allow the determination of the sum of the toxins. On the contrary, the FP immunoassay based on the use of 8H2/T2-FL combination (Fig. 3c) exhibited a lower cross-reactivity for HT2 (2.8%, $IC_{50}=35 \text{ ng mL}^{-1}$) as compared with T2 ($IC_{50}=0.98 \text{ ng mL}^{-1}$) leading to under-estimation of the sum of the toxins content.

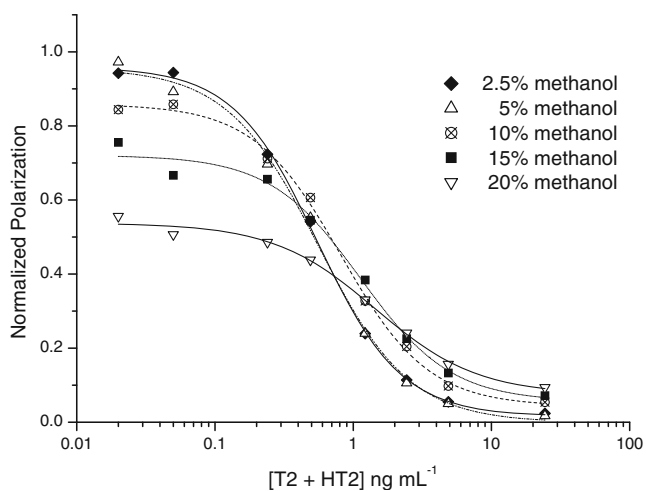


Fig. 5 Normalized calibration curve of the optimized FP immunoassay carried out with T2/HT2 standard solutions in presence of different methanol contents: 2.5% (filled diamond), 5% (triangle), 10% (circled times), 15% (filled square) and 20% (inverted triangle) v/v, in PBS-A solution

Calibration curves were performed by mixed standard solutions of T2 and HT2 at different concentrations (expressed as the sum of the two toxins) for FP immunoassays using H10-A10/HT2-FL_{1a} and AK-HT2/HT2-FL_{1a} antibody-tracer combinations. The FP immunoassay using H10-A10/HT2-FL_{1a} combination showed a midpoint concentration ($IC_{50}=0.54\pm 0.04 \text{ ng mL}^{-1}$) lower than the assay using AK-HT2/HT2-FL_{1a} combination ($IC_{50}=1.61\pm 0.22 \text{ ng mL}^{-1}$). Due to the better sensitivity of the assay, the combination antibody-tracer H10-A10/HT2-FL_{1a} was used in all subsequent experiments.

Figure 4 shows the calibration curve obtained for the FP immunoassay with H10-A10/HT2-FL_{1a} combination in the

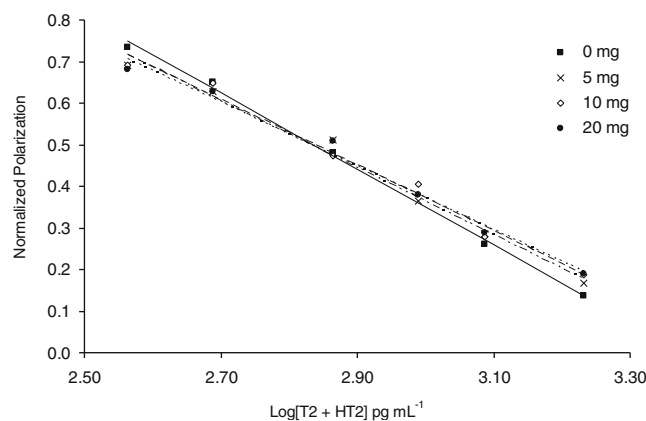


Fig. 6 Calibration curves in the linearity range of concentration (0.37 to 1.71 ng mL^{-1}) obtained by using either T2/HT2 standard solutions (filled square) and spiked diluted extracts with a matrix equivalent analysed of: 5 mg (multiplication sign), 10 mg (diamond) and 20 mg (filled circle)

Table 2 Average recoveries of T2 and HT2, expressed as the sum of the toxins, from spiked durum wheat obtained by FP immunoassay and HPLC method

Spiking level ($\mu\text{g kg}^{-1}$)	FP immunoassay			HPLC		
	Recovery	SD	RSD (%)	Recovery	SD	RSD (%)
50	96	8	8	78	10	12
100	89	4	4	80	6	8
200	103	5	5	82	6	7
Overall average	96	8	8	80	7	8

SD standard deviation ($n=3$ replicates), RSD relative standard deviation

concentration range $0.02\text{--}24.4 \text{ ng mL}^{-1}$. A good correlation (coefficient of correlation, $r=0.9973$) between polarization values and logarithm of T2/HT2 concentrations was observed in the concentration range $0.37\text{--}2.20 \text{ ng mL}^{-1}$ with good repeatability of the FP responses (relative standard deviation $<10\%$; $n=3$).

Cross-reactivity of the FP immunoassay

Antibody cross-reactivity was tested, in the optimized FP immunoassay, against structurally related mycotoxins such as deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, neosolaniol, nivalenol and other mycotoxins commonly occurring in wheat such as zearalenone and ochratoxin A. The antibody demonstrated high specificity for T2 and HT2, a very low cross-reactivity for neosolaniol ($\text{CR}\%=0.12\%$; $\text{IC}_{50}=461.6 \text{ ng mL}^{-1}$) and no cross-reactivity was observed for all tested mycotoxins.

Matrix effect and recovery experiments

To evaluate the compatibility of the optimized FP immunoassay for organic solvents, the assay performance was evaluated using different methanol contents. When the FP immunoassay was carried out with mixed T2/HT2 standard solutions ($0.02\text{--}24.4 \text{ ng mL}^{-1}$), the methanol content increase from 2.5% (methanol content of the optimized FP immunoassay) to 20% caused a reduction of the polarization range of the calibration curve, affecting negatively the precision of the method (Fig. 5). However, no significant difference in the FP response was observed between 2.5% and 5% methanol.

In order to evaluate the possible matrix interferences in the FP measurements, calibration curves in the concentration range $0.37\text{--}1.71 \text{ ng mL}^{-1}$ were determined by using either T2/HT2 standard solutions or spiked diluted extract of uncontaminated wheat samples (Fig. 6). Spiked extracts were analysed by using different amounts of matrix equivalent, namely 5, 10 and 20 mg (corresponding to 2.5%, 3.75% and 5% methanol, respectively). No significant differences were observed between slopes ($t_{\text{calc}} < 2.306$; $p < 0.05$) and positions ($t_{\text{calc}} < 2.262$; $p < 0.05$) of the regression lines obtained with T2/HT2 standard solutions in buffer and those obtained in the

presence of spiked diluted extracts. This indicated the whole absence of detectable matrix effects that could produce an overestimation of toxins content. For the optimized FP immunoassay, analysing 20 mg of matrix equivalent (corresponding to 5% methanol), a LOD of 0.15 ng mL^{-1} (equivalent to $8 \mu\text{g kg}^{-1}$ in wheat samples) was calculated.

Results of the recovery experiments in durum wheat with T2 and HT2 in the spiking range $50\text{--}200 \mu\text{g kg}^{-1}$ are shown in Table 2. Average recovery for FP immunoassay was 96% with relative standard deviation lower than 8%, whereas average recovery for HPLC method (with immunoaffinity cleanup) was 80% with relative standard deviation lower than 12%.

Comparison of FP immunoassay and HPLC/FLD for contaminated wheat samples

For comparison, a total of 55 durum wheat samples, of which 22 naturally contaminated with T2 and HT2 at concentrations from 10 to $100 \mu\text{g kg}^{-1}$ (expressed as the sum and determined by HPLC), 23 spiked samples at levels of from 100 to $300 \mu\text{g kg}^{-1}$ and 10 uncontaminated samples, were tested by both FP immunoassay and HPLC/FLD with immunoaffinity clean-up method. No false positive result was observed by FP

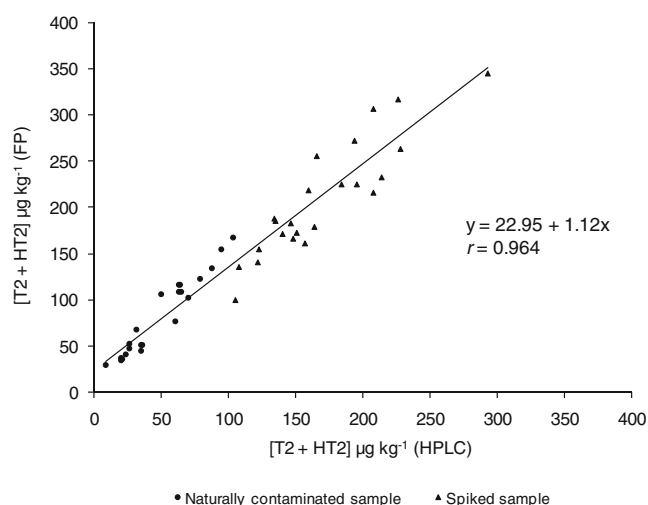


Fig. 7 Comparison of T2/HT2 contents in naturally contaminated (filled circle) and spiked (filled triangle) samples analysed by HPLC and FP immunoassay (data corrected for relevant average recoveries)

immunoassay for uncontaminated samples. A good correlation (coefficient of correlation, $r=0.964$) between T2/HT2 concentrations obtained by FP immunoassay and HPLC method was found for the 45 contaminated samples (Fig. 7). The linear regression fit was of the form $[T2+HT2 \text{ by FP}] = 22.95 + 1.12 [T2+HT2 \text{ by HPLC}]$, where all data were previously corrected for average recoveries.

Discussion

In our study a rapid, easy-to-perform and sensitive FP immunoassay has been developed for the determination of the total content of T2 and HT2 in wheat. Important FP immunoassay parameters such as speed, precision and sensitivity are depending upon the selection of the appropriate antibody-tracer combination. Although the antibody is the key reagent for specificity and sensitivity of immunoassays, the structure of the tracer may also have a significant influence on the assay sensitivity. The interactions between four fluorescein-labelled T2/HT2 tracers, ad hoc synthesized, and seven monoclonal antibodies have been studied. Two competitive FP immunoassays using the antibody-tracer combination H10-A10/HT2-FL_{1a} and AK-HT2/HT2-FL_{1a}, having 100% cross-reactivity for both T2 and HT2, allowed the determination of the sum of the toxins. The sensitivity of the FP immunoassays using H10-A10/HT2-FL_{1a} ($IC_{50} = 0.54 \pm 0.04 \text{ ng mL}^{-1}$) and AK-HT2/HT2-FL_{1a} ($IC_{50} = 1.61 \pm 0.22 \text{ ng mL}^{-1}$) combinations was better than that of other published antibody-based assay for T2/HT2 determination.

The H10-A10/HT2-FL_{1a} combination was selected for the method developed herein because of its better sensitivity (LOD, $8 \mu\text{g kg}^{-1}$ expressed as the sum) that was comparable with that of HPLC/FLD immunoaffinity cleanup method [14].

In addition, the absence of long incubation times and washing steps reduces the time of analysis as compared to other antibody-based methods. In fact, the overall time consumed in T2/HT2 analysis with the optimized immunoassay procedure was less than 10 min, thus allowing the performance of the analysis with a very high sample throughput.

Due to the absence of significant matrix effect (in the tested conditions) and cross-reactivity against the mycotoxins most frequently occurring in wheat, the developed FP immunoassay avoids overestimation of the toxins content. Analytical performance in terms of accuracy and precision values of FP immunoassay was better than that HPLC method and fulfil the criteria established by the European Union for acceptance of an analytical method for the determination of T2 and HT2 [32]. The accuracy of the

assay was confirmed by the good correlation ($r=0.996$) of the results obtained by FP immunoassay and HPLC/FLD method for 45 naturally contaminated or spiked wheat samples. In addition, no false positive result was observed for uncontaminated samples.

Furthermore the proposed assay is inexpensive, easy-to-perform, suitable for automation and it uses a portable instrument. These findings indicate that this method is suitable for high throughput screening as well as for quantitative determination of T2 and HT2 in wheat and can be used as a valid alternative method to HPLC/FLD. Moreover, the concept of determining the total content of T2 and HT2 in cereal samples, for both official control purposes and risk assessment studies, is definitely in line with incoming EU legislation requirements [33].

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