JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of Qualitative and Semiquantitative Immunoassay-Based Rapid Strip Tests for the Detection of T-2 Toxin in Wheat and Oat

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Novel qualitative as well as semiquantitative rapid strip tests for screening of T-2 mycotoxin in agricultural commodities were developed. Colloidal gold particles were coated with monoclonal anti-T-2 antibodies and used as detector reagent, indicating the strip test results by formation of up to two colored lines in a competitive assay format. The test line comprises a protein conjugate of the T-2 mycotoxin and the control line an antispecies-specific antibody to confirm the correct test development. To perform the test, 5 g of sample was extracted in a ratio of 1:5 with methanol/water (70:30) by shaking for 3 min and the extract directly used without further cleanup steps. The T-2 toxin lateral flow device (LFD) presented has a cutoff level around 100 μg/kg for naturally contaminated wheat and oat. The semiquantitative test may be used in the lower micrograms per kilogram range and allows for rapid semiquantitative photometric classification of the level of sample contamination. For both tests, results were obtained within 4 min. The developed LFDs therefore allow for the first time fast and on-site screening for the determination of T-2 toxin in cereals.

KEYWORDS: Lateral flow device; immunostrip test; colloidal gold; mycotoxins; T-2 toxin; on-site screening; qualitative; semiquantitative; cereals

INTRODUCTION

Mycotoxins are toxic natural secondary metabolites produced by several species of fungi on agricultural commodities in the field or during storage. T-2 toxin is a type A trichothecene produced by *Fusarium* species and may be found in grains such as wheat, oat, maize, barley, and rice, occurring mainly in temperate to cold areas. Because T-2 toxin is rapidly metabolized to HT-2 toxin, these two are often given as a sum parameter. A combined temporary tolerable daily intake of 0.06 μg/kg of body weight was defined for T-2 and HT-2 (*1*). It has been shown that T-2 toxin has a high acute toxicity, is hematotoxic and immunotoxic (*2*), and inhibits protein and nucleic acid synthesis (*3*). In the European Community, a maximum level for the sum of T-2 and HT-2 will be fixed, if appropriate, according to regulation EC 856/2005. Nevertheless, reliable occurrence data are very limited, and no maximum level has been proposed. In the United States there are currently no levels for T-2 and HT-2 toxins recommended by regulatory agencies.

Besides classical analytical methods based on chromatographic techniques, fast and inexpensive tools that do not require often tedious and time-consuming sample preparation and cleanup steps are needed for screening mycotoxins in susceptible crops on-site. Extraction of type A trichothecenes is usually performed with acetonitrile and water or methanol and water. Solid phase extraction materials (SPE) are available in disposable plastic cartridges for cleanup ranging from C18, silica gel, cyano, and Florisil SPE cartridges to multifunctional MycoSep columns (*4*). Immunoaffinity cleanup columns are also available for the cleanup step for T-2 and HT-2 analysis (*5*). Due to the lack of a conjugated keto group at the C-8 position, a derivatization step is necessary for quantitative analysis by HPLC or gas chromatography (GC). Common approaches for type A trichothecenes include GC−electron capture detection (ECD) and GC-MS methods (*6*). Furthermore, LC-MS/MS-based methods such as multimycotoxin methods allowing for simultaneous detection of a wide range of mycotoxin classes such as type A and type B trichothecenes are increasingly being used (*7, 8*). For the screening of T-2 and HT-2 toxins, ELISA-based immunoassays and thin layer chromatography (TLC) are used. Other immunoassay-based approaches targeting the T-2 toxin are scarce and include the development of a membrane-based immunofiltration assay (*9*) and a dipstick enzyme immunoassay for the detection of T-2 toxin in wheat (*10*). The dipstick assay included several steps such as incubation of the antibody-coated dipstick in a mixture of sample and T-2 toxin−horseradish peroxidase conjugate and dipping in a chromogen-containing

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10.1021/jf800393j CCC: $40.75
Published on Web 03/28/2008

substrate solution for color reaction, with a test time of 45 min and a dot color intensity visually distinguishable from blank material starting at 12 μg/kg spiked wheat. In the past few years, immunodiagnostic strip tests or lateral flow devices (LFDs) have increasingly gained interest in the field of mycotoxin analysis due to the potential for fast and simple on-site application. LFDs have been developed for the mycotoxins fumonisin B_1 (*11*), ochratoxin A (*12*), and aflatoxin B_1 (*13–15*). The detector reagents in these strip tests range from the most commonly used colloidal gold (*13, 14*) to dye-containing liposomes (*15*). Moreover, a lateral-flow immunoassay for the rapid simultaneous detection of zearalenone and deoxynivalenol has been reported recently (*16*). The cutoff levels of the qualitative strip test with spiked wheat samples were 1500 and 100 μg/kg for deoxynivalenol and zearalenone, respectively. Nevertheless, successful approaches remain limited, with only a few LFDs being available on the market, such as for the mycotoxins deoxynivalenol, total aflatoxins, and fumonisins.

Increasing data and therefore awareness of the high relevance of the T-2 and HT-2 toxins together with recent regulations have pointed out the importance of developing not only sensitive and accurate quantitative analytical methods but also fast qualitative methods for on-site use. The aim of the presented work was to develop novel qualitative as well as semiquantitative immunochromatographic strip tests for the rapid screening of T-2 toxin in cereal commodities. The strip tests should allow fast and selective sample screening within a few minutes, requiring no skilled personnel.

MATERIALS AND METHODS

Materials. T-2 toxin and T-2 toxin−bovine serum albumin (BSA) conjugate were purchased from Biopure Referenzsubstanzen (Tulln, Austria). A T-2 toxin stock solution of 100 μg/mL in acetonitrile was kept at 4 °C for further dilution. Rabbit anti-mouse IgG (whole molecule, 2 mg/mL), Tween 20, BSA, tetrachloroauric(III) acid trihydrate, citric acid trisodium salt, and sodium azide were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membranes (AE100, AE99, AE98, FF85) and wick materials (470 grade cotton linter, CF5) were from Whatman (Middlesex, U.K.). White vinyl backing cards with one-sided GL-187 medical grade adhesive coating and release liner were from G&L Precision Die Cutting (San Jose, CA).

Equipment. The equipment used for spraying and cutting strip tests was purchased from BioDot (Irvine, CA). A ZX1000 dispensing platform with two Frontline contact tips and one Airjet Quanti 3000 were used for lateral flow device production together with a CM4000 guillotine cutter for strip test cutting. A benchtop-sized BioScan BSR1000 reflectance photometer (BioDot) was used for reading the semiquantitative test strips. Grinding of agricultural samples was performed with a series II mill from Romer Labs Diagnostic (Tulln, Austria). For the extraction, an overhead mixer from Labor-Brand (Giessen, Germany) was used. Also, an Avanti 30 centrifuge from Beckman Coulter (Krefeld, Germany) was used for centrifugation of gold colloid conjugates.

Production of Monoclonal Antibodies against T-2 Toxin. Anti-T-2 toxin monoclonal antibodies (Mabs) were produced and characterized according to the slightly modified method of Kohler et al. (*17*). The selected T2-B1 antibodies (EC BioCop Project FOOD-CT-2005-006988) showed high sensitivity and specificity to T-2 toxin. The cross-reactivity of the monoclonal T2-B1 antibodies was determined with an indirect competitive ELISA for deoxynivalenol, nivalenol, zearalenone, neosolaniol, and HT-2 toxin. Only a cross-reactivity of 5.4% with HT-2 was found; no cross-reactivity was found with deoxynivalenol, nivalenol, zearalenone, and neosolaniol. The 50% inhibition concentration for T-2 toxin was 26.6 μg/kg (PBS assay buffer with 25% methanol).

Protein and Antibody Quantification. Protein quantification was performed by using the BCA protein assay from Pierce (Rockford, IL). Immunoglobulin concentration was also measured photometrically using the absorption at 280 nm (*18*).

Synthesis of Colloidal Gold. Colloidal gold particles with an absorption maximum at 526 nm were prepared by controlled reduction of tetrachloroauric(III) acid trihydrate with citric acid trisodium salt using the procedure described by Frens (*19*) and Turkevitch (*20*).

Conjugation of Anti-T-2 Mab with Colloidal Gold. The pH of the gold colloid solution was adjusted to 8.5 with 0.2 M potassium carbonate. The required concentration of antibody was determined prior to conjugation according to the method of Horisberger et al. (*21*): increasing amounts of antibody adjusted to 0.2 mg/mL in phosphate buffer saline (PBS) were titrated to a fixed amount of 500 μL of colloidal gold solution. The addition of 100 μL of 10% (w/v) sodium chloride led to gold colloid aggregation of insufficiently coated particles, which was measured photometrically. For conjugation, 8 μL/mL gold colloid was mixed in 50 mL centrifugation tubes and incubated for 90 min at room temperature on a shaker. The remaining binding sites were blocked by the addition of BSA and incubation of the mixture as previously described for a further 90 min. The solution was centrifuged for 30 min at 8000*g* to separate unbound antibody from the gold colloid conjugate. The colorless supernatant was removed and the dark red conjugate washed twice with 30 mL of distilled water. After the addition of 6% BSA (20% v/v) and 10% sodium azide (0.5% v/v) solutions, the centrifuged conjugate (OD 13) was stored at 4 °C until further use.

Manufacturing of Lateral Flow Devices. The control and test lines were sprayed onto a nitrocellulose membrane of 300 mm × 25 mm with full-contact Frontline tips (BioDot). Nitrocellulose membranes of various pore sizes were tested for their suitability as analytical membrane. For the control line, rabbit anti-mouse antibodies at 1 mg/mL were used. For the test line, the T-2 toxin−BSA conjugate was sprayed at various concentrations ranging from 0.125 to 0.75 mg/mL depending on the qualitative or semiquantitative application of the strip test. After drying for 60 min at 25 °C, the nitrocellulose membranes were blocked with 1% BSA in 0.05 M PBS by incubation for 30 min on a rocker. The membranes were then washed three times with 0.05 M PBS and dried for 60 min at 25 °C. Both membrane (300 mm × 25 mm) and sample wick (300 mm × 27 mm) were assembled onto a 0.010 in. plastic backing card overlapping 2 mm. The assembled test was then cut into 5 mm strips with the BioDot guillotine cutter.

Cereal Samples. Naturally contaminated wheat samples were obtained from the Institute for Biotechnology in Plant Production, Department for Agrobiotechnology IFA-Tulln, University of Natural Resources and Applied Life Sciences, Vienna, Austria. Naturally contaminated oat samples were obtained from Romer Labs Diagnostic (Tulln, Austria). Ground blank samples were fortified by adding T-2 toxin standard solution to the desired concentration and stored for 48 h at 36 °C for solvent evaporation. Blended samples were furthermore obtained by blending naturally contaminated samples with blank material.

Sample Preparation for Strip Test. Agricultural commodities used were wheat and oats. Five grams of a ground sample was extracted in a ratio of 1:5 (w:v) with methanol/water (70:30, v/v) using an overhead mixer (30 rpm) for 3 min. The extract was then filtered using a folded filter (Whatman Schleicher & Schuell) if necessary and further diluted 1:5 with 0.05 M PBS before use.

Test Procedure. Each single test was performed in a well of a nonbinding ELISA microtiter plate. For the qualitative strip test, 25 μL of the test reagent mixture containing 80% gold conjugate (previously diluted 1:11 with 0.05 M PBS buffer) and 20% Tween 20 solution (5% in 0.05 M PBS) was added to a microwell. Fifty microliters of sample extract (previously diluted 1:5 after filtering) was then added to the test reagent mixture. The content in each well was mixed by pipetting it up and down a few times. After introduction of one test strip per well, the test was allowed to develop color for 4 min at room temperature, and results were read immediately thereafter. It was important to adhere to development time before reading the strips as the strips darken when either left dipped into the solution in the well or after drying out.

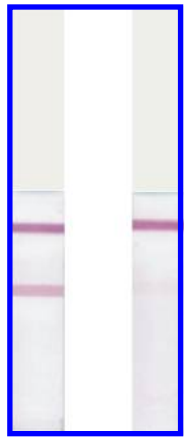

Figure 1. Qualitative strip tests for the detection of T-2 toxin in agricultural commodities. Two lines indicate a negative result, whereas one line indicates a positive result equal to or above the cutoff level.

For the semiquantitative strip test used with the benchtop-sized photometric reflectance reader, 40 μL of the test reagent mixture containing 87.5% gold conjugate (previously diluted 1:7.5 with 0.05 M PBS buffer) and 12.5% Tween 20 solution was inserted in a microwell. Fifty microliters of sample extract (previously diluted 1:5) was then added to the test reagent mixture. The content in each well was mixed by pipetting it up and down a few times. After introduction of one test strip per well, the test was allowed to develop color for 4 min. The strips were then inserted into a cassette holder, which was positioned into the reflectance photometer for immediate reading.

Reference Method. A validated liquid chromatographic/tandem mass spectrometric multitoxin method as described by Sulyok et al. (*7*) was used for the characterization of naturally contaminated samples and for the determination of the extraction efficiency using naturally contaminated and blended samples. Five grams of sample was extracted respectively with acetonitrile/water/acetic acid, 79:20:1, in a ratio of 1:4 (w:v) for 90 min (*7*) using an overhead mixer (30 rpm) and in a ratio of 1:5 (w/v) with methanol/water, 70:30 (v/v), for 3 min using an overhead mixer (30 rpm). Three hundred microliter aliquots of the extracts were transferred without cleanup steps into glass vials and diluted 1:1 with acetonitrile/water/acetic acid, 20:79:1, before injection of 5 μL into the LC-MS/MS system. Measurements were performed with a QTrap 4000 LC-MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ESI source and a 1100 series HPLC system (Agilent, Waldbronn, Germany) with a 150 $\times$ 4.6 mm i.d., 5 μm, Gemini C18 column (Phenomenex, Torrance, CA).

RESULTS AND DISCUSSION

Development and Optimization of the Strip Test. The developed strip tests are one-step lateral flow immunochromatographic tests based on a competitive immunoassay format determining a qualitative level (cutoff) or semiquantitative level for the presence of mycotoxin and are intended for on-site use with agricultural commodities. Two lines of reagent (test and control line) are immobilized onto a nitrocellulose membrane (25 mm length) at a 5 mm distance from each other using a contact-tip reagent dispenser (see **Figure 1**). The test line comprises a BSA conjugate of the T-2 mycotoxin and the control line an anti-mouse specific antibody. Colloidal gold particles coated with monoclonal mouse anti-T2 antibodies are used as detector reagent. The antibody−colloidal gold particle complex is dissolved in assay diluent and mixed with 50 μL of sample extract in a microwell. The strip test is inserted into the well, and the mixed content then migrates onto the membrane. Mycotoxin−protein conjugate coated on the test zone captures free antibody−colloidal gold particle complex, allowing color particles to concentrate and form a visible line. A positive sample with a contamination greater than the cutoff level will result in no visual line in the test zone. Alternatively, a negative sample containing less than the cutoff level will form a visible line in the test zone. The control line will always be visible regardless of the presence of T2-toxin, confirming correct test development.

Table 1. Determination of Strip Test Cutoff Level for Naturally Contaminated and Blended Wheat and Oat Samples[a]

	T-2 toxin[b] (μg/kg)	no. of replicates	positive results	negative results
wheat sample				
W1	349 $\pm$ 13	10	10	0
W2	202 $\pm$ 9	10	10	0
W3	179 $\pm$ 9	10	10	0
W4	121 $\pm$ 5	10	10	0
W5	124 $\pm$ 26	10	6	4
W6	66.6 $\pm$ 8.9	10	3	7
W7	36.7 $\pm$ 4.5	10	0	10
blank	<LOD[c]	10	0	10
oat sample				
O1	162 $\pm$ 18	10	10	0
O2	79.2 $\pm$ 4.3	10	10	0
O3	87.5 $\pm$ 15.6	10	1	9
O4	46.1 $\pm$ 11.2	10	0	10
blank	<LOD	10	0	10

[a] For cutoff determination, 10 of 10 strip test replicates must be positive. [b] T-2 toxin concentration as measured with reference method. [c] Limit of determination.

The flow properties of the strip test were optimized by the selection of type and pore size of membrane; type and concentration of blocking agent for blocking membrane binding sites after spraying of reagents (test and control line); type of buffer, pH range, and ionic strength; and the use of surfactants and modifiers for pre or post treatment of strip test materials as well as in reagent mix solutions. The unsupported nitrocellulose (NC) membranes of increasing pore size (5−12 μm) showed different protein-binding properties when spraying the T-2 toxin−BSA protein conjugate. Best results were obtained with the AE100 membrane, giving a strong test signal when incubated with anti-T-2 toxin antibody−colloidal gold conjugate as detector reagent. Concentrations and applied amounts of T-2 toxin−BSA protein conjugate and detector reagent, as well as contact dispenser application speed, were optimized, allowing test sensitivity to be adjusted to obtain a strip test with a cutoff around 100 μg/kg. The cutoff value was selected on the basis of available T-2 surveys showing T-2 concentrations mostly within 1–100 μg/kg but also >100, >200, and >500 μg/kg (*22, 23*). The detector reagent (OD 13) was diluted 1:11 with PBS buffer, adjusting the concentration to obtain during test performance a reproducible and color intense control line; a visual detection limit of approximately 120 μg/kg in wheat and 100 μg/kg in oat, respectively, with no test line visible at sample concentrations at and above the cutoff (**Table 1**); and no background coloring of membrane due to nonspecific binding of excess colloidal gold particles. Strip test production showed that the blocking procedure of the NC membrane after spraying of the reagent lines was a critical step for obtaining reproducible test results. Best results were obtained with 1% BSA protein solution as compared to fish gelatin or conalbumin A. Nonspecific binding of matrix components or colloidal gold particles to the membrane was prevented by membrane blocking. In addition, blocking allowed stabilization of the sprayed reagents, ensuring longer stability of the strip tests. Nonspecific binding of colloidal gold particles to matrix components that may bind to the membrane, further contributing to background coloring, was also strongly reduced.

Table 2. LC-MS/MS Characterization of the T-2 Toxin Concentration in the Investigated Blended Wheat (W) and Oat (O) Samples

	T-2 toxin (μg/kg)					
	1–1	1–2	2–1	2–2	mean ± s (μg/kg)	RSD[a] (%)
wheat sample						
W1	349	330	362	354	349 ± 13	3.7
W2	200	190	209	210	202 ± 9	4.5
W3	188	184	169	174	179 ± 9	5.0
W4	118	120	128	118	121 ± 5	4.1
W5	106	98	148	146	124 ± 26	21.0
W6	59.4	58.6	74.6	73.9	66.6 ± 8.9	13.4
W7	33.5	32.3	39.9	41.2	36.7 ± 4.5	12.3
W8	61.1	61.4	30.4	25.5	44.6 ± 19.3	43.3
W9	27.8	25.4	40.8	38.9	33.2 ± 7.8	23.5
oat sample						
O1	142	152	171	182	162 ± 18	11.1
O2	74.8	81.6	76.6	84.0	79.2 ± 4.3	5.4
O3	100.8	100.8	70.7	77.6	87.5 ± 15.6	17.9
O4	34.3	38.9	53.7	57.4	46.1 ± 11.2	24.3
O5	57.4	68.5	111.2	121.6	89.7 ± 31.5	35.1

[a] Relative standard deviation (n = 4).

The use of Tween 20 as surfactant in 0.05 M PBS in the test reagent mixture greatly improved sample flow on the membrane, allowing homogeneous solvent frontlines to be obtained. Tween 20 may furthermore contribute to reducing hydrophobic attraction between sample matrix and gold particles.

Although a good extraction solvent for the T-2 toxin, acetonitrile was avoided due to incompatibility with the anti-T-2 toxin Mab. Furthermore, because the extraction should also be performed on-site in a nonlaboratory environment by unskilled personnel, acetonitrile was avoided as extraction solvent. A mixture of methanol/water (70:30, v/v) was therefore selected for extraction of T-2 toxin from cereal commodities. Results showed that when a strip test was performed, false-negative signals were obtained with a methanol content over 15% in the final test mix solution. Therefore, the sample extract was diluted 1:5 with PBS buffer prior to use, resulting in a final methanol concentration of 9% for the strip test. Large differences were furthermore observed between spiked samples and naturally contaminated samples with shifts of the cutoff level in the strip test. The strip test optimization was therefore performed using only naturally contaminated material, which was blended with blank material for obtaining samples with a range of T-2 concentrations. The obtained samples were characterized by LC-MS/MS (**Table 2**). Each sample was extracted twice with acetonitrile/water/acetic acid, 79:20:1, in a ratio of 1:4 (w/v) for 90 min (*7*) and measured in duplicate. Results are shown in **Table 2** for the two extractions, where each of the duplicate measurements is shown separately. Because the reference method was validated for wheat and maize (*7*), a matrix standard addition was performed with oats to ensure the applicability of the method with the oat matrix as well. For this purpose, six concentration levels in the range of 5−1600 μg/kg were used (n = 3). A standard calibration curve (linear calibration, weighted 1/x) for T-2 toxin was constructed by plotting the signal intensity versus the T-2 toxin concentration and showed a r^2 value of 0.9989, confirming the applicability of the method for the oat matrix. **Table 2** shows that although T-2 concentrations from consecutive injections from one extraction are highly reproducible, values may vary when two extractions are compared, especially in the lower concentration range, due to the large dilution factor of the contaminated material with blank material, besides integration uncertainties of the LC-MS/MS peak at low concentration levels. Mixing ratios ranged for wheat from 1:2 to 1:25 and for oat from 1:2 to 1:10. Higher RSDs were found in oat as compared to wheat. Recoveries were monitored using the reference method. Relatively low extraction efficiencies of ∼57% in wheat and ∼43% in oat were obtained, which are in agreement with the 55 ± 1% (n = 2) apparent recovery found by Sulyok et al. (*7*) with a 75% methanol extraction. These extraction efficiencies are much lower as compared to when a mixture containing acetonitrile was used, such as performed in the reference method using acetonitrile/water/acetic acid (79:20:1, v/v/v), which yielded apparent recoveries for, for example, spiked wheat samples of 107 ± 6% (n = 3). Data obtained by performing the extraction with methanol/water, 70:30 (v/v), were correlated with T-2 toxin concentration from reference measurements. The low extraction efficiencies were taken into account and did not impair the development of the strip test kit for T-2 toxin.

Screening of Naturally Contaminated Samples and Test Validation. The performance characteristics of the LFDs for the detection of T-2 toxin were determined using naturally highly contaminated wheat and oat samples, which were blended with blank material for obtaining samples containing distinct concentrations of T-2 toxin at different concentration levels in the lower micrograms per kilogram range. Eight wheat samples and five oat samples were screened with the strip test developed. Test and control line formation was observed visually. Results showed that the strip test was selective and sensitive for the determination of T-2 toxin in wheat and oat with cutoff levels around 120 and 100 μg/kg, respectively, as shown in **Table 1**. There are no general validation protocols available for qualitative test methods. Nevertheless, 10 replicates are usually needed and were measured at each concentration level (*24*). With a cutoff level defined as the concentration threshold below which positive identification becomes unreliable (*24*), and 100% of the 10 replicates being positive for sample W4 (121 ± 5 μg/kg T-2 toxin concentration from LC-MS/MS reference method), the cutoff level for wheat was around 120 μg/kg (**Table 1**). As shown by sample W5 with higher RSD (124 ± 26%) as compared to W4 (RSD = 3.9%), the cutoff can only be given as an approximate value. Nevertheless, when standard solutions as well as spiked sample extracts were used, which would allow the cutoff range to be narrowed, shifts were observed in the cutoff range as compared to naturally contaminated samples. Therefore, only the naturally contaminated blended samples were used. Similarly, 10 of 10 replicates were positive at a concentration of approximately 100 μg/kg for the oat samples tested (sample O2, 79.2 ± 4.3 μg/kg) (**Table 1**). Due to the specific cross-reactivity with HT-2 of 5.4%, the HT-2 contamination was also measured with the reference LC-MS-MS method as shown in **Table 3**. Seven-fold higher amounts of HT-2 as compared to T-2 toxin were found in the investigated wheat samples, and a 3-fold higher amount of HT-2 as compared to T-2 toxin was found in the investigated oat samples (**Table 3**). The qualitative strip tests were nevertheless developed using these naturally contaminated samples, therefore taking into account the high HT-2 concentrations. The qualitative strip test allowed for the selective determination of T-2 toxin in blended naturally contaminated samples with a cutoff at the 100 μg/kg level in wheat and oat.

Development and Optimization of the Semiquantitative Strip Test. In a semiquantitative approach, the intensity of the color of the test line on the strip test was analyzed by measuring the relative light reflectance from the test zone. The composition and concentration of test line reagent and color reagent was modified and optimized accordingly because a darker test line was needed for photometric line screening. Furthermore, to

Table 3. LC-MS/MS Characterization of the HT-2 Toxin Concentration in the Investigated Blended Wheat (W) and Oat (O) Samples

	HT-2 toxin (μg/kg)					
	1–1	1–2	2–1	2–2	mean ± s (μg/kg)	RSD[a] (%)
wheat sample						
W1	2504	2344	2480	2464	2448 ± 71	2.9
W2	1368	1344	1448	1320	1370 ± 56	4.1
W3	1256	1136	1192	1152	1184 ± 54	4.6
W4	864	816	816	864	840 ± 28	3.3
W5	650	646	732	832	715 ± 87	12.2
W6	362	436	403	437	410 ± 35	8.5
W7	250	268	266	281	266 ± 13	4.9
W8	202	220	206	211	210 ± 8	3.8
W9	183	184	270	298	234 ± 59	25.2
oat sample						
O1	355	363	508	538	441 ± 95	21.5
O2	210	208	263	290	243 ± 41	16.9
O3	352	373	173	181	270 ± 108	40.0
O4	92.8	102	133	142	117 ± 24	20.5
O5	121	146	202	213	170 ± 44	25.9

[a] Relative standard deviation (n = 4).

better differentiate between different T-2 toxin concentrations in the sample, a larger color intensity scale of the test line was needed, ranging from an almost invisible test line (higher T-2 toxin concentration in the sample) to a strong test line (no T-2 toxin in the sample). To validate the developed strip tests, eight blended wheat samples were extracted with methanol/water as previously reported. The extracts used for performing the LFD tests were also analyzed with LC-MS/MS for reference measurements. The relative reflectance of the test line from the measured wheat samples was plotted against the T-2 toxin concentration measured with LC-MS/MS (no recovery correction for extraction procedure shown here), showing a linear correlation (**Figure 2A**). Error bars represent the standard deviation (1s) of 10 measurements (n = 10). The absence of T-2 toxin (blank samples) or low T-2 toxin concentrations resulted in high color intensities, as expected for competitive assays. Coefficients of variation (CV) were obtained ranging from 1.1 to 8.2% (n = 10), showing high reproducibility of strip-to-strip performance and photometric read out. Similarly, the relative reflectance of the test line from five blended oat samples, extracted in a ratio of 1:5 (w/v) with methanol/water, 70:30 (v/v), was plotted against the T-2 toxin concentration, showing a linear correlation (**Figure 2B**), with CVs for the photometric read out ranging from 1.8 to 5.1% (n = 5). Results already show the high matrix dependence of the strip test results, confirming that the developed strip test may not be suited for every commodity and must be validated for each commodity separately. A steeper regression line as seen in **Figure 2A** for the wheat matrix will allow for more accurate semiquantitative analysis, where a calibration curve obtained using a large data set of naturally contaminated samples may be used. The read out of the relative reflectance of the test line of unknown samples may then be given, providing a level of confidence of 95% when using the 95% prediction interval around the regression line. The semiquantitative strip test will therefore enable rapid screening for T-2 toxin in the two relevant matrices, wheat and oats, delivering results in <10 min.

Strip Test Stability. To ensure maintenance of the biological activity of the antibodies used, the conditions for storage of strip tests were investigated in real-time/real-temperature stability studies. The strip test stability was evaluated by monitoring the test performance using strip tests from the same batch stored at room temperature in a light-shielding container for a period of several months. Both the test and control line were stable

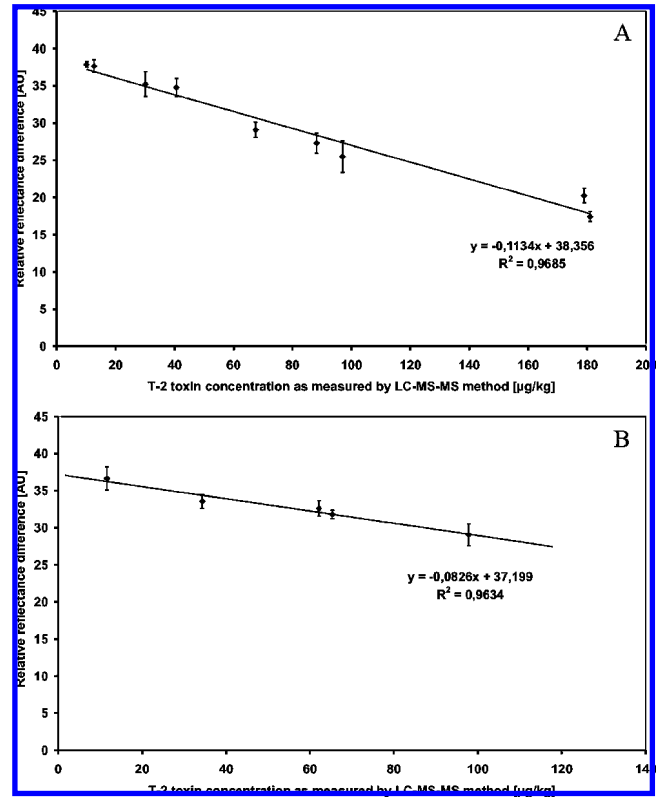

Figure 2. Relative reflectance value of the screened test line on the strip test versus T-2 toxin concentration of measured naturally contaminated and blended (**A**) wheat samples (n = 10) and (**B**) oat samples (n = 5).

and showed reproducible color line intensity after 4 months. The blocking procedure during strip test preparation was shown to be critical for the long-term stability of the reagents sprayed onto the nitrocellulose membrane. Without blocking or with BSA concentrations lower than 1%, the sprayed reagents quickly lost their biological activity, showing no or strongly reduced test color intensities.

The novel strip tests developed for the T-2 mycotoxin allowed a fast qualitative and/or semiquantitative result, indicating the level of sample contamination in naturally contaminated wheat and oat commodities. Results were obtained within 4 min with no sample preparation required other than an extraction by shaking 5 g of sample for 3 min. The strip tests therefore allow for simple and fast screening of agricultural commodities. The cutoff level of the qualitative strip test was set around 100 μg/kg. A semiquantitative format was furthermore developed using a benchtop-sized photometric strip reader allowing screening T-2 toxin within a linear working range of up to 350 μg/kg for wheat and 250 μg/kg for oat. The conditions selected for sample extraction were chosen as a compromise between extraction efficiency and solvent compatibility of the antibodies used in this immunoaffinity-based test. A 3 min extraction procedure with methanol/water (70:30, v/v) instead of acetonitrile/water/acetic acid (79:20:1, v/v/v) with no further sample pretreatment allowed the fulfillment of requirements for a rapid screening test. The high sensitivity and specificity of the applied anti-T-2 toxin Mab allowed the lower extraction recoveries obtained with methanol/water to be taken into account. Results have furthermore shown that multisubstrate methods are more demanding than single-substrate methods and require single matrix-matched calibrations for each analyzed matrix as was shown by the different matrix effects obtained with wheat and oat.

The presented tests were accurate and sensitive for qualitative and semiquantitative measurements of T-2 toxin, respectively, in agricultural commodities such as wheat and oat. Due to the lack of reliable occurrence data on T-2 and HT-2 toxins, tools for the fast detection of both T-2 and HT-2 toxin as single analytes, as well as tools for the detection of the sum of T-2 and HT-2 toxins, are greatly needed. Ongoing work developing a strip test for the sum of T-2 and HT-2 will allow testing for both T-2 and HT-2 toxins in wheat and oat, complementing the T-2 strip test and providing a fast method for the screening of agricultural commodities.

ABBREVIATIONS USED

LFD, lateral flow device; TLC, thin layer chromatography; SPE, solid phase extraction; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Mab, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; OD, optical density; rpm, revolutions per minute; HPLC, high-pressure liquid chromatography; LC-MS/MS, liquid chromatography−tandem mass spectrometry; ESI, electrospray ionization.

ACKNOWLEDGMENT

We acknowledge the EC BioCop Project (New Technologies to Screen Multiple Chemical Contaminants in Foods, FOOD-CT-2005-006988) within which the T2-B1 monoclonal antibodies were produced. We thank Marc Lemmens, Biotechnology in Plant Production, from the IFA-Tulln for providing the wheat samples and Romer Labs Diagnostic (Tulln, Austria) for providing the oat samples.

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Received for review February 8, 2008. Accepted February 8, 2008. This work is supported by the Christian Doppler Society and the Lower Austrian Government.

JF800393J