

Fast detection of atrazine in corn using thermometric biosensors

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Fast detection is important in screening large-scale samples. This study establishes a direct competitive ELISA method (dcTELISA) based on an enzyme thermistor for fast atrazine (ATZ) detection. ATZ competes with β -lactamase-labeled ATZ (ATZ-E) for the binding sites on anti-ATZ monoclonal antibody (mAb). The mAb are covalently bound to Controlled Pore Glass (CPG) in an immunoreactor to form immunocomplexes with ATZ and ATZ-E. Several parameters of biosensor performance were optimized, such as the ATZ-E concentration, concentration and nature of the substrate, flow rate, and effect of temperature on the sensor response. After optimization, the assay time for a single sample was 12 min. The work process and result were compared with those of high-performance liquid chromatography (HPLC). The detection results exhibited a recovery rate of 88% to 107% in ATZ-spiked fresh cut corn stalks and silage samples. The results obtained via dcTELISA had good correlation with that of HPLC, and the biosensor response was reproducible and stable even when used continuously for over 4 months. All these properties suggested that the fast detection method, dcTELISA, may be used to detect pesticide residue in large-scale samples.

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1 Introduction

Triazine herbicides are widely used in agriculture to protect crops from undesirable broadleaf weeds by inhibiting photosynthesis. This practice saves billions of dollars per year in China. The herbicides persists in the environment and could be taken up by plants and transferred to higher trophic levels. ATZ is the most commonly detected herbicide in surface water and groundwater because of its high solubility (33 mg L^{-1} in water) and low absorption (soil absorption coefficient $K_{oc} = 100$).¹ These conditions increase exposure and combined with the bioaccumulation of ATZ in the trophic chain, increase the risk of cancer and endocrine disruption. Additionally, ATZ residue detection over trophic chain samples during entry–exit inspection quarantine and domestic food security surveillance is increasingly required. Therefore, establishing a fast ATZ detection method in plants is crucial.

The current techniques for ATZ detection in trophic chain samples include chromatographic and immunological assays. Given that ATZ is determined using chromatographic methods in various samples, a range of different procedures should be developed. These procedures include extraction, clean-up and

preconcentration, followed by the selection of the analytical method. Immunoassays are usually performed on microtiter plates.^{2–4} This method is highly sensitive, but it requires extensive pipetting, washing, and incubation. Thus, an immunoassay is time-consuming and has limited application in fast detection. Moreover, the sample color is another important factor that interferes with the assay results. These drawbacks may be circumvented with the aid of biosensors based on immunological methods, allowing fast analyte determination.^{5,6} Thus, we combined the simplicity of calorimetry with the accuracy and sensitivity of direct competitive ELISA.

In dcTELISA,⁷ the analyte and a constant amount of labeled derivative compete for the binding sites on the Ab, which are covalently immobilized onto CPG. After competition, the immunocomplexes form on an immunosorbent packed in a reactor. The amount of labeled antigens bound to the Ab is proportional to the enthalpy change recorded as peak height and is related to the analyte concentration in the sample. Finally, the immunosorbent is regenerated for new use through the application of a desorbent solution that dissociates the Ab from the antigen–antibody (Ag–Ab) complexes without affecting the supporting characteristics. Contrary to spectrophotometry and titrimetry, thermometric biosensors that exploit the fundamental property of biological reactions, *i.e.*, enthalpy change, are more flexible and do not require monitoring changes in color and pH during reactions. Hence, the assay results are not affected by the optical properties of samples.

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This study primarily demonstrates fast ATZ detection in corn stalk fodder through dcTELISA and compares the results with the data obtained by HPLC. The optimized dcTELISA is simple, convenient, and meets the USA requirement on the regulation of maximum residue limits (MRLs) of ATZ in corn (15 mg kg^{-1}).⁸

2 Experimental

2.1 Reagents

Analytical grade ATZ, simazine (SIM), and chlorpyrifos were provided by Sigma (St. Louis, MO, USA). Stock solutions of the herbicides (10 g L^{-1}) were prepared in dimethyl sulfoxide (DMSO) and stored at 4°C . The ATZ standard solutions for the immunoassay calibration purposes were prepared daily by diluting the stock solution in phosphate-buffered saline (PBS; 10 mmol L^{-1} , pH 7.4) that contained 2% DMSO. *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl), and ethanolamine hydrochloride (ethanolamine) were provided by Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade. Anti-ATZ mAbs were prepared. The β -lactamase used to determine the enzymatic reactions was purchased from Aladdin (Shanghai, China). Penicillin and ampicillin trihydrate were purchased from Sigma Chemical (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The immunosorbent CPG (Trisoperl, $125 \mu\text{m}$ to $14 \mu\text{m}$ diameter, 54.3 nm pore size) for Abs immobilization were provided by Schuller (Steinach, Germany).

2.2 Instruments

The setup for the dcTELISA biosensor for ATZ analysis is presented in Fig. 1. The setup consists of a peristaltic pump purchased from Shenchen (Hebei, China), an injection valve (type 50 from Rheodyne, Cotati, USA), a sample loop (0.37 mL), a thermostat unit, a Wheatstone bridge equipped with an amplifier, and a data recorder N2000 chromatography data system. Teflon tubing (1.0 mm i.d.) was used for the connections. The carrier buffer was 10 mmol L^{-1} PBS at pH 7.4.

2.3 Preparation of β -lactamase-ATZ conjugate (ATZ-E)

ATZ-E was prepared using a previous method, with some modifications.⁹ Carboxylic ATZ (ATZ-COOH; 10 mg), NHS (6 mg), and EDC-HCl (10 mg) were dissolved subsequently in

$400 \mu\text{L}$ of DMF and stirred in darkness for 8 h at room temperature. The obtained carboxyl activated ATZ solution was added dropwise to 6 mg L^{-1} β -lactamase solution in PBS at a β -lactamase:ATZ-COOH molar ratio of 1 : 30. The mixture was stirred at 4°C for 12 h and then dialyzed (pore size of the membrane = 8000 Da) against PBS for 3 days. After preparation, the ATZ-E conjugates were identified, and the molar ratio was determined *via* mass spectrometry (MS).

The coupling ratios of the conjugate were calculated using eqn (1).

$$\text{Coupling ratio} = \frac{M_{W_{\text{conjugate}}} - M_{W_{\beta\text{-lactamase}}}}{M_{W_{\text{Hap}}}} \quad (1)$$

2.4 Covalent immobilization of anti-ATZ mAb to CPG

The glutaraldehyde activation was performed according to the standard method on presilanized spherical CPG beads.¹⁰ CPG (0.35 g) was treated with 8 mL of 2.5% glutaraldehyde solution (50%, Fluka, Switzerland) diluted in PBS and allowed to react at a normal temperature under reduced pressure for 30 min (using a water aspirator) and then at normal pressure for 30 min. The product, which became brick red, was washed exhaustively on a core sand funnel with 200 mL of distilled water. The protein, 3.5 mg of anti-ATZ mAb, was added to the activated beads in 8 mL of coupling buffer and allowed to immobilize for 2 h at 20°C and left overnight at 4°C . The excess activated aldehyde groups on the CPG were blocked with 0.2 mol L^{-1} ethanolamine for 1 h and then washed exhaustively on a core sand funnel with 200 mL of PBS. The unbound amino group on the CPG was blocked with 8 mL of 1% OVA in coupling buffer for 1 h. The beads were finally washed on a core sand funnel with coupling buffer, followed by the buffer used for the ATZ determination. The solid support with the immobilized antibodies was stored in PBS at 4°C until use.

2.5 Measuring procedure

ATZ determination was based on the inhibition of immobilized β -lactamase in the presence of its substrate penicillin. Penicillin was catalyzed, and the reaction caused a change in enthalpy, which led to an increase in the peak signal recorded by the data system. The enzymatic activity decreased in the presence of increasing amounts of ATZ and a constant substrate concentration. Therefore, a smaller change in enthalpy occurred, and another small change in the peak signal of the sensor was measured. CPG beads with the immobilized anti-ATZ mAb were packed in an enzyme column in dcTELISA. A Wheatstone bridge was used to monitor the change in the enthalpy signal.

Inhibition measurements, shown schematically in Fig. 2, were performed according to the following procedure: a carrier solution of PBS that contained 2% DMSO was initially pumped into the system using a peristaltic pump to obtain a baseline. The substrate solution was injected manually through a six-channel valve to evaluate background enthalpy change, followed by sample solution and substrate solution injection to determine the residual enzymatic activity.

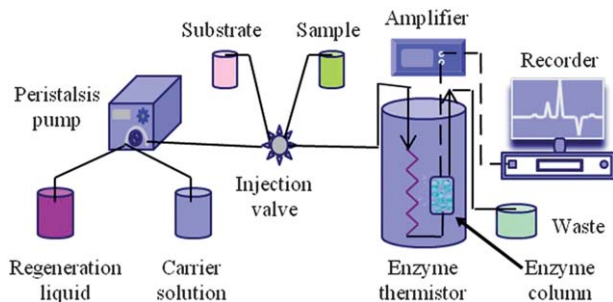


Fig. 1 dcTELISA manifold for ATZ determination.

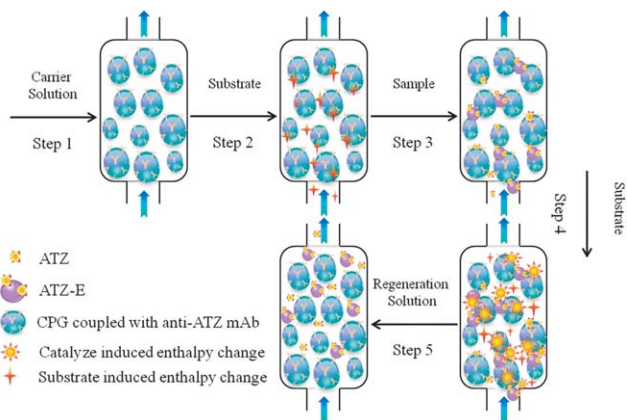


Fig. 2 Schematics of the inhibition procedure in an immunoreactor. Carrier solution flow for a baseline (step 1); substrate injection for the background value (step 2); sample injection for direct competitive reaction (step 3); substrate injection to reveal residual enzyme activity (step 4); and regeneration solution injection for the desorption of the analyte and ATZ-E (step 5).

After obtaining the peak, the reactor was washed to desorb the analyte and ATZ-E by passing through 0.1 mol L^{-1} Gly-HCl (pH 2.3) buffer that contained 1% DMSO (v/v) and 0.1% Triton X-100 (v/v). The immunoreactor exhibited remarkable reproducibility, and the system was usable for more than 4 months. The enzyme column was stored in the carrier solution at 4°C when not in use.

The experimental signals were normalized using eqn (2)

$$\text{Normalized response} = \frac{B - B_\infty}{B_0 - B_\infty} \quad (2)$$

where B is the signal (peak height) measured in the presence of increasing analyte concentration, B_∞ is the background signal obtained in the presence of an excess of ATZ, and B_0 is the signal in the absence of ATZ. The normalized response was plotted as a function of the analyte concentration, and the experimental data were fitted to eqn (3)

$$\text{Inhibition} = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{X}{X_0}\right)^{AP}} \quad (3)$$

2.6 Selectivity studies

Cross-reactivity studies were performed by measuring the competitive inhibition curves for other chemically related and unrelated pesticides under optimized conditions. The cross-reactivity rate (CR) was calculated as the percentage of the IC_{50} for the ATZ over the IC_{50} for the interfering compounds according to eqn (4)

$$\%CR = \frac{\text{IC}_{50}(\text{ATR})}{\text{IC}_{50} \text{ of cross-reacting compound}} \times 100 \quad (4)$$

where %CR is the percentage of cross-reactivity.

2.7 Analysis of spiked corn stalk fodder samples

ATZ determination was conducted in spiked corn stalk fresh cut and silage samples. The homogenate samples of chopped corn

stalk fresh cut and silage (1 g) were spiked with $50 \mu\text{L}$ of increasing ATZ concentrations (0.075 , 0.150 , and 0.300 g L^{-1}) in DMSO. The samples were mixed with 5 mL of PBS that contained 10% DMSO, mixed with an oscillator for 5 min, and equilibrated for at least 30 min. The supernates were collected. The pH was adjusted to 7.4 using 1 mol L^{-1} hydrochloric acid, and filtered through a $0.22 \mu\text{m}$ PTFE filter (Phenomenex, Torrance, CA) to remove suspended matter. Blank samples were prepared following the previous procedure without pesticide spiking. The samples were also checked to exclude the presence of naturally contaminating herbicide and then spiked with increasing ATZ concentrations to evaluate the matrix effect on the dcTELISA.

The samples were also analyzed *via* HPLC for validation. The homogenate samples of chopped corn stalk fresh cut and silage (1 g) were respectively spiked with $42 \mu\text{L}$ of increasing ATZ concentrations (0.075 , 0.150 , and 0.300 g L^{-1}) in methanol. After shaking for 1 min, chloroform (CHCl_3 , 5 mL) was added. The mixtures were extracted with ultrasonication for 15 min and then centrifuged at 4000 rpm for 5 min. The supernate was collected. The liquid was dried *via* mild N_2 drying, and 70% methanol-water (5 mL) was subsequently added. The samples were continuously shaken until the solid was completely dissolved. Blank samples were prepared as described above without ATZ spiking. The liquids were filtered with a $0.22 \mu\text{m}$ PTFE filter, followed by HPLC detection (Waters Alliance 2695, USA). The optimized chromatographic conditions were as follows: column, C_{18} ; column temperature, 25°C ; mobile phase, methanol-water ($70:30$, v/v); flow rate, 1.0 mL min^{-1} ; and detection wavelength, 224 nm . The analysis was performed in triplicate.

3 Results and discussion

3.1 ATZ-E conjugate verification

The molecular weights of β -lactamase and ATZ-E conjugate were 33 757 and 34 141, as determined by MS (Fig. 3). The coupling ratio was approximately 2 : 1. The ATZ was changed into ATZ-E. Thus, the ATZ-E conjugate was successfully prepared.

3.2 Optimization of experimental conditions

Several parameters that affected the performance of the dcTELISA were evaluated, such as concentration and nature of the enzyme substrate, ATZ-E concentration, efficiency of the regeneration liquid, effect of temperature on the sensor response, effect of organic solvent DMSO in the buffer solution, flow rate, and interval between two immediate steps during detection.

ATZ detection was performed using penicillin as a substrate. Ampicillin trihydrate had been previously tested for assay development. The thermal signals obtained with 2 mmol L^{-1} ampicillin trihydrate were 49.6 mV on average, which was lower than 62.8 mV on average with 2 mmol L^{-1} penicillin. Moreover, the latter was more cost-effective and was consequently selected as the enzyme substrate.

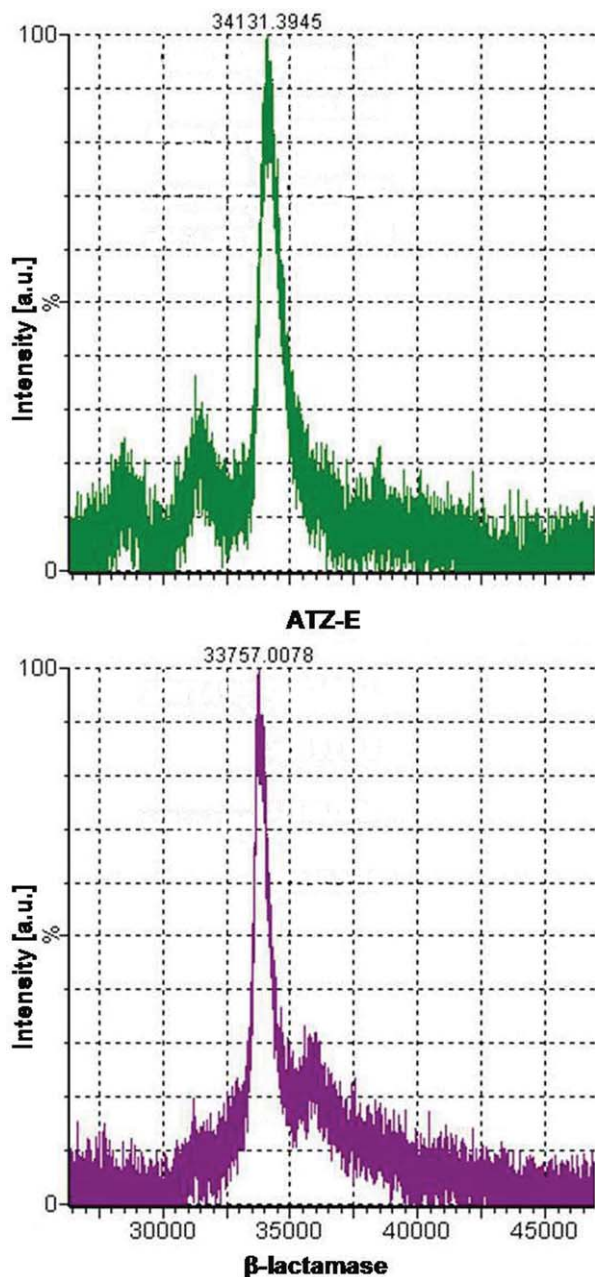


Fig. 3 Mass spectra of the ATZ-E conjugate and β -lactamase.

With constant ATZ-E concentration, various concentrations of penicillin were assessed for maximal enthalpy values (peak height). The penicillin concentration was tested from 2 mmol L^{-1} to 32 mmol L^{-1} . The maximal signal was achieved at 16 mmol L^{-1} . Higher concentrations yielded a constant peak height. The reproducibility of the biosensor response in terms of the relative standard deviation for repeated injections of 16 mmol L^{-1} of penicillin was 5% ($n = 3$).

The effect of the ATZ-E concentration was investigated from 0.05 g L^{-1} to 1 g L^{-1} , using a 16 mmol L^{-1} substrate concentration. The amount of ATZ-E applied in the assay should be kept low enough to achieve good sensitivity, but must be

sufficient to provide an acceptable signal. The catalytic capacity increased with increasing ATZ-E concentration. An ATZ-E concentration of 0.2 g L^{-1} was chosen as the compromise between sensitivity and efficacy.

Effective Ag dissociation and Ab regeneration are the most important factors in the reuse of the Ab in the biosensor application. The binding efficiency and interaction between the Ag and Ab vary with each Ag-Ab complex. Gly-HCl (pH 2.3) buffer that contains 1% DMSO (v/v) is a highly efficient dissociation buffer, which enables multiple reuse of the immobilized Ab for biosensor applications.¹¹ In this study, we observed that the combination of Gly-HCl (pH 2.3) buffer that contains 1% DMSO (v/v) with 0.1% Triton X-100 (v/v) showed a comparatively better dissociation for the ATZ-Ag-Ab complex.

The effect of DMSO in the carrier solution was tested from 1% (v/v) to 5%, and 2% was chosen as a compromise between solubility enhancement and mAb-injury minimization.

Considering that temperature influences both the conjugation property of mAb and the enzymatic activity of β -lactamase during sensor response, 37 °C was chosen as a compromise. An optimum flow rate of 0.8 mL min^{-1} was selected as the compromise between the peak height and the contact time of the substrate with the immobilized enzyme. The selected sample injection volume was 0.37 mL. The optimized dcTELISA

Table 1 Optimized parameters for ATZ analysis based on dcTELISA

Parameter	Optimized value
ATZ-E concentration (g L^{-1})	0.2
Substrate and concentration (mmol L^{-1})	Penicillin 16
Carrier solution	PBS (pH 2.3) 0.01 mol L^{-1} containing 2% DMSO (v/v)
Regeneration solution	Gly-HCl (pH 2.3) 0.1 mol L^{-1} containing 1% DMSO (v/v) with 0.1% Triton X-100 (v/v)
Temperature (°C)	37
Flow rate (mL min^{-1})	0.8
Sample injection volume (mL)	0.37
Immunesupport	CPG

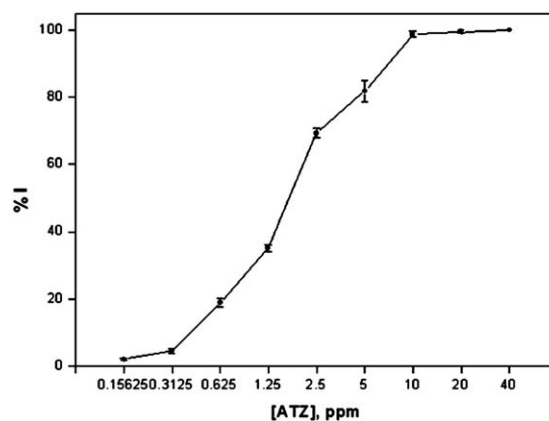


Fig. 4 Calibration curve for ATZ determination based on dcTELISA.

Table 2 Cross-reactivity of some s-triazines and their analogs with the ATZ biosensor

Compound	Structural formula	IC ₅₀ (mg L ⁻¹)	Cross-reactivity (%)
ATZ		1.51	100
SIM		4.15	36.39
Melamine		NC	NC
Tricyanic acid		NC	NC
Chlorpyrifos		NC	NC

parameters for ATZ determination are summarized in Table 1. With the given optimized parameters, the assay time per sample is 12 min.

3.3 Analytical characteristics of the sensor for ATZ determination

The biosensor that resulted from anti-ATZ mAb immobilization was tested for ATZ detection. ATZ and ATZ-E competed for the active binding site on the antibodies.

The calibration plot was obtained to determine ATZ (Fig. 4). The percentage inhibition increases linearly with increasing ATZ concentration that ranged from 0.7 mg L⁻¹ to 3.9 mg L⁻¹

(%I = 101.444 - $\frac{101.946}{1 + \left(\frac{X}{1.704}\right)^{1.602}}$, $R^2 = 0.9958$, each point was the average of three injections).

3.4 Cross-reactivity

Cross-reactivity was calculated as a percentage of the ratio between the IC₅₀ of ATZ and that of the interfering compound. The results are shown in Table 2.

The relative cross-reactivity was most pronounced in the other s-triazine, SIM. The high cross-reactivity exhibited by SIM could be attributed to the structural similarity of the ATZ and the SIM, except for an extra methyl group in the SIM. No recognition was obtained for melamine. These lower or zero CRs could be attributed to the presence of a free amino group that interfered in the formation of Ag-Ab immunocomplexes.¹² Furthermore, no tricyanic acid CR could be attributed to the lack of the chloride group (instead of -OH), which seems to be essential for good recognition of the s-triazines with the mAb. Unrelated pesticides, such as chlorpyrifos, were not recognized by the mAb, even though free chloride and ethyl groups were also present in their structures.

3.5 Corn stalk fodder samples spiked with ATZ

Immunoassays are known to suffer from matrix effects because of nonspecific binding and denaturation of the Ab and the enzyme, which results in false-positive responses.¹³ Fresh cut corn stalk and silage samples were spiked with increasing ATZ concentrations and analyzed using the optimized dcTELISA to investigate the matrix effect. The samples were checked previously and found to contain undetectable herbicide levels. Comparison of the repeatedly obtained signal using the blank matrix and that using Milli-Q water showed no statistically significant difference. Good recovery rates were also obtained with the spiked samples. The results are shown in Table 3(a) and (b). Thus, the proposed biosensor allows direct ATZ analysis in buffered fresh cut and silage corn stalk samples.

The samples were also analyzed by HPLC for validation. No significant differences were obtained (confidence level, 95%) in

Table 3 (a) ATZ analysis in corn stalk fresh cut samples using the biosensor developed (mean ± SD, $n = 3$); (b) ATZ analysis in corn stalk silage samples using the biosensor developed (mean ± SD, $n = 3$)

Spiked concentration (mg kg ⁻¹)	dcTELISA (mean ± SD ^a) (mg kg ⁻¹)	Recovery ratio (%)	HPLC (mean ± SD) (mg kg ⁻¹)	Recovery ratio (%)
(a)				
3.75	3.74 ± 0.08	99.68	3.71 ± 0.03	98.96
7.50	7.00 ± 0.07	93.31	8.20 ± 0.15	109.20
15.00	14.01 ± 0.22	93.39	15.83 ± 0.12	105.54
(b)				
3.75	3.61 ± 0.02	96.22	3.95 ± 0.07	105.35
7.50	6.61 ± 0.08	88.14	7.26 ± 0.12	96.81
15.00	16.08 ± 0.21	107.23	16.73 ± 0.65	111.54

^a Standard deviation.

any of the samples tested, which demonstrates the good performance of the optimized biosensor.

4 Conclusions

We described a dcTELISA-based fast and simple method for ATZ pesticide determination and successfully applied the method to spiked corn stalk fodder (fresh cut and silage).

The proposed method is advantageous because it does not depend on the optical properties of the sample, which evidently simplifies the sample pretreatment. The 12 min assay time per sample makes the dcTELISA suitable for fast detection of large-scale samples. The biosensor produces reproducible and stable responses even when used continuously for over 4 months.

The ATZ detection limit of the dcELISA was as low as 0.4 mg L⁻¹, which meets the USA requirement on the regulation of MRLs of ATZ. The β -lactamase-based biosensor was tested by analyzing ATZ-spiked corn stalk fodder samples, and recovery rates between 88% and 107% were obtained at different concentrations. The results of ATZ detection by dcTELISA were as good as those using HPLC. However, the LOD was relatively high compared with that of ELISA when we tested ATZ in the spiked food samples in our laboratory (data not shown). The difference may be attributed to the decreased activity of the specific mAb in the organic solvent during coupling of mAb to CPG.

Future studies may improve the sensitivity of the manifold and expansion to multichannel detection. The methods, which may increase the sensitivity of the manifold, can be based on SPA introduction, because it specifically and reversibly binds all immunoglobulin G subclasses through Fc domains. Coupled with CPG as the immunosupport, the new method may facilitate the retention of Ab activity. Moreover, the competitive binding reaction in the solution may ensure sufficient incubation time for Ag-Ab binding and facilitate Ab concentration optimization and sensitivity improvement. Meanwhile, problems related to fluid stability should be resolved. Furthermore, several Ab mixtures may be applied to construct a multichannel manifold to detect the total or respective pesticide residual toxicity of complex samples.

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References

- 1 G. G. Ying, R. S. Kookana and M. Mallavarpu, Release behavior of triazine residues in stabilised contaminated soils, *Environ. Pollut.*, 2005, **134**, 71–77.
- 2 G. A. Bennet, T. C. Nelsen and B. M. Miller, Enzyme-linked immunosorbent assay for detection of zearalenone in corn, wheat, and pig feed: collaborative study, *J. AOAC Int.*, 1994, **77**(6), 1500–1509.
- 3 D. Thouvenot and R. F. Morfin, Radioimmunoassay for zearalenone and zearalanol in human serum: production, properties, and use of porcine antibodies, *Appl. Environ. Microbiol.*, 1983, **45**(1), 16–23.
- 4 K. M. Cooper, L. Ribeiro, P. Alves, V. Vozikis, S. Tsitamis, G. Alfredsson, T. Lövgren, M. Tuomola, H. Takalo, A. Itia, S. S. Strerck and D. G. Kennedy, Interlaboratory ring test of time-resolved fluoroimmunoassays for zeranone and alpha-zearalenone and comparison with zeranone test kits, *Food Addit. Contam.*, 2003, **20**(9), 804–812.
- 5 M. A. Gonzalez-Martinez, S. Morais, R. Puchades, A. Maquieira, A. Abad and A. Montoya, Development of an automated controlled-pore glass flow-through immunosensor for carbaryl, *Anal. Chim. Acta*, 1997, **347**, 199–205.
- 6 M. A. Gonzalez-Martinez, R. Puchades, A. Maquieira, I. Ferrer, M. P. Macro and D. Barceló, Reversible immunosensor for the automatic determination of atrazine, selection and performance of three polyclonal antisera, *Anal. Chim. Acta*, 1999, **386**, 201–210.
- 7 C. Borrebaeck, J. Börjeson and B. Mattiasson, Thermometric enzyme linked immunosorbent assay in continuous flow system: optimization and evaluation using human serum albumin as a model system, *Clin. Chim. Acta*, 1978, **86**(3), 267–278.
- 8 W. J. Zhuang, W. Liu, J. M. Ye and W. I. Shan, *The Global Regulations On Maximum Residue Limits (MRLs) For Pesticides In Food Stuffs And Feedstuffs*, Chemical Industry Press, Peking, 1st edn, 2010, vol. 4, ch. 1, pp. 122–123.
- 9 N. Sai, Y. P. Chen, N. Liu, G. G. Yu, P. Su, Y. Feng, Z. J. Zhou, X. Y. Liu, H. Y. Zhou, Z. X. Gao and B. A. Ning, A sensitive immunoassay based on direct hapten coated format and biotin–streptavidin system, *Talanta*, 2010, **82**, 1113–1121.
- 10 H. H. Weetall, Covalent coupling methods for inorganic support materials, *Methods Enzymol.*, 1976, **44**, 134–148.
- 11 A. Farjam, A. E. Brugman, H. Lingeman and U. A. Brinkman, On-line immunoaffinity sample pre-treatment for column liquid chromatography: evaluation of desorption techniques and operating conditions using an anti-estrogen immuno-precolumn as a model system, *Analyst*, 1991, **116**(9), 891–896.
- 12 M. A. Gonzalez-Martinez, S. Morais, R. Puchades, A. Maquieira, A. Abad and A. Montoya, Development of an automated controlled-pore glass flow-through immunosensor for carbaryl, *Anal. Chim. Acta*, 1997, **347**, 199–205.
- 13 M. Hennion and D. Barcelo, Strengths and limitations of immunoassays for effective and efficient use for pesticide analysis in water samples: a review, *Anal. Chim. Acta*, 1998, **362**, 3–34.