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A collaborative study to validate novel field immunoassay kits for rapid mycotoxin detection

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Abstract

Kits designed to detect ochratoxin A (OA) and T-2 toxin by a membrane-based flow-through enzyme immunoassay were studied collaboratively by screening cereals (wheat, rye, maize and barley) for the presence of these mycotoxins. Sample preparation and test procedure were clearly described in the instruction leaflets included in the kits. A simple methanol-based extraction followed by filtration and dilution steps was prescribed. Reagents were successively pipetted to the membrane of the device, then colour development was evaluated visually. Limits of detection for the ochratoxin A and T-2 toxin tests were 4 and 50 μg kg^{-1}, respectively. Five laboratories took part in the first stage of this study, and five more joined the second stage. Cereal samples (blank, spiked or inoculated) were shipped with the kits to the participating laboratories, while results obtained were confirmed by high-performance liquid chromatography with fluorescence detection and by gas chromatography-mass spectrometry for ochratoxin A and T-2 toxin, respectively. Some initial difficulties were encountered. In the second stage, four ochratoxin A and four T-2 toxin kits were used by 10 collaborators to analyse 21 cereal samples. For the ochratoxin A kits, the percentage of false positive and false negative results were 2% and 4%, respectively. The results of one T-2 toxin kit were outliers and when excluded, the overall percentage false positive and false negative results were 6% and 3%, respectively.

Keywords: Ochratoxin A; T-2 toxin; Cereals; Field immunoassay; Collaborative study

1. Introduction

Ochratoxin A (OA) is a mycotoxin mainly produced by *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum*. OA contamination has been reported in cereals, coffee, wines, dried fruit and animal feeds, as well as in pig tissues and blood. OA has carcinogenic, genotoxic, immunosuppressive, nephrotoxic and teratogenic properties (Kuiper-Goodman and Scott, 1989; Smith et al., 1995). T-2 toxin is a trichothecene mycotoxin formed by various species of *Fusarium*, which occur in cereals. It is immunosuppressive and has negative gastrointestinal and hematopoetic effects (Richard, 1998).

Many analytical methods for the detection of these mycotoxins in food and feed have been described (Chu, 1995; Trucksess, 2000). However, most of them are time-consuming and require sophisticated equipment. We developed rapid membrane-based enzyme immunoassays for OA, T-2 and aflatoxin M_1 detection (De Saeger and Van Peteghem, 1999a,b; Sibanda et

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al., 1999, 2000). No expensive instruments are needed as results are visually evaluated. In the initial stages, these mycotoxin assays were performed in our laboratory. However, as our intention was to use them in the field, kits consisting of all necessary materials were produced and sent to several laboratories for evaluation. This paper reports this interlaboratory validation.

2. Materials and methods

2.1. Materials and reagents

A plastic snap-fit device from Trosley Equipment, Dover, Kent, England was used as a flow-through device. The bottom member of this device was filled with 100% cotton wool as absorbent material. A piece (2 by 2 cm) of Immunodyne ABC membrane (Pall France, Saint Germain-en-Laye, France; pore size, 0.45 μm), coated with 3-μl undiluted rabbit anti-mouse immunoglobulins (Ig) (Dako, Glostrup, Denmark; no. Z259; protein concentration 3.5 g/l), was placed on the absorbent material. The top lid of the device was fitted on the bottom lid. The membrane was placed on the absorbent pad such that the center of the membrane, where the antibody spot was located, was accessible through the aperture of the top member (De Saeger and Van Peteghem, 1999a,b). Immunodyne ABC membranes, spotted side-by-side with 3-μl undiluted rabbit anti-mouse Ig and 2-μl goat anti-horseradish peroxidase (HRP) (Sigma; no. P-5774; protein concentration 52.7 mg/ml; diluted 1: 20,000) as internal control spot, were also prepared (Sibanda et al., 2000).

Monoclonal antibodies against T-2 toxin (mouse anti-T-2 IgG; protein concentration 1 mg/ml) and against OA (mouse anti-OA IgG; protein concentration 2.2 mg/ml) were produced and characterized by the Agricultural Biotechnology Center, Diagnostic Laboratory, Gödöllö, Hungary (Gyöngyösi et al., 1994; Gyöngyösi-Horvath et al., 1996). T-2–HRP conjugate and OA–HRP were produced by the same Hungarian institute (Barna-Vetro et al., 1994, 1996).

The substrate–chromogen solution was made prior to the assay by mixing equal volumes of solutions A and B (De Saeger and Van Peteghem, 1999a). Solution A (pH 5.0) contained 1 g urea hydrogen peroxide (Sigma, no. U-1753), $Na_2HPO_4 \cdot 2H_2O$, 18 g, citric acid·H_2O, 10.3 g (Merck), and Proclin 300 (500 μl) as preservative (Supelco, Bellefonte, PA, USA) per litre of water. Solution B (pH 2.4) contained 3,3′, 5, 5′ -tetramethylbenzidine, 500 mg (TMB; Sigma), dimethyl sulphoxide, 40 ml, citric acid·H_2O, 10.3 g, and Proclin 300 (500 μl) per litre of water. OA and T-2 toxin were purchased from Sigma.

OA and T-2 kits consisted of all the reagents and materials necessary to perform the flow-through enzyme immunoassays. The different components of the OA kit are listed in Table 1. The content of the T-2 kit was similar. As a positive control, a T-2 solution of 5 ng ml^{-1} in methanol (15%) plus PBS/Tween (0.05%) was included. Anti-T-2 IgG and T-2–HRP were prediluted 1:1000 and 1:250, respectively.

2.2. Sample preparation

Extraction of cereal samples (wheat, rye, maize and barley) was clearly described in the instruction leaflet included in the kits. Cereals were to be ground in a household coffee grinder to a mesh size between 0.05 and 2 mm. A 5-g portion was mixed with 15 ml of

Table 1
Composition of the ochratoxin A kit

Reagent	Amount	Packaging
Flow-through devices filled with cotton wool and coated membranes	10 pieces	plastic vacuum-sealed bag with desiccant
Wash solution (PBS/Tween 0.05%)	2 × 15 ml	plastic bottle
Negative control (15% methanol in PBS/Tween 0.05%)	4 ml	glass vial
Positive control (OA 0.4 ng ml^{-1} in 15% methanol in PBS/Tween 0.05%)	4 ml	brown glass vial
Anti-OA IgG (diluted 1:100)	1200 μl	glass vial
OA-HRP (diluted 1:200)	1200 μl	brown glass vial
Colour reagent A	500 μl	brown glass vial
Colour reagent B	500 μl	brown glass vial
Whatman no. 4 filters	6 pieces	plastic bag
Plastic 3-ml syringes	6 pieces	–
0.45-μm filters (chromafil disposable filter, Macherey-Nagel, Düren, Germany)	6 pieces	–
Instruction leaflet	–	–

methanol/water (8:2; v/v) for 15 min on a horizontal shaker at room temperature. The suspension was filtered through Whatman no. 4 filter paper. Wash solution (1000 μl) was added to 250 μl of the filtrate. This dilution was filtered through the 0.45-μm filter and a 600-μl volume was used in the flow-through test.

Cereal samples (wheat, rye, maize and barley) with a range of OA and T-2 levels were obtained from Prof. P. Golinski (August Cieszkowski Agricultural University, Poznan, Poland). To prepare the OA positive cereal samples, 250 g of grain kernels (water content 40%) was incubated with *A. ochraceus* at 25 °C. The OA yield depended on the incubation time (24 h up to 3 weeks). T-2 positive wheat samples were obtained by inoculating the cereals with a *Fusarium sporotrichioides* culture at ambient temperature for periods ranging from 1 to 14 days (Sibanda et al., 2000).

Wheat samples spiked with T-2 were prepared by adding the appropriate volume of T-2 toxin in methanol (20 ng μl^{-1}) to 10 g of ground wheat.

2.3. Test procedure

The procedure for the OA and T-2 flow-through tests were identical. Assay reagents were successively dropped on the membrane through the aperture of the flow-through device with a micropipette. First, anti-OA or anti-T-2 IgG solution (100 μl) was dropped onto the membrane, followed by wash solution (300 μl), negative or positive control or extracted sample (600 μl), OA–HRP or T-2–HRP (100 μl), and finally wash solution (600 μl). Between each step it was necessary to wait for the liquid to be absorbed by the absorbent pad. Finally, substrate–chromogen (H_2O_2–TMB) solution (50 μl) was dropped onto the membrane. After 1 min the dot colour intensity was evaluated. When membranes with one spot (anti-mouse Ig) were used, visual evaluation required comparison of the dot colour intensity of the test membrane with that of the negative control. The negative control test was performed externally but at the same time as the OA or T-2 assays. The negative control test resulted in the most intense blue-coloured spot because of the inverse relationship between toxin concentration and colour development. Using membranes with an internal control spot, visual evaluation was done by comparing the colour development of the test spot with that of the internal control spot. A portable colourimeter (Minolta Chroma Meter CR-321, Minolta, Osaka) was used to quantify the colour of the membrane spots (De Saeger and Van Peteghem, 1999a). It took less than 15 min to analyse a flow-through test. Up to 10 tests could be performed simultaneously.

The assay was valid if a clear blue spot appeared on the negative control membrane or if the internal control spot was coloured blue. If, for the OA test, a blue-coloured spot appeared that was more, equally or less intensive than the blue colour of the control spot, the assay was considered to be negative for OA, i.e. the OA concentration being less than 4 $\mu g\ kg^{-1}$. If no coloured spot appeared for the OA assay, the test was considered to be positive for OA, i.e. the OA concentration being equal or more than 4 $\mu g\ kg^{-1}$. For T-2 toxin the limit of detection was 50 $\mu g\ kg^{-1}$.

2.4. Collaborative study

Five Central European laboratories participated in the first stage of the collaborative study and another five Central European laboratories were in the second stage. Every month each partner received one OA and one T-2 kit sent by express mail. For the first time, however, only one OA kit was sent (OA kit I). Kits were stored refrigerated (2–8 °C) after receipt. Participants were asked to follow the instructions written in the leaflet included in the kits. In one laboratory the Minolta colourimeter was used. The other partners evaluated the colour visually. Cereal samples (blanks, spiked or inoculated, each 10 g) were sent together with the kits. Concerning blank and inoculated samples, each partner received 10 g of the same batch. For each kit, two or three samples were analysed in duplicate, as well as the appropriate negative and positive controls. Partners were asked to send their results within 1 month after receiving the kits by describing the colour development on the membrane and stating whether the cereal samples were positive or negative. A control test of the same samples was performed in our laboratory with both kits. Results obtained with the kits were confirmed by high-performance liquid chromatography (HPLC) with fluorescence detection for OA and by gas chromatography-mass spectrometry (GC-MS) for T-2 by the August Cieszkowski Agricultural University (Sibanda et al., 2000). At the end of stage one, a workshop was organized in our lab-

oratory. The first five partners presented their results and the use of the flow-through assay was demonstrated to the five new participants.

3. Results and discussion

Tables 2 and 3 include the results obtained during the first stage of the collaborative study. Although samples were analysed in duplicate, most of the participants reported only one result per sample because no differences were observed between the duplicate results. The results of OA kit III and T-2 kit II are not included in the tables due to a production error (incorrect buffer for immunoreagent dilution). It is clear from Table 2 that there were problems with OA kits I, II and IV. For almost 50% of the assays, no colour development was observed in the negative controls. There were different reasons for this. Glass vials which contained 15 μl of undiluted anti-OA IgG or OA–HRP were too small to take out quantitatively the volume for diluting. Participants had to dilute the immunoreagents by themselves, which was a source of error because some of them did not have appropriate micropipettes available. Instructions in the leaflets were sometimes ambiguous and could be misinterpreted. Some glass vials leaked during transport and therefore, laboratory 1 could not analyse the samples in OA kit IV. However, by delivering anti-OA IgG and

Table 2
Results of the collaborative study: ochratoxin A kit (first stage—five laboratories involved)

Kit no.	Samples[a]	Laboratory identification					Control test
		1	2	3	4	5	
OA kit I	wheat 1 μg kg^{-1}	–[b]	–	0[c]	0	–	–
	wheat 150 μg kg^{-1}	+[d]	+			+	+
OA kit II	wheat 1 μg kg^{-1}	–	0	0	–	–	–
	wheat 150 μg kg^{-1}	+			+	+	+
OA kit IV	wheat 1 μg kg^{-1}	Na[e]	0	0	0	–	–
	wheat 150 μg kg^{-1}					+	+
OA kit V	wheat 1 μg kg^{-1}	–	–	–	–	–	–
	wheat 35 μg kg^{-1}	+	+	+	+	+	+
OA kit VI	wheat 1 μg kg^{-1}	–	–	–	–	–	–
	wheat 35 μg kg^{-1}	+	+	+	+	+	+
OA kit VII	wheat 1 μg kg^{-1}	–	0	–	–	–	–
	wheat 1 μg kg^{-1}	–		–	–	–	–

[a] OA level determined by HPLC.
[b] Sample was reported being negative, i.e. with an OA level less than 4 μg kg^{-1}. A blue-coloured spot was seen on the membrane.
[c] Assay was not valid: no colour development for negative control.
[d] Sample was reported being positive, i.e. with an OA level equal or more than 4 μg kg^{-1}. No coloured spot was seen on the membrane.
[e] Not analysed.

Table 3
Results of the collaborative study: T-2 toxin kit (first stage—five laboratories involved)

Kit no.	Samples[a]	Laboratory identification					Control test
		1	2	3	4	5	
T-2 kit I	wheat < 20 μg kg^{-1}	–[b]	–	0[c]	–	–	–
	wheat spike 50 μg kg^{-1}	+[d]	–[e]		+	+	+
T-2 kit III	wheat < 20 μg kg^{-1}	Na[f]	+[g]	0	0	–	–
	wheat spike 50 μg kg^{-1}		+			+	+
T-2 kit IV	wheat < 20 μg kg^{-1}	–	–	–	–	–	–
	wheat spike 100 μg kg^{-1}	+	+	+	+	+	+
T-2 kit V	wheat < 20 μg kg^{-1}	0	+[g]	+[g]	+[g]	–	–
	wheat spike 80 μg kg^{-1}		–[e]	–[e]	+	–[e]	+
T-2 kit VI	wheat < 20 μg kg^{-1}	–	–	–	–	–	–
	wheat spike 100 μg kg^{-1}	+	+	+	+	+	+

[a] T-2 level determined by GC-MS.
[b] Sample was reported being negative, i.e. with a T-2 level less than 50 μg kg^{-1}. A blue-coloured spot was seen on the membrane.
[c] Assay not valid: no colour development for negative control.
[d] Sample was reported being positive, i.e. with a T-2 level equal or more than 50 μg kg^{-1}. No coloured spot was seen on the membrane.
[e] False negative result.
[f] Not analysed.
[g] False positive result.

OA–HRP in their final dilutions these problems were solved. Instruction leaflets were modified according to the remarks of the collaborators. Glass vials with wider apertures and tight screw caps were used. These improvements led to good results for OA kits V, VI and VII. No false positive or false negative results were obtained. Only laboratory 2 had no colour development with OA kit VII.

T-2 kits I and III were sent together with OA kit II and IV, respectively. Laboratory 1 could not perform the assays with T-2 kit III due to leakage of the immunoreagent vials (Table 3). Some assays with T-2 kit I and III resulted in no colour development for negative controls. This was due to the same problems as for the first OA kits. So, the same measures were taken. Better results were obtained for the next T-2 kits (IV, V and VI) and only laboratory 1 had no colour development with T-2 kit V. Four false negative and four false positive results were seen with these T-2 kits. Because positive results must be confirmed by a confirmatory method, false positive results will therefore be detected. One of the false negative results was situated around the cut-off level. The other false negative results were obtained with wheat samples spiked with 80 μg kg^{-1} T-2 and sent in T-2 kit V. Spiked wheat samples were individually prepared as described in the Materials and methods. According to the AOAC recommendations for the design of a collaborative study (AOAC, 1989), the use of such individually prepared spiked materials is allowed. Three of the false positive results were also obtained with this T-2 kit V. Maybe it can be explained by incorrect spiking, nonhomogeneous spiked samples or erroneous labeling of the samples.

Tables 4 and 5 include the results obtained during the second stage of the collaborative study. OA kit VIII and T-2 kit VII were used at the workshop. Laboratory 2 could not perform a successful assay with OA kit VIII, while no colour development was obtained by laboratories 3, 4 and 8 with T-2 kit VII. Three of these four partners were already involved in the first stage of the study. Laboratories 2, 3 and 4 had also a number of problems during the first part of the collaborative study. It was clear they had misinterpreted some instructions. During the workshop they were shown how to analyse the tests correctly. Instruction leaflets were again modified following the remarks of the

Table 4
Results of the collaborative study: ochratoxin A kit (second stage—10 laboratories involved)

Kit no.	Samples[a]	Laboratory identification										Control test
		1	2	3	4	5	6	7	8	9	10	
OA kit VIII[b]	wheat 1 μg kg^{-1}	−[c]	0[d]	−	−	−	−	−	−	−	−	−
	wheat 1 μg kg^{-1}	−		−	−	−	−	−	−	−	−	−
OA kit IX	rye 320 μg kg^{-1}	+[e]	−[f]	+	+	+	+	+	+	+	+	+
	wheat 2 μg kg^{-1}	−	−	−	−	−	−	−	−	−	−	−
	wheat 1 μg kg^{-1}	−	−	−	−	−	−	−	−	−	−	−
OA kit X[g]	barley 230 μg kg^{-1}	+	+	Na[h]	−[f]	+	+	+	+	+	+	+
	wheat 1 μg kg^{-1}	+[i]	−		+[i]	−	−	−	−	−	−	−
	maize 45 μg kg^{-1}	0	+		+	+	+	+	+	+	+	+
OA kit XI[g]	barley 210 μg kg^{-1}	+	−[f]	+	+	+	+	+	+	+	+	+
	wheat 15 μg kg^{-1}	+	+	+	+	+	+	+	+	+	+	+
	wheat 2 μg kg^{-1}	−	−	−	−	−	−	−	−	−	−	−

[a] OA level determined by HPLC.
[b] Used during workshop.
[c] Sample was reported being negative, i.e. with an OA level less than 4 μg kg^{-1}. A blue-coloured spot was seen on the membrane.
[d] Assay was not valid: no colour development for negative control or internal control.
[e] Sample was reported being positive, i.e. with an OA level equal or more than 4 μg kg^{-1}. No coloured spot was seen on the membrane.
[f] False negative result.
[g] Test with internal control spot.
[h] Not analysed.
[i] False positive result.

Table 5
Results of the collaborative study: T-2 toxin kit (second stage—10 laboratories involved)

Kit no.	Samples[a]	Laboratory identification										Control test
		1	2	3	4	5	6	7	8	9	10	
T-2 kit VII[b]	wheat spike 50 μg kg^{-1}	+[c]	+	0[d]	0	+	+	+	0	+	−[e]	+
	wheat < 20 μg kg^{-1}	−[f]	−			−	−	−		−	−	−
T-2 kit VIII	wheat < 20 μg kg^{-1}	−	−	0	−	−	−	−	−	−	−	−
	wheat spike 100 μg kg^{-1}	+	+		+	+	+	+	+	+	+	+
T-2 kit IX[g]	wheat spike 100 μg kg^{-1}	+	+	Na[h]	+	+	+	+	0	0	+	+
	wheat spike 25 μg kg^{-1}	+[i]	−		−	−	−	−			−	−
	wheat < 20 μg kg^{-1}	+[i]	−		−	−	−	−			−	−
T-2 kit X[g]	wheat 800 μg kg^{-1}	+	+	+	+	+	+	+	+	Na	+	+
	wheat 500 μg kg^{-1}	+	+	+	+	+	+	+	+		+	+
	wheat 35 μg kg^{-1}	−	−	+[i]	−	−	−	−	−		−	−

[a] T-2 level determined by GC-MS.
[b] Used during workshop.
[c] Sample was reported being positive, i.e. with a T-2 level equal or more than 50 μg kg^{-1}. No coloured spot was seen on the membrane.
[d] Assay was not valid: no colour development for negative control or internal control.
[e] False negative result.
[f] Sample was reported being negative, i.e. with a T-2 level less than 50 μg kg^{-1}. A blue-coloured spot was seen on the membrane.
[g] Test with internal control spot.
[h] Not analysed.
[i] False positive result.

participants. One false negative result was obtained when analysing wheat spiked with 50 μg kg^{-1} T-2 in T-2 kit VII.

OA kit IX and T-2 kit VIII were sent after the workshop. Laboratory 2 reported a false negative result for a rye sample with an OA level of 320 μg kg^{-1}. Laboratory 3 could not perform a valid assay with T-2 kit VIII.

The last kits (OA kit X and XI; T-2 kit IX and X) were sent containing flow-through tests with internal control spots as described in the materials and methods section. The use of an internal control spot resulted in a higher sample throughput, because no separate negative controls had to be analysed. For these OA kits two false positive results were reported as well as two false negative results. For T-2 kits IX and X three false positive results were seen but no false negatives.

The total %false positive[1] and false negative results[2] in this study were calculated. For the OA kits 2% false positive and 4% false negative results were obtained. For the T-2 kits 10% false positive and 7% false negative results were reported.

However, if T-2 kit V was treated as an outlier—most of the results with that kit being false results—the total % false positive and false negative results for the T-2 kits would be 6% and 3%, respectively. According to the AOAC (1989), analyses become uninterpretable from lack of confidence in the presence or absence of the analyte when false positives and/or false negatives exceed about 10% of all values, unless all positive laboratory samples are reanalysed by a confirmatory method with a lower limit of determination than the method under study. For the OA kit, 10% was not exceeded. If T-2 kit V was treated as an outlier, this criterion would also be fulfilled for the T-2 kit. According to the European Commission (2000), analytical techniques for which a false negative rate of < 5% at the level of interest can be demonstrated are suitable for screening purposes.

The Minolta Chroma Meter CR-321 was used for the control test in our laboratory and in one of the partner laboratories. The same instrumental settings such as light source, colour space and calibration data were used in both laboratories. However, colour measurement is still dependent on other factors such as the colour of the material behind the specimen during the

[1] %False positives = false positives × 100/total known negatives.
[2] %False negatives = false negatives × 100/total known positives.

measurement process and the time of measuring after colour reaction (Hunter R.S. Color Institute, 1998). Therefore, it is obvious that different colour values were obtained for the same tested samples, making it difficult to use these colourimeter values for interlaboratory validation of the kits. Visual evaluation was much more reliable for this particular study.

4. Conclusions

This collaborative study has shown that OA and T-2 kits can be used for the screening of cereals (wheat, rye, maize and barley) for the presence of these mycotoxins.

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