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Distribution of T-2 and HT-2 Toxins in Milling Fractions of Durum Wheat

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ABSTRACT

The effect of processing on mycotoxin content in milling fractions has been investigated in 10 samples of durum wheat contaminated with T-2 and HT-2 toxins at levels ranging from 97 to 5,954 μg/kg (sum of T-2 and HT-2 toxins). Either naturally contaminated samples or samples artificially inoculated with *Fusarium sporotrichioides* under field conditions were used. A method based on liquid chromatography–tandem mass spectrometry coupled with immunoaffinity column cleanup was validated in-house for the simultaneous analysis of both toxins in a variety of matrices, including uncleaned wheat, cleaned wheat, screenings, bran, red dog, fine middlings, and semolina. Mean recoveries from samples spiked with T-2 and HT-2 toxins at levels of 100 μg/kg ranged from 85 to 107%, with relative standard deviations (RSDs) lower than 14%. The milling process led to an increase of T-2 and HT-2 toxin contents up to 13- and 5-fold in screenings and bran, respectively, compared with occurrence in the uncleaned wheat; however, an overall reduction of T-2 and HT-2 toxins by 54% (RSD, 20%) and 89% (RSD, 3%) was observed in cleaned wheat and in semolina, respectively.

T-2 toxin (T-2) and HT-2 toxin (HT-2) are trichothecene mycotoxins produced mainly by *Fusarium sporotrichioides, F. poae,* and *F. langsethiae,* which can contaminate a wide variety of cereals such as wheat, oats, corn, barley, and derived products *(2, 15, 26).* Toxic effects of these mycotoxins are well documented in animals. In particular, T-2 is a potent inhibitor of protein, DNA, and RNA synthesis, both in vitro and in vivo, and has immunosuppressive and hematotoxic effects *(2, 26, 27).* Dermal exposure studies have shown that T-2 causes extremely toxic effects on the skin and mucosal surface *(21).* The toxicity of both T-2 and HT-2 was evaluated in 2001 by the Scientific Committee for Food of the European Union and by the Joint Food and Agriculture Organization and World Health Organization Expert Committee on Food Additives; it was concluded that the toxic effects of T-2 and its metabolite HT-2 could not be differentiated and that the in vivo toxicity of T-2 might be due at least in part to HT-2. A provisional maximum tolerable daily intake of 60 ng/kg of body weight per day for combined T-2 and HT-2 was established *(2, 17).*

The maximum permitted levels of these toxins in unprocessed cereals and cereal-based commodities intended for human consumption are currently under discussion by the European Commission. Reports of T-2 and HT-2 occurrence are mainly restricted to cereal grains in Europe *(12, 15, 20, 25, 26).* A survey carried out within the European project ''SCOOP'' on the occurrence of *Fusarium* toxins showed contamination by T-2 and HT-2 in 20% of 3,490 cereal samples and in 14% of 3,032 samples of cereal-based products tested. In particular, maize (28%), wheat (21%), oats (21%), and rye (16%) were contaminated by T-2; oats (41%), maize (24%), rye (17%), and wheat (12%) were contaminated by HT-2 *(15).* More recent data showed that oats were frequently contaminated with T-2 and HT-2 at levels up to 9,990 μg/kg, particularly for samples from northern Europe, while T-2 and HT-2 contamination of wheat was less common and occurred at lower levels (up to 214 μg/kg) *(4, 5, 20, 25, 26).*

With an estimated production for the 2010 to 2011 marketing year of approximately 30 Mt, durum wheat (*Triticum turgidum* var. *durum* Desf.) represents a ''secondary'' cereal in regard to total world production *(24).* Used as raw material for the manufacture of pasta, durum wheat is of primary importance in Italy. A recent survey carried out in Italy showed T-2 and HT-2 contamination in durum wheat to be widespread (59% of 229 samples tested), although at negligible levels (up to 122 μg/kg, mean 27.3 μg/kg in 2008 and 24.8 μg/kg in 2009) *(12).* T-2 was also found at low levels in pasta samples in Germany and Spain *(6, 26).*

It has been shown that processing of cereals generally reduces mycotoxin contamination in the final products *(3, 9, 10, 19).* For the durum wheat–pasta production chain, previous investigations have shown that there is a

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redistribution of deoxynivalenol through the milling fractions *(28)*. The fate of T-2 and HT-2 has been well investigated in oats processing; most of the mycotoxins are found in the hulls, and the dehulling step leads to over 90% toxin reduction *(16, 18, 20)*. On the contrary, very little information on the change in T-2 and HT-2 concentrations in wheat during the manufacture of retail products is available, due to the low levels of mycotoxins found in the starting material *(10, 20)*.

This study investigated the effect that milling of durum wheat had on T-2 and HT-2 contamination in cleaned wheat, screenings, bran, fine middlings, red dog, and semolina. A robust analytical method was optimized to obtain reliable data for T-2 and HT-2 in all investigated milling fractions.

MATERIALS AND METHODS

Origin of samples. Ten durum wheat samples (cv. Levante), either naturally contaminated or artificially inoculated under field conditions according to a procedure reported elsewhere *(7)*, were provided by Syngenta Crop Protection (Milan, Italy). Sample 1 was naturally contaminated with T-2 and HT-2 at levels of 97 $\pm$ 12 μg/kg (sum of T-2 and HT-2, $n = 4$). Samples 2 through 10 were derived from field experiments after inoculation of durum wheat with a strain of *F. sporotrichioides* that produces T-2 and HT-2 (Item 707, Consiglio Nazionale delle Ricerche, Istituto di Scienze delle Produzioni Alimentari, Bari, Italy) and treatments with fungicides (prochloraz, tebuconazole, or prothioconazole).

Fungal conidia were obtained by growing the fungal strain in liquid shake cultures in 1-liter Erlenmeyer flasks that contained 400 ml of liquid medium (200 ml of V8 juice [Campbell Grocery Products LTD, King's Lynn, Norfolk, UK] plus 3 g of $CaCO_3$ brought to 1 liter with distilled water) that had been previously sterilized by autoclaving for 15 min at 121°C. After 7 days in an incubator shaker in the dark at 25°C and 150 rpm, the flask contents were filtered through two layers of cheesecloth to obtain a conidial suspension. The concentration of the inoculum was measured with a Thoma camera (HBG Henneberg-Sander GmbH, Lutzellinden, Germany) at the light microscope.

Three field trials were carried out in northern Italy (Parma and Bologna) and southern Italy (Foggia). Two replicate plots for each treatment (fungicide treatments and inoculated or untreated controls) were prepared. All plots were cultivated according to normal agronomic practices, which were standardized across sites. Each plot had a surface area of about 18 m^2 (2 by 9 m or 2.5 by 7 m). For the artificial inoculation, each plot was sprayed at the beginning of anthesis (BBCH scale 61) with 450 ml of a suspension containing conidia of *F. sporotrichioides* (about 3×10^5 conidia per ml) using an Echo motorized pump (SHR 4100, Kioritz Corporation, Tokyo, Japan). Delivery pressure at the nozzle (TeeJet 8002 VS, TeeJet LH Agro South Europe, Olivet Orleans, France) was 250 kPa, and the distance of the nozzle from the ears was 4 to 5 cm in order to avoid conidial dispersion. Fungicide treatments were performed within 24 h before the fungal inoculation, using the same Echo pump with a delivery pressure at the nozzle (TeeJet 8003 VS) of 300 kPa and a delivery rate of 500 liters ha^{-1}. Both inoculation and treatments were performed in the evening, with relative humidity above 75% and in the absence of wind. At the maturity stage (BBCH 89), ears were mechanically harvested using a small plot combine harvester (Hege model 125B, Hege Maschinenbau GmbH, Waldenburg, Germany) and analyzed for T-2 and HT-2 content. Samples collected had concentrations (based on the analysis performed within this study, $n = 4$) of 221 $\pm$ 10, 235 $\pm$ 32, 443 $\pm$ 29, 507 $\pm$ 29, 589 $\pm$ 32, 637 $\pm$ 31, 720 $\pm$ 9, 1,436 $\pm$ 163, and 5,950 $\pm$ 363 μg/kg, as the sum of T-2 and HT-2. The samples covered a wide range of T-2 and HT-2 contamination levels, allowing us to achieve robust and representative experiments.

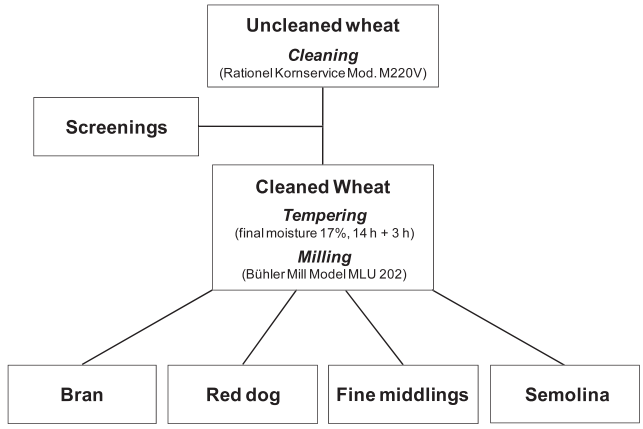

FIGURE 1. *Simplified scheme of the milling process and relevant milling fractions.*

An uncontaminated durum wheat sample of the same cultivar was processed to obtain milling fraction samples to be spiked for recovery experiments.

Milling process. Figure 1 shows a simplified scheme of the milling process. Durum wheat samples (about 10 kg each) were first cleansed of foreign seeds, seeds that were broken or inadequate for milling, and glumes with a Rationel Kornservice model M220V sifter (A/S Rationel Kornservice, Esbjerg, Denmark) equipped with an aspiration system and two sieves (aperture sizes: 5 by 15 mm and 2 by 19 mm, respectively). Cleaned wheat was transferred into polypropylene bags (maximum 5 kg each). Grain moisture content was measured with a Tecator 1229 Grain Analyzer (Foss Tecator AB, Hoganas, Sweden) and then brought to 17% in two steps. First, 80% of the required amount of water was added, and then, after gentle shaking of the material in the closed bag for 14 h, the remaining 20%. The material was left to stand for at least 3 h before milling with a Bühler Laboratory Mill with pneumatic product transport (model MLU 202, Bühler, Uzwil, Switzerland) equipped with three break and three stripping systems. Semolina was separated from gross material (waste semolina also containing parts of bran) by treatment with a Namad purifier (Namad, Rome, Italy). Uncleaned wheat, cleaned wheat, screenings, bran, red dog, fine middlings, and semolina were analyzed for T-2 and HT-2 as reported below.

Materials and chemicals. High-pressure liquid chromatography (HPLC)–grade methanol was purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced with a Milli-Q system (Millipore, Bedford, MA). T-2 and HT-2, sodium chloride (NaCl), and ammonium acetate were purchased from Sigma-Aldrich (Milan, Italy). Internal standards $^{13}C_{24}$ T-2 and $^{13}C_{22}$ HT-2, as liquid calibrants (25 μg/ml) in acetonitrile, were obtained from Biopure (Tulln, Austria). T-2 test HPLC immunoaffinity columns were obtained from Vicam (Milford, MA), and glass microfiber filters (Whatman GF/A) and paper filters (Whatman no. 4) from Whatman (Maidstone, UK).

Preparation of standard solutions. T-2 and HT-2 stock solutions (1 mg/ml each) were prepared by dissolving T-2 and HT-

2 solid commercial toxins in acetonitrile (HPLC grade). A standard solution containing both T-2 and HT-2 at a concentration of 2 μg/ml each was prepared for spiking purposes by diluting the T-2 and HT-2 stock solutions with acetonitrile and mixing them.

For calibration purposes, two mixed internal standard solutions of $^{13}C_{24}$ T-2 and $^{13}C_{22}$ HT-2 in acetonitrile were prepared by dilution of commercial solutions to obtain concentrations of 1 μg/ml and 10 μg/ml of both ^{13}C-labeled toxins.

Sample extraction and cleanup. Sample extraction and cleanup were performed in quadruplicate ($n = 4$) according to the method reported by Visconti et al. *(29)*. Ground wheat samples and milling fractions (50 g of uncleaned wheat, cleaned wheat, red dog, and semolina, 25 g of fine middlings and screenings, 15 g of bran), with 1 g of NaCl and 100 ml of methanol-water (90:10, vol/vol) added, were blended at high speed for 2 min in a Sorvall Omnimixer (Dupont Instruments, Newton, CT). The extracts were filtered through filter paper (Whatman no. 4) and diluted with distilled water (10 ml plus 40 ml). The diluted extracts were filtered through a glass microfiber filter (Whatman GF/A), and 10 ml of filtrate was passed through T-2 test HPLC columns at a flow rate of about one drop per second. Before loading on immunoaffinity columns, appropriate dilutions were necessary for samples contaminated with T-2 and HT-2 at levels higher than 1,400 μg/kg (sum of toxins), to avoid saturation of the T-2 antibody binding sites *(29)*. Subsequently, columns were washed with 10 ml of distilled water at one to two drops per second. T-2 and HT-2 were eluted from the column with 1.5 ml of methanol (2 × 0.75 ml) at a flow rate of 1 drop per s. Cleaned-up extracts were collected in a 4-ml screw-cap amber vial, and 10 μl of mixed $^{13}C_{24}$ T-2 and $^{3}C_{22}$ HT-2 calibrant solution was added before drying them under a nitrogen stream at 50°C in a heating block. For cleaned-up extracts of uncleaned wheat, cleaned wheat, fine middlings, red dog, and semolina, 10 μl of 1 μg/ml ^{13}C-calibrant solution was added; for cleaned-up extracts of screenings and bran, 10 μl of 10 μg/ml ^{13}C-calibrant solution was added. Dried residues were reconstituted with 200 μl of HPLC mobile phase (methanol-water, 40:60, containing 5 mM ammonium acetate) prior to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Recovery experiments. Recovery experiments were performed in triplicate ($n = 3$); uncontaminated wheat kernels and screenings, bran, red dog, fine middlings, and semolina samples obtained by milling the uncontaminated wheat sample were spiked with T-2 and HT-2 (100 μg/kg of each toxin). Spiked samples were left overnight at room temperature to allow solvent evaporation prior to extraction with methanol-water (90:10, vol/vol).

LC-MS/MS analysis. LC-MS/MS analyses were performed according to Lattanzio et al. *(11)* with a QTrap LC-MS/MS system (Applied Biosystems, Foster City, CA) equipped with an atmospheric pressure chemical ionization interface and an 1100 Series micro-LC system comprising a binary pump and a micro-autosampler from Agilent Technologies (Waldbronn, Germany). The analytical column was a Synergi Hydro (150 by 3 mm, 4-μm particles; Phenomenex, Torrance, CA), preceded by an Aqua C18 guard column (4 by 2 mm, 10-μm particles; Phenomenex). The flow rate of the mobile phase was 350 μl/min, and the injection volume was 20 μl. The column effluent was directly transferred into the atmospheric pressure chemical ionization interface without splitting. Eluent A was water and eluent B was methanol, both containing 5 mM ammonium acetate. The elution was performed by changing the mobile phase composition as follows. After 3 min at 60%, eluent B was increased to 80% in 1 min, kept constant for 5 min, then increased to 100% in 1 min and kept constant for 5 min. For column re-equilibration, eluent B was decreased to 60% in 1 min and kept constant for 6 min.

For LC-MS/MS analyses, the atmospheric pressure chemical ionization interface was used in positive ion mode with the following settings: temperature, 380°C; curtain gas (nitrogen), 30 psi; nebulizer gas (air), 50 psi; auxiliary gas (air), 40 psi; corona discharge needle current, +3 μA. The mass spectrometer operated in multiple reaction monitoring mode using parameters reported by Lattanzio et al. *(11)*. Monitoring fragments *m/z* used for individual analytes were, for T-2, 484.0 (precursor ion), 305.2, 215.1 (quantifier ion), and 185.3; for $^{13}C_{24}$ T-2, 508.0 (precursor ion), 322.2, 229.2 (quantifier ion), and 198.2; for HT-2, 442.0 (precursor ion), 263.3 (quantifier ion), 245.2, and 215.1; and for $^{13}C_{22}$ HT-2, 464.2 (precursor ion), 278.2 (quantifier ion), 260.2, and 229.2, with a dwell time of 100 ms. Quantification of T-2 and HT-2 was performed by measuring peak areas in the multiple reaction monitoring chromatogram and comparing them with peak areas of the relevant internal standard, i.e., $^{13}C_{24}$ T-2 and $^{13}C_{22}$ HT-2.

For screenings, bran, and red dog, recovery values were calculated by subtracting the relevant endogenous value (this was lower than 10% of the spiked toxin amount for all matrices).

Statistical analysis. Data of toxin distribution in the milled fractions were processed by one-way analysis of variance at $P = 0.05$ to indicate statistically significant differences between means (Student-Newman-Keuls test). Data were processed using Sigma Plot 11 statistical software (Systat Software Inc, London, UK).

RESULTS AND DISCUSSION

In-house validation of the LC-MS/MS method for the determination of T-2 and HT-2. The HPLC method with fluorescence detection developed by Visconti et al. *(29)* for the simultaneous analysis of T-2 and HT-2 in cereals, based on the purification of the extracts with immunoaffinity columns and precolumn derivatization with 1-anthroylnitrile, was not applicable to all milling fractions of durum wheat. For instance, red dog and bran extracts showed interfering chromatographic peaks at the retention time of HT-2 that did not allow an accurate quantification of the toxin. For this reason a liquid chromatographic method, based on extract purification by immunoaffinity columns and LC-MS/MS detection, was optimized for the simultaneous analysis of T-2 and HT-2 in all investigated matrices.

Recovery values, relative standard deviations (RSDs), and limits of detection of the full analytical procedure for all tested matrices are reported in Table 1. Mean recoveries from samples spiked with T-2 and HT-2, at levels of 100 μg/kg each, were greater than 85% (up to 107%) with RSDs lower than 14%. Recovery and repeatability values meet the performance criteria established by the European Union for acceptance as 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 RSDs ≤ 40% for T-2 and HT-2 concentrations in the range of 50 to 250 μg/kg and 100 to 200 μg/kg, respectively *(1)*. Limits of detection of the method for the different matrices (based on a signal-to-noise ratio of 3:1) ranged from 0.1 to 0.9 μg/kg for T-2 and from 0.2 to 1.7 μg/kg for HT-2 (Table 1). Typical extracted ion chromatograms (quantifier ions) of spiked clean wheat,

TABLE 1. *Validation data of the LC-MS/MS method*

Matrix or milling fraction	% recovery (% RSD)[a]		LOD (μg/kg)[b]	
	T-2	HT-2	T-2	HT-2
Wheat kernels	104 (3)	107 (2)	0.5	0.7
Fine middlings	92 (5)	85 (5)	0.9	1.5
Red dog	103 (14)	91 (7)	0.9	1.4
Bran	90 (5)	92 (9)	0.6	0.9
Screenings	98 (3)	85 (6)	1.5	1.7
Semolina	102 (3)	101 (5)	0.5	0.7

[a] Spiking levels, 100 μg/kg of T-2 and 100 μg/kg of HT-2; RSD, relative standard deviation ($n = 3$).
[b] LOD, limit of detection (signal-to-noise ratio of 3:1).

contaminated clean wheat, semolina, and bran after purification of extracts by immunoaffinity columns are shown in Figure 2. Similar chromatograms were observed for the other milling fractions (uncleaned wheat, fine middlings, red dog, and screenings).

Distribution of T-2 and HT-2 in milling fractions. Because T-2 and HT-2 are generally found in low levels in naturally contaminated wheat, in order to obtain useful and reliable information on the fate of T-2 and HT-2 during the milling process, we used one naturally contaminated sample and nine wheat samples artificially inoculated with *F. sporotrichioides* under field conditions, from different test-plot experiments.

These unprocessed samples were contaminated with T-2 and HT-2 in a wide range of concentrations (from 35 to 785 μg/kg for T-2 and from 62 to 5,169 μg/kg for HT-2). In agreement with occurrence data previously reported for T-2 and HT-2 in naturally contaminated cereals *(4, 5, 15, 20, 25, 26),* levels of T-2 in unprocessed wheat samples (naturally and artificially contaminated) used in this study were lower than HT-2 levels.

The levels of T-2 and HT-2 in the different milling fractions obtained from the processing of 10 durum wheat samples are reported in Tables 2 and 3, respectively. For example, toxin distribution in the milling fractions obtained from a sample of durum wheat contaminated with T-2 and HT-2 at levels that occur naturally in wheat (sample 2: 58 ± 5 μg/kg of T-2 and 163 ± 13 μg/kg of HT-2 in uncleaned wheat) is discussed. A significant reduction of toxins after cleaning was observed due to the removal of all foreign materials, shrunken and lightweight kernels, and dust. This is the material (screenings) in which most of the toxins accumulated (sum of T-2 and HT-2, 834 ± 80 μg/kg). Also, T-2 and HT-2 were concentrated in the bran (317 ± 34 μg/kg) and the red dog (245 ± 11 μg/kg). A notable reduction in the levels of toxins was observed in fine middlings (46 ± 3 μg/kg) and semolina (23 ± 2 μg/kg). A similar toxin distribution was observed during the processing of the other contaminated wheat samples.

Tables 4 and 5 show the relative concentrations of T-2 and HT-2 in milling fractions after processing of the 10 contaminated durum wheat samples as compared with concentrations in the unprocessed wheat. Average levels of

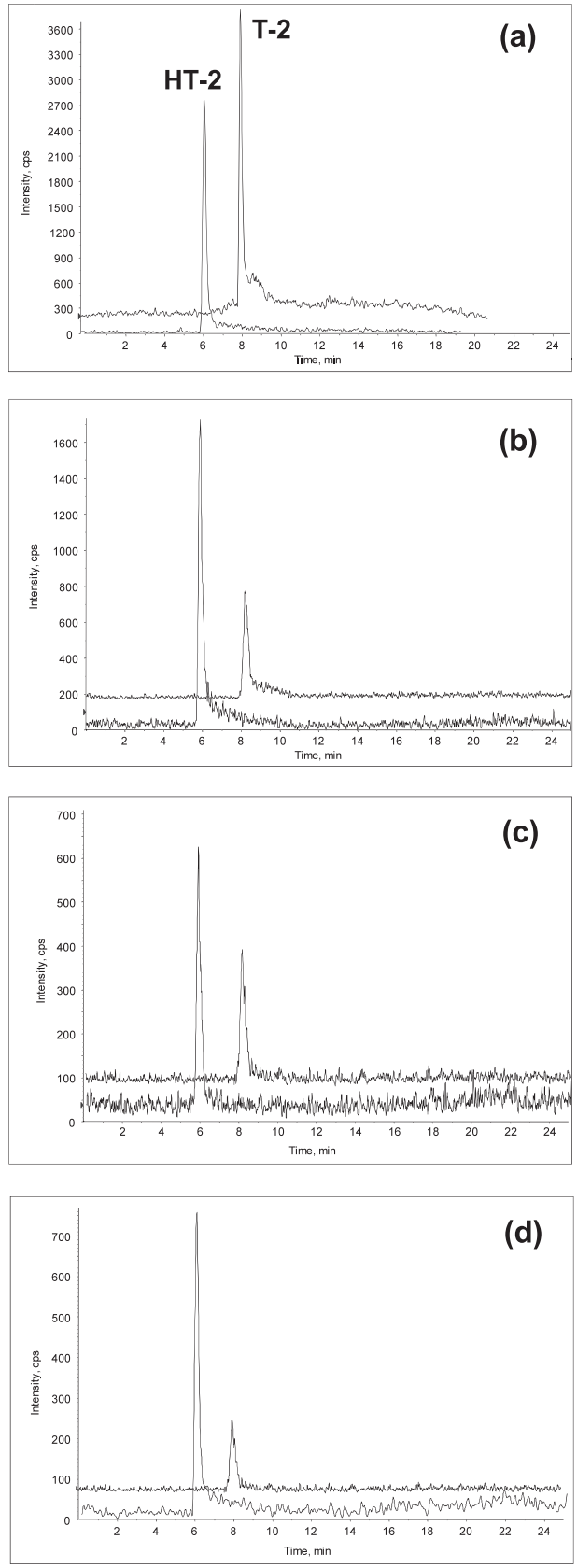

FIGURE 2. *Extracted ion chromatograms* (m/z = *442.0 to 263.3 for HT-2 and* m/z = *484.0 to 215.1 for T-2) of (a) clean wheat spiked with T-2 and HT-2 (100 μg/kg each), (b) contaminated clean wheat (T-2, 12 μg/kg; HT-2, 51 μg/kg), (c) semolina (T-2, 6 μg/kg; HT-2, 15 μg/kg), and (d) bran (T-2, 65 μg/kg; HT-2, 256 μg/kg) after extract cleanup by immunoaffinity column. Experimental conditions are reported in ''Materials and Methods.''*

TABLE 2. *Levels of T-2 in milling fractions obtained from the processing of contaminated durum wheat*[a]

Matrix or milling fraction	T-2 toxin (SD) per sample (μg/kg)									
	1	2	3	4	5	6	7	8	9	10
Uncleaned wheat	35 (4)	58 (5)	83 (8)	150 (11)	139 (12)	205 (16)	167 (11)	181 (12)	132 (14)	785 (72)
Cleaned wheat	—[b]	13 (1)	—	—	43 (4)	—	35 (2)	136 (19)	71 (2)	184 (15)
Fine middlings	8 (0)	14 (0)	15 (1)	23 (1)	38 (2)	28 (2)	43 (1)	51 (2)	48 (4)	156 (3)
Red dog	57 (1)	64 (2)	127 (11)	259 (5)	171 (18)	351 (14)	201 (21)	323 (31)	181 (17)	488 (34)
Bran	51 (2)	61 (14)	157 (6)	324 (35)	149 (22)	377 (45)	298 (39)	350 (39)	269 (20)	1,485 (95)
Screenings	348 (41)	487 (68)	282 (31)	308 (34)	498 (36)	246 (21)	806 (76)	1,947 (216)	3,389 (357)	7,998 (335)
Semolina	4 (1)	6 (0)	7 (1)	12 (1)	16 (0)	13 (2)	20 (1)	25 (1)	27 (2)	70 (6)

[a] Data refer to mean values of four replicated measurements from the processing of durum wheat (uncleaned) containing T-2 in the range of 35 to 785 μg/kg.
[b] —, sample not available.

T-2 dropped to 37.8% (range, 21.0 to 75.1%) in cleaned wheat and to 11.1% (range, 6.3 to 20.5%) in semolina, with RSDs of 22.0 and 4.0%, respectively. A similar effect of processing was observed for HT-2: toxin levels dropped to 47.4% (range, 29.9 to 72.3%) in cleaned wheat and to 11.6% (range, 9.1 to 17.2%) in semolina, with RSDs of 18.7 and 2.5%, respectively. Very high levels of T-2 and HT-2 and a high variability of results were observed in the screenings, with mean values (sum of toxins) for the different wheat samples ranging from 1.8- to 13.2-fold greater than the toxin levels of the corresponding raw wheat, and an overall mean value sixfold greater than the original levels. T-2 and HT-2 also accumulated in bran and red dog, in which average toxin increases of 3.2-fold and 1.3-fold were observed with respect to the corresponding unprocessed wheat. In bran, HT-2 accumulation (3.7-fold increase) was higher than T-2 accumulation (1.7-fold increase). The use of these by-products in feed production should be carefully assessed to prevent possible animal health problems.

The average distribution of T-2 and HT-2 in the milling fractions (fine middlings, red dog, bran, and semolina) of six wheat samples cleaned using a Bühler pilot mill is shown in Table 6. Distribution values were derived from toxin mass balance in the milling fractions by considering the relevant weights and contamination levels. T-2 and HT-2 mainly concentrated in the bran (50% or more), followed by fine middlings and red dog, whereas a lower percentage of toxins was found in semolina (about 10%). These results show a high accumulation of T-2 and HT-2 in the peripheral grain layers, presumably due to the low capacity of toxin-producing fungi to penetrate to the inner part of the grains, as previously shown for deoxynivalenol and deoxynivalenol-producing fungi *(8, 13, 14)*. Despite the similar order of distribution among milling fractions for both T-2 and HT-2, some significant differences between the percentage values of the two toxins within the same fraction were observed: bran accumulated more HT-2 than T-2 (62 versus 48%) and fine middlings and semolina more T-2 than HT-2 (23 versus 14% and 12 versus 8%, respectively). The presence of carboxylesterase enzymes with specific activity toward T-2 at the C-4 position to produce HT-2 in cereals, including wheat, as well as in crude extracts of *Fusarium* species has recently been shown, and the potential role of this hydrolytic activity in the detoxification plant response to T-2 cannot be excluded *(11)*. The greater accumulation of HT-2 in the bran (outer layers of wheat kernels) could tentatively be explained by the longer contact time that carboxylesterase enzymes have with T-2 (deacetylating T-2 into HT-2); T-2 is produced by the fungus first and prevalently on the peripheral layers.

Our findings support previous reports that show an increase in levels of other mycotoxins (deoxynivalenol, nivalenol, ochratoxin A, and zearalenone) in wheat bran and low levels in flour *(9, 10, 19, 22, 28)*. Most of the studies relevant to the fate of T-2 and HT-2 during processing refer to oats. It has been reported that cleaning and sieving reduce T-2 and HT-2 levels in oat kernels and that the dehulling stage further reduces toxin levels in the production of oat flakes or bread *(16, 18, 20)*. A recent study on the fate of trichothecene mycotoxins (including T-2 and HT-2) in the bread production chain, which used naturally contaminated wheat samples, showed no contamination of T-2 and HT-2 in the uncleaned grains, whereas low amounts of T-2 and HT-2 were detected in impurity, dust, and bran samples *(10)*.

Our study showed that the milling process led to a significant overall reduction (i.e., 89%) of T-2 and HT-2 levels in semolina, in comparison with levels in the raw material, whereas toxin levels significantly increased in screenings and bran. Note that most of the wheat samples used in these experiments were from crops that were artificially inoculated with *F. sporotrichioides* and that contained T-2 and HT-2 levels not commonly found in natural conditions. In addition, the processing of samples contaminated at levels lower than 250 μg/kg (levels that can occur in naturally contaminated wheat) resulted in semolina with negligible levels of T-2 and HT-2. The significant reduction of T-2 and HT-2 levels observed in semolina (generally used to produce pasta or bread) should reassure the consumer about the possible health risk associated with exposure to these mycotoxins. These data were presented at the VII Fusarium Forum 2010 of the European Commission, Directorate-General for Health and Consumer Protection

TABLE 3. *Levels of HT-2 in milling fractions obtained from the processing of contaminated durum wheat*[a]

Matrix or milling fraction	HT-2 toxin (SD) per sample (μg/kg)									
	1	2	3	4	5	6	7	8	9	10
Uncleaned wheat	62 (8)	163 (13)	152 (27)	293 (19)	368 (19)	384 (23)	470 (27)	539 (9)	1,304 (100)	5,169 (298)
Cleaned wheat	—[b]	55 (5)	—	—	110 (7)	—	169 (13)	374 (29)	943 (89)	2,221 (406)
Fine middlings	19 (1)	32 (3)	24 (3)	39 (3)	86 (6)	54 (3)	101 (9)	108 (9)	381 (18)	1,145 (41)
Red dog	50 (9)	181 (11)	112 (25)	254 (6)	532 (42)	433 (74)	572 (51)	889 (65)	2,733 (308)	7,764 (484)
Bran	362 (49)	256 (24)	584 (32)	1,241 (59)	760 (75)	1,672 (115)	1,073 (231)	2,010 (237)	4,642 (186)	28,626 (2,101)
Screenings	890 (132)	347 (23)	792 (81)	849 (69)	423 (33)	825 (73)	1,208 (90)	3,563 (125)	15,495 (1,309)	28,596 (1,718)
Semolina	9 (2)	17 (6)	15 (2)	27 (3)	42 (2)	35 (2)	50 (3)	66 (5)	224 (23)	570 (40)

[a] Data refer to mean values of four replicated measurements from the processing of durum wheat (uncleaned) containing HT-2 in the range of 62 to 5,169 μg/kg.
[b] —, sample not available.

TABLE 4. *Relative concentrations of T-2 in milling fractions, with respect to the uncleaned wheat, obtained from the processing of contaminated durum wheat*[a]

Matrix or milling fraction	% T-2 toxin per sample										Mean ± SD (%)
	1	2	3	4	5	6	7	8	9	10	
Uncleaned wheat (μg/kg T-2)[b]	100 (35)	100 (58)	100 (83)	100 (150)	100 (139)	100 (205)	100 (167)	100 (181)	100 (132)	100 (785)	100.0 ± 0.0
Cleaned wheat	—[c]	22.4	—	—	30.9	—	21.0	75.1	53.8	23.4	37.8 ± 22.0
Fine middlings	22.9	24.1	18.1	15.3	27.3	13.7	25.7	28.2	36.4	19.9	23.2 ± 6.8
Red dog	162.9	110.3	153.0	172.7	123.0	171.2	120.4	178.5	137.1	62.2	139.1 ± 36.3
Bran	145.7	105.2	189.2	216.0	107.2	183.9	178.4	193.4	203.8	189.2	171.2 ± 38.8
Screenings	994.3	839.7	339.8	205.3	358.3	120.0	482.6	1,075.8	2,567.4	1,018.9	800.2 ± 715.8
Semolina	11.4	10.3	8.4	8.0	11.5	6.3	12.0	13.8	20.5	8.9	11.1 ± 4.0

[a] Data refer to mean values of four replicated measurements from the processing of durum wheat (uncleaned) containing T-2 in the range of 35 to 785 μg/kg.
[b] Initial contamination level in parentheses.
[c] —, sample not available.

TABLE 5. *Relative concentrations of HT-2 in milling fractions, with respect to the uncleaned wheat, obtained from the processing of contaminated durum wheat*[a]

Matrix or milling fraction	% HT-2 toxin per sample										Mean ± SD (%)
	1	2	3	4	5	6	7	8	9	10	
Uncleaned wheat (μg/kg HT-2)[b]	100 (62)	100 (163)	100 (152)	100 (293)	100 (368)	100 (384)	100 (470)	100 (539)	100 (1,304)	100 (5,169)	100.0 ± 0.0
Cleaned wheat	—[c]	33.7	—	—	29.9	—	36.0	69.4	72.3	43.0	47.4 ± 18.7
Fine middlings	30.6	19.6	15.8	13.3	23.4	14.1	21.5	20.0	29.2	22.2	21.0 ± 5.8
Red dog	80.6	110.0	73.7	86.7	144.6	112.8	121.7	164.9	209.6	150.2	125.5 ± 42.5
Bran	583.9	157.1	384.2	423.5	206.5	435.4	228.3	372.9	356.0	553.8	370.2 ± 141.1
Screenings	1,435.5	212.9	521.1	289.8	114.9	214.8	257.0	661.0	1,188.3	553.2	544.9 ± 443.9
Semolina	14.5	10.4	9.9	9.2	11.4	9.1	10.6	12.2	17.2	11.0	11.6 ± 2.5

[a] Data refer to mean values of four replicated measurements from the processing of durum wheat (uncleaned) containing HT-2 in the range of 62 to 5,169 μg/kg.
[b] Initial contamination level in parentheses.
[c] —, sample not available.

TABLE 6. *Distribution of T-2 and HT-2 in the milling fractions obtained from contaminated durum wheat (cleaned wheat)*[a]

Matrix or milling fraction	T-2 ± SD (%)	HT-2 ± SD (%)
Cleaned wheat	100.0 ± 0.0	100.0 ± 0.0
Fine middlings	23.0 ± 4.3 A	14.2 ± 4.2 B
Red dog	17.3 ± 3.4 A	15.5 ± 2.8 A
Bran	48.3 ± 8.2 A	62.3 ± 8.2 B
Semolina	11.5 ± 1.9 A	8.0 ± 1.9 B

[a] Data refer to mean values of four replicated measurements from the processing of six samples of cleaned durum wheat (samples 2, 5, 7, 8, 9, and 10) contaminated with T-2 (13 to 184 μg/kg) and HT-2 (55 to 2,221 μg/kg). Values followed by the same letter in a row are not significantly different at $P = 0.05$ according to the Student-Newman-Keuls test.

(Brussels, 1 to 2 February 2010) and contributed to the debate on maximum admissible limits for T-2 and HT-2 in the European Union in order to safeguard the health of European citizens without penalizing the durum wheat–pasta production chain.

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