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Validation of an ELISA test kit for the detection of ochratoxin A in several food commodities by comparison with HPLC

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Abstract

An ELISA Microtiter Plate, Ochratoxin Test called AgraQuant® was validated to measure ochratoxin A in a range from 2 to 40 ppb in corn, milo, barley, wheat, soybeans and green coffee. The test is performed as a solid phase direct competitive ELISA using a horseradish peroxidase conjugate as the competing, measurable entity. For the test method, ochratoxin A is extracted from ground samples with 70% methanol and sample extracts plus conjugate are mixed and then added to the antibody-coated microwells. After 10 min incubation at room temperature, the plate is washed and enzyme substrate is added and allowed to incubate for an additional 5 min. Stop solution is then added and the intensity of the resulting yellow color is measured optically with a microplate reader at 450 nm. Results obtained from internal validation studies assessing accelerated stability indicate a 1 year shelf life; accuracy and precision are comparable to HPLC from 0 to 80 ppb and limit of detection in corn is 1.9 ppb and other food commodities is up to 3.8 ppb. Comparison of the method to HPLC, ability to detect individual ochratoxins, and ruggedness of the test kits determined this test to be rugged from 18 to 30 °C, sensitive, accurate, precise and effective comparable to HPLC for measuring ochratoxin A ranging from 2 to 40 ppb in several commodities.

Key words: AgraQuant®, ELISA, HPLC, ochratoxin A, test kit, validation

Introduction

Ochratoxin A, produced primarily by the fungi *Aspergillus ochraceus* and *Penicillium verrucosum,* can be found in a wide variety of food commodities such as corn, wheat, sorghum, oats, rice, wine and green coffee [1–5]. Ochratoxin A is a nephrotoxic mycotoxin that primarily affects the kidneys in animals exposed to naturally occurring levels [6]. If the concentration of ochratoxins is high, it can cause liver damage. Ochratoxin A is a carcinogen in rats and mice and is suspect as the causation of a human disease, Balkan Endemic Nephropathy, which affects the kidneys. Often, tumors are associated with this disease [7]. Although the ochratoxin amounts may be relatively low, the levels may accumulate in the body of either humans or animals consuming contaminated food. Ochratoxin is often not rapidly removed from the body and significant amounts may accumulate in the blood and other selected tissues. Since March 2001, the commission of the European communities has set ochratoxin A maximum levels of 5 ppb for raw cereal grains (including raw rice and buckwheat) and 3 ppb for all products derived from cereals (including processed cereal products and cereal grains intended for direct human consumption).

Commonly used analytical methods for the determination of ochratoxin A are high-performance liquid chromatography (HPLC) with fluorescence detection, thin layer chromatography (TLC) and immunochemical methods such as enzyme linked immunosorbent assay (ELISA). A cleanup step is needed for both HPLC and TLC

methods using either solid-phase extraction or immunoaffinity column. ELISA test kits are well favored as high through-put assays with low sample volume requirements and less sample clean-up procedures compared to conventional methods such as TLC and HPLC. They are rapid, simple, specific, sensitive and field-portable and have become the most common quick methods for the detection of mycotoxins in foods and feeds [8]. However, since the antibodies produced often show cross-reactivity to compounds similar to mycotoxins, an extensive study on the accuracy and precision of the ELISA method over a range of commodities is essential and critical before the method can be commercially used [Z.M. Zheng et al., submitted for publication].

The aim of this study was to validate the AgraQuant® Ochratoxin (2–40 ppb) 96 well microtiter plate ELISA test kit. This article reports full validation results of the test kits including accelerated stability, accuracy, precision and limit of detection in corn and other food commodities, comparison of method to HPLC, ability to detect individual ochratoxins, and ruggedness of the test kits. The criteria used in the study were modified from USDA/GIPSA design criteria for total aflatoxin test kit because currently no such criteria are available for ochratoxin test kits [Z.M. Zheng et al., submitted for publication, 9].

Materials and methods

Chemicals and test kits

Crystalline ochratoxin A and B were purchased from Sigma-Aldrich Co. (St. Louis, MO); 5 $\mu g\ ml^{-1}$ of ochratoxin A and B spiking standards were prepared and analyzed according to the HPLC method (Romer HPLC Method code: AM000072-0) by Romer Labs Inc®. Two lots of AgraQuant® ochratoxin ELISA test kits (COKAQ2000), produced in Romer Labs Asia c/o Biomin Laboratory Singapore Pte Ltd, were used in the study.

Validation commodities and preparation

Six commodities were used in the study: corn, milo, barley, wheat, soybeans and green coffee. All commodity samples were ground with a Mikro-Samplmill (MikroPul, Summit, NJ) such that over 95% of the material passed through a 20-mesh sieve. Ground portions were mixed until homogenous and then divided into 50 g sub-portions. Prior to fortification, at least four 50 g sub-portions of each commodity were analyzed for the presence of ochratoxin residues using the HPLC method and only those containing less than 1 ppb of ochratoxin A were used to produce fortified test samples or ochratoxin-free controls. Besides ochratoxin-free commodities, two levels of naturally ochratoxin contaminated corn samples i.e., 5.2 and 26.9 ppb (confirmed with HPLC method) were also used in the study.

Test kit assay method

Fifty gram ground sample was extracted with 250 ml of 70% methanol by using an orbital shaker (Heidolph, Germany) at a speed of 250 rpm for 3 min. The sample was allowed to settle, then solvent was filtered through a Whatman #1 filter paper and the filtrate was collected. Sample extract (100 μl) or ochratoxin test kit standard were each mixed with 200 μl of conjugate in individual dilution wells, and then 100 μl from each dilution well was transferred to a respective antibody-coated microwell. After 10 min incubation at room temperature, the plate was washed 5× with distilled water and 100 μl of enzyme substrate was added to each well and allowed to incubate for an additional 5 min. Stop solution (100 μl for each well) was then added and the intensity of the resulting yellow color was measured optically with a microplate reader at a wavelength of 450 nm with a differential filter of 630 nm. The ELISA readers used in the study were Stat Fax® 303 microwell readers (Awareness Technology Inc.) and BioRad 550 microplate reader (BioRad Laboratories Inc.). The total incubation time of the test kit assay was 15 min.

Accelerated stability study

Twelve unopened test kits from same production lot were grouped into three sets of four kits. One set was stored at 4 °C, a set was stored at 21 °C and the last set was stored at 37 °C. One test kit from each temperature was used by one analyst every week for 4 weeks to test an ochratoxin-free corn sample (i.e. <1 ppb) and two naturally contami-

nated corn samples (i.e. 5.2 and 26.9 ppb) using one Stat Fax 303 microwell reader.

Accuracy, precision and limit of detection (LOD) in corn

Eight concentration levels of ochratoxin A were investigated in this experiment: 0, 2, 5, 10, 20, 30, 40 and 80 ppb. Six sets of ochratoxin-free corn samples for each concentration level were spiked with $5\,\mu g\,ml^{-1}$ ochratoxin A standard. Five sets were extracted for ELISA test kit analyses and the sixth set was extracted for HPLC analyses. The 80 ppb extracts for test kit analyses were diluted at a 1:8 ratio of sample to 70% methanol before test kit analyses. Each extract from five replicate sets was assayed by three analysts in triplicate using two lots of test kits, two Stat Fax® 303 readers and one BioRad 550 microplate reader resulting in 180 data points for each concentration level with Stat Fax® 303 readers and 90 data points for each concentration level with BioRad 550 microplate reader. The LOD of the test kit for corn was determined from the mean (plus 2 standard deviations) of 10 ochratoxin-free corn samples.

Comparison to reference HPLC method using naturally contaminated corn

Two levels of naturally contaminated corn samples i.e., 5.2 and 26.9 ppb were used in this experiment. Five sub-portions (of samples ground as above) of each level of naturally contaminated corn were extracted as above. The filtered extracts were assayed by three analysts in triplicate using one lot of test kits and one Stat Fax® 303 reader. A second set of five sub-portions for each level of naturally contaminated corn were extracted similarly and analyzed by HPLC. The 30 individual test kit averages were compared to HPLC results.

Ability of test kit to detect individual ochratoxins

Ten ochratoxin-free corn samples were spiked with 20 ppb of ochratoxin A, and 10 samples were spiked with 20 ppb ochratoxin B. Each of the 10 spiked samples for each individual ochratoxin was assayed by one analyst in triplicate using one lot of test kits and one Stat Fax® 303 reader. The 30 data points for each individual ochratoxin were averaged and compared to the LOD determined in the above experiment.

Accuracy, precision and LOD in other food commodities

Two sets of four 50 g sub-portions of each commodity matrix were spiked at four levels of ochratoxin A (i.e. 0, 5, 20 and 80 ppb). One set was assayed by three analysts in triplicate using two lots of test kits and one Stat Fax® 303 reader. The second set of sub-portions was used for HPLC analyses. LOD for each commodity was determined from the mean (plus 2 standard deviations) of 10 ochratoxin-free samples.

Ruggedness of test kits

Three sets of 50 g corn samples for each of eight concentrations (i.e. 0, 2, 5, 10, 20, 40, 80 ppb) were spiked with $5\,\mu g\,ml^{-1}$ of ochratoxin A standard. Each sample was extracted and all extracts, equipment and test kits for the analyses were placed in an 18 °C environment and allowed to equilibrate for 1 h. Each extract was then assayed by one analyst using one lot of test kits and one Stat Fax® 303 reader. This process was repeated for two other temperatures, 24 and 30 °C. The range of the means of the three values for each level of ochratoxin A at each temperature was determined.

Results and discussion

Accelerated stability study

Table 1 shows summarized results for accelerated stability study after 25 days storage. Rigorous criteria have been employed in this study: 1 day test kit storage at 21 and 37 °C is equivalent to 5 days and 15 days storage at 4 °C, respectively. Test kits stored at 21 and 37 °C have similar performance with those stored at 4 °C. Test kit results of ochratoxin-free and naturally contaminated corn samples at all testing conditions are comparable with HPLC pre-determined results. The results indicate that the test kits have a minimum 1-year shelf life.

Table 1. Summarized results for accelerated stability experiment after 25 days storage of test kits at three different temperatures and applied to testing ochratoxin-free and naturally contaminated corn

Storage temp. (°C)	Equal to days below at 4 °C (days)[b]	Sample 1 (<1 ppb)		Sample 2 (5.2 ppb)		Sample 3 (26.9 ppb)	
		Ochratoxin A[a] (ppb) Accuracy	Standard deviation (ppb) Precision	Ochratoxin A[a] (ppb) Accuracy	Standard deviation (ppb) Precision	Ochratoxin A[a] (ppb) Accuracy	Standard deviation (ppb) Precision
4 °C	25	2.3	0.1	7.3	0.5	32.5	1.1
21 °C	125	1.3	0.7	8.5	0.4	32.1	0.7
37 °C	375	2.7	0.2	9.0	0.1	30.2	0.1
Study Criteria		≤7.0	4.0	5.2 ± 4.0	5.0	26.9 ± 11.4	9.4
Pass/Fail		Pass	Pass	Pass	Pass	Pass	Pass

[a] ELISA results are the mean of triplicate analyses.
[b] One day test kit storage at 21 and 37 °C is estimated to be equivalent to 5 and 15 days storage at 4 °C, respectively.

Accuracy, precision and limit of detection (LOD) in corn

The correlation between ELISA test kits and HPLC results for corn samples spiked with eight concentration levels of ochratoxin A (i.e. 0, 2, 5, 10, 20, 30, 40 and 80 ppb) was good (Figure 1). The ELISA method has a slope of 0.97 compared to a slope of 1.0 for HPLC method. Hence, the test kits have very good accuracy in the ochratoxin A concentrations ranging from 0 to 80 ppb. The precision profile shows % coefficient variances (CV) are less than 20% when ochratoxin A concentrations are equal to/above 5 ppb (Figure 2). The LOD of test kits for corn has been determined to be 1.9 ppb (Table 2).

Comparison to reference HPLC method using naturally contaminated corn

There was good correlation between ELISA test kits and HPLC results for two levels of naturally

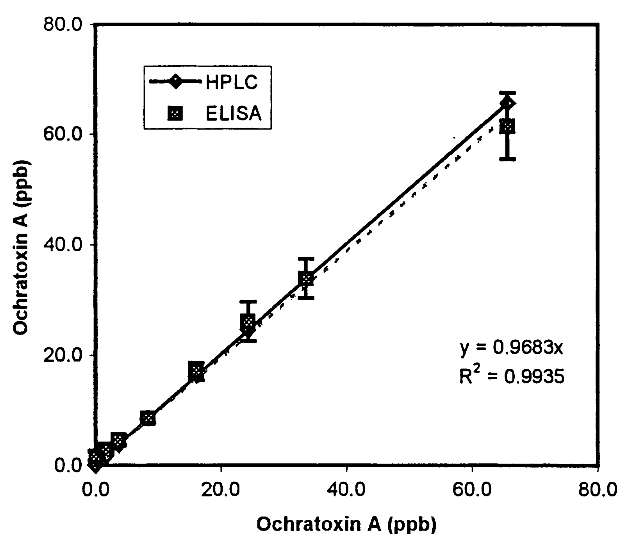

Figure 1. Correlation between ELISA test kits and HPLC results in corn matrix.

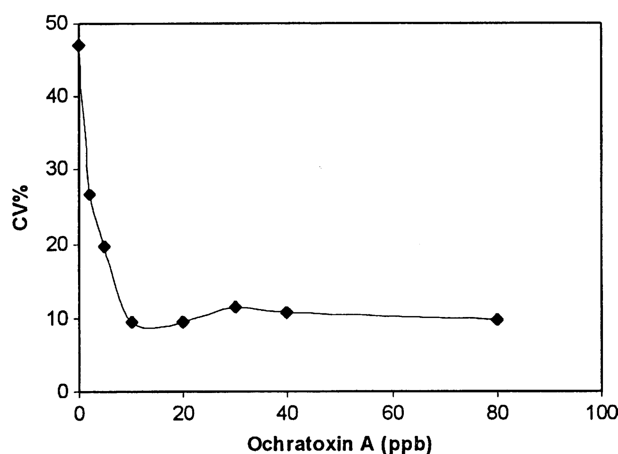

Figure 2. The precision profile of the test kits, expressed as % coefficient of variation (CV) versus ochratoxin A concentrations in corn.

Table 2. Summarized results for LOD of test kits applied to the analysis of ochratoxin in corn

Sample ID	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10	Average	Standard deviation	LOD
Ochratoxin by StatFax®303 reader (ppb)[a]	0.7	0.0	0.2	0.3	0.0	0.0	1.5	1.7	1.1	1.0	0.65	0.6	1.9

[a] Each value is the mean of triplicate results.

contaminated corn samples (Figure 3). For naturally contaminated corn samples of 5.2 and 26.9 ppb (determined by HPLC), the ELISA test kit results were 7.0 ± 1.5 and 31.9 ± 4.4 ppb respectively.

Ability of test kit to detect individual ochratoxins

The results for ability of test kits to detect individual ochratoxins are summarized (Table 3). ELISA results for ochratoxin A and B are much

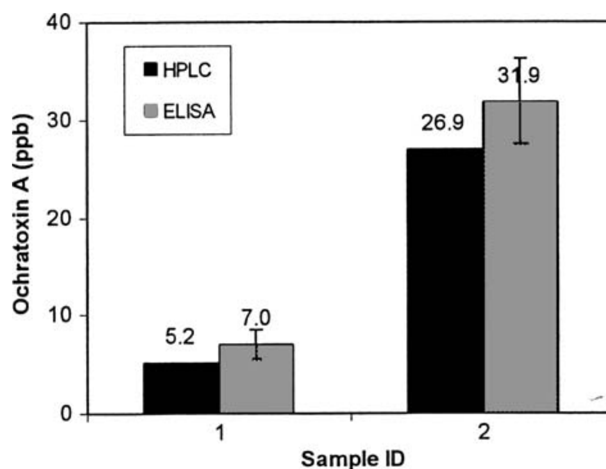

Figure 3. Correlation between ELISA test kits and HPLC results for naturally contaminated corn samples.

Table 3. Summarized results for ability of test kits to detect individual ochratoxins

Individual ochratoxin	Fortified conc. (ppb)	Experimental results (ppb)[a]	(Experimental fortified)%[b]	Test kit cross reactivity
A	20	18.4	92.0	100%
B	20	19.9	99.7	108%

[a] Each value is the mean of 30 experimental results.
[b] Percentage of experimental result in fortified concentration.

greater than the test kit LOD value for corn (i.e. 1.9 ppb). Assuming the test kit cross reactivity for ochratoxin A being 100% (which is true because the recovery for ochratoxin A is about 100% (Figure 1)), its cross reactivity for ochratoxin B can be calculated to be 108%. Hence, the test kit can detect total ochratoxins (A and B).

Accuracy, precision and LOD in other food commodities

The ELISA results for six commodities are comparable to the HPLC results at all four tested levels (Table 4). Test kits have good accuracy on six tested commodities (i.e. corn, milo, wheat, barley,

Table 4. Summarized accuracy and precision for six commodities tested using the ELISA test kits for the analysis of ochratoxin A

Commodity	Fortified concentration (ppb)	HPLC confirmation (ppb)	Experimental results for accuracy (ppb)	Allowable limits accuracy (ppb)	Pass/Fail	Experimental results for precision S_i (ppb)	Allowable maximum S_i precision (ppb)	Pass/Fail
Corn[a]	0	<1.0	1.7	≤7	Pass	0.8	≤4	Pass
	5	3.7	4.6	1–9	Pass	0.9	≤5	Pass
	20	16.1	17	10–30	Pass	1.6	≤8	Pass
	80	65.6	61.5	60–100	Pass	3.6	≤16	Pass
Milo	0	<1.0	2.6	≤7	Pass	1.1	≤4	Pass
	5	4.6	5.9	1–9	Pass	1.4	≤5	Pass
	20	18.7	20.6	10–30	Pass	5.6	≤8	Pass
	80	72.4	68.2	60–100	Pass	3.8	≤16	Pass
Wheat	0	< 1.0	1.9	≤7	Pass	1.2	≤4	Pass
	5	4.6	4.6	1–9	Pass	1.3	≤5	Pass
	20	16.6	13.0	10–30	Pass	1.4	≤8	Pass
	80	69.8	65.4	60–100	Pass	7.0	≤16	Pass
Barley	0	<1.0	1.9	≤7	Pass	0.5	≤4	Pass
	5	4.6	4.3	1–9	Pass	0.4	≤5	Pass
	20	17.6	13.0	10–30	Pass	0.7	≤8	Pass
	80	68.2	51.5	60–100	Failed	3.7	≤16	Pass
Soybeans	0	<1.0	1.6	≤7	Pass	0.5	≤4	Pass
	5	4.8	3.5	1–9	Pass	0.5	≤5	Pass
	20	19.9	12.2	10–30	Pass	1.2	≤8	Pass
	80	80.1	57.2	60–100	Failed	3.3	≤16	Pass
Green Coffee	0	<1.0	2.5	≤7	Pass	0.8	≤4	Pass
	5	4.1	6.2	1–9	Pass	1.1	≤5	Pass
	20	16.8	18.8	10–30	Pass	1.3	≤8	Pass
	80	52.8	61.9	60–100	Pass	5.0	≤16	Pass

[a] As reported in Figure 2.

Table 5. Limit of detection (LOD) for ochratoxin A using the test kits applied to six commodities

Commodity	Test kit average results (ppb)	Standard deviation (ppb)	LOD (ppb)
Milo	2.9	0.4	3.8
Barley	2.2	0.3	2.8
Green coffee	2.8	0.2	3.3
Wheat	2.2	0.7	3.5
Soybeans	1.5	0.5	2.5
Corn[a]	0.6	0.6	1.9

[a] As reported in Table 2.

Table 6. Ruggedness (temperature sensitivity) of test kits evaluated at three different temperatures

Fortified level of ochratoxin A conc. (ppb)	Results from the environmental temperature			Experimental results for temperature sensitivity range (ppb)	Criteria for temperature sensitivity range (ppb)	Pass/Fail
	18 °C	24 °C	30 °C			
0	<LOD	<LOD	<LOD	–	4	Pass
2	3.0	3.1	3.0	0.1	5	Pass
5	4.4	4.8	5.2	0.8	6	Pass
10	8.1	9.1	9.7	1.6	8	Pass
20	17.3	19.0	19.5	2.2	12	Pass
30	27.0	29.0	28.8	2.0	16	Pass
40	35.4	37.4	36.4	2.0	20	Pass
80	59.7	65.9	72.5	12.8	32	Pass

soybeans and green coffee) with the exception for barley and soybeans at the fortified level of 80 ppb, for which the test kits give underestimates. The test kit has excellent precision on all tested levels for all six commodities and results meet study criteria. The test kit LOD values for all six commodities are shown in Table 5. The LODs are in a range of 1.9 to 3.8 ppb.

Ruggedness of test kits

The ELISA results for temperature sensitivity range were lower than 2.2 ppb for fortified levels of ochratoxin A from 0 to 40 ppb (Table 6), and it was 12.8 ppb for the fortified level of 80 ppb. ELISA test kit results of temperature sensitivity range for all eight fortified levels meet the study criteria. Hence, the test kit can be used in the environmental temperatures ranging from 18 to 30 °C.

Conclusions

Conclusions from the validation study of the described ELISA test kits is that they have:

- at least a 1-year shelf life.
- good accuracy and precision in spiked corn from 2 to 80 ppb. The limit of detection in corn is 1.9 ppb.
- good accuracy and precision for analysis of naturally contaminated corn, and the results are consistent with HPLC results.
- the capability to detect total ochratoxins (A and B).
- good accuracy and precision for several food commodities, specifically: corn, barley, green coffee, milo, soybeans and wheat in its quantitation range.
- demonstrated validity when used in environmental temperatures ranging from 18 to 30 °C.
- demonstrated validity when read using the Awareness Technology Inc. Stat Fax® 303 microwell strip reader and BioRad Laboratory Inc. BioRad 550 model microplate reader, using an operating filter of 450 nm with a differential filter of 630 nm.

Overall, the AgraQuant® Ochratoxin ELISA test kits are effective in measuring ochratoxin A and B

in several commodities in which ochratoxin occurs in the quantitation range of 2–40 ppb.

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