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Optimization and Validation of a Fluorescence Polarization Immunoassay for Rapid Detection of T-2 and HT-2 Toxins in Cereals and Cereal-Based Products

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Abstract A fluorescence polarization (FP) immunoassay has been optimized and validated for rapid quantification of T-2 and HT-2 toxins in both unprocessed cereals, including oats, barley and rye, and cereal-based products for direct human consumption, such as oat flakes, oats crispbread and pasta. Samples were extracted with 90 % methanol, and the extract was filtered and diluted with water or sodium chloride solution prior to the FP immunoassay. Overall mean recoveries from spiked oats, rye, barley, oat flakes, oats crispbread and pasta ranged from 101 to 107 %, with relative standard deviations lower than 7 %. Limits of detection (LODs) of the FP immunoassay were 70 μg/kg for oats, 40 μg/kg for oat flakes and barley, 25 μg/kg for pasta and 20 μg/kg for rye and oats crispbread. The trueness of the immunoassay was assessed by using two oat and oat flake reference materials for T-2 and HT-2 toxins, showing good accuracy and precision. Good correlations ($r > 0.953$) were observed between T-2 and HT-2 toxin contents in naturally and artificially contaminated samples determined by both FP immunoassay and ultra-high-performance liquid chromatography (UHPLC) with immunoaffinity column cleanup used as reference method. These results, combined with rapidity and simplicity of the assay, show that the optimized assay is suitable for high-throughput screening, as well as for reliable quantitative determination of T-2 and HT-2 toxins in cereals and cereal-based products.

Keywords T-2 and HT-2 toxins · Fluorescence polarization immunoassay · Oats · Barley · Rye · Cereal-based products

Introduction

T-2 toxin (T-2) and HT-2 toxin (HT-2) are type A trichothecene mycotoxins produced by several *Fusarium* species, mainly *Fusarium sporotrichioides*, *Fusarium langsethiae* and *Fusarium poae*. Generally, these *Fusarium* species can grow on cereals and produce T-2 and HT-2 under moist cool conditions already prior to harvest (Canady et al. 2001; Schothorst and van Egmond 2004; van der Fels-Klerx 2010; EFSA 2011).

The major toxic effect of T-2 is inhibition of protein, DNA and RNA synthesis. In addition, T-2 induces haematotoxicity, dermal toxicity and immunosuppression in several animal species and cytotoxic effects both in vitro and in vivo. Acute toxicity of T-2 is quite high, with LD50 values for rodents in the range 5–10 mg/kg body weight. T-2, in vivo, is rapidly metabolized to HT-2 that also induces adverse effects similar to T-2, with an acute toxicity in the same range (Canady et al. 2001; EFSA 2011).

Occurrence data collected in the scientific opinion on the risk for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed showed that the highest mean concentrations for the sum of T-2 and HT-2 were observed in oats and oat products followed by barley (EFSA 2011). Levels in unprocessed grains were higher than in grain products for human consumption, suggesting that processing applied to grains results in lower T-2 and HT-2 concentrations. Several studies demonstrated that during cleaning, sorting and milling processes of grains, T-2 and HT-2 are not destroyed but unevenly redistributed between fractions (Scudamore et al. 2007, 2009; Pascale et al. 2011). Moreover, T-2 and HT-2

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are relatively stable compounds during baking and cooking (Beyer et al. 2009; Schwake-Anduschus et al. 2010).

Among cereals, rye is, in addition to wheat, the major bread grain in northern Europe where it is the second key dietary source of T-2 exposure (Eriksen and Alexander 1998; Bondia-Pons et al. 2009). Furthermore, rye plays an important role in whole-grain food production, of which consumption can be associated with reduced incidence of chronic diseases such as diabetes, cardiovascular diseases and some types of cancer. Thus, it is particularly important to ensure the safety of the consumer so that the positive nutritional effects of these food grains and products derived from them are not compromised by the presence of mycotoxins (Błajet-Kosicka et al. 2014).

Therefore, basing on the available data concerning toxicity and exposure to T-2- and HT-2-contaminated food, the EFSA panel on Contaminants in Food Chain (CONTAM) has established a group tolerable daily intake (TDI) of 0.1 μg/kg body weight per day for the sum of T-2 and HT-2 toxins (EFSA 2011).

Although maximum permitted levels have been set by the European Commission in several food products for various mycotoxin-matrix combinations (European Commission 2006a), legal levels for T-2 and HT-2 were discussed for several years but not established yet. In an attempt to assess changes and trends in human and animal exposure to T-2 and HT-2 toxins, the European Commission has recommended to collect more monitoring data on the presence of these mycotoxins in cereals and cereal-based products. Therefore, in the Recommendation no. 165/2013 (European Commission 2013), the commission has reported indicative levels for the sum of T-2 and HT-2 (μg/kg) in cereals and cereal-based products, from which/above which investigations should be performed. In particular, for unprocessed oats, barley and rye, indicative levels were fixed at 1000, 200 and 100 μg/kg, respectively, while for cereal-based products, for direct human consumption, such as oat flakes, the indicative level was fixed at 75 μg/kg; for bread, small bakery wares, pastries, biscuits, cereal snacks and pasta, the indicative level was 25 μg/kg.

A rapid monitoring of T-2 and HT-2 in both unprocessed cereals and cereal-based products is highly demanded in order to provide safe food to the consumers. Rapid, sensitive and reliable methods for the determination of T-2 and HT-2 are highly required. Although no official methods for the determination of T-2 and HT-2 are available, several analytical methods have been developed and extensively reviewed (Lattanzio et al. 2009; Meneely et al. 2011; Krska et al. 2014). These methods, which allow the detection of T-2 and HT-2 alone or in combination with other trichothecenes, are mainly based on chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC). GC and HPLC methods require a pre-column derivatization step to provide higher sensitivity allowing the determination of T-2 and HT-2 at levels established by the European Commission (European Commission 2006b). The derivatization is a long step with relevant problems of precision and matrix interferences (Jiménez et al. 2000; Petterson and Langseth 2002; Visconti et al. 2005; Lippolis et al. 2008). The use of the ultra-high-performance liquid chromatography (UHPLC) coupled with a photodiode array (PDA) for the simultaneous determination of T-2 and HT-2 in unprocessed cereals without pre-column derivatization has been recently proposed (Pascale et al. 2012). Furthermore, LC-MS/MS has become the most frequently used approach for the analysis of T-2 and HT-2 due to its sensitivity, applicability to simultaneous determination of co-occurring mycotoxins and absence of derivatization steps (Berthiller et al. 2005; Lattanzio et al. 2007; Lattanzio et al. 2009; Krska et al. 2014).

Generally, rapid methods for screening mycotoxins do not require tedious and time-consuming sample preparations, as well as matrix-matched standards or the use of internal standards. The majority of screening assays employed to determine T-2 and HT-2 are immunochemical methods and include enzyme-linked immunochemical assays (ELISA) (Yoshizawa et al. 2004), lateral flow devices (FLD) (Molinelli et al. 2008), biosensor assay (Meneely et al. 2010) and FP immunoassay (Lippolis et al. 2011).

Fluorescence polarization (FP) immunoassay is a homogeneous technique that is getting attention as a screening tool in food safety control due to its simplicity, rapidity, cheapness and reliability. Several FP immunoassays have been reported and reviewed for mycotoxin analysis, including aflatoxins, fumonisins, deoxynivalenol, ochratoxin A, zearalenone and T-2 and HT-2 toxins, in different food commodities (Lippolis and Maragos 2014). These immunoassays are based on the competition between the mycotoxin and the mycotoxin labelled with a fluorophore (tracer) to a specific antibody. The binding of the tracer to the antibody affects the rotation of the tracer molecule and increases the FP value. The amount of bound tracer is inversely related to the amount of free mycotoxin present in the sample, and as a result, the polarization value is inversely related to the analyte concentration.

An FP immunoassay for the determination of the sum of T-2 and HT-2 in wheat has been recently developed (Lippolis et al. 2011). The proposed method coupled performances in terms of sensitivity, accuracy and precision comparable to those of a chromatographic technique with rapidity (10 min), costs and simplicity typical of a high-throughput screening method. The concept of determining the total content of T-2 and HT-2 in cereal samples for both official control purposes and risk assessment studies results in line with EC Recommendation (European Commission 2013).

The aim of present work was to evaluate the applicability of the abovementioned FP immunoassay to unprocessed cereals, such as oats, barley and rye, and cereal-based products, such as pasta, oat flakes and oats crispbread, for the

quantitative determination of the total content of T-2 and HT-2. An extensive validation of this FP immunoassay was also performed on all tested matrices by using naturally and/or artificially contaminated samples and reference materials, when commercially available.

Materials and Methods

Chemicals and Reagents

T-2 and HT-2 toxins, phosphate-buffered saline (PBS), sodium azide (NaN_3), sodium chloride (NaCl) and ovalbumin (OVA) were purchased from Sigma-Aldrich (Milan, Italy). The monoclonal antibody specific for HT-2 (clone H10-A10) was purchased from the University of Natural Resources and Life Science, Vienna, Department for Agrobiotechnology IFA-Tulln (Tulln, Austria). 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). T-2 and HT-2 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 Standard and Immunoreagent Solutions

T-2 and HT-2 stock solutions were individually prepared by dissolving solid commercial toxins in acetonitrile at a concentration of 1 mg/mL. Diluted T-2 and HT-2 solutions were prepared in acetonitrile at a concentration of 0.1 mg/mL. A mixed standard solution of T-2 and HT-2 was prepared in acetonitrile, at a concentration of 10 μg/mL each toxin, for spiking purposes in recovery experiments and for preparation of standard solutions for UHPLC and FP calibrations. Standard solutions of mixed T-2 and HT-2 for FP immunoassay calibration curves were prepared by dissolving in PBS (sodium phosphate 10 mM, 0.85 % of NaCl, pH = 7.4) containing 0.1 % of sodium azide (PBS-A) adequate amounts of the diluted solutions, previously evaporated to dryness under nitrogen stream.

FP measurements were conducted by using the tracer, HT-2 conjugated with 4′-(aminomethyl)-fluorescein (HT2-FL), prepared according to Lippolis et al. (2011). The tracer working solution was prepared daily by diluting aliquots of the tracer stock solution with a dilution ratio 1:3000 *v*/*v* in PBS-A. Antibody working solution was prepared daily by diluting aliquots of the stock solution of HT-2 monoclonal antibody, clone H10-A10, at a ratio of 1:450 (*v*/*v*) in PBS-OVA (PBS-A containing 0.1 % OVA).

Samples Preparation

Barley samples were obtained from various fields in Italy. Pasta and oat flake samples were purchased by Italian retail markets. Rye, oats and oats crispbread were derived from northern Europe countries' suppliers and local markets. Samples were ground with the Ultra Centrifugal Mill ZM 200 (Retsch Technology GmbH, Haan, Germany) laboratory mill equipped with a 500-μm sieve. Sample extraction was performed according to the method originally developed by Visconti et al. (2005) for the analysis of T-2 and HT-2 in cereals and subsequently optimized by Trebstein et al. (2008) for oats and cereal-based products, with minor modifications. In particular, ground samples (50 g for barley and pasta; 25 g for oats, rye, oat flakes and oats crispbread) were weighed into a blender jar, added with NaCl (1 g for barley and pasta; 2.5 g for oats, rye, oat flakes and oats crispbread) and extracted with 100 mL methanol/water 90:10 (*v*/*v*) by blending at high speed for 3 min with the Steril Mixer 12 blender (International PBI, Milan, Italy). Extracts were filtered through a filter paper and diluted in a ratio 1:5 (*v*/*v*) with water for barley and pasta, with 1 % NaCl solution for oats, oat flakes and oats crispbread and with 4 % NaCl solution for rye. To let precipitation of proteins and matrix insoluble compounds after dilution with NaCl solutions, extracts were left to rest for 5 min and then mixed by shaking for 3 min and left again to rest for additional 5 min. Then the diluted extracts were filtered through a glass microfibre filter and analyzed by FP immunoassay (without further treatment) as described below. The matrix effect on the FP signal was determined by spiking diluted extracts of uncontaminated barley, oats, rye, pasta, oat flakes and oats crispbread samples at different T-2/HT-2 levels (expressed as sum of toxins) in concentration range 4.2–150 ng/mL for barley and pasta, 4.7–45 ng/mL for oats, 2.1–75 ng/mL for rye and oats crispbread and 1.7–37.5 ng/mL for oat flakes and analyzing them by FP immunoassay.

FP Immunoassay

FP measurements were performed by a Sentry 100 portable system (Diachemix Corporation, Milwaukee, WI, USA), a manual single-well instrument which uses glass culture tubes and excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 485 and 535 nm, respectively. FP immunoassays were performed according to the method proposed by Lippolis et al. (2011), with minor modifications. Briefly, in the test tube, 100 μL of antibody working solution and 60 μL of oat diluted extract (equivalent to 3 mg of matrix) or 100 μL of barley or pasta diluted extract (equivalent to 10 mg of matrix) or 200 μL of rye or oats crispbread diluted extract (equivalent to 10 mg of matrix) or 240 μL of oat flake diluted extract (equivalent to

12 mg of matrix) and PBS-A up to 1000 μL were added. The test solution was mixed by vortex and placed in the instrument, and the signal was considered as blank. Then, 25 μL of tracer working solution was added and test solution was mixed by vortex and replaced in the instrument. The polarization value was measured after 5 min (incubation time). The polarization values were normalized to fit the range 0–1 with the equation $Y_{obs} = (mP_{obs} - mP_0)/(mP_1 - mP_0)$. The values of mP_{obs}, mP_0 and mP_1 are the polarization of the test solution, the polarization of clone H10-A10-free control solution and the polarization of toxin-free control solution, respectively, and Y_{obs} is the normalized result for the test solution (Maragos and Kim 2004). T-2 and HT-2 content (expressed as sum of two toxins) in the sample extracts was quantified by comparing normalized polarization value with FP calibration curve in the range 0.5–7.5 ng/mL.

UHPLC Analysis

T-2 and HT-2 analyses of unprocessed cereals and cereal-based products tested were performed according to the method developed by Pascale et al. (2012), with minor modifications. After filtration through a filter paper, extracts were diluted in a ratio 1:5 (*v*/*v*) with 4 % NaCl solution for each matrix tested. The extracts were left to rest for 5 min, mixed by stirring for 3 min and left again to rest for additional 5 min, and then, the diluted extracts were filtered through a glass microfibre filter. Ten millilitres of barley or pasta diluted extracts (equivalent to 1-g sample) or 25 mL of oats, rye, oat flakes or oats crispbread diluted extracts (equivalent to 1.25-g sample) was passed through the immunoaffinity columns at a flow rate of about one drop per second. To avoid saturation of the antibody sites, for contaminated samples with both toxins at levels equal or higher than 1000 μg/kg (sum of toxins), 5 mL of diluted extracts was loaded on the immunoassay 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 –one to two drops per second. T-2 and HT-2 were eluted from the column with methanol (2 × 1 mL) at a flow rate of –one to two drops per second. Cleaned-up extracts were collected in a 4-mL-screw cap amber vial and evaporated under a stream of nitrogen at 50 °C in a heating block. Dried residues were reconstituted with 200 μL of water/acetonitrile (80:20 *v*/*v*), and 10 μL was injected into the UHPLC apparatus by full loop injection. The liquid chromatography (LC) 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, and 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 and then returned to the initial conditions in 1 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. 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 detection limit (signal to noise ratio of 3:1) of the method was 8 μg/kg for both mycotoxins.

In-House Validation of the Optimized FP Immunoassay

Recovery experiments were performed in triplicate by spiking uncontaminated barley, rye, pasta, oat flakes and oats crispbread samples with a mixed spiking solution of T-2 and HT-2 at levels of 100, 200 and 400 μg/kg (sum of the two toxins) and uncontaminated oat samples at levels of 250, 500 and 1000 μg/kg (sum of the two toxins). Spiked samples were left overnight at room temperature to allow solvent evaporation prior to extraction and analysis by both FP immunoassay and UHPLC method. Two reference materials, i.e. FAPAS® T2261 oat test material (the Food and Environment Research Agency, Sand Hutton, York, UK) and ERM®—BC720 oat flake test material (BAM Federal Institute for Materials Research and Testing, Berlin, Germany), were analyzed to assess the trueness of the FP immunoassay. Each experiment was conducted in triplicate. The FAPAS® T2261 oat test material had assigned values of 164 μg/kg for T-2 (satisfactory range 95–233 μg/kg) and 257 μg/kg for HT-2 (satisfactory range 156–358 μg/kg). The ERM®—BC720 oat flake test material had assigned values of 82 μg/kg for T-2 (satisfactory range 78–86 μg/kg) and 81 μg/kg for HT-2 (satisfactory range 77–85 μg/kg).

A total of 52 oat samples, of which 5 uncontaminated and 47 naturally or artificially contaminated with T-2 and HT-2; a total of 35 barley samples, of which 10 uncontaminated and 25 naturally or artificially contaminated with T-2 and HT-2; and a total of 30 rye, pasta, oat flakes and oats crispbread samples, of which 10 uncontaminated and 20 artificially contaminated, were tested for comparison by both FP immunoassay and UHPLC analysis. No naturally contaminated samples of rye, pasta, oat flakes and oats crispbread were found.

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: $y = A2 + [A1 - A2/1 + (x/x_0)P]$, where A1 and A2 represent the initial (left horizontal asymptote) and final

values (right horizontal asymptote), respectively, x_0 is the centre (inflection point), and P is the power. Comparisons between linear regression curves were performed by parallelism and position statistical tests (Soliani 2007). Limits of detection (LODs) of the FP immunoassay were calculated from the mean FP signals of representative uncontaminated samples ($n = 10$) minus 3 standard deviations of the mean signal (Miller and Miller 1984). Limits of quantification (LOQs) were calculated by measuring the lowest amount of the sum of T-2 and HT-2 that was quantitatively determined by the calibration curve within the FP linearity range. 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.

Results and Discussion

A rapid, sensitive and reliable FP immunoassay has been recently reported for the determination of the sum of T-2 and HT-2 toxins in wheat (Lippolis et al. 2011). The used monoclonal antibody (clone H10-A10) showed 100 % cross-reactivity for both T-2 and HT-2 together with a very low cross-reactivity for neosolaniol (CR% = 0.12 %) and no cross-reactivity for other trichothecenes (i.e. DAS, NIV, DON, 3-Ac-DON, 15-Ac-DON) and ochratoxin A and zearalenone (Lippolis et al. 2011). The developed FP immunoassay has been applied to the determination of T-2 and HT-2 in other unprocessed cereals, including oats, barley, rye and cereal-based products for direct human consumption, such as oat flakes, oats crispbread and pasta. These matrices were selected both for their high incidence of T-2 and HT-2 contamination and for their significance as products for human consumption in Europe. As with other types of immunoassays, matrix interferences can exist in the FP immunoassays and they can affect assay performance. In the case of sensitive assays, matrix interferences can often be reduced to negligible levels through simple dilution. Where the assays do not possess sufficient sensitivity or where the assays need to detected low levels in food products, further sample cleanup, concentration and/or background subtraction may be needed (Lippolis and Maragos 2014).

Sample Preparation Optimization and Matrix Effect Experiments

Three different diluting conditions were investigated for each selected matrix in order to test the matrix interference and to obtain the target sensitivity of the FP immunoassay. An acceptable sensitivity should permit the quantification of the total content of T-2 and HT-2 in unprocessed cereals and cereal-based products intended for human consumption below the indicative levels established by the European Commission (European Commission 2013).

In order to minimize matrix interferences, sample extracts of the tested matrices were diluted with water and 1 and 4 % NaCl solutions and analyzed by FP immunoassay. The dilution with salts (i.e. NaCl) lets the precipitation of interfering compounds, such as proteins, which may contribute to increasing the matrix effect in FP immunoassay. The evaluation of matrix effect in the FP measurements was performed, at the three different diluting conditions, by comparing the regression line obtained with T-2/ HT-2 standard solutions in the range 0.5–7.5 ng/mL with the regression lines obtained by adding spiked diluted extracts of barley, oats, rye, pasta, oat flakes and oats crispbread uncontaminated samples at different amounts of analyzed matrix equivalent by means of parallelism and position statistical tests. The best results in terms of sensitivity and reduction of matrix interference were obtained respectively, diluting barley and pasta samples with water, oats, oat flakes and oats crispbread samples with 1 % NaCl solution and rye samples with 4 % NaCl solution. Figure 1 shows, as an example, regression lines obtained using both T-2/ HT-2 standard solutions and spiked sample extract of oats, using the optimal diluting conditions. In the optimal diluting conditions, the amounts of matrix equivalent analyzed by FP immunoassay were 2.5, 3 and 4 mg for spiked extracts of oats; 5, 10 and 12 mg for barley, pasta and rye; and 10, 12 and 15 mg of matrix equivalent for oat flakes. No significant differences were observed between slopes ($t_{calc} < 2.306$; $p < 0.05$) and positions ($t_{calc} < 2.262$; $p < 0.05$) of the regression lines obtained with T-2/HT-2 standard solutions in buffer and those obtained in the presence of spiked diluted extracts by using amounts of matrix equivalent up to 3 mg for oats; 10 mg for barley, rye, oats crispbread and pasta; and 12 mg for oat flakes.

In addition for the optimized FP immunoassay, LODs of 0.45 ng/mL (equivalent to 40 μg/kg in oat flakes), 0.40 ng/mL (equivalent to 40 μg/kg in barley), 0.20 ng/mL (equivalent to 20 μg/kg in rye), 0.25 ng/mL (equivalent to 25 μg/kg in pasta), 0.20 ng/mL (equivalent to 20 μg/kg in oats crispbread) and 0.20 ng/mL (equivalent to 70 μg/kg in oats) were calculated, respectively. Even though different diluting conditions were required for the tested matrices, LODs obtained in the experimental conditions were lower than or equal to the indicative levels suggested by the European Commission for a sensitive detection of the total content of T-2 and HT-2 toxins in unprocessed cereals and cereal-based products tested (European Commission 2013).

In-House Validation

An in-house validation of the optimized FP immunoassay was performed on all tested matrices by determining recovery values, by analyzing reference materials and by comparative analysis using both naturally and artificially contaminated samples.

Recoveries of T-2/HT-2 in all tested matrices carried out on the same sample extracts for both FP immunoassay and

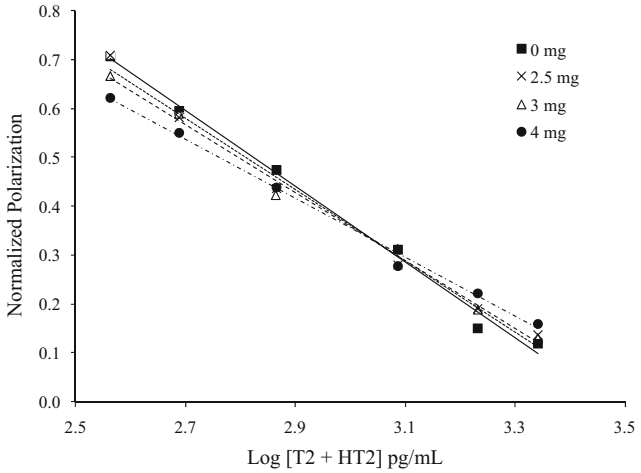

Fig. 1 Regression lines obtained by using both T-2/HT-2 standard solutions (*filled squares*) and spiked extract of oats uncontaminated samples and corresponding to different amounts of analyzed matrix

UHPLC with immunoaffinity cleanup are shown in Table 1. Recovery experiments were performed for barley, rye, pasta, oats crispbread and oat flake samples in the range 100–400 μg/kg and for oat samples in the range 250–1000 μg/kg (sum of toxins). Overall mean recoveries of the optimized FP immunoassay were 107, 105, 104, 104, 103 and 101 % for oats crispbread, rye, oats, pasta, barley and oat flakes, respectively, with relative standard deviations lower than 7 %, whereas overall mean recoveries for the UHPLC method (after immunoaffinity cleanup) were in the range 85–99 %, with relative standard deviations lower than 8 % (Table 1). The analytical performances of the optimized FP immunoassay in terms of accuracy and precision fulfil the criteria established by the European Commission for the acceptability of analytical method for T-2 and HT-2 determination (European Commission 2006b).

Furthermore, the trueness of the method was confirmed by the analysis of two reference materials, a FAPAS® T2261 oat test material and an ERM®-BC720 oat flake reference material. The results (after correction for recovery) were 387 ± 41 and 168 ± 2 μg/kg ($n = 3$ replicates) for oat and oat flake reference materials, respectively, which are very close to the assigned value of 421 and 163 μg/kg (sum of toxins), respectively, and within the FAPAS® T2261 satisfactory range of 251–591 μg/kg and ERM®-BC720 satisfactory range of 155–171 μg/kg (sum of toxins). These results demonstrated the good accuracy

Table 1 Average recoveries of T-2 and HT-2 toxins, expressed as the sum of the toxins, from spiked oats, barley, rye, oat flakes, oats crispbread and pasta obtained by FP immunoassay and UHPLC methods

Matrix	Spiking level (μg/kg)	FP immunoassay		UHPLC	
		Recovery	RSD (%)	Recovery	RSD (%)
Oats	250	101	6	99	3
	500	102	6	97	3
	1000	109	1	100	6
	Overall average	104	5	99	4
Barley	100	98	6	82	8
	200	106	1	91	6
	400	104	2	81	6
	Overall average	103	3	85	7
Rye	100	110	1	99	3
	200	107	4	93	3
	400	99	1	99	2
	Overall average	105	3	97	3
Oat flakes	100	102	3	99	1
	200	102	5	93	4
	400	100	4	105	1
	Overall average	101	4	99	2
Oats crispbread	100	108	6	98	3
	200	109	2	92	1
	400	103	3	91	2
	Overall average	107	4	94	2
Pasta	100	104	5	97	3
	200	108	7	101	4
	400	100	5	98	3
	Overall average	104	6	99	3

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

and precision of the FP immunoassay on naturally contaminated samples used as reference materials. A similar analysis could not be performed with barley, rye, oats crispbread and pasta due to the lack of relevant reference materials.

In addition, a comparative analysis of the levels of contamination in naturally contaminated samples and spiked samples was performed by both FP immunoassay and UHPLC method. In particular, a total of 35 barley samples, of which 9 were

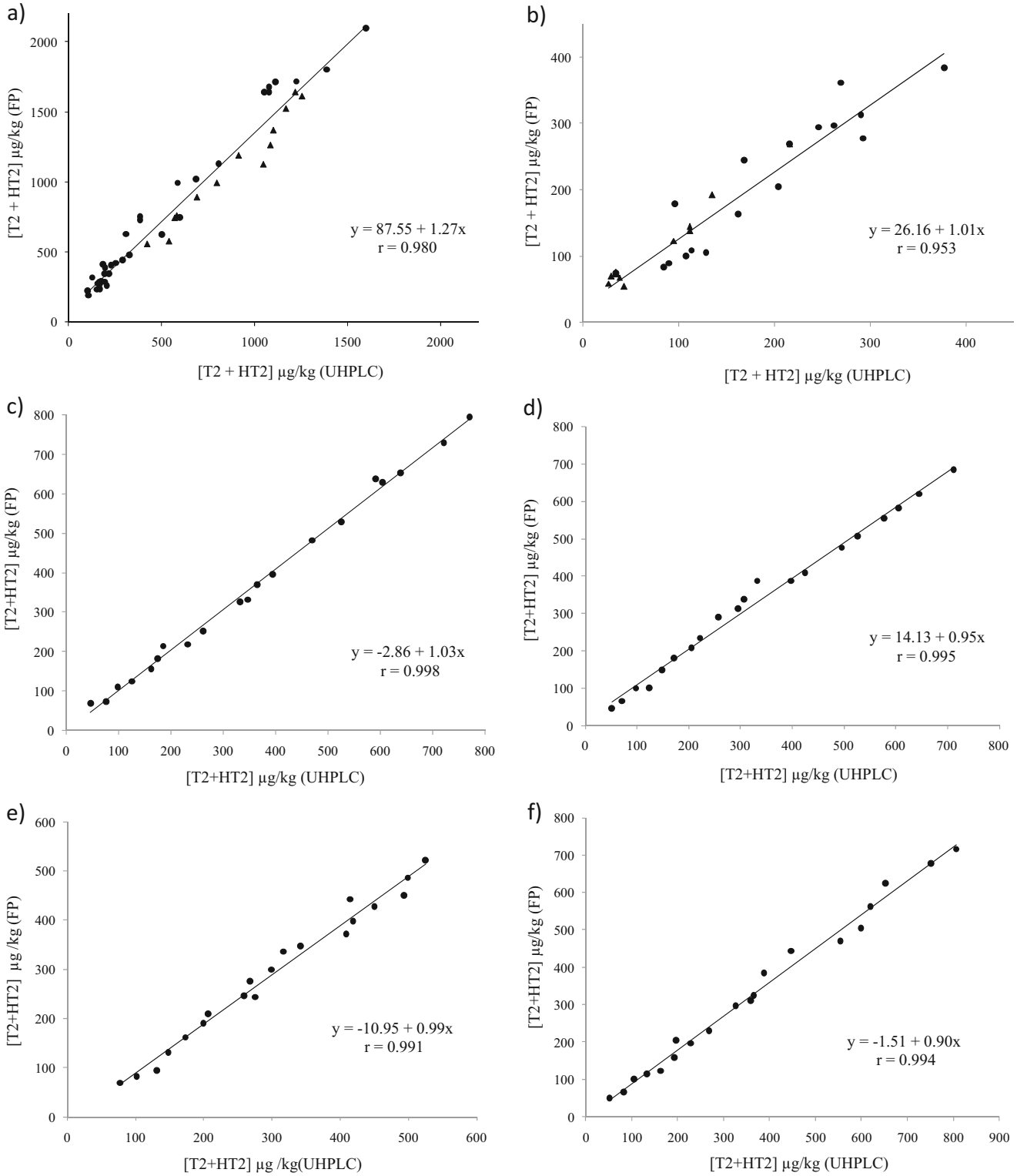

Fig. 2 Comparison of T-2/HT-2 contents in naturally contaminated (*filled triangles*) and spiked (*filled circles*) oats (**a**), barley (**b**), rye (**c**), pasta (**d**), oat flake (**e**), and oats crispbread (**f**) samples analyzed by UHPLC and FP immunoassay (data corrected for average recoveries)

naturally contaminated with T-2 and HT-2 at concentrations from 26 to 135 μg/kg (sum of toxins), 16 spiked samples at levels from 80 to 250 μg/kg and 10 uncontaminated samples; a total of 52 oat samples, of which 34 were naturally contaminated with T-2 and HT-2 at concentrations from 102 to 1598 μg/kg, 13 spiked samples at levels from 423 to 1253 μg/kg and 5 uncontaminated samples; and a total of 30 rye, pasta, oats crispbread and oat flake samples, of which 20 spiked samples at levels from 50 to 700 μg/kg and from 50 to 600 μg/kg (oat flakes) and 10 uncontaminated samples, were analyzed. Good correlations ($r = 0.953$ for barley; $r = 0.980$ for oats; $r = 0.998$ for rye; $r = 0.995$ for pasta; $r = 0.991$ for oat flakes; $r = 0.994$ for oats crispbread) were found between T-2 and HT-2 concentrations obtained by FP immunoassay and those obtained by the UHPLC method (Fig. 2). The linear regression fit was of the form [T-2 + HT-2 by FP] = 26.16 + 1.01 [T-2 + HT-2 by UHPLC] for barley, [T-2 + HT-2 by FP] = 87.55 + 1.27 [T-2 + HT-2 by UHPLC] for oats, [T-2 + HT-2 by FP] = 2.86 + 1.03 [T-2 + HT-2 by UHPLC] for rye, [T-2 + HT-2 by FP] = 14.13 + 0.95 [T-2 + HT-2 by UHPLC] for pasta, [T-2 + HT-2 by FP] = 10.95 + 0.99 [T-2 + HT-2 by UHPLC] for oat flakes and [T-2 + HT-2 by FP] = 1.51 + 0.90 [T-2 + HT-2 by UHPLC] for oats crispbread, where all data were previously corrected for average recoveries. No false-positive result was observed by FP immunoassay for uncontaminated samples. This comparative analysis confirmed the good accuracy of the optimized FP immunoassay.

Conclusions

A rapid and accurate FP immunoassay previously described for the determination of the total content of T-2 and HT-2 in wheat was applied to the analysis of other unprocessed cereals, including barley, oats and rye, and cereal-based products, including pasta, oat flakes and oats crispbread. No purification step of extracts was required, although in order to reduce the matrix effect for oats, rye, oat flakes and oats crispbread, a dilution step with NaCl solution was necessary to let precipitation of proteins and matrix interfering compounds. The optimized protocols were rapid and easy to perform, allowing the FP immunoassay to be carried out in a total time less than 10 min for barley and pasta and 20 min for oats, rye, oat flakes and oats crispbread. Furthermore, LODs of the FP immunoassay were lower than or equal to the indicative levels reported by the European Commission for a sensitive detection of the total content of T-2 and HT-2 toxins in the tested unprocessed cereals and cereal-based products.

Analytical performances of optimized FP immunoassay in terms of accuracy and precision fulfil the criteria establish by the European Union (European Commission 2006a). The trueness of the assay was confirmed by the analysis of two reference materials for T-2 and HT-2 (sum of toxins) and by comparison of T-2 and HT-2 levels in naturally contaminated samples by the FP immunoassay and a reliable UHPLC reference method.

Moreover, the proposed assay is inexpensive, suitable for automation, uses a portable instrument and does not require high technical skills. These findings indicate that the optimized FP immunoassay is suitable for screening purposes for quantitative determination of T-2 and HT-2 in unprocessed cereals and cereal-based products and can be used as a valid alternative to more expensive and time-consuming LC methods.

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Compliance with Ethical Standards

Conflict of Interest Anna Chiara R. Porricelli declares that she has no conflict of interest. Vincenzo Lippolis declares that he has no conflict of interest. Stefania Valenzano declares that she has no conflict of interest. Marina Cortese declares that she has no conflict of interest. Michele Suman declares that he has no conflict of interest. Sandro Zanardi declares that he has no conflict of interest. Michelangelo Pascale declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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