JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2011, 59, 809–813 809
DOI:10.1021/jf103261u

Comparison of Enzyme-Linked Immunosorbent Assays with Chemiluminescent and Colorimetric Detection for the Determination of Ochratoxin A in Food

Feng-Yih Yu,[†,‡] Marina M. Vdovenko,[§] Jing-Jhih Wang,[†,‡] and Ivan Yu. Sakharov*[,§,#]

[†]Department of Biomedical Sciences, Chung Shan Medical University, Taichung, Taiwan, [‡]Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan, [§]Chemical Enzymology Department, Faculty of Chemistry, M. V. Lomonosov Moscow State University, Moscow 119991, Russia, and [#]Division of Chemistry, G. V. Plekhanov Russian Economic Academy, Moscow 115998, Russia

A direct competitive chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) for the determination of ochratoxin A (OTA) was developed using soybean peroxidase (SbP) in combination with 3-(10′-phenothiazinyl)propane-1-sulfonate (SPTZ) and 4-morpholinopyridine (MORPH) as a detection system. By varying the concentrations of the capture monoclonal anti-OTA antibody, a conjugate of OTA with SbP, and the composition of blocking buffers, the conditions of the immunoassay were optimized. Advantages of CL-ELISA were demonstrated by comparison with ELISA with colorimetric detection (COL-ELISA). The values of IC_{10}, IC_{50}, and working range ($IC_{20}-IC_{80}$) for CL-ELISA and COL-ELISA were 0.01, 0.08, and 0.02−0.3 ng/mL and 0.08, 0.58, and 0.17−2.2 ng/mL, respectively. The recovery values of CL-ELISA from three soybean spiked samples with OTA concentrations of 0.07, 0.1, and 0.15 ng/mL ranged from 72 to 125%. Determination of OTA in 21 various agricultural commodities showed that OTA in 8 examined samples was not detected by COL-ELISA. Furthermore, it was found that in 4 of these 8 samples the developed CL-ELISA determined OTA at levels from 0.96 to 4.64 ng/g.

KEYWORDS: Ochratoxin A; ELISA; chemiluminescence; enhancement; peroxidase; soy

INTRODUCTION

Ochratoxin A (OTA) was originally described as a secondary metabolite of *Aspergillus ochraceus* (*1*) and later as one of other *Aspergillus* and *Penicillium* species (*2*). It was found in a wide variety of cereals (wheat, barley, maize, oat), dried fruits, spices, coffee, fermented beverages (beer and wine), and even milk (*3*−*6*). As it is spread among some agricultural commodities and has potential health risks, mainly toward humans, OTA has been classified by different international and national organizations as a possible carcinogen (*7*), nephrotoxic agent (*8*), and immunosuppressor (*3*). In 1997 the International Agency for Research on Cancer (IARC) classified OTA as a class 2B carcinogen (*9*).

International and governmental organizations in many countries regulate the OTA content in food products. For example, the European Commission has set limits of 10.0 μg/kg for OTA contamination in dried nuts, 5.0 μg/kg in soluble coffee and spices, raw cereal grains, and roasted coffee, 3.0 μg/kg in cereals intended for human consumption, 2.0 μg/kg in wine and grape juice, and 0.5 μg/kg in baby food and cereal-based food products intended for young children (*10*, *11*).

To minimize the risk of human exposure by OTA and to control the content of OTA in food and feed samples, various analytical techniques have been developed. These include thin-layer chromatography, high-performance liquid chromatography, immunoassay, and their combinations. Liquid chromatography with fluorescence detection was employed to detect OTA because of its low limit of detection (LDL) (*12*−*14*). Thin-layer chromatography is still very popular in screening, despite its lower performance and relatively high LDL values (*15*). Direct competitive enzyme-linked immunosorbent assay (ELISA) is a specific and highly sensitive assay (*16*, *17*). Moreover, it allows the analysis of a massive number of samples, may be automatized easily, and does not require time-consuming procedures and sophisticated equipment as chromatographic methods (*18*, *19*).

Recently we have produced and characterized monoclonal anti-OTA antibodies that allowed the development of an ELISA with colorimetric detection (COL-ELISA) for OTA determination in food (*16*). The IC_{50} for this immunoassay was 0.32 ng/mL. Because upon the preparation of food samples the content of OTA is decreased (*17*) not more than in 40 times, we could conclude that the developed immunochemical method allows measuring regulatory concentrations of OTA in food. However, some food samples showed a high matrix effect that led to overestimation of the OTA concentration values and, hence, limited the use of the COL-ELISA in practice.

*Address correspondence to this author at the Faculty of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russia [fax 7 (495) 9395417; e-mail sakharovivan@gmail.com].

Published on Web 01/04/2011

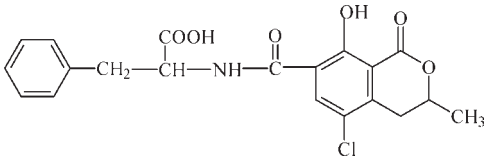

Figure 1. Chemical structure of ochratoxin A (OTA).

One of the approaches to prevent the matrix effect is an additional dilution of analyte samples. In this case a more sensitive immunoassay should be applied for its determination. It is well-known that a replacement of reaction of chromogenic substrates oxidation, used for the measurement of enzymatic activity of peroxidase conjugates, with enhanced chemuliminescence reaction (ECR) may sharply decrease the LDL value of the immunoassay (*20*−*22*). This fact is widely used in the production of highly sensitive CL-ELISA kits.

Simultaneously, it was shown that soybean peroxidase (SbP), even in the absence of any enhancers, may effectively oxidize luminol, forming a long-term chemiluminescent signal (*23*). In examples of CL-ELISAs for the determination of mouse IgG and sulfamethoxypyridazine (*24*, *25*) it was demonstrated that the use of SbP as an enzyme label has clear advantages in comparison with HRP. Recently we found that 3-(10′-phenothiazinyl)propane-1-sulfonate (SPTZ) in combination with 4-morpholinopyridine (MORPH) is a potent enhancer for SbP (*26*). Their addition to luminol solution allowed a sharp increase in the detectability of SbP in ECR. To the best our knowledge, the SbP/SPTZ/MORPH system is the most sensitive of chemiluminescent detection systems based on luminol oxidation by peroxidases so far. Therefore, the use of SbP opens good possibilities to increase CL-ELISA sensitivity.

In this paper we describe the development of an ultrasensitive direct competitive CL-ELISA for the determination of OTA using the SbP/SPTZ/MORPH detection system. Also, the developed assay was applied for the determination of OTA in some commercially available food products. Comparison of results of OTA measurement in food samples performed by both chemiluminescent and colorimetric ELISAs clearly demonstrated the advantages of practical application of SbP-based CL-ELISA.

MATERIALS AND METHODS

Materials. OTA (**Figure 1**) was purchased from Sigma (St. Louis, MO). A standard solution of OTA at 1 μg/mL in toluene/acetic acid (99:1) was prepared and assayed according to the Association of Official Analytical Chemists (AOAC) method (*27*). SbP (RZ 1.5) was also purchased from Sigma and used without further purification. Horseradish peroxidase (HRP) (RZ 3.0.) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). The monoclonal antibodies specific to OTA (anti-OTA-mAb) were produced according to the method of Liu et al. (*16*).

SPTZ was prepared as described in ref *28*. Luminol, bovine serum albumin (BSA), gelatin, ammonium biocarbonate, Tween-20, dimethylformamide, 1,1′-carbonyldiimidazole, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide, and *N*-hydroxysuccinimide were obtained from Sigma; MORPH was from Aldrich (Milwaukee, WI); Tris and H_2O_2 (30%) were from J. T.Baker (Phillipsburg, NJ). 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was obtained from Neogen Corp. (Lexington, KY). Ammonium sulfate, absolute ethanol, and methanol were obtained from Merck (Darmstadt, Germany). Black polystyrene plates and F-16 strips (high protein binding) were obtained from Nunc (Roskilde, Demark).

Conjugation of OTA with Peroxidases. (a) Conjugation of OTA to HRP (OTA−HRP) was achieved by using a 1,1′-carbonyldiimidazole method (*17*). Briefly, 0.2 mg of OTA in 0.05 mL of acetone was mixed with 0.4 mg of 1,1′-carbonyldiimidazole, and then a HRP solution (0.8 mg of HRP in 0.3 mL of 0.1 M, pH 9.6, carbonate buffer) was added. After being stirred at room temperature for 2 h, the mixture was dialyzed against 10 mM phosphate buffer with 0.15 M NaCl, pH 7.5 (PBS) for 72 h and then lyophilized.

(b) The conjugate of OTA to SbP (OTA−SbP) was prepared as follows: 0.4 mg of OTA in 40 μL of dimethylformamide was mixed with 0.4 mg of 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide and 0.3 mg of *N*-hydroxysuccinimide and incubated for 2 h at room temperature. Then 0.8 mg of SbP in 0.24 mL of 0.1 M carbonate buffer, pH 9.5, was added drop by drop. After being stirred at room temperature for 2 h, the mixture was dialyzed against PBS overnight. The OTA−SbP conjugate was stored at 4 °C.

Determination of OTA by COL-ELISA. One hundred microliters of monoclonal anti-OTA antibody (dilution 1:10000) was coated into each well of transparent F-16 strips. After incubation at 4 °C overnight and washing with 0.05% of Tween-20 in PBS (PBST) four times, 100 μL of 0.1% of BSA in PBS was added into each well, and the plate was incubated at 37 °C for 30 min. Then the plate was washed again with PBST four times, and OTA standard (0.05 mL/well in PBS) concentrations from 0.01 to 100 ng/mL or samples were added simultaneously with the OTA−HRP conjugate (1:10000 in PBS, 0.05 mL/well) and incubated at 37 °C for 1 h. The plate was washed four times with PBST, and 0.1 mL of TMB substrate solution was added. After incubation at room temperature in the dark for 10 min, the reaction was terminated by adding 0.1 mL of 1 N HCl. In each plate well the absorbance at 450 nm was determined in the ELISA reader (Menlo Park, CA), using the absorbance at 650 nm in the same wells as background value. Each experiment represents the mean of duplicates.

Determination of OTA by CL-ELISA. CL-ELISA for the determination of OTA was carried out using 96-well black polystyrene plates (MaxiSorb, Nunc). The capture antibodies were coated by adding 100 μL of the solution of the monoclonal anti-OTA antibody in the PBS to each plate well and incubated at 4 °C overnight. The plate was then washed by PBST four times and blocked by adding 100 μL of PBS containing 0.1% casein, gelatin, or BSA for 30 min at 37 °C. The plate was washed four times with PBST. Subsequently, 50 μL of the OTA standard in PBS concentrations from 0.01 to 100 ng/mL or samples simultaneously with 50 μL of the OTA−SbP conjugate (1:5000 in PBS) were added and incubated at 37 °C for 1 h. After washing of the plate as described above, 100 μL of freshly prepared substrate solution were added to each well and stirred. Chemiluminescence intensity was monitored at room temperature on a luminescence reader (FlexStation 3, Molecular Devices). Each experiment represents the mean of duplicates.

The substrate solution for chemiluminescence detection of SbP activity optimized previously (*26*) was prepared by adding 90 μL of 100 mM luminol dissolved in 2 M NaOH, 120 μL of 100 mM SPTZ dissolved in DMSO, 120 μL of 100 mM MORPH dissolved in DMSO, and 0.8 μL of 8 M hydrogen peroxide solution to 11.67 mL of 50 mM Tris-HCl buffer, pH 8.3.

Data Analysis. Standards and samples were run in triplicates, and the mean values were processed. Standard curves were obtained by plotting the light intensity against the logarithm of the analyte concentration and fitted to a four-parameter logistic equation using the Origin 7.5 software (OriginLab Corp.):

$$Y = [(A - D)/(1 + (x/C)^B)] + D$$

A is the asymptotic maximum (intensity in the absence of an analyte, I_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point, and D is the asymptotic minimum (background signal).

Using the obtained calibration curves the values of a detection limit (IC_{10}) and IC_{50}, as well as working range (IC_{20}−IC_{80}), maximum CL intensity (A_{max}), and ratio A_{max}/IC_{50} were calculated.

Preparation of Real Food Samples. Twenty-one food samples purchased from stores in Taiwan were evaluated to determine the OTA levels. The preparation of the samples was performed in accordance with a protocol described in ref *17*. Briefly, each sample (10 g) was homogenized with 100 mL of extraction solvent (methanol/water 1:1, v/v) and incubated for 2 h with shaking (200 rpm) at 37 °C. After centrifugation at 14000*g* for 10 min, the extract was passed through a 0.45 μm syringe filter. The obtained extract was diluted 2 times with PBS and then directly subjected to ELISA.

RESULTS AND DISCUSSION

Optimization of OTA CL-ELISA. Previously the conditions of direct competitive COL-ELISA for OTA determination were optimized by us (*17*). Because the plates used for CL-ELISA and

Table 1. Determination of the Optimal Concentrations of Coating Antibody (Anti-OTA-mAb) and Conjugate OTA−SbP in CL-ELISA[a]

anti-OTA antibody dilution	OTA−SbP conjugate dilution	IC_{10}, ng/mL	IC_{50}, ng/mL	IC_{20}−IC_{80}, ng/mL	A_{max}	A_{max}/IC_{50}
1:5000	1:5000	0.25	0.81	0.46−2.0	12450	15400
	1:10000	0.3	0.83	0.4−2.0	6060	7300
	1:20000	0.25	0.68	0.35−1.4	2960	4350
1:10000	1:5000	0.13	0.55	0.2−1.4	8600	15650
	1:10000	0.15	0.45	0.2−1.0	4500	9990
	1:20000	0.1	0.24	0.14−0.4	2570	10700
1:20000	1:2500	0.013	0.2	0.04−1.0	5200	24900
	1:5000	0.01	0.12	0.025−0.47	3570	29750
	1:10000	0.01	0.08	0.024−0.36	1930	24200
1:40000	1:1250	0.7	1.0	0.8−1.0	1650	1700
	1:2500	0.35	0.7	0.5−1.0	700	1000
	1:5000	0.006	0.06	0.02−0.2	500	8700

[a] CL-ELISA was carried out using PBS with 0.1% BSA as a blocking buffer.

Table 2. Analytical Parameters of CL-ELISA for OTA Determination by Using Different Blocking Buffers

blocking buffer	IC_{10}, ng/mL	IC_{50}, ng/mL	IC_{20}−IC_{80}, ng/mL
PBS	0.05	0.17	0.08−0.4
0.1% casein in PBS	0.01	0.08	0.02−0.3
0.1% gelatin in PBS	0.03	0.19	0.05−0.6
0.1% BSA in PBS	0.01	0.12	0.025−0.47

Table 3. Effect of Composition of Blocking Buffers on Recovery from Soybean Samples Containing Different OTA Concentrations by CL-ELISA

blocking buffer	spiked concentration of OTA, ng/mL	recovered concentration of OTA, ng/mL	recovery, %
PBS	0.07	0.156	223
	0.1	0.2	200
	0.15	0.33	220
0.1% casein in PBS	0.07	0.05	72
	0.1	0.1	100
	0.15	0.187	125
0.1% gelatin in PBS	0.07	0.53	757
	0.1	0.71	710
	0.15	1.01	673
0.1% BSA in PBS	0.07	0.17	243
	0.1	0.27	270
	0.15	0.49	327

COL-ELISA had different absorption properties, we first estimated favorable conditions for performance of OTA CL-ELISA. By varying the concentrations of anti-OTA antibody (capture antibody) and conjugate of SbP and OTA, a set of calibration curves was produced. All curves had a form of typical calibration curve obtained in performance of direct competitive ELISA. The values of IC_{10}, IC_{50}, working range (IC_{20}−IC_{80}), the highest analytical signal (A_{max}), and the ratio of A_{max} to IC_{50} (A_{max}/IC_{50}) were selected as the parameters used for estimation of the assay efficiency.

As seen in **Table 1**, with an increase of dilution of anti-OTA antibody from 1:5000 to 1:20000, the values of IC_{10} and IC_{50} decreased significantly. In turn, the working range was shifted in the area of lower concentrations of OTA. At that time, A_{max} decreased, but remained acceptable for the ELISA. The highest A_{max}/IC_{50} ratio was obtained at the antibody dilution of 1:20000.

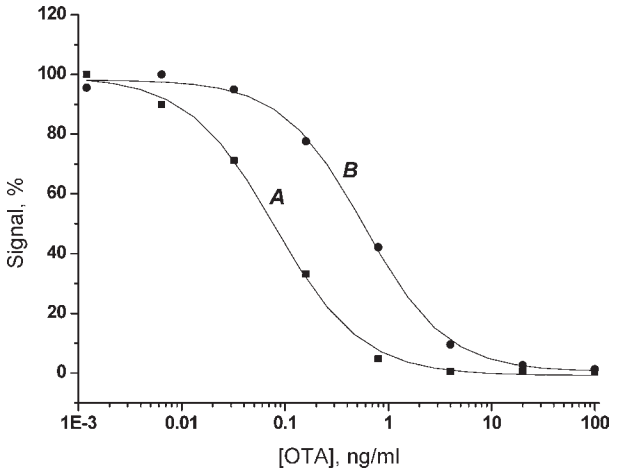

Figure 2. Calibration curves for determination of OTA by CL-ELISA (A) and by COL-ELISA (B).

Additional dilution of the antibody made all analytical parameters of the assay worse.

Fixing the antibody dilution (1:20000), we used OTA−SbP conjugates in three dilutions: 1:2500, 1:5000, and 1:10000. In the first case the lowest value of the working range (0.04 ng/mL) and detection limit value (0.013 ng/mL) were higher than the analogue parameters obtained for two other dilutions. In the case of OTA−SbP dilutions of 1:5000 and 1:10000 the lowest values of the working range and detection limit values were identical, but we had higher values of A_{max} and A_{max}/IC_{50} for the dilution of 1:5000. Therefore, the solutions of anti-OTA antibody and OTA−SbP conjugate with dilutions of 1:20000 and 1:5000, respectively, were selected as the optimal ones for CL-ELISA.

Choice of Blocking Buffer. To prevent nonspecific adsorption of enzyme conjugates on the well surface, inert proteins may be introduced to assay solution as blocking agents. In this work the effect of the presence of BSA, gelatin, and casein in blocking buffer on the analytical parameters of CL-ELISA was analyzed (**Table 2**). The obtained results demonstrated that at the determination of OTA dissolved in PBS the addition of the blocking proteins decreased the IC_{10} and IC_{50} values (in the case of casein and BSA) and the lowest value of the working range. Therefore, the use of blocking proteins improved the detectability of OTA in CL-ELISA.

Matrix Effect. The matrix effect is a phenomenon well-known to bioanalysts. It appears at replacement of aqueous or buffer

Table 4. Content of Ochratoxin in Food Samples Measured by Colorimetric and Chemiluminescent ELISAs

		COL-ELISA		CL-ELISA	
no.	food sample	concentration of OTA in extract, ng/mL ± SD	content of OTA in food sample, ng/g ± SD	concentration of OTA in extract, ng/mL ± SD	content of OTA in food sample, ng/g ± SD
1	roasted coffee	1.34 ± 0.14	53.6 ± 5.6		
2	instant coffee	0.38 ± 0.02	15.2 ± 0.8		
3	corn	0.395 ± 0.015	15.8 ± 0.6		
4	corn	0.26 ± 0.02	10.4 ± 0.8		
5	Job's tears	0.34 ± 0.05	13.6 ± 2.0		
6	black grape	0.39 ± 0.00	15.6 ± 0.0		
7	yellow rice	0.455 ± 0.065	18.2 ± 2.6		
8	rice in husk	0.24 ± 0.03	9.6 ± 1.2		
9	white rice	0.535 ± 0.005	21.4 ± 0.2		
10	yellow rice	0.91 ± 0.08	36.4 ± 3.2		
11	black rice	0.925 ± 0.001	37.0 ± 0.1		
12	red rice	1.025 ± 0.025	41.0 ± 1.0		
13	rice in husk	0.20 ± 0.02	8.0 ± 0.7		
14	unroasted coffee	0	0	0	0
15	unroasted coffee	0	0	0.024 ± 0.004	0.96 ± 0.16
16	instant coffee	0	0	0.084 ± 0.046	3.36 ± 1.84
17	oat	0	0	0	0
18	corn	0	0	0	0
19	red kidney beans	0	0	0.116 ± 0.024	4.64 ± 0.96
20	black raisins	0	0	0.105 ± 0.005	4.2 ± 0.2
21	rice in husk for birds	0	0	0	0

solutions of a measuring analyte with extracts of real samples containing the same analyte. The presence of various substances in real samples often results in an overestimation of analyte concentration. The matrix effect in the CL-ELISA was studied for OTA spiked in soybean extract (dilution 1:2). To prevent the matrix effect we used blocking buffers with different proteins. As seen in **Table 3**, by using PBS as blocking buffer, the analytical recovery from OTA samples was in the range of 200−223%. Also, the addition of proteins such as gelatin and BSA to PBS made the recovery values worse. Contrary to these proteins, the introduction of casein in PBS showed good recovery values (72−125%). Thus, the use of casein as a component of blocking buffer led to the improvement of the detectability of OTA in CL-ELISA and simultaneously the prevention of matrix effect of real samples.

Comparison of COL-ELISA and CL-ELISA. At the favorable conditions the analytical parameters of CL-ELISA and COL-ELISA developed for OTA determination were compared. It should be noted that the experimental conditions for COL-ELISA determined previously (*17*) and CL-ELISA are not identical. Therefore, COL-ELISA was performed using the anti-OTA antibody with dilution of 1:10000 and OTA−HRP conjugate with dilution of 1:10000. To compare the two ELISA formats, the magnitudes of signals obtained in each immunoassay were expressed in percentage terms. As seen in **Figure 2**, the curve for CL-ELISA is in an area of lower concentrations than that for COL-ELISA. The values of IC_{10}, IC_{50}, and IC_{20}−IC_{80} for CL-ELISA and COL-ELISA were 0.01, 0.08, and 0.02−0.3 ng/mL and 0.08, 0.58, and 0.17−2.2 ng/mL, respectively. Thus, the obtained results demonstrated that the replacement of COL-ELISA for the determination of OTA with CL-ELISA significantly improved the analytical parameters of the assay.

Analysis of Real Samples with CL-ELISA and COL-ELISA. ELISA was used to measure the content of OTA in 21 samples of food purchased from Taiwan. All samples were divided into two groups. The first group included 13 food samples. The content of OTA in these foods was too high and easily measured by COL-ELISA (samples 1−13 in **Table 4**).

The other eight food samples belong to the second group. Using COL-ELISA the OTA content in these food samples could not be detected (samples 14−21 in **Table 4**). Thus, we were faced with a dilemma: either the food samples of the second group did not really contain OTA or the detection limit of COL-ELISA was too low to detect this analyte in these samples. To determine the answer, we applied the ultrasensitive CL-ELISA developed in this work to determine the OTA content in the food samples of group 2. Our results demonstrated that in samples 14, 17, 18, and 21 the presence of OTA was found neither by COL-ELISA nor by CL-ELISA, whereas in samples 15, 16, 19, and 20 OTA was present and its content in these samples was measured by CL-ELISA (**Table 4**).

In this work we have developed an ultrasensitive direct competitive CL-ELISA for the determination of OTA. The high sensitivity was achieved by using a chemiluminescent method of measurement of peroxidase activity and application of an ultrasensitive detection system based on SbP, luminol, SPTZ, and MORPH. The improved sensitivity of CL-ELISA revealed a higher number of food samples contaminated by OTA, which could not be detected by COL-ELISA.

ABBREVIATIONS USED

OTA, ochratoxin A; SbP, soybean peroxidase; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; CL, chemiluminescence; SPTZ, 3-(10′-phenothiazinyl)propane-1-sulfonate; MORPH, 4-morpholinopyridine; BSA, bovine serum albumin; ECR, enhanced chemuliminescence reaction.

ACKNOWLEDGMENT

We thank Dr. O. S. Sokolova (Biology Faculty, MSU, Russia) for providing the luminometer.

LITERATURE CITED

(1) Van der Merwe, K. J.; Steyn, P. S.; Fourie, L. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. *Nature* **1965**, *205*, 1112−1113.

(2) Cabañes, F. J.; Accensi, F.; Bragulat, M. R.; Abarca, M. L.; Castellá, G.; Minguez, S.; Pons, A. What is the source of ochratoxin A in wine? *Int. J. Food Microbiol.* **2002**, *79*, 213−215.

(3) IARC. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC Monogr. Evaluation Carcinogenic Risks Hum.* **1993**, *No. 56*, 489−521.
(4) Battaglia, R.; Hatzold, T.; Kroes, R. Occurrence and significance of ochratoxin A in food. *Food Addit. Contam.* **1996**, *13*, 1−3.
(5) Trucksess, M. W.; Gilbert, J.; Young, K.; White, K. D.; Page, S. W. Determination and survey of ochratoxin A in wheat, barley, and coffee. *J. Assoc. Off. Anal. Chem. Int.* **1999**, *82*, 85−89.
(6) Jorgensen, K. Survey of pork, poultry, coffee, beer, and pulses for ochratoxin A. *Food Addit. Contam.* **1998**, *15*, 550−554.
(7) WHO. Ochratoxin A: Toxicological evaluation of certain food additives and contaminants. *WHO Food Addit. Ser.* **1996**, *No. 35*, 363−376.
(8) Castegnaro, M.; Plèstina, R.; Dirheimer, G.; Chernozemsky, I. N.; Bartsch, H. Mycotoxins, endemic nephropathy and urinary tract tumours. *IARC Sci. Publ.* **1991**, *No. 115*.
(9) International Agency for Research on Cancer. The evaluation of carcinogenic risks to human. *IARC Monogr.* **1997**, *No. 56*.
(10) European Commission Regulation No. 472/2002. *Off. J. Eur. Communities* **2002**, *L75*, 18−20.
(11) European Commission Regulation 123/2005 of 26 January 2005 amending EC 466/2001 as regards ochratoxin A. *Off. J. Eur. Communities* **2005**, *L25*, 3−5.
(12) Blesa, J.; Berrada, H.; Soriano, J. M.; Molto, J. C.; Manes, J. Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. *J. Chromatogr., A* **2004**, *1046*, 127−131.
(13) Zimmerli, B.; Dick, R. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. *J Chromatogr., B* **1995**, *666*, 85−99.
(14) Domijan, A.-M.; Peraica, M.; Jurjević, Ž.; Ivić, D.; Cvjetković, B. Fumonisin B1, fumonisin B2, zearalenone and ochratoxin A contamination of maize in Croatia. *Food Addit. Contam.* **2005**, *22*, 677−80.
(15) Klaric, M. Š.; Cvetnic, Z.; Pepeljnjak, S.; Kosalec, I. Co-occurrence of aflatoxins, ochratoxin A, fumonisins, and zearalenone in cereals and feed, determined by competitive direct enzyme-linked immunosorbent assay and thin-layer chromatography. *Arh. Hig. Rada Toksikol.* **2009**, *60*, 427−434.
(16) Liu, B. H.; Tsao, Z. J.; Wang, J. J.; Yu, F. Y. Development of a monoclonal antibody against ochratoxin A and its application in enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip. *Anal. Chem.* **2008**, *80*, 7029−7035.
(17) Yu, F. Y.; Chi, T. F.; Liu, B. H.; Su, C. C. Development of a sensitive enzyme-linked immunosorbent assay for the determination of ochratoxin A. *J. Agric. Food Chem.* **2005**, *53*, 6947−6953.
(18) Reinhard, H.; Zimmerli, B. Reversed-Phase liquid chromatographic behavior of mycotoxins citrinin and ochratoxin A. *J. Chromatogr., A* **1999**, *862*, 147−159.
(19) Romani, S.; Sacchetti, G.; Chaves, L.; Pinnavaia, G. G.; Dalla, R. M. Screening on the occurrence of ochratoxin A in green coffee beans of different origins and types. *J. Agric. Food Chem.* **2000**, *48*, 3616−3619.
(20) Knopp, D. Immunoassay development for environmental analysis. *Anal. Bioanal. Chem.* **2006**, *385*, 425−427.
(21) Roda, A.; Pasini, P.; Mirasoli, M.; Michelini, E.; Guardigli, M. Biotechnological applications of bioluminescence and chemiluminescence. *Trends Biotechnol.* **2004**, *22*, 295−303.
(22) Marquette, C. A.; Blum, L. J. Applications of the luminol chemiluminescent reaction in analytical chemistry. *Anal. Bioanal. Chem.* **2006**, *385*, 546−554.
(23) Alpeeva, I. S.; Sakharov, I. Yu. Soybean peroxidase-catalysed oxidation of luminol by hydrogen peroxide. *J. Agric. Food Chem.* **2005**, *53*, 5784−5788.
(24) Sakharov, I. Yu.; Alpeeva, I. S.; Efremov, E. E. Use of soybean peroxidase in chemiluminescent enzyme-linked immunosorbent assay. *J. Agric. Food Chem.* **2006**, *54*, 1584−1587.
(25) Sakharov, I. Yu.; Berlina, A N.; Zherdev, A. V.; Dzantiev, B. B. Advantages of soybean peroxidase over horseradish peroxidase as the enzyme label in chemiluminescent enzyme-linked immunosorbent assay of sulfamethoxypyridazine. *J. Agric. Food Chem.* **2010**, *58*, 3284−3289.
(26) Vdovenko, M. M.; Della Ciana, L.; Sakharov, I. Yu. 3-(10′-Phenothiazinyl)propane-1-sulfonate is a potent enhancer of soybean peroxidase-induced chemiluminescence. *Anal. Biochem.* **2009**, *392*, 54−58.
(27) Stoloff, L.; Scott, P. M. Natural poisons. In *Official Methods of Analysis*, 16th ed.; Williams, S., Ed.; AOAC: Arlington, VA, 1995; Chapter 49, pp 37−39.
(28) Marzocchi, E.; Grilli, S.; Della Ciana, L.; Prodi, L.; Mirasoli, M.; Roda, A. Chemiluminescent detection systems of horseradish peroxidase employing nucleophilic acylation catalysts. *Anal. Biochem.* **2008**, *377*, 189−194.

Received for review August 26, 2010. Revised manuscript received December 11, 2010. Accepted December 13, 2010. We thank the Russian Foundation for Basic Research for financial support (09-03-92008-NNS_*a*) and the National Science Council of the Republic of China (Taiwan) for Taiwan−Russia Cooperation Grant NSC-98-2923-B-040-001-MY2.