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Optimization of a Fluorescence Polarization Immunoassay for Rapid Quantification of Deoxynivalenol in Durum Wheat–Based Products

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ABSTRACT

A fluorescence polarization immunoassay previously described for deoxynivalenol (DON) screening in wheat was optimized for the rapid quantification of DON in durum wheat kernels, semolina, and pasta. A background signal was observed in both spiked and naturally contaminated samples, strictly depending on the testing matrix. After subtracting the background DON level for durum wheat (0.27 μg of DON per g), semolina (0.08 μg of DON per g), and pasta (0.04 μg of DON per g), an accurate quantification of DON was possible at levels greater than 0.10 μg/g for all matrices. Average recoveries from spiked samples (0.25 to 1.75 μg/g) were 98, 102, and 101% for wheat, semolina, and pasta, respectively. Comparative analyses of 35 naturally contaminated durum wheat samples, 22 semolina samples, and 26 pasta samples performed by both the fluorescence polarization method and high-pressure liquid chromatography/immunoaffinity cleanup showed a good correlation ($r > 0.995$). The fluorescence polarization method showed better accuracy and precision with respect to the high-pressure liquid chromatography method and is suitable for the rapid and quantitative determination of DON in durum wheat–based products at levels foreseen by existing or coming international regulations.

Fusarium culmorum and *Fusarium graminearum* are worldwide fungal pathogens of wheat and other small-grain cereals that cause a disease known as *Fusarium* head blight *(16).* These fungi may produce deoxynivalenol (DON), a trichothecene mycotoxin frequently occurring in wheat kernels and derivative products at levels generally lower than 1.0 μg/g, although higher levels have been found in samples affected by *Fusarium* head blight, with durum wheat generally more contaminated than soft wheat *(2, 17).* About 8 million tons of durum wheat were produced annually in the European Union during the years 2000 to 2003, of which 6 million tons were intended for pasta production *(28).*

The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives showed that 62% of 14,200 wheat samples were contaminated with DON *(2).* Similar results were found in the European Union, within an ad hoc SCOOP (Scientific Cooperation) project on the occurrence of *Fusarium* toxins, showing an incidence of DON-positive samples for 57% of 11,022 samples *(22).*

DON has been shown to inhibit DNA, RNA, and protein synthesis and to cause hematic and anorexic syndromes as well as neurotoxic and immunotoxic effects in mammals *(20).* To protect consumers from exposure to DON through the consumption of wheat-based food products, the U.S. Food and Drug Administration has issued an advisory level of 1.0 μg/g for DON in milled wheat products (e.g., flour, bran, germ) intended for human consumption. The following maximum levels for DON will apply beginning 1 July 2006 within the European Community: 1.75 μg/g for unprocessed durum wheat, maize, and oats (1.25 μg/g for other cereals); 0.75 μg/g for cereal flour and pasta (dry); 0.50 μg/g for cereal products intended for direct consumption (e.g., bread, pastries, biscuits, cereal snacks, and breakfast cereals); and 0.20 μg/g for cereal-based food intended for infants and use in baby food *(5).*

The development of rapid, sensitive, and accurate methods for the determination of DON in wheat and wheat-based food products is required to ensure that these mandatory regulatory levels are met and to protect consumer health from the risk of exposure to this toxin *(21).* Gas-chromatographic methods based on electron-capture detection and mass-spectrometric (MS) detection and high-pressure liquid chromatographic (HPLC) methods based on UV and MS detection are the most widely used methods for the quantitative determination of DON in foodstuffs and feedstuff *(1, 7, 8, 18, 25).* However, these methods require a preliminary cleanup of extracts to obtain good sensitivity and are time-consuming, expensive, and unsuitable for screening purposes.

Several publications are available that describe spectrophotometric enzyme-linked immunosorbent assays (ELISAs) that use polyclonal or monoclonal antibodies against DON *(3, 11, 13, 15, 24, 27, 29).* Competitive ELISA kits for the quantitative analysis of DON in foods are commercially available that allow the simultaneous analysis of up to 40 samples with good sensitivity and application ranges. However, most immunoassays show

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strong cross-reactivity against acetyl derivatives of DON, involve multiple washing steps, and require long incubation times for complete antigen–antibody reaction.

The fluorescent polarization (FP) immunoassay is a simple technique that measures interactions between a fluorescently labeled antigen (tracer) and a specific antibody, and its application to mycotoxin analysis is emerging. 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 and horizontal axes, respectively. The polarization value is inversely proportional to the free unlabeled 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. FP immunoassays have been reported for the determination of zearalenone, fumonisins, DON, aflatoxins, and ochratoxin A in solution, although when applied to the analysis of cereal samples, low accuracy and sensitivity were obtained *(9, 10, 12, 14, 23).* Recently, a new DON-fluorescein tracer (DON-FL2) was synthesized and used in a competitive FP immunoassay for the rapid screening of DON in wheat. However, the FP immunoassay, when compared with an HPLC-UV method, showed an overestimation of DON in naturally contaminated samples at high levels, which did not allow an accurate measurement of DON at levels close to the regulatory limits *(12).* The goals of the present study were to identify the problem that leads to biased results in the determination of DON in durum wheat by FP immunoassay at levels commonly found in naturally contaminated samples and to optimize the method for its application throughout the entire durum wheat chain (wheat kernels, semolina, and pasta). A comparison of the newly developed FP immunoassay with a widely used HPLC/immunoaffinity method indicated that this FP assay for the determination of DON in wheat and wheat-derivative products was a rapid and cheap method that presented an alternative to the more robust chromatographic methods. A new approach for the synthesis of the DON-FL2 that leads to a selective product is also reported.

MATERIALS AND METHODS

Chemicals and materials. DON, 3-acetyl-DON, 15-acetyl-DON, 1,1′-carbonyldiimidazole, 1-butylboronic acid, phosphate-buffered saline (PBS), and sodium bicarbonate were purchased from Sigma-Aldrich (Milan, Italy). 4′-(Aminomethyl)fluorescein hydrochloride was purchased from Molecular Probes (Eugene, Oreg.). The monoclonal antibody for DON (clone 22) was provided by the U.S. Department of Agriculture–Agricultural Research Service–National Center for Agricultural Utilization Research (USDA-AGS-NCAUR; Peoria, Ill.) and has been described by Maragos and McCormick *(11).* DON immunoaffinity columns (DONtest HPLC) were obtained from VICAM (Watertown, Mass.). All other chemicals and solvents were reagent grade or better and were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Waters Milli-Q system (Waters, Milford, Mass.).

Preparation of DON-FL2. The DON-FL2 was prepared according to Maragos and Plattner *(12),* with the additional step of protecting the C7- and C15-hydroxyl groups of DON by reaction with 1-butylboronic acid to form a cyclic boronate ester *(3).*

1-Butylboronic acid (68 mg) was added to 20 mg of DON in 200 μl of pyridine and stirred overnight at room temperature to yield 7,15-O-(butylboronil)-DON. An aliquot of the reaction mixture (58 μl) was added to 160 mg of 1,1′-carbonyldiimidazole dissolved in 560 μl of dry acetone and kept at room temperature for 90 min. Water (50 μl) was added, and then 1.15 ml of 4′-(aminomethyl)fluorescein (10 mg/ml in dimethylformamide) and 1.2 ml of 0.1 M sodium bicarbonate, pH = 8.2, were added. The reaction, held for 4 days at 4°C, was periodically monitored by analytical HPLC. Chromatographic separation was performed by an Agilent 1100 Series system (Agilent Technologies, Palo Alto, Calif.) equipped with a fluorescence detector ($\lambda_{ex.}$ = 485 nm, $\lambda_{em.}$ = 531 nm) and a Simmetry column (5 μm, 150 by 4.6 mm inside diameter [i.d.]; Waters) with a methanol:water (60:40, vol/vol) ratio containing 1% acetic acid as the mobile phase at a flow rate of 1 ml/min (DON-FL2 retention time, 17.6 min). DON-FL2 was isolated by semipreparative HPLC with a PerkinElmer Series 200 pump (PerkinElmer, Norwalk, Conn.), a Simmetry semipreparative column (7 μm, 150 by 7.8 mm i.d.; Waters), and an HP 1040A diode array detector set at λ = 220 nm (Hewlett Packard, Waldbronn, Germany). Aliquots of the reaction mixture (100 μl) were injected. The following binary gradient was applied at a flow rate of 5.0 ml/min: (i) 45% methanol–55% water containing 1% acetic acid for 35 min, and then (ii) 75% methanol for 5 min, followed by (iii) 5 min with 75% methanol. Fractions eluting between 28 and 32 min (containing DON-FL2) were collected from the column, and the solvent was removed by a combination of vacuum evaporation and lyophilization.

The identity of DON-FL2 was confirmed by HPLC-MS (positive chemical ionization mode) by means of a Varian 9012 chromatographic system interfaced to a QqTOF/MS QSTAR (Applied Biosystem/MSD Sciex, Concord, Ontario, Canada) equipped with a turbo–ionspray interface. Interface conditions were as follows: nebulizer gas (air), 1.3 liters/min; curtain gas (nitrogen), 1.2 liters/min; heater gas (air, 300°C), 6 liters/min; mass range, 100 to 800 amu; scan time, 1 s; needle voltage, 5,000 V; focusing potential, 150 V; and declustering potential, 30 V. Accurate mass measurements (four decimal figures) were carried out by obtaining the averaged spectra of the peaks and then by calibrating them with two ions of known chemical structure present in the same spectra.

A DON-FL2 stock solution was prepared by reconstituting the isolated compound with 10 ml of deionized water. DON-FL2 working solutions were prepared daily by diluting 1:1,000 (vol/vol) aliquots of stock solution with phosphate-buffered saline (PBS: 10 mM sodium phosphate, 0.85% sodium chloride, pH 7.4) containing 0.1% sodium azide (PBS-A).

Samples. Durum wheat kernels were obtained from different experimental fields in Italy. A total of 83 naturally contaminated samples (35 durum wheat, 22 semolina, and 26 pasta) were analyzed both by FP immunoassay and HPLC. Durum wheat inoculated under field conditions with DON-producing strains of *F. culmorum* and *F. graminearum (6)* was used to obtain samples of semolina and pasta with the highest DON concentrations (concentrations of DON in durum wheat, semolina, and pasta for the two strains were 1.06, 1.11, and 1.12 μg/g and 0.36, 1.09, and 2.36 μg/g, respectively, as determined by HPLC analysis). The processing of these samples was performed as described by Visconti et al. *(28).*

Wheat and pasta samples were finely ground by a model MLI 204 Bühler mill (Bühler S.p.A, Milan, Italy) to pass a 1-mm sieve. Semolina samples were prepared by milling cleaned wheat kernels

with a Bühler Laboratory Mill model MLU 202 equipped with three break and three stripping systems and then treating the samples with a Namad purifier (Namad, Rome, Italy).

To determine the matrix effect on the FP signal, different amounts of 10 cultivars of durum wheat (Duilio, Simeto, Bracco, Orobel, Svevo, Iride, Ionio, Meridiano, San Carlo, and Colosseo) and soft wheat (Bilancia, Centauro, Sagittario, Artico, Bolero, Bramante, Carisma, Agadir, Amarok, and Blasco), free of DON and 3-acetyl-DON, were used, representing different compositions of durum and soft wheat. In addition, extracts of durum wheat samples were spiked at four DON levels (7.5, 10, 30, and 60 ng of DON) and analyzed by the FP immunoassay.

Extraction of samples for FP immunoassay and HPLC analysis. Ground samples (25 g) were weighed into a blender jar and extracted with 100 ml of PBS by blending at high speed for 2 min with a Sorvall Omnimixer (Sorvall Instruments, Norwalk, Conn.). Extracts were filtered through both filter paper (Whatman no. 4) and a glass microfiber filter (Whatman GF/A) and analyzed by FP immunoassay, without further treatment, and by HPLC, after immunoaffinity column cleanup, as described below.

Samples free of DON and related metabolites (3-acetyl-DON, 15-acetyl-DON), used to evaluate the matrix effect and used in recovery experiments, were processed according to Trucksess et al. *(26)* and analyzed by HPLC according to Quarta et al. *(19).*

FP immunoassay. The FP instrument was a Spectrofluorometer LS 55 (PerkinElmer). Antibody working solutions were prepared by diluting DON monoclonal antibody clone 22 at a rate of 1:300 (vol/vol) in PBS containing 0.1% bovine serum albumin. Glass cuvettes (10-mm optical pathway) were used for the assays. Measurements of spiked or naturally contaminated samples were performed by adding 0.10 ml of antibody working solution and then 240 μl of filtered extract (equivalent to 60 mg of matrix) to 1.75 ml of the PBS-A present in the cuvette. After being mixed for 2 min by a magnetic stirrer, the test solution was placed in the instrument, and this signal was measured as the blank. The cuvette was removed from the instrument, and tracer (25 μl of the DON-FL2 working solution) was added to the test solution. This mixture was vigorously mixed for 2 min (incubation time). The test solution containing the tracer was then returned to the fluorometer, and the signal (in millipolarization units) was measured. Measurement conditions of the spectrofluorometer were as follows: $\lambda_{ex.} = 496$ nm and $\lambda_{em.} = 525$ nm, $slit_{ex.} = 15$ nm and $slit_{em.} = 15$ nm, integration time = 10 s. Polarization measurements were performed after reaching a constant value, generally between 1 and 3 min.

The polarization value (P) was normalized to fit the range of 0 to 1 with the equation $Y_{obs} = (mP_{obs} - mP_0)/(mP_1 - mP_0)$, where mP_{obs} is the signal from the test sample, mP_0 is the signal from a control that does not contain an antibody, mP_1 is the signal from a control that does not contain a toxin, and Y_{obs} is the normalized result for the test sample.

The DON content in sample extracts was calculated by measuring polarization values and comparing them with the relevant calibration curve from 0.1 to 2.0 μg/g. The calibration curve was obtained by adding PBS-A (1.75 ml) to each cuvette and then adding 0.10 ml of the antibody working solution and 240 μl of appropriate DON standard solutions (in PBS-A) from 12.5 to 2,500 ng/ml.

HPLC analysis. DON analyses of the different matrices were performed according to the method described by Visconti et al. *(28),* with minor modifications. In particular, ground samples were extracted with PBS as described above, and 2 ml of the filtered extracts (equivalent to 0.5 g of matrix) was cleaned up as described by Visconti et al. *(28)* and analyzed for DON by HPLC as described by Quarta et al. *(19).*

Recovery experiments. Recovery experiments were performed in quadruplicate by spiking blank samples (durum wheat, semolina, and pasta) with DON at levels of 0.25, 0.75, 1.25, and 1.75 μg/g. Spiked samples were left overnight at room temperature to allow solvent evaporation prior to extraction with PBS and analysis by both FP immunoassay and HPLC/immunoaffinity.

Statistical analysis. Linear and sigmoidal fits of FP immunoassay data were performed by means of the unweighted least-squares method by Origin version 6.0 (OriginLab Corporation, Northampton, Mass.). A SigmaStat for Windows version 3.0 (Sigma, St. Louis, Mo.) was used for the statistical evaluation of data (means and standard deviations) relevant to recovery experiments and the matrix effect; for the latter, an analysis of variance was also performed.

RESULTS AND DISCUSSION

DON-FL2 approach. Monoclonal antibody 22 was previously shown to have a good interaction with DON-4′-(aminomethyl)fluorescein (DON-FL2) when used as tracer in the FP immunoassay for DON detection in wheat and maize *(12).* A new approach to the synthesis of the DON-FL2 was performed following the scheme shown in Figure 1. DON-FL2 was synthesized by the reaction of DON with 4′-(aminomethyl)fluorescein after protection of the C7- and C15-hydroxyl groups of DON by reaction with 1-butylboronic acid to form a cyclic boronate ester.

Figure 2 shows the chromatogram of the reaction mixture obtained after 4 days of reaction. The peak at 17.6-min retention time corresponded to DON-FL2. Peaks due to residual 4′-(aminomethyl)fluorescein and to secondary reaction products occur between 1 and 10 min of retention time. The identity of DON-FL2 was confirmed by HPLC-MS analysis (positive chemical ionization mode) of the reaction mixture. The total ion chromatogram of the reaction products showed the presence, at the DON-FL2 retention time, of a molecular ion of *m/z* 684.2034 ($C_{37}H_{34}NO_{12}$ calculated *m/z,* 684.2075; error, −6.1 ppm), corresponding to the $[(DON\text{-}FL2)\text{-}H]^+$ adduct, and of a molecular ion of *m/z* 706.1609 corresponding to the $[(DON\text{-}FL2)\text{-}Na]^+$ adduct. Fluorescence spectra in PBS-A (pH 7.4) of the tracer isolated and purified by semipreparative HPLC showed a maximum excitation wavelength at 496 nm and a maximum emission wavelength at 519 nm.

Two major advantages can be observed by using the described DON-FL2 synthesis when compared to the previously described procedure *(12).* In particular, the protection of the C7- and C15-hydroxyl groups of DON led to a significant improvement in the yield of the targeted reaction product, thus, in turn, leading to a single fluorescent derivative of DON with fluorescein bound selectively to the C3-hydroxyl group. This allowed the tracer to be easily isolated by semipreparative HPLC. Moreover, the synthesis of the DON-FL2 was completed after only 4 days, compared to the 18 days required by the previous procedure *(12).*

FP immunoassay for DON standard solution. The FP immunoassay measures competition between the DON-

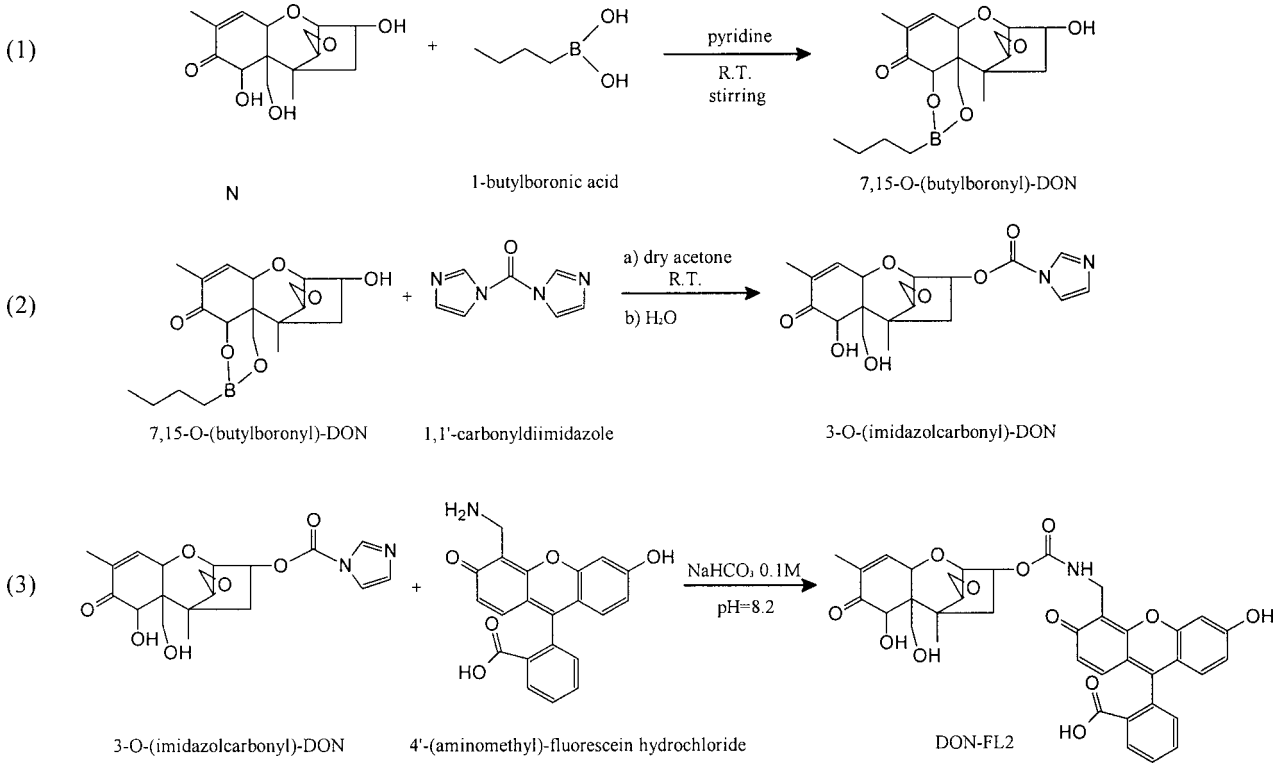

FIGURE 1. *Pathway of the DON-fluorescein tracer (DON-FL2) synthesis: (1) protection of DON with 1-butylboronic acid; (2) activation of the C3-hydroxyl group with 1,1′-carbonyldiimidazole (a) and hydrolysis at C7- and C15-hydroxyl groups of DON (b); and (3) reaction of the activated DON with 4′-(aminomethyl)fluorescein hydrochloride to form DON-FL2.*

FL2 and the unlabeled DON in solution for binding to the DON-specific antibody. The DON calibration curve obtained under our experimental conditions from 12.5 to 2,500 ng/ml is shown in Figure 3. The IC_{50} concentration, representing the DON concentration giving an FP response of 50%, was 105 ng/ml. A good linearity response (coefficient of correlation, $r = 0.9968$) between polarization values (P) and logarithm of DON concentrations (expressed as nanograms per milliliter) was observed from 25 to 500 ng/ml. The limit of detection, calculated as the DON concentration giving an FP signal equal to threefold the standard deviation of that observed for a DON-free solution, was 20 ng/ml.

FP immunoassay: evaluation of the matrix effect. A rapid FP immunoassay for the determination of DON in wheat and maize has been previously described *(12).* Nevertheless, by comparison with a widely used HPLC method, an overestimation of the DON content in naturally contaminated samples was observed. The authors speculated that, in the case of wheat, the cross-reactivity of antibody 22 with fungal metabolites present in the wheat extract, in particular 3-aceyl-DON, influenced the FP determination of DON *(11, 12).*

Experiments on the evaluation of the matrix effect showed that the DON concentration calculated from the FP signal was directly proportional to the amount of matrix analyzed and was independent of the actual DON concentration in the durum wheat sample. Figure 4 shows five curves, with curve 1 corresponding to an extract free of DON and 3-acetyl-DON and curves 2 through 5 corresponding to extracts with four different DON concentrations, showing the absolute DON concentrations (in nano-

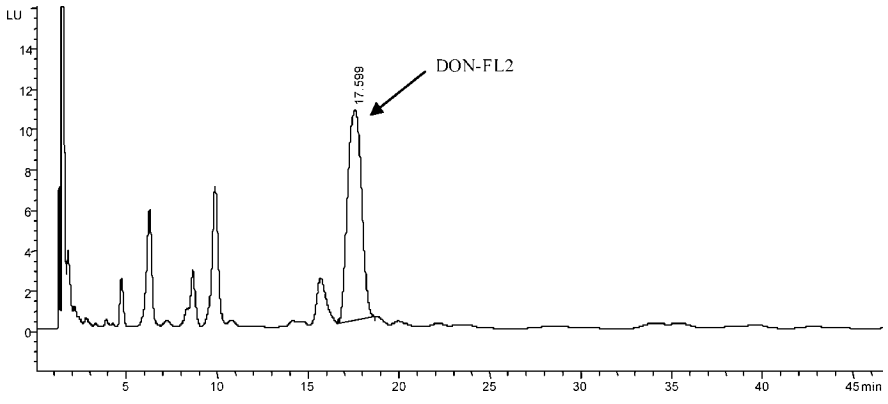

FIGURE 2. *HPLC chromatogram of the reaction mixture for the preparation of DON-FL2 after 4 days. Chromatographic conditions are described in "Materials and Methods" (preparation of DON-fluorescein tracer).*

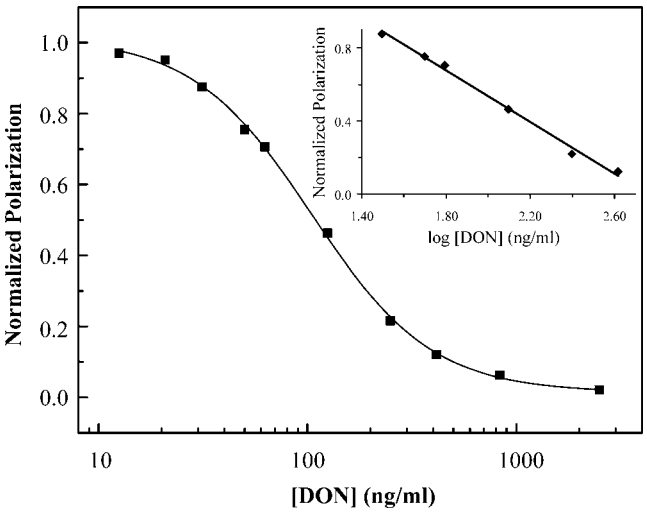

FIGURE 3. *Normalized FP calibration curve for DON in PBS-A solution. The FP linearity range versus log [DON] is shown in the inset.*

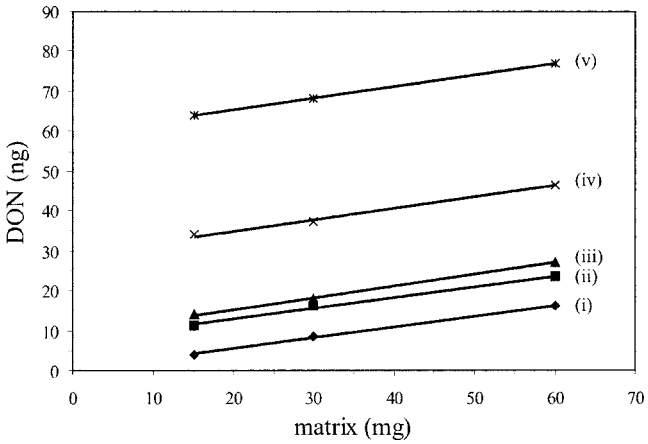

FIGURE 4. *Effect of amount of matrix on DON concentration as determined by FP immunoassay: (i) curve obtained with durum wheat extracts free of DON and 3-acetyl-DON; (ii, iii, iv, and v) curves obtained with durum wheat extracts spiked with 7.5, 10, 30, and 60 ng of DON, respectively.*

grams) (calculated from the FP signals) obtained by increasing the amount of durum wheat matrix (in milligrams) analyzed. All five regression plots showed the same slope (average value = 0.27 ± 0.01 μg/g), indicating that DON overestimation was constant and directly related to the matrix. Moreover, the intercept of the regression plot corresponded to the spiked DON concentration for each experiment.

In Table 1, average values of DON overestimation in measurements performed on durum wheat at different DON spiking levels and different amounts of analyzed matrix are reported as micrograms per gram (±SD, $n = 10$). No significant differences ($P > 0.1$) were observed between the values recorded. The mean of means value was 0.27 ± 0.01 μg/g with a relative standard deviation (coefficient of variation [CV]) of 4%; considering all individual measurements as independent ($n = 150$), the overall average value of the DON background (overestimation) was 0.27 ± 0.03 μg/g (CV = 11%). Considering the high number of measurements (150 total) performed with 10 different cultivars of durum wheat, it can be stated that the background value is highly reproducible for this matrix. A higher DON background value was observed when similar experiments were carried out with 10 cultivars of soft wheat free of DON and 3-acetyl-DON. In particular, the average value of DON overestimation ($n = 30$) was 0.39 μg/g, and the CV was 15%, both of which confirmed the good reproducibility of the background value observed for this matrix.

These results show that the DON overestimation is due to a matrix effect and that it cannot be attributed to the presence of other fungal metabolites that cross-react with the DON antibody, as previously hypothesized by Maragos and Plattner *(12).* This is also supported by HPLC analysis, showing the absence in the wheat extract of 3-acetyl-DON, the major compound that may cross-react with the DON-specific antibody. Under our chromatographic conditions *(19),* a good separation was obtained between 3-acetyl-DON (37.8 min) and 15-acetyl-DON (39.8 min).

Similar experiments carried out with semolina and pasta free of DON and 3-acetyl-DON showed overall mean values ($n = 30$) of DON overestimation to be 0.08 ± 0.01 μg/g (CV = 13%) and 0.04 ± 0.01 μg/g (CV = 25%), respectively. This indicated that the matrix effect for semolina and pasta was lower than that observed for durum and soft wheat, probably due to lower amounts of compounds cross-reacting with the antibody that remains in the wheat products after milling and processing.

Figure 5 shows regression plots obtained for four dif-

TABLE 1. *Overestimation average values, expressed as micrograms of DON per g of matrix, for different DON spiking levels and different matrix amounts of 10 different cultivars of durum wheat samples analyzed by FP immunoassay*

DON spiking level (ng)	Avg overestimation ± SD (μg/g) for different amt of matrix (mg)[a]			Mean of means ± SD (μg/g)
	15	30	60	
0	0.27 ± 0.03	0.27 ± 0.03	0.29 ± 0.03	0.27 ± 0.01
7.5	0.27 ± 0.04	0.28 ± 0.03	0.27 ± 0.03	0.27 ± 0.01
10	0.27 ± 0.02	0.28 ± 0.03	0.29 ± 0.03	0.28 ± 0.01
30	0.27 ± 0.03	0.27 ± 0.05	0.28 ± 0.05	0.27 ± 0.01
60	0.27 ± 0.03	0.28 ± 0.04	0.28 ± 0.05	0.28 ± 0.01
Mean of means ± SD	0.27 ± 0.01	0.27 ± 0.01	0.28 ± 0.01	0.27 ± 0.01

[a] Average values ± standard deviations obtained by FP immunoassay of 10 different cultivars of durum wheat extracts free of DON and 3-acetyl-DON and spiked with DON ($n = 10$ measurements).

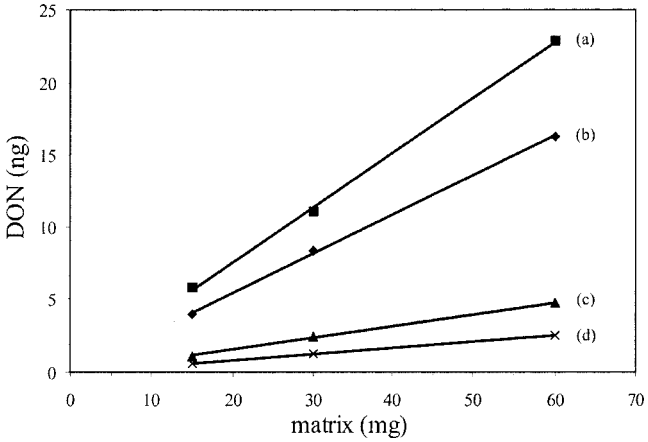

FIGURE 5. *Effect of amount of matrix on DON concentration as determined by FP immunoassay. Regression lines obtained with soft wheat (curve a), durum wheat (curve b), semolina (curve c), and pasta (curve d). All matrices were free of DON and 3-acetyl-DON.*

ferent matrices (soft wheat, durum wheat, semolina, and pasta) and gives the absolute DON concentrations (in nanograms) calculated by FP immunoassay versus the amount (in milligrams) of matrix free of DON and 3-acetyl-DON. The different regression slopes indicated the various DON background values, strictly related to the specific matrices, that should be subtracted from the measured DON content to calculate the correct DON contents.

Under the FP immunoassay conditions, described in ''Materials and Methods,'' a good linearity response was observed from 0.10 to 2.0 μg of DON per g of matrix.

The limit of detection, calculated as the DON content associated with an FP signal corresponding to three standard deviations ($n = 10$) from that of the DON-free control, was 0.08 μg/g for all matrices.

A correct FP quantification of DON contents in durum wheat, semolina, and pasta was also achieved by measuring polarization values and comparing them with the relevant calibration curve obtained with DON standard solutions diluted in DON-free extracts (data not shown).

Recovery of DON from durum wheat, semolina, and pasta. Recoveries of DON from durum wheat, semolina, and pasta were determined by both FP immunoassay and HPLC with immunoaffinity cleanup. The same extract after immunoaffinity cleanup was used to compare the two methods for toxin determination to avoid possible differences in DON recovery relevant to the extraction step.

Results of the recovery experiments (quadruplicate measurements) of both analytical procedures carried out on different matrices spiked with DON at levels ranging from 0.25 to 1.75 μg/g are shown in Table 2. Average recoveries (obtained after subtracting the background DON level) from durum wheat, semolina, and pasta determined by FP immunoassay were 98, 102, and 101%, respectively, with relative standard deviations less than 5%. Average recoveries from durum wheat, semolina, and pasta determined by HPLC method were 82, 86, and 87%, respectively, with relative standard deviations less than 10%.

Recovery and repeatability values of both methods for all matrices were within the criteria established by CEN (European Committee for Standardization) for acceptance of an analytical method for DON; these criteria foresee recoveries between 70 and 110% and relative standard deviations for repeatability (RSD_r) less than 20% for DON content above 0.1 μg/g *(4).*

Naturally contaminated samples. For comparison, PBS extracts of 35 samples of naturally contaminated durum wheat, 22 samples of semolina, and 26 samples of pasta were analyzed by both FP immunoassay and HPLC. A good correlation between DON concentrations obtained by FP immunoassay (after subtracting the background DON level) and HPLC was found for all matrices (Figure 6). Correlation coefficients (r values) for durum wheat, semolina, and pasta were 0.9972, 0.9955, and 0.9994, respectively. Data fitted the linear regression by the following equations: [DON by FP] = −0.017 + 1.298 [DON by HPLC] for durum wheat; [DON by FP] = 0.019 + 1.151 [DON by HPLC] for semolina; and [DON by FP] = 0.010 + 1.155 [DON by HPLC] for pasta. After correction of the HPLC data for recovery artifacts, slopes of the regression lines were 1.038, 0.990, and 1.004 for durum wheat, semolina, and pasta, respectively.

The FP method showed better sensitivity and accuracy than the previously described FP immunoassay and better accuracy and precision than the HPLC/immunoaffinity method.

A major disadvantage of the proposed method is the presence of the DON background level, which is mainly

TABLE 2. *Average recoveries of DON from spiked durum wheat, semolina, and pasta obtained by FP immunoassay (FPIA) and the HPLC method*

Spiking level (μg/g)	% recovery ± SD[a]					
	Durum wheat		Semolina		Pasta	
	HPLC	FPIA	HPLC	FPIA	HPLC	FPIA
0.25	82.5 ± 9.9	97.2 ± 3.6	83.5 ± 4.4	100.7 ± 3.5	87.2 ± 1.9	99.8 ± 5.3
0.75	79.9 ± 6.8	96.1 ± 5.5	87.4 ± 3.5	106.6 ± 4.8	87.5 ± 2.0	101.5 ± 1.6
1.25	84.2 ± 8.0	100.1 ± 2.9	87.6 ± 2.8	98.8 ± 1.7	87.6 ± 1.3	102.3 ± 2.8
1.75	83.2 ± 8.1	97.5 ± 4.1	85.6 ± 3.1	102.1 ± 3.0	86.3 ± 3.0	99.5 ± 4.1
Overall avg ± SD	82.2 ± 7.5	97.8 ± 4.0	86.2 ± 3.7	102.0 ± 4.7	87.4 ± 1.5	101.2 ± 3.3

[a] SD, standard deviation (n = four replicates).

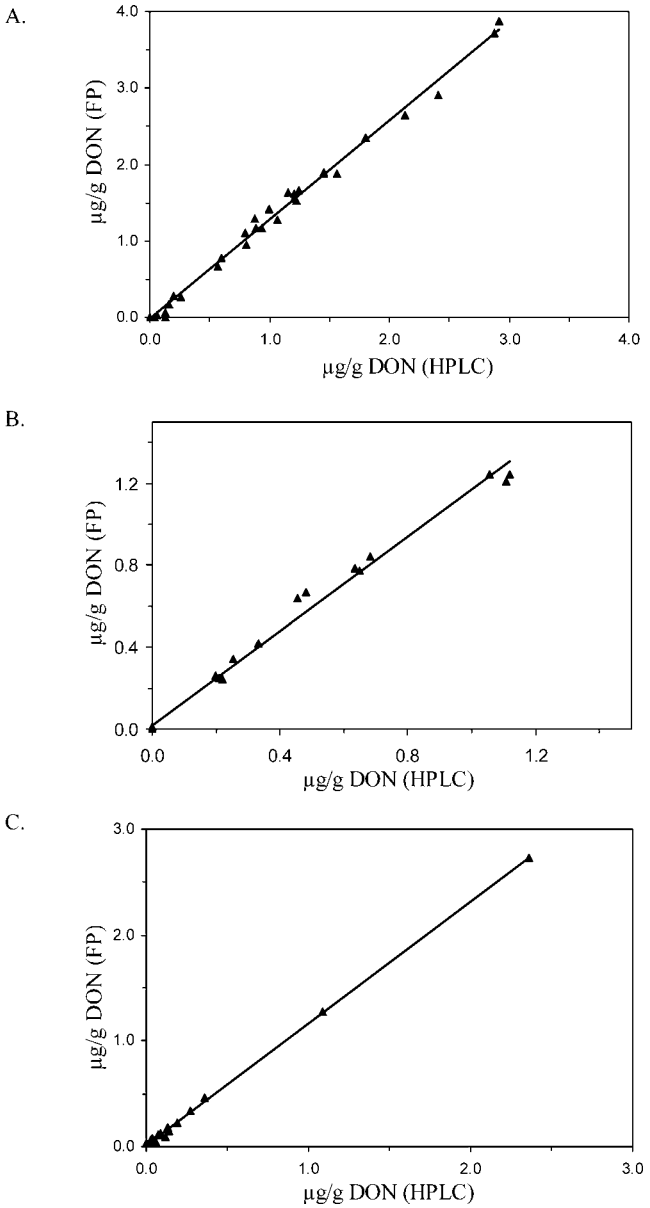

FIGURE 6. *A comparison of DON contents in naturally contaminated samples analyzed by HPLC and FP immunoassay. (A) Durum wheat, (B) semolina, and (C) pasta. Extracts of samples containing DON at concentrations higher than 2.0 µg/g were diluted prior to the FP immunoassay.*

related to the presence of compounds cross-reacting with the DON-specific antibody. This implies the need to subtract the FP background value from the measured DON value. However, the FP background value obtained with a specific matrix was highly reproducible, and this background value decreased as the wheat went through various milling procedures from wheat kernels (soft or durum) to wheat-derived products. In particular, it was close to the detection limit or almost irrelevant for semolina and pasta, which suggests that a decrease in interfering compounds occurred because of the milling and processing procedures used for durum wheat.

A major advantage of the FP assay with respect to ELISA procedures is the absence of undefined DON overestimations, leading to incorrect DON quantifications. In addition, the absence of long incubation times and washing steps reduces the time of analysis.

In conclusion, the FP immunoassay is an inexpensive, easy-to-perform method that allows the rapid and quantitative determination of DON at levels that naturally occur in wheat and wheat-based products and can be used as an alternative method to HPLC/immunoaffinity cleanup.

The overall time consumed in DON analysis with this immunoassay procedure for wheat-based products, including sample preparation and FP immunoassay measurement, was less than 10 min, thus allowing analysts to perform a much higher number of analyses than when HPLC or gas-chromatographic methods are used. This advantage positively affects food control procedures at various levels and, in fact, affects the entire production chain, including trading and enforcement processes and risk assessment monitoring, thus increasing the level of protection for human health.

The sensitivity of the FP immunoassay, although lower than the HPLC method, was sufficient for the quantitative determination of DON at concentrations below the maximum admissible levels proposed by the European Community for durum wheat, semolina, and pasta.

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