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Chemiluminescent enzyme-linked immunosorbent assay using monoclonal antibodies for zearalenone in food and feed

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ORIGINAL RESEARCH PAPER

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ABSTRACT

A chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) for the detection of zearalenone (ZEN) in maize and feed was developed by optimising antigen and antibody concentrations, as well as incubation times. The results indicated that the CL-ELISA exhibited a linear working range of 0.039–1.027 ng mL^{-1}, with a half-maximal inhibitory concentration (IC_{50}) of 0.187 ng mL^{-1} and a limit of detection (LOD) of 0.039 ng mL^{-1}. The method exhibited cross-reactivity ranging from 7.95 to 102.19% for five ZEN structural analogues, with no detectable cross-reactivity toward other mycotoxins or the carrier protein. The intra- and inter-assay coefficients of variation were both below 10% and recovery rates in spiked maize and feed samples ranged from 86.68 to 103.86%. In conclusion, the developed CL-ELISA is suitable for the rapid, group-specific screening of ZEN in maize and feed.

KEYWORDS

zearalenone, CL-ELISA, mycotoxin detection, rapid screening

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1. INTRODUCTION

Zearalenone (ZEN) is a thermally stable estrogenic mycotoxin produced by *Fusarium* species, frequently contaminating cereals and feeds (Cai et al., 2024). Upon ingestion, ZEN poses serious reproductive and carcinogenic risks to humans and animals (Rogowska et al., 2019). To mitigate these risks, strict maximum residue limits (MRLs) have been established worldwide (Zhang et al., 2018). For instance, the Chinese national standards stipulate MRLs of 60 μg kg^{-1} for maize and 250 μg kg^{-1} for swine compound feeds (National Health and Family Planning Commission of the PRC, 2017; Standardization Administration of China, 2017).

To date, numerous analytical methods have been reported for the detection of ZEN residues. Among these, high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) are widely utilised (Huang et al., 2018; Xu et al., 2020). However, their high cost and long analysis times limit their routine or on-site use (Yan et al., 2020). In contrast, immunoassays such as ELISA provide rapid, cost-effective screening but frequently suffer from low sensitivity (Merrill and Matson, 2023). To address this limitation, CL-ELISA employs chemiluminescent signal amplification to achieve superior sensitivity (Hendrickson et al., 2016). This study developed a CL-ELISA method for the detection of ZEN in maize and feed, with critical conditions optimised to maximise assay sensitivity and reliability.

2. MATERIALS AND METHODS

2.1. Reagents and instruments

ZEN, α/β-zearalanol (α/β-ZAL), α/β-zearalenol (α/β-ZEL), zearalanone (ZAN), ochratoxin A (OTA), and deoxynivalenol (DON) were purchased from Qingdao Pribolab Bioengineering (Qingdao, China). Aflatoxin B1 (AFB1) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). The horseradish peroxidase (HRP)-labelled goat anti-mouse IgG was purchased from Thermo Fisher Scientific (Waltham, USA). Maize and swine feed samples were obtained from the Institute of Agricultural Products Quality and Safety at the Henan Academy of Agricultural Sciences (China). ZEN-BSA and the ZEN monoclonal antibody were preserved and provided by the Institute for Animal Health, Henan Academy of Agricultural Sciences (China). All other reagents used were of analytical grade.

A Multimode microplate reader was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

2.2. ZEN CL-ELISA steps

The assay was performed in strict accordance with Ge et al. (2025). Briefly, microplates were coated overnight with ZEN-BSA at 4 °C and subsequently blocked for 2 h at 37 °C. Samples and the ZEN monoclonal antibody (mAb) were then sequentially added and incubated at 37 °C. After washing, the HRP-labelled goat anti-mouse IgG antibody was added and incubated for a further period. Following a final washing step, a luminol-based chemiluminescent substrate was added at room temperature, and the relative light units (RLU) were measured at 425 nm using a multimode microplate reader to quantify the chemiluminescent signal.

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2.3. Optimisation of ZEN CL-ELISA working conditions

A checkerboard titration was performed by coating the plates with varying concentrations of ZEN-BSA to determine the optimal combination of coating concentration and antibody dilution. An orthogonal design was used to optimise incubation times (the ZEN mAb and HRP-labelled goat anti-mouse IgG) and substrate reaction duration to enhance assay sensitivity (Ge et al., 2025).

2.4. Establishment of the CL-ELISA standard curve

A series of ZEN standard solutions was prepared at concentrations of 0.032, 0.062, 0.125, 0.25, 0.5, and 1 ng mL^{-1}. The sensitivity of the mAb was evaluated using the developed CL-ELISA, with all assays performed in triplicate (Wang et al., 2024). Data were processed and analysed using Origin 2022 software.

2.5. Determination of CL-ELISA performance

2.5.1. Limit of detection and linear range. The LOD was determined by performing 16 replicate measurements of blank samples using the established ZEN CL-ELISA. The LOD was calculated as the concentration corresponding to the mean signal minus two standard deviations (mean - 2SD). Furthermore, the linear range was defined as the ZEN concentrations corresponding to B/B_0 values between 0.100 and 0.900 (Wu et al., 2023).

2.5.2. Specificity. To evaluate the specificity of the developed assay, cross reactivity (CR) was determined. Structural analogues of ZEN (including α-ZAL, β-ZAL, α-ZEL, β-ZEL, ZAN), other major mycotoxins (AFB1, DON, and OTA), and BSA were employed as competitors in the CL-ELISA. The CR values were then calculated using the following equation (Liu et al., 2021).

$$CR = \frac{IC_{50} \text{ of ZEN}}{IC_{50} \text{ of other compounds}} \times 100\%$$

2.5.3. Precision. To evaluate the precision of the CL-ELISA, three different batches of microplates were used to analyse the same positive sample in duplicate for 12 consecutive days. Intra- and inter-assay variations were quantified as CVs, calculated using the following formula (Zhu et al., 2022).

$$CV = SD/\overline{X} \times 100\%$$

2.6. Detection of food and feed samples

2.6.1. Minimisation of matrix effects and optimisation of extraction conditions. Aqueous methanol solutions at various concentrations were employed as assay buffers, with PBS serving as the control, to determine the effect of the organic solvent on the ZEN CL-ELISA.

Blank and spiked samples (5 g, spiked with 500 μg kg^{-1} ZEN) were extracted with 5 mL of methanol-water mixtures at various concentrations by shaking for 20 min, followed by

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centrifugation at 6,000 r.p.m. for 10 min. Recovery rates were calculated using matrix-matched standard curves prepared from blank sample extracts (Yu et al., 2012).

2.6.2. Accuracy. The accuracy of the CL-ELISA was validated by determining the recovery rates of ZEN spiked into blank samples at varying concentrations.

2.7. Comparison of CL-ELISA and HPLC results

To assess the accuracy and reliability of the method, one maize and one feed sample containing varying ZEN concentrations, previously confirmed *via* HPLC by an independent institute (the Institute of Agricultural Products Quality and Safety at the Henan Academy of Agricultural Sciences), were selected. After extraction and dilution following the optimised sample preparation protocol, each sample was analysed in triplicate using the developed CL-ELISA. The results from both methods were compared to verify the suitability of the CL-ELISA for routine sample monitoring.

3. RESULTS AND DISCUSSION

3.1. Optimisation of ZEN CL-ELISA working conditions

To prevent chemiluminescent signal saturation, antibody concentrations yielding sub-saturating signals were selected for sensitivity assessment. As illustrated in Fig. 1a, the optimal sensitivity for the CL-ELISA was obtained using a ZEN-BSA coating concentration of 0.25 μg mL^{-1} combined with a primary antibody dilution of 1:256,000.

Statistical analysis of the orthogonal experimental results (Table 1) indicated that the incubation time for the HRP-labelled goat anti-mouse IgG had the most significant impact on the assay performance. Consequently, the optimal conditions were established as follows: 20 min for the primary antibody, 50 min for the secondary antibody, and 3 min for substrate development.

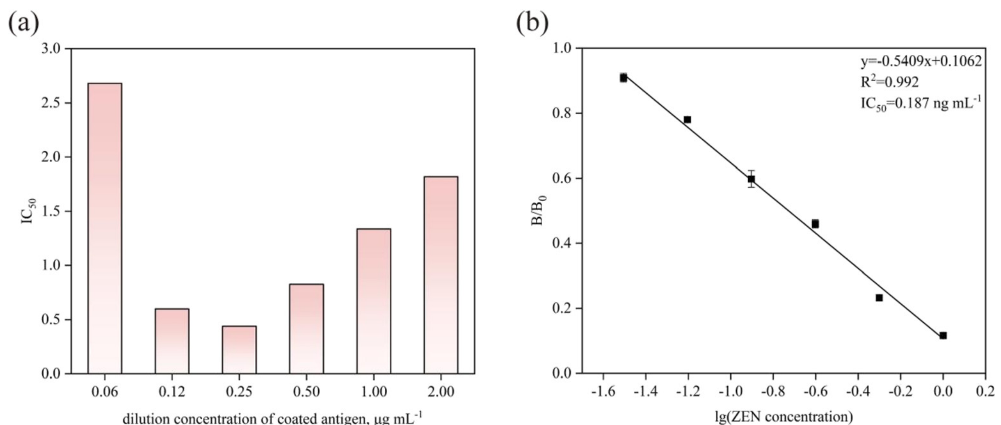

Fig. 1. (a) IC_{50} values at varying coating concentrations, (b) Standard curve for the ZEN CL-ELISA

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Table 1. Results of orthogonal experiments

Test number	mAb incubation time	HRP-labelled goat anti-mouse IgG incubation time	Colour development time	P/N*
1	10	10	1	88.803
2	10	30	3	290.563
3	10	50	5	292.315
4	15	10	3	138.063
5	15	30	5	270.913
6	15	50	1	394.937
7	20	10	5	103.554
8	20	30	1	335.024
9	20	50	3	604.479
K1	223.894	110.140	272.921	
K2	267.971	298.833	344.368	
K3	347.685	430.577	222.260	
R	123.792	320.437	122.108	
preferred combination	A3	B3	C2	
major and minor factors		B>A>C		

*P: The OD_{425} value under the specified assay conditions. N: The OD_{425} value of the blank control under identical conditions.

3.2. Establishment of standard curve

Under the optimised conditions, the established ZEN CL-ELISA exhibited excellent linearity (Fig. 1b). The regression equation of the standard curve was $y = -0.5409x + 0.1062$, $R^2 = 0.992$, yielding a half-maximal inhibitory concentration (IC_{50}) value of 0.187 ng mL^{-1}.

3.3. Evaluation of CL-ELISA performance

3.3.1. Limit of detection and linear range. The LOD was determined to be 0.039 ng mL^{-1} based on the standard curve (Fig. 1b). The calculated ZEN concentrations were 1.027 and 0.034 ng mL^{-1}, corresponding to B/B_0 values of 0.100 and 0.900, respectively. Consequently, taking the LOD into account, the linear range of the CL-ELISA was determined to be 0.039–1.027 ng mL^{-1}.

Table 2 summarises the analytical performance of recently reported methods for ZEN detection. The developed CL-ELISA exhibits a low LOD and an appropriate linear range, without the need for sophisticated analytical instruments. Compared with conventional colorimetric ELISAs and various fluorescent immunoassays, the present method achieved a sensitivity of 0.039 ng mL^{-1} *via* systematic optimisation of the chemiluminescent signal amplification system. Furthermore, its detection capability is comparable to or even superior to that of some instrument-dependent methods, such as surface-enhanced Raman spectroscopy and surface plasmon resonance. These results confirm that the proposed assay maintains high analytical sensitivity without relying on complex materials or auxiliary instruments, retaining the practical advantages of microplate-based assays for high-throughput detection.

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Table 2. Comparative analytical performance of reported methods for ZEN detection

Method	Linear range, ng mL^{-1}	LOD, ng mL^{-1}	Reference
ELISA	0.92–82.24	0.76	(Wang et al., 2022)
ELISA	13.8–508.9	9.3	(Qiu et al., 2023)
Fluorescent ELISA	0.19–1.51	0.05	(Chen et al., 2025)
Fluorescent Immunochromatographic Test Strip	0.488–15.996	0.273	(Chen et al., 2025)
Quantum Dot Fluorescent Immunochromatography	0.264–23.55	0.125	(Yang et al., 2024)
Surface-Enhanced Raman Spectroscopy	5–400	3	(Yin et al., 2023)
Surface Plasmon Resonance	1–480	0.102	(Lee et al., 2018)
This method	0.039–1.027	0.039	This work

3.3.2. Specificity. The specificity study of the CL-ELISA demonstrated that the cross-reactivities (CRs) with α-ZAL, β-ZAL, α-ZEL, β-ZEL, and ZAN were 102.19, 7.95, 46.29, 15.56, and 46.75%, respectively, whereas the CRs with other mycotoxins and BSA were all less than 0.01% (Table 3). Studies have shown that structural analogues of ZEN exhibit higher reproductive toxicity than ZEN itself (Liu et al., 2021). Therefore, the simultaneous monitoring of ZEN and its derivatives is of critical importance. Wang et al. (2013) and Hendrickson et al. (2016) also developed CL-ELISAs for ZEN, but they either did not report or exhibited insufficient CR (<35%) for ZEN structural analogues. In contrast, the CL-ELISA developed in this study demonstrates a more favourable CR profile, making it suitable for the broad-spectrum screening of ZEN and its structural analogues.

3.3.3. Precision. The intra-assay coefficients of variation were determined to be 8.89, 8.97, and 9.13%, while the inter-assay coefficient of variation was 9.81%. As all CVs were below 10%, the method demonstrated adequate precision.

Table 3. CR of ZEN mAb with its structural analogues, other mycotoxins, and BSA

Inhibitors	IC_{50}, ng mL^{-1}	CR, %
ZEN	0.187	100
ZAN	0.400	46.75
α-ZAL	0.183	102.19
β-ZAL	2.351	7.95
α-ZEL	0.404	46.29
β-ZEL	1.202	15.56
AFB1	1.0×10^4	<0.01
OTA	1.0×10^4	<0.01
DON	1.0×10^4	<0.01
BSA	1.0×10^4	<0.01

ZEN: zearalenone; ZAN: zearalanone; ZAL: zearalanol; ZEL: zearalenol; AFB1: aflatoxin B1; OTA: ochratoxin A; DON: deoxynivalenol; BSA: bovine serum albumin; CR: cross-reactivity.

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3.4. Detection in food and feed samples

3.4.1. Minimisation of matrix effects and optimisation of extraction conditions. The results indicated that methanol concentrations below 6.25% did not interfere with the CL-ELISA (Fig. 2a). The highest extraction efficiency for ZEN in maize and feed was obtained with an 80% methanol-water solution, yielding recoveries of 98.89 and 98.53%, respectively (Table 4). Matrix-matched standard curves were established for both matrices using the 80% methanol extracts. For maize (Fig. 2b), the linear regression equation was $y = -0.6737x + 0.1360$ ($R^2 = 0.993$) with an IC_{50} value of 0.288 μg kg^{-1}. For feed (Fig. 2c), the equation was $y = -0.5309x + 0.1913$ ($R^2 = 0.994$) with an IC_{50} of 0.262 μg kg^{-1}. Since the final methanol concentration required was ≤ 6.25%, samples extracted with 80% methanol had to be diluted at least 13-fold prior to detection. Consequently, the LOD for maize and feed samples using this method was calculated to be 0.507 μg kg^{-1}.

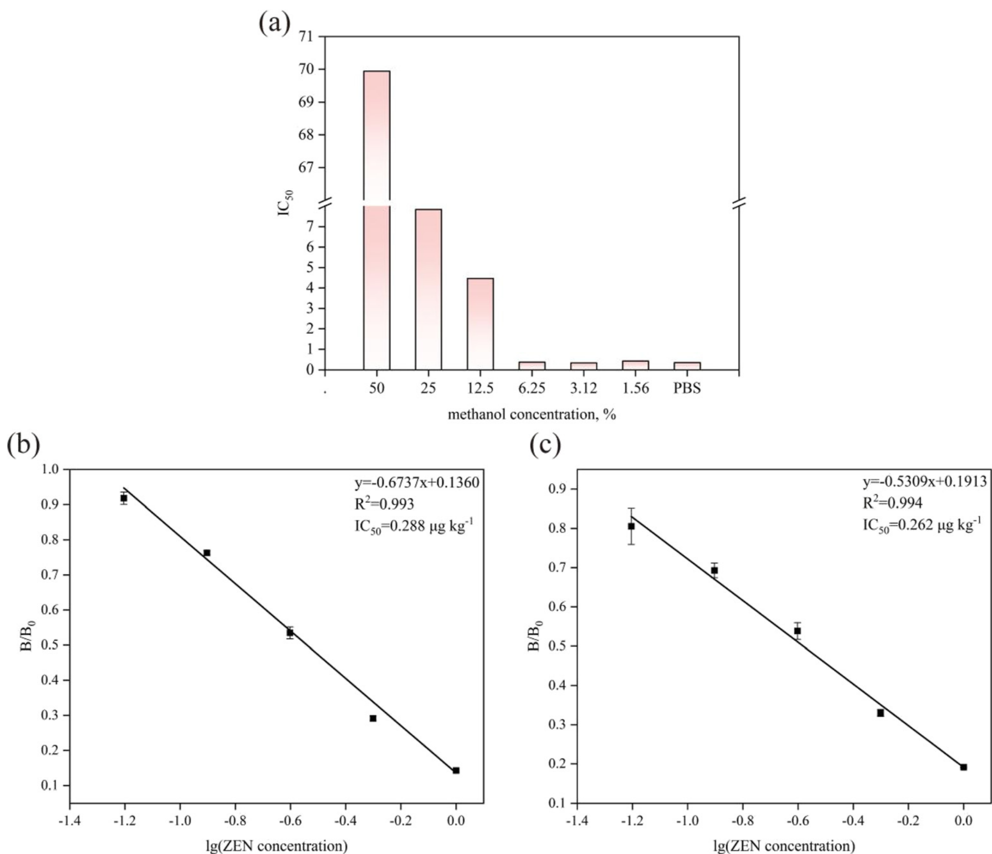

Fig. 2. (a) Effect of varying methanol concentrations on the sensitivity of the ZEN CL-ELISA; (b) Calibration curve for the ZEN CL-ELISA in a maize matrix, (c) Calibration curve for the ZEN CL-ELISA in a swine feed matrix

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Table 4. Extraction efficiencies of zearalenone (ZEN) from spiked maize and swine feed using varying aqueous methanol concentrations

Sample	Methanol content	ZEN content, μg kg^{-1}	Extraction rate, %
Maize	100%	408.193	81.64
	80%	494.427	98.89
	60%	418.820	83.76
	40%	189.556	37.11
	20%	127.145	25.43
Swine feed	100%	322.875	64.58
	80%	492.643	98.53
	60%	401.331	80.27
	40%	245.240	49.05
	20%	60.847	24.33

3.4.2. Accuracy. As shown in Table 5, the recoveries for maize and feed samples ranged from 86.68 to 103.86%, with CVs below 10%, demonstrating high accuracy of the developed method.

3.5. Comparison of CL-ELISA and HPLC results

The data presented in Table 6 indicate that the ZEN concentrations in naturally contaminated samples determined by the developed CL-ELISA were highly consistent with the results obtained using the standard HPLC method. This strong correlation verifies the practical applicability of the CL-ELISA for routine sample monitoring.

Table 5. Accuracy and recovery of ZEN CL-ELISA in spiked samples

Sample	ZEN additives, μg kg^{-1}	Measured value, μg kg^{-1}	Recovery, %	CV, %
Maize	5	5.193 ± 0.462	103.86	8.90
	10	9.692 ± 0.261	96.92	2.69
	20	18.054 ± 0.421	90.27	2.33
Swine feed	5	4.334 ± 0.227	86.68	5.24
	10	9.598 ± 0.248	95.98	2.59
	20	19.997 ± 0.649	99.98	3.25

Table 6. Comparison of the analytical results obtained by CL-ELISA and HPLC

Sample	HPLC Measured, μg kg^{-1}	CL-ELISA Measured, μg kg^{-1}
Maize	140	138.358 ± 2.853
Swine feed	9.64	9.346 ± 0.278

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4. CONCLUSIONS

A sensitive, group-specific CL-ELISA was established for the detection of ZEN in maize and feed. The method exhibited a linear range from 0.039 to 1.027 ng mL^{-1}, with an IC_{50} value of 0.187 ng mL^{-1}. The recoveries ranged from 86.68 to 103.86%, with CVs below 10%. Overall, the developed method enables the simultaneous detection of ZEN and its structural analogues, making it suitable for rapid screening in food and feed safety monitoring.

Disclosure statement: The authors declare no conflicts of interest.

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