

Highly sensitive electrochemical immunoassay for zearalenone in grain and grain-based food

Yuan-Kai Wang · Ya-Xian Yan · Zi-Wei Mao ·
Heng-an Wang · Qi Zou · Qian-Wen Hao ·
Wen-Hui Ji · Jian-He Sun

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Abstract We have developed a highly sensitive electrochemical immunoassay for the quantitation of zearalenone (ZEN), a mycotoxin produced by *Fusarium* species. In this enzyme linked immunosorbent assay, the enzymatic conversion of the substrate *p*-nitrophenylphosphate is detected by a microplate reader and the signal subsequently converted into an electrical signal. The concentrations of coating antigen (ZEN-ovalbumin), of monoclonal antibody, and of goat anti-mouse antibody labeled with alkaline phosphatase were optimized. In terms of electrochemical detection, the types and pH values of the buffers, the conditions for agitating, and scanning frequency were optimized. The effective detection range of this immunoassay is quite wide (0.004 to 9.5 ngmL⁻¹), and the limit of detection is 2 pgmL⁻¹. ZEN-free corn, wheat, and grain-based food samples were spiked with ZEN and analyzed by this method, and recoveries were found to range from 91.6 % to 113.0 %. Unlike previously described electrochemical methods, this method is both highly sensitive and has a wide working range. The method is fast and thus provides a platform for high-throughput analysis that meets the current need to monitor trace levels of analytes in grain and grain-based food.

Keywords Zearalenone · Electrochemical analysis · Immunoassay · Highly sensitive · Broad dynamic range

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Yuan-Kai Wang and Ya-Xian Yan contributed to the work equally and should be regarded as co-first authors

Y.-K. Wang · Y.-X. Yan · Z.-W. Mao · H.-a. Wang · Q. Zou ·
Q.-W. Hao · W.-H. Ji · J.-H. Sun (✉)
Key Laboratory of Veterinary Biotechnology, School of
Agriculture and Biology, Shanghai Jiao Tong University, Shanghai
200240, China
e-mail: sunjhe@sjtu.edu.cn

Introduction

Zearalenone (ZEN), a secondary metabolites from *Fusarium* species, is a mycotoxin found throughout the world, and primarily in grain crops, such as corn, wheat, barley, and oats [1, 2]. Classified as a nonsteroidal estrogen or mycoestrogen [3] with immune toxicity [4] and reproductive toxicity [5] towards humans and animals, ZEN has been causally linked to abortion and stillbirth in livestock. To decrease the incidence of ZEN-based toxicity towards humans, European Commission regulations specify a maximum level of 20 µg of ZEN per kg of cereal-based foods intended for consumption by infants and young children [6]. This upper limit is considerably lower than the maximum amount reported in cereals (100 µg.kg⁻¹) for adults. The need for more stringent control of ZEN levels in the food of infants and young children has necessitated urgent measures to develop highly sensitive, rapid, and simple instrumentation, with a wide dynamic range in order to monitor the content of the ZEN in food samples.

Increasing expectations for the highly sensitive detection for mycotoxins in food samples has led to the development of many traditional analytical methods, such as gas chromatography–mass spectrometry [7], high-performance liquid chromatography–fluorescence detection [8], high-performance liquid chromatography–mass spectrometry [9], enzyme linked immunosorbent assay (ELISA) [10], radioimmunoassay [11], and fluorescent immunoassay [12]. Nonetheless, these methods still did not meet the current trace level and high-throughput demands simultaneously for the detection of ZEN in certain foodstuffs.

Electrochemical sensors respond to specific analytes by converting the signal generated by a chemical reaction to an electrical signal. Owing to the high sensitivity of electrochemical sensing methods, different kinds of electrochemical analysis have been reported, including methods that

involve either glass carbon electrodes [13] or screen-printed electrodes [14]. However, these methods require immobilization of antigen conjugates or antibodies on the electrodes that provide a solid phase for the immune reaction, and this immobilization reduces the generation of electrochemical signals and hinders electron transfer [15]. Another type of electrochemical immunoassay, which uses magnetic beads, enables highly sensitive detection of ZEN in baby food [15, 16], although the narrow detection range cannot be adjusted to detect different levels of sample contamination.

Alkaline phosphatase (ALP) converts the colorless substrate *p*-nitrophenylphosphate (*p*NPP) to the yellow product *p*-nitrophenol (*p*NP), which can be detected by a microplate reader set to monitor the change in 405 nm of optical density (OD). Therefore, the level of an analyte in a sample is proportional to the OD_{405 nm} value, and can be quantified using a calibration curve prepared using an analyte standard. Given that *p*NP has electrochemical activity, whereas *p*NPP does not, residual unreacted *p*NPP in the substrate buffer does not affect the detection of *p*NP. This permitted the development of electrochemical analytical method based on the catalytic conversion of *p*NPP for the detection of drug residues [17]. In initial studies, sensitivity was insufficient to detect trace amounts of analyte, and the working range was narrow because each electrochemical analysis could not be coupled with other methods, such as ELISA. Moreover, this approach has not yet been used to detect mycotoxins, for which there is an urgent need for a rapid, highly sensitive method with a wide dynamic range.

In this study, a highly sensitive electrochemical immunoassay that involved *p*NP was established. Combined use of ELISA and electrochemical analysis improved the sensitivity and working range of the approach to enable large-scale detection. Advantages of the strategy relative to the current alternatives are that it is highly sensitive and has a wider detection range, enabling high throughput, rapidity, and accuracy. This new platform permits trace detection across a wide working range for analysis of food samples.

Materials and methods

Reagents

Whereas ZEN and *p*NPP were purchased from the Sigma Chemical Co. (St. Louis, MO, USA, <http://www.sigmaldrich.com>), ovalbumin (OVA) and dry skimmed milk were obtained from Sangon Biotech (Shanghai, China, <http://www.sangon.com/>), Tween 20 was obtained from Generay Biotech (Shanghai, China, <http://www.generay.com.cn/>), a specific monoclonal antibody against ZEN (2C9) was prepared in our laboratory [18], and ALP-labeled goat anti-mouse antibody was obtained from ProteinTech (Chicago, IL, USA, <http://www.ptglab.com/>).

Equipment

Microtiter plates were purchased from Nunc (Roskilde, Denmark, <http://www.nuncbrand.com>), the microplate reader was obtained from Bio-Tek (Winooski, VT, USA, <http://www.biotek.com/>) and the CHI630C multifunction-voltammograph and three-electrode systems were obtained from Chenhua (Shanghai, China, <http://www.chinstr.com/>).

Development and optimization of electrochemical immunoassay

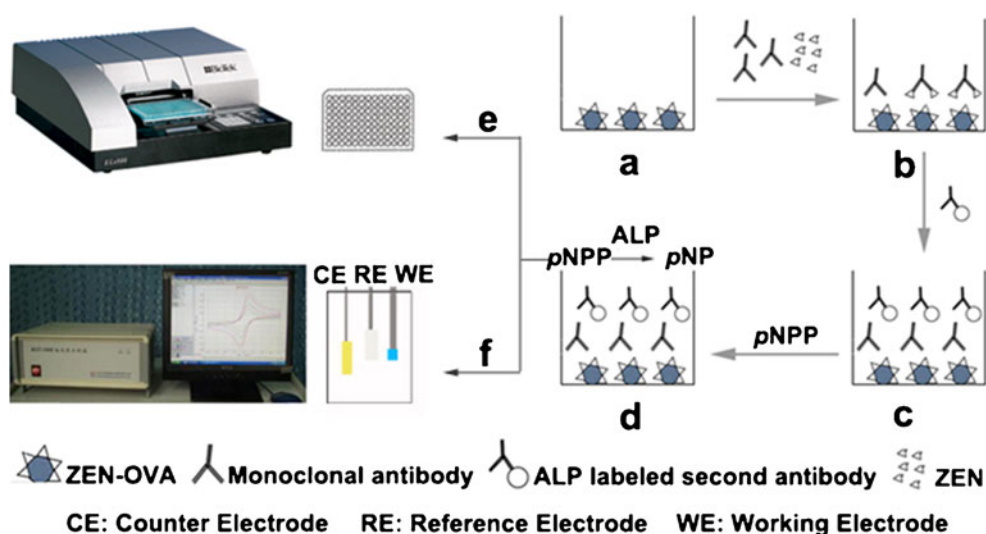
After conjugation of ZEN and OVA as described previously [19], the ZEN-OVA conjugates were diluted in 0.05 M carbonate buffer (pH 9.6) and used as the coating antigen. The 96-well microplate was coated with the conjugates for 4 h at 37 °C, and blocked using skimmed milk for 2 h at 37 °C. We added 100 µL of a mixture of diluted monoclonal antibody and standard solution of ZEN (1:1, v/v) to the wells after they were washed three times with 0.01 M phosphate buffer saline (PBS, pH 7.4) containing 0.05 % Tween-20 (PBST). Plates were then incubated for 1 h at 37 °C. After washing the plates with PBST, ALP-labeled goat anti-mouse antibody was added to the wells, and the plates were incubated for 1 h at 37 °C. After washing with PBST, the substrate (*p*NPP) solution was added, and the plates were incubated for 30 min at 37 °C before adding stop solution. The plates were then transferred to the microplate reader to record absorbance values, and then analyzed using the three-electrode system provided with the multifunction-voltammograph work station.

Different concentrations of coating antigen (ZEN-OVA), monoclonal antibody, and ALP-labeled goat anti-mouse antibody were assessed by checkerboard titration involving ELISA and electrochemical methods to improve sensitivity [16]. In order to enhance the performance of electrochemical analysis, the reaction buffers, such as Britton-Robison buffer (BR), Tris-HCl, and borate buffer (BB) with different pH values (1.0, 3.0, 5.0, 7.0, and 9.0) were investigated. Different conditions of agitating (0.1 V, 0 V, -0.1 V for 30 s, 90 s, and 180 s respectively) and scanning frequency were also determined to improve the sensitivity of electrochemical analysis.

Sample preparation

The ZEN-free corn and wheat samples and grain-based food samples (noodles and biscuit) were milled and provided by the Shanghai Entry-Exit Inspection and Quarantine Bureau. To spike and extract the samples, standard ZEN solution (in methanol) was added to 5 g samples at different levels (0.2, 1, 2, 10, 20, 100, and 200 µg.kg⁻¹), then homogenized and allowed to stand at room temperature overnight. First, each 5 g sample was added to 25 ml methanol:H₂O (80:20, v/v), and shaken vigorously for 15 min at room temperature. Second,

Fig. 1 Scheme of test procedure of electrochemical immunoassay (procedure of immune-reaction: from a to f)



the supernatants were purified by centrifugation at 2000g for 10 min. Finally, extracts were diluted five times in 0.01 M PBS before subsequent analysis.

Test procedure

First, 96-well microplates were coated with ZEN-OVA conjugates (diluted 1/4,000) for 4 h at 37 °C, and blocked using 5 % skimmed milk for 2 h at 37 °C. Extracts of spiked samples or standard solutions of ZEN (50 μL , range from 0 to 50 $\text{ng}\cdot\text{mL}^{-1}$) were then mixed with the same volume of monoclonal antibody (50 μL , 1/81,000), and incubated for 1 h at 37 °C. The mixtures (100 μL) were then added in the wells of microtiter plates, which had already been washed with 0.01 M PBST three times. The plates were incubated for 1 h at 37 °C, and washed with 0.01 M PBST three times. Then, 100 μL of ALP-labeled goat anti-mouse antibody (1/2,000) was added to the plates and incubated for 1 h at 37 °C. After washing three times, the substrate solution (100 μL of 10 mM *p*NPP in 0.1 M diethanolamine buffer, containing 1 mM MgCl, pH 9.8) was added, and the plates were incubated for 30 min at 37 °C. 50 μL of 2.0 M NaOH was added to terminate reaction. Finally, the plates were

analyzed by transfer to a microplate reader to record the $\text{OD}_{405\text{ nm}}$ of the contents of the wells. Samples (100 μL) from each well were added to 6 mL BR buffer (0.2 M, pH 3.0), and agitated for 90 s in -0.1 V. The parameters used for the electrochemical immunoassay included the scanning range (from 0.9 to 1.5 V), pulse amplitude (50 mV), pulse duration (20 s), pulse period (200 s) and scanning speed (50 $\text{mV}\cdot\text{s}^{-1}$), as described previously [17]. The oxidation peak current was observed by differential pulse voltammetry (DPV) using the CHI630c work station. Each sample was evaluated three times in duplicate. The scheme of the test procedure is shown in Fig. 1.

Results and discussion

Optimization of the electrochemical immunoassay

Concentrations of ZEN-OVA, ZEN-specific monoclonal antibody, and ALP-labeled goat anti-mouse antibody needed to be optimized using ELISA and electrochemical analysis,

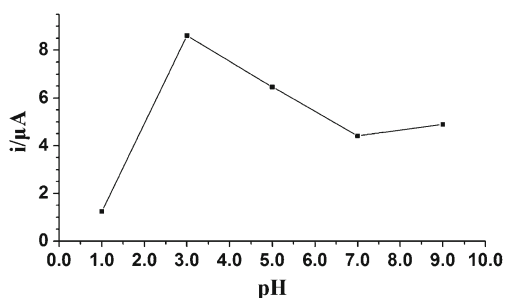


Fig. 2 Oxidation peak currents of *p*NP at different pH values of BR buffer

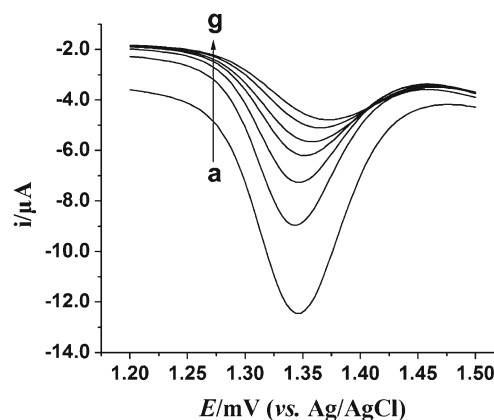


Fig. 3 Oxidation peak currents of *p*NP of scanning frequencies curves (from a to g, the scanning frequencies were 1, 2, 3, 4, 5, 6, and 7 times)

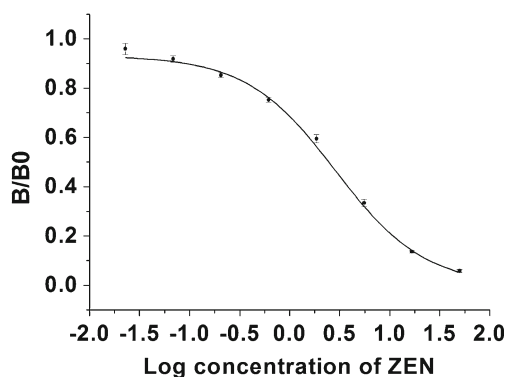


Fig. 4 Calibration curve of ELISA for the immunoassay

because each detection method has a different detection range, and could not detect all concentrations of ZEN in the range from 0 to 50 ng.mL⁻¹. The optimum concentrations of coating antigen (1/4,000, 0.25 μg.mL⁻¹), monoclonal antibody (1/81,000, 0.12 μg.mL⁻¹) and ALP labeled goat anti-mouse antibody (1/2,000, 0.5 μg.mL⁻¹) were determined by checkerboard titration performed through ELISA and electrochemical analysis. For the electrochemical part of this immunoassay, several parameters were optimized to improve the performance of detection. First, the effects of using different reaction buffers (BR, Tris-HCl, and BB) were determined. The BR buffer was chosen on the basis that it could conduct more current than either Tris-HCl or BB and did not have the base peak. The Tris-HCl buffer had an oxidation peak current at 1.20 V, whereas the *p*NP peak was at 1.2–1.4 V. Thus, Tris-HCl could affect the current of the *p*NP when used as the reaction buffer. For BB, the current associated with *p*NP was not stable at a given concentration.

Next, the effects of modifying the pH of BR buffer were investigated. The oxidation peak currents of *p*NP when different pH values of BR buffer (pH values of

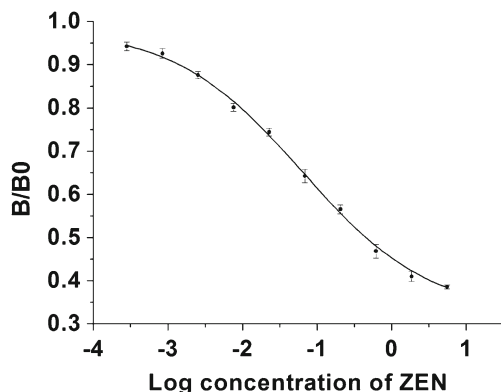


Fig. 5 Calibration curve of electrochemical analysis for the immunoassay

1.0, 3.0, 5.0, 7.0, and 9.0) were tested, are shown in Fig. 2. The current was largest at pH 3.0. Different conditions of agitating were also determined. The results indicated that 90 s at -0.1 V is the largest current (data not shown). The optimum scanning frequency was determined by seven continuous scans. The oxidation peak currents recorded are shown in Fig. 3. Data from the first scan was used, because it had a larger current than recorded using the other scans.

Calibration curves for the detection of ZEN

The calibration curves for the ELISA and electrochemical parts of the immunoassay are shown in Figs. 4 and 5. For ELISA ($y = -0.4418x + 0.6776, R^2 = 0.9921$), as the

Table 1 Recovery rates of ZEN-free corn, wheat, and grain-based food samples spiked with the indicated concentrations of ZEN

Samples	Concentration of ZEN		Recovery rates (%)	RSD ^a (%)
	Spiked (μg.kg ⁻¹)	Detected (μg.kg ⁻¹)		
Corn	200	217.5	108.8	5.4
	100	104.5	104.5	4.6
	20	19.4	97.0	4.8
	10	9.63	96.3	6.7
	2	2.04	102.0	5.9
	1	1.13	113.0	7.8
	0.2	0.211	105.5	7.4
	Wheat	200	208.4	104.2
100		107.2	107.2	5.4
20		19.7	98.5	7.2
10		10.7	107.0	6.8
2		1.95	97.5	9.2
1		0.947	94.7	7.5
0.2		0.205	102.5	7.8
Noodles		200	207.6	103.8
	100	104.6	104.6	4.1
	20	20.3	101.5	5.7
	10	9.16	91.6	5.6
	2	2.05	102.5	7.9
	1	1.07	107.0	8.1
	0.2	0.189	94.5	6.4
	Biscuit	200	215.4	107.7
100		104.6	104.6	3.7
20		21.3	106.5	5.8
10		11.1	111.0	5.4
2		2.14	107.0	8.6
1		1.05	105.0	9.1
0.2		0.212	106.0	7.8

^a RSD indicates relative standard deviation

concentrations that gives 20 % and 80 % inhibition (IC 20-IC 80) of color development, the detection range was from 0.35 to 9.5 ng.mL⁻¹, EC50 (concentration for 50 % of maximal effect) was 2.7 ng.mL⁻¹. Based on the concentration, which results in a 10 % inhibition (IC 10) of color development, the limit of detection (LOD) was 0.16 ng.mL⁻¹. For the electrochemical analysis ($y = -0.1558x + 0.46$, $R^2 = 0.9943$), the detection range was from 0.004 to 0.55 ng.mL⁻¹, EC50 was 0.054 ng.mL⁻¹, and the detection limit was 0.002 ng.mL⁻¹.

According to the overlap in the detection ranges of the ELISA and electrochemical components, the detection range of our immunoassay ranged from 0.004 to 9.5 ng.mL⁻¹, with a detection limit of 0.002 ng.mL⁻¹. Considering the dilution ratio (5×5) during sample extraction, the limit of detection was 0.05 µg.kg⁻¹, and the working range was 0.1–237.5 µg.kg⁻¹ in samples. The relative standard deviations (RSD) were below 10 % for both ELISA and electrochemical analysis.

Recovery studies

Levels of ZEN in the spiked corn and wheat samples, and grain-based food samples (0.2, 1, 2, 10, 20, 100, and 200 µg.kg⁻¹) were determined using the electrochemical immunoassay. The results are shown in Table 1. Each sample was evaluated three times in duplicate to verify the repeatability. The recovery rates range from 91.6 % to 113.0 %, indicating the accuracy of this method in both grain and grain-based food samples.

Based on the overlap concentrations (0.4 and 0.5 ng.mL⁻¹) of dynamic ranges in the two different detection methods and the dilution ratio of sample extraction (5×5), ZEN-free samples (corn, wheat, noodles and biscuit) were spiked in 10 and 12.5 µg.kg⁻¹. The spiked samples were determined by ELISA

and electrochemical analysis, respectively. The results were shown on Table 2. For both methods the recovery rates were almost identical and statistical analyses using Pearson correlation by SPSS software (11.5 version) show a significant correlation at the $P < 0.01$ level between the two methods. The recovery rates for detection of ZEN in samples have a good correlation between the two different methods, which means the dynamic ranges obtained from two different detection methods overlap.

Comparison of different methodologies for determination of ZEN

An outline of recent studies about ZEN detection is presented in Table 3. These methods were mainly immunoassays with the advantages of rapidity and accuracy. High sensitivity (0.011 and 0.007 ng.mL⁻¹) was reported, albeit with narrow working ranges [15, 16]. A method that used surface plasmon resonance involved a complicated procedure with additional clean-up steps was not suitable for detection by various farms or companies, although it had an even wider dynamic working range [20].

Owing to their rapidity and sensitivity, sensors based on colorimetry [21], fluorescence [12], and electrochemistry [15, 16, 22] have been employed in recently published detection methods. Along with the wider applications of sensors in analytical methods and increased concerns about food safety, the need for higher sensitivity and a wider dynamic range has become a particularly important request. In this study, the highly sensitive detection capacity (0.002 ng.mL⁻¹) and wide working range (0.004–9.5 ng.mL⁻¹) of our immunoassay was achieved by combining ELISA and electrochemical analysis, which only involved an additional 1–2 min of assay time compared with electrochemical analysis alone [16].

Table 2 Recovery rates of ZEN-free corn, wheat, and grain-based food spiked samples within the overlap concentrations of calibration curves in ELISA and electrochemical analysis

Samples	ELISA				Electrochemical analysis			
	Concentration of ZEN		Recovery rates (%)	RSD ^a (n=3) (%)	Concentration of ZEN		Recovery rates (%)	RSD (n=3) (%)
	Spiked (µg.kg ⁻¹)	Detected (µg.kg ⁻¹)			Spiked (µg.kg ⁻¹)	Detected (µg.kg ⁻¹)		
Corn	10	9.58	95.8	5.9	10	9.66	96.6	6.8
	12.5	11.5	92	6.3	12.5	12.1	96.8	6.9
Wheat	10	10.8	108	6.6	10	10.9	109	6.4
	12.5	12.9	108	6.1	12.5	13.2	106	7.9
Noodles	10	10.3	103	7.1	10	9.34	93.4	6.7
	12.5	13.4	107	5.8	12.5	11.7	93.6	6.5
Biscuit	10	11.5	115	7.4	10	10.8	108	8.1
	12.5	13.1	105	6.9	12.5	12.7	102	7.8

^a RSD indicates relative standard deviation

Table 3 Recent studies describing methods for the detection of ZEN

Analytical methods	Matrices	Limit of detection	EC50 ^a	Dynamic working range	References
This method	Corn, wheat, noodles, biscuit	0.002 ng.mL ⁻¹	0.054 ng.mL ⁻¹	0.004–9.5 ng.mL ⁻¹	This study
ELISA	Corn, wheat, barley, oat, coix seed, rodent feed	1.56 ng.mL ⁻¹	- ^b	1.56–100 ng.mL ⁻¹	Gao et al. [23]
Lateral flow immunoassay	Cereal-based baby food, maize, wheat, feed stuffs	1 ng.mL ⁻¹	-	1–50 ng.mL ⁻¹	Liu et al. [21]
Capillary electrophoresis	Maize	5 µg.kg ⁻¹	-	5–500 µg.kg ⁻¹	Maragos et al. [24]
Fluorescence polarization immunoassay	Corn	137 µg.kg ⁻¹	-	150–1,000 µg.kg ⁻¹	Chun et al. [12]
Electrochemical sensor (glass carbon electrode)	Maize, baby food	0.011 ng.mL ⁻¹	0.079 ng.mL ⁻¹	-	Hervás et al. [16]
Electrochemical sensor (screen-printed electrode)	Maize, baby food	0.007 ng.mL ⁻¹	0.088 ng.mL ⁻¹	-	Hervás et al. [15]
Microfluidic immunosensor	Maize, baby food	0.4 ng.mL ⁻¹ in standard solution, 20 µg.kg ⁻¹ in solid sample, 20 ng.mL ⁻¹ in liquid sample	-	-	Hervás et al. [22]
Surface plasmon resonance	Feed stuffs	0.41 µg.kg ⁻¹	-	-	Panini et al. [25]
Flow-through immunosensor	Corn	0.3 µg.kg ⁻¹	-	0.3–3,000 ng.mL ⁻¹	Choi et al. [20]
Chemiluminescence immunoassay	Corn, wheat, swine feed	0.007 ng.mL ⁻¹	0.087 ng.mL ⁻¹	0.019–0.422 ng.mL ⁻¹	Urraca et al. [26]
Fluorescent-labeled immunosorbent assay	Corn	0.02 ng.mL ⁻¹	2.93 ng.mL ⁻¹	0.05–54.34 ng.mL ⁻¹	Wang et al. [27]
	Wheat	0.03 ng.mL ⁻¹	0.1 ng.mL ⁻¹	0.05–0.24 ng.mL ⁻¹	Beloglazova et al. [28]

^a EC 50 means concentration for 50 % of maximal effect

^b “-” means not mentioned in studies

Conclusions

In this study, a highly sensitive electrochemical immunoassay with a wide dynamic working range was developed for the detection of ZEN in foodstuffs. The limit of detection was 0.002 ng.mL⁻¹, which could ensure detection of trace amounts of ZEN in grain and grain-based foods. The detection range (0.004–9.5 ng.mL⁻¹) was wider than most broadly comparable detection methods, and the approach is compatible with high-throughput analysis, saving time, labor, and money.

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