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Immunoenzyme Assay of Zearalenone using Magnetic Nanoparticles and Chemiluminescent Detection

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Abstract

In this study, the competitive enzyme-linked immunosorbent assay of zearalenone, with magnetic nanoparticles (MNP) as a solid support and chemiluminescent detection of peroxidise label activity, was developed. Using magnetic nanoparticles allows for rendering the immunoassay in a pseudo-homogeneous format through the rapid formation of immune complexes in the solution, their rapid separation and their efficient heterogeneous detection. MNP provides a considerable increase of surface area for the immobilization of reagents and their uniform distribution over the reaction medium. Because of this, immunochemical interactions accelerated significantly. Instead of the traditional colorimetric detection of peroxidase-labeled specific complexes, highly sensitive chemiluminescent detection was used. In the developed assay, the detection limit of zearalenone was 0.04 ng/mL, and the duration was 30 min; the achieved characteristics significantly exceeded those of the conventional enzyme-linked immunosorbent assay. The applicability of the reported approach for mycotoxin detection in wheat was demonstrated. Thus, the developed method can be proposed as an analytical tool for the rapid control of mycotoxins.

Keywords: mycotoxins, zearalenone, ELISA, magnetic nanoparticles, chemiluminescent peroxidase reaction

INTRODUCTION

Nowadays, there is an urgent need to monitor food products that come into the market and ensure their compliance with quality standards. Contamination with mycotoxins, which are very toxic mould metabolites, significantly reduces the quality of food and animal feed and can have serious adverse effects on human and animal health [1, 2]. Human consumption of food contaminated with mycotoxins leads to the development of both acute intoxication and chronic life-threatening diseases [3]. The presence of mycotoxins in animal feed is one of the main reasons for the deterioration of farm animals' health, violation of their reproductive functions, decrease in productive parameters and immune status [4, 5]. Both of these events lead to large-scale economic damage (only in respect of agriculture) due to the costs of the prevention and treatment of animals, decontamination of the harvest, organization of control measures, etc. Therefore, it is necessary to develop effective analytical methods for mycotoxin detection and apply them at all stages of the process chain – from harvesting and storage to the sales of finished products.

According to the main requirements for the analysis of food contaminants (high sensitivity and specificity), immunochemical methods based on highly affine antigen-antibody interactions seem to be very promising for determining mycotoxin content [6]. Microplate enzyme-linked immunosorbent assay (ELISA) is the most widely used immunochemical method for the detection of mycotoxins [6-8]. The main advantages of this ELISA are ease of implementation, small volume of tested samples, low cost of used reactants and equipment and the ability to simultaneously test a large number of samples. However, the main limitation of the microplate ELISA is the necessity of prolonged incubations in order to facilitate heterogeneous interactions.

In our previous study, we used magnetic particles as a solid phase for antibody immobilization in the detection of aflatoxin B1 [9]. ELISA is implemented in the pseudo-homogeneous mode when immobilized antibodies react with native and labelled antigens in solution, allowing for a significant reduction in interaction time without impairing the analytical characteristics. The adsorption of immunoglobulins on the surface of magnetic nanoparticles increases protein stability in aqueous-organic media, thus minimizing the degree of sample dilution required. As a result, we achieved a detection limit of 20 pg/mL for aflatoxin B1 and a total assay duration of 20 min. In this study, we also applied the developed technique for the detection of another mycotoxin – zearalenone (ZEN), which is one of the relevant contaminants in food and agricultural products containing grains [10].

The ELISA of zearalenone has been developed and reported in a number of studies; see, for example, [11-13]. Besides the conventional microplate ELISA schemes with colorimetric detection of the enzyme label (usually, horseradish peroxidise, HRP), some original approaches for the high-sensitive detection of ZEN were also proposed. Zhang *et al*. developed fluorescent ELISA with catalase as an enzyme label and CdTe quantum dots for signal transduction [14]; the achieved detection limit was 4.1 pg/mL. Wang *et al.* used the variable domain of heavy-chain antibodies (VHH) to

produce anti-idiotypic antibodies that act as non-toxic mycotoxin-surrogate reagents in immunoassay [15]; this ELISA demonstrated a ZEN detection limit of 0.08 ng/mL. A similar approach was applied in the study of Wang *et al.* to determine the content of zearalenone in corn [16]. The authors used non-toxic single-stranded DNA aptamer mimicking ZEN epitopes and achieved a detection limit of 0.01 ng/mL.

Currently, only a few ELISA of mycotoxins based on magnetic nanoparticles (MNP–ELISA) can be found in the literature, including our previous work. Ochratoxin A, aflatoxins B1 and M1 and deoxynivalenol are among the mycotoxins detected by this approach [17-20]. Only one study deals with zearalenone [21]. Zhang *et al.* performed ELISA with magnetic nanoparticles and biotin/streptavidin–HRP conjugate to detect zearalenone in cereal and feed. As an enzyme label, peroxidase was used. The detection limit of ZEN was 0.04 ng/mL.

In this study, a competitive immunoenzyme assay based on magnetic nanoparticles and chemiluminescent detection of the peroxidase label was developed for zearalenone. Instead of the traditional chromogenic substrates of the peroxidase label, we use a highly sensitive chemiluminescent luminol-based substrate for the detection of specific complexes. Luminol oxidation catalysed by HRP results in a cascade of redox reactions, causing the formation of unstable products, including 3-aminophthalate. Upon its transition from electronically excited to a stable state, visible light is emitted [22]. The unique enhancer in the used substrate results in rapid kinetic light output and high signal intensity. Among the obvious advantages of chemiluminescence analysis, a very low ‘noise-to-signal’ ratio is notable.

The substitution of chemiluminescent measurements (instead of colorimetric ones) is known to cause a substantial enhancement in ELISA sensitivity [23]. However, the format of MNP–ELISA, with the chemiluminescent detection of zearalenone, was proposed for the first time in this study. This assay mode helped us to improve sensitivity and rapidity and avoid using toxic chromogenic HRP substrates.

EXPERIMENTAL

Materials, reagents and equipment

Zearalenone (ZEN; Khromresurs, Russia), 3,3′,5,5′-tetramethylbenzidine (TMB), Triton X-100, iron(III) chloride, iron(II) chloride, ethanolamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) (Sigma–Aldrich, USA), bovine serum albumin (BSA; MP Biomedicals, USA) and luminol-based SuperSignal® ELISA Pico Chemiluminescent Substrate (Thermo Scientific, USA) were used. Monoclonal antibodies against zearalenone were from the All-Russian Center of Molecular Diagnostics and Therapy (Moscow, Russia), and zearalenone conjugated with horseradish peroxidase (ZEN–HRP) was from the State Research Center for Applied Microbiology & Biotechnology (Obolensk, Russia). All other reagents were of analytical or chemical purity.

A MagnetoPURE 96 magnetic separator (Chemicell, Germany) was used for the separation of magnetic nanoparticles in 96-well plates. A neodymium magnet (MAGNET-MSCs, Russia) was used for magnetic separations in tubes. For ELISA, Costar 9018 transparent microplates (Corning, USA) and MaxiSorp black microplates (Nunc, Denmark) were used for colorimetric and chemiluminescent detection, respectively. Optical density and chemiluminescence were measured using a microplate photometer Zenyth 3100 (Anthos Labtec Instruments, Austria).

Synthesis of magnetic nanoparticles (MNP)

MNP were synthesized according to [24-25], with slight modifications. An aqueous 0.5% solution of iron (III) and (II) chlorides in a molar ratio of 2:1 was prepared. Then, a 30% ammonia hydrate solution was added dropwise to a final concentration of 8%. The resultant mixture was incubated for 15 min at room temperature with stirring. The obtained particles were collected with a magnet and, after removal of the supernatant, resuspended in bidistilled water, washed five times with excess distilled water and washed one time with 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM NaCl (phosphate-buffered saline, PBS). The suspension of MNP was stored at 4 °C. The obtained preparation was stable for at least three months. The concentration of the obtained MNP suspension was determined according to Urusov *et al*. [9].

Absorption of antibodies on magnetic nanoparticles

The immobilization of antibodies on MNP by physical adsorption was carried out as described previously [9]. Briefly, 500 µL of MNPs in PBS at 3 mg/mL was mixed with a solution of anti-ZEN antibodies (2 mg/mL) to obtain a final antibody concentration of 70 µg/mL. The mixture was incubated for 30 min with vigorous stirring. Then, MNP were collected with a magnet and washed three times with PBS. The resulting suspension was stored at 4 °C.

Covalent immobilization of antibodies on magnetic nanoparticles

The immobilisation of antibodies on MNP was carried out by a two-step procedure according to Puertas *et al.* [26]. First, MNP coated with oleic acid were prepared. For this purpose, 2 mL of MNP suspension in PBS at a concentration of 3 mg/mL was separated by a magnetic field, and 2 mL of oleic acid was added. The mixture was incubated for 30 min at room temperature with stirring. Then, the modified particles were separated by a magnetic field and washed three times with PBS.

Solutions of EDC and NHS in bidistilled water were added to 500 µg of oleic acid-coated MNP, making the final concentration of each activator 2.5 mg/mL. Then, a solution of anti-ZEN antibodies was added to the final concentration of 500 µg/mL, and the mixture was incubated for 2 h at room temperature with stirring. The unreacted activated groups on the surface of the MNP were blocked by incubation with 100 mM ethanolamine solution for 10 min in the same mode. Particles were deposited by means of magnetic field and washed three times with PBS.

Preparation of plant extracts

Milled wheat grains were mixed with an extraction solution (methanol–water, 70:30) at a ratio of 1:5 and incubated with gentle stirring at room temperature for 2 h (in accordance with [27], with modifications). After centrifugation, the supernatant was collected and stored at 4 ºC. The extracts were analysed by HPLC according to [28], and no zearalenone was detected.

Evaluation of antigen-binding properties of the antibody–MNP conjugates

First, 50 µL of ZEN−peroxidase conjugate in PBS containing 0.05% Triton X-100 and 0.1% BSA (PBST-BSA) at several dilutions between 500 and 0.05 ng/mL was added to the microplate wells. Then, 50 µL of antibody–MNP conjugates at concentrations of 50, 25, 10 or 5 µg/mL in PBST-BSA was added to the wells. The resulting solution was stirred for 30 min at room temperature. Then, the MNP were collected by magnet and washed four times with 100 µL of PBST-BSA and one time with distilled water.

The formed immune complexes were detected by chemiluminescent peroxidase reaction. For this purpose, 100 µL of SuperSignal® substrate was added to each well, and the chemiluminescence was immediately measured.

MNP–ELISA of zearalenone

First, 50 µL of ZEN in PBST-BSA at several dilutions between 10 and 0.001 ng/mL was added to the microplate wells. Alternatively, an extraction solution or extract spiked with ZEN (10–0.001 ng/ml) was added. Then, 50 µL of ZEN–HRP conjugate (75 ng/mL in PBST-BSA) was added to the wells. The resulting solution was stirred for 10 s, and 50 µL of the MNP-antibody conjugate at a concentration of 25 µg/mL (based on the MNP concentration) in PBST-BSA was added to the wells. The incubation was performed at room temperature with stirring. The incubation time was varied for 5–30 min, and finally, 30 min incubation was chosen for the analysis. The MNP were collected by magnet and washed four times with 100 µL of PBST-BSA and one time with distilled water. Peroxidase activity was measured by chemiluminescent reaction, as described above. For colorimetric detection, 100 µL of substrate solution containing 0.42 mM TMB and 1.8 mM H_2O_2 in 0.1 M citrate buffer, pH 4.0, was added to wells. Then, the microplate was incubated for 15 min at room temperature. The reaction was stopped by adding 1 M H_2SO_4 (50 µL per well). The optical density of the reaction product was measured at 450 nm.

The plots of the relative chemiluminescent units or optical density (y) on the antigen concentration (x) were fitted using the Origin 7.5 software (OriginLab, USA) to a four-parameter logistic function:

$$y = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p},$$

where A_1 is the maximal optical density in the absence of the antigen, A_2 is the minimal optical density, p is the slope of the calibration curve and x_0 is the antigen concentration causing 50% inhibition of the antibody binding (IC_{50}).

The antigen concentration resulting in 10% inhibition (IC_{10}) was calculated by the following formula:

$$IC_{10} = \left(\frac{1}{9}\right)^{\frac{1}{p}} * x_0$$

and then regarded as the detection limit of the assay [29].

RESULTS AND DISCUSSION

Synthesis and characterization of magnetic nanoparticles and their conjugates with anti-ZEN antibodies

In this study, a co-precipitation technique was used to obtain magnetic nanoparticles from iron chlorides (II) and (III). In order to determine the size and aggregation state of nanoparticles, the transmission electron microscopic (TEM) study was performed. The TEM investigation indicated that the obtained suspension consisted of aggregates of various sizes – from ca. 35×50 nm to 300×600 nm – formed by individual particles of 10–15 nm diameter with a shape close to spherical (Fig. 1). It should be noted that small sizes of MNP enhance the stability of their aqueous suspension and lead to the increase of the total surface area of the respective antibody–MNP conjugates for interaction with mycotoxin molecules.

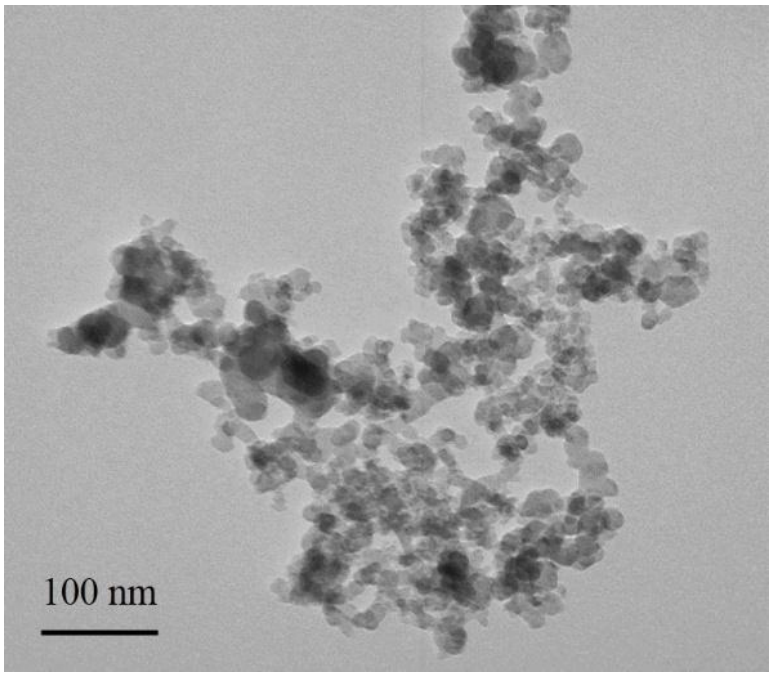

Figure 1. Microphotograph of the magnetic nanoparticles.

In our previous study, a comparative study of antibody–MNP conjugates prepared by the physical adsorption of antibodies against mycotoxin aflatoxin B1 on native MNP and their covalent binding to oleic acid-coated MNP was performed [30]. It was demonstrated that, in the latter case, the detection limits of aflatoxin were somewhat lower, namely 2.6 and 0.4 ng/mL, respectively. However, the one-step procedure of physical adsorption is much easier and less time-consuming. Therefore, for MNP conjugation with antibodies, we initially used a physical adsorption as the simplest method of immobilization based on the hydrophobic, electrostatic and van-der-Walls forces between nanoparticles' surfaces and antibodies. For this purpose, a plain short-term incubation of nanoparticles with immunoglobulins was carried out.

A more complex method of covalent antibody–MNP binding is based on the formation of amide bonds between primary amines of immunoglobulins and carboxyl groups introduced into nanoparticles' surfaces. Amphiphilic oleic acid is used to form multilayers on the particle surface [31]. Thus, the conjugates were formed using EDC and NHS activators [32].

To study the antigen-binding properties of the prepared immunosorbents, they were incubated at the same fixed concentration (namely, 5, 10, 25 or 50 μg/mL), with the ZEN−peroxidase conjugate at different dilutions. After the incubation and washing, the catalytic activity of the bound enzyme label was measured with the help of the SuperSignal® substrate. The obtained dependencies showed that the antigen-binding properties of immunosorbents obtained by two approaches substantially differed: in the case of the antibody–MNP conjugate prepared by adsorption, antigen-binding properties were incomparably lower. Thus, at the ZEN–HRP concentration, which was further applied for competitive MNP–ELISA (of about 100 ng/mL), the chemiluminescence signals were >100000 and <100 relative units in the case of covalent and non-covalent conjugates (data not shown). As the latter value was not satisfactory for further implementation of the high-sensitive immunoassay, the covalent antibody–MNP conjugate was applied in MNP–ELISA. The concentration dependencies of the interaction of ZEN–HRP conjugate with covalent antibody–MNP conjugate are shown in Fig. 2. For the ELISA, a 25 μg/mL concentration of antibody–MNP conjugate (providing high amplitude of chemiluminescent signal) was chosen.

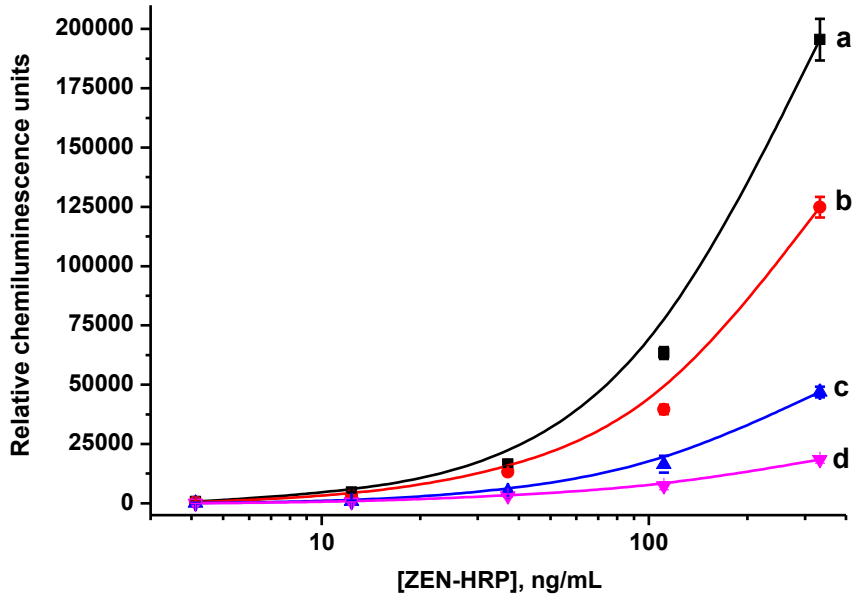

Figure 2. Concentration dependency of the interaction of ZEN–HRP conjugate with antibody–MNP conjugate at concentrations of 50 (a), 25 (b), 10 (c), and 5 (d) μg/mL (n=3).

Principle of the assay

Both homogeneous and heterogeneous kinds of immunoassay have a number of drawbacks. A convenient microplate ELISA is characterized by a long duration of stages (up to several hours), which is attributed to the heterogeneous interaction between reagents immobilized in microplate wells and solution. The possibility to decrease the assay duration is limited by the slow diffusion of dissolved reagents to a solid support [33]. In diffusion unlimited homogeneous systems, the formed immune complexes cannot be separated from unbound molecules and, thus, avoid the influence of the matrix effect when analyzing biological probes.

In the proposed MNP–ELISA, these circumstances are partially overcome. MNP, themselves, serve as a solid support by means of which the formed immune complexes can be simply and rapidly separated from unbound components by a magnetic field and washed. The main advantage of MNP as a separating support is a large surface area for antibody immobilization and uniform distribution throughout the whole volume of the reaction medium. Therefore, the developed pseudo-homogeneous immunoassay consists of the following stages: (a) competitive interaction of free ZEN and ZEN−peroxidase conjugates for antigen-binding sites on the surfaces of MNP; (b) magnetic separation; (c) washing of the MNP−antibody−mycotoxin complexes from unreacted components; and (d) interaction with the substrate in order to detect the activity of the bound peroxidase marker.

Development of the MNP–ELISA

The development of the ELISA included the selection of the assay regime, namely optimization of specific components' concentrations and duration of the immune interactions, in order to decrease the detection limit of zearalenone while retaining a high amplitude of the detected signal. Competitive curves (chemiluminescence *versus* ZEN concentration) for different incubation times are presented in Fig. 3.

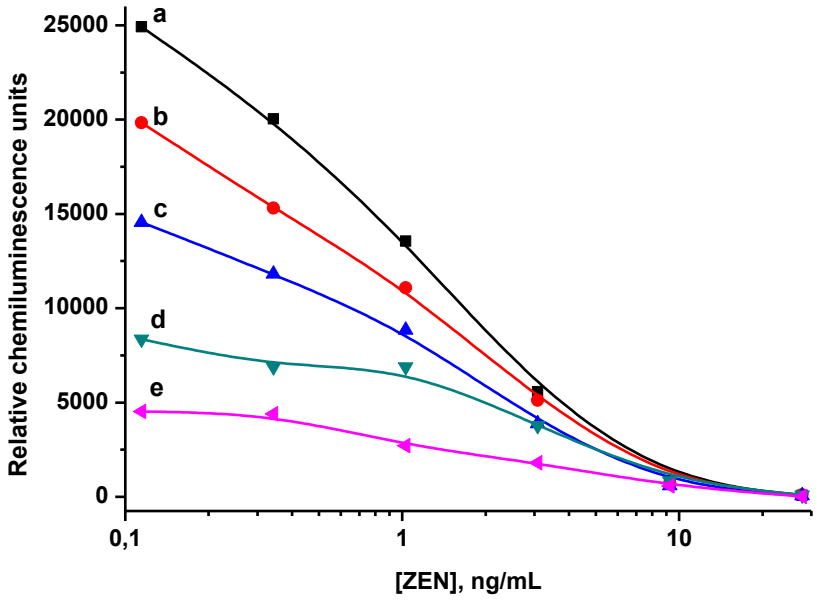

Figure 3. Competitive curves for MNP–ELISA of ZEN with the incubation time of 30 (a), 20 (b), 15 (c), 10 (d), and 5 (e) min.

The detection limits of zearalenone (presented in Table 1) were comparable in terms of 20 and 30 min durations of the interaction. However, when incubating immunoreagents for 30 min, chemiluminescence increased up to 25%. Therefore, the competitive interaction was further prolonged for 30 min.

Table 1. Limits of ZEN detection by the developed MNP–ELISA for different incubation times.

Incubation time, min	IC_{10}, ng/mL
5	0.2
10	0.8
15	0.2
20	0.1
30	0.07

The parameters of MNP–ELISA were compared when using three concentrations of ZEN–HRP conjugate, namely 100, 75 and 50 ng/mL. The corresponding competitive curves are demonstrated in Fig. 4. ZEN detection limits are given in Table 2. The lowest detection limit of ZEN, 0.04 ng/mL, was achieved using ZEN–HRP at the concentration of 75 ng/mL.

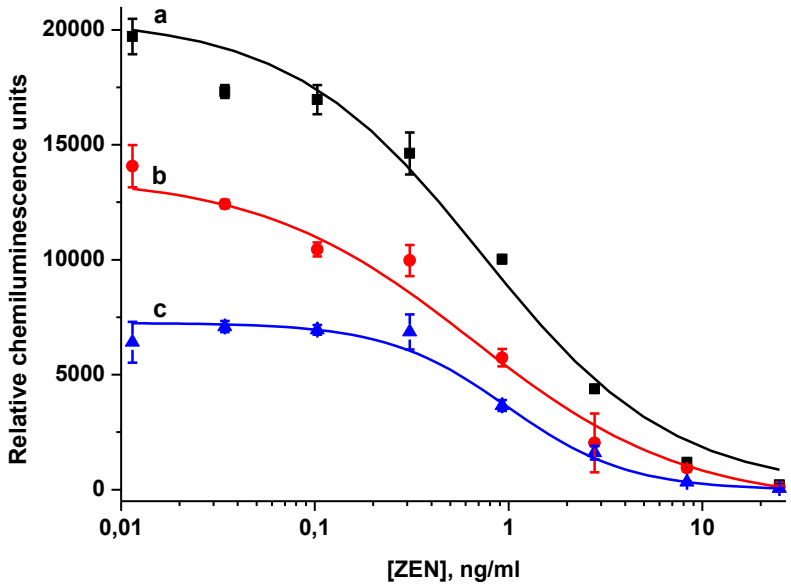

Figure 4. Competitive curves for the MNP–ELISA of ZEN. The ZEN–HRP concentration is 100 (a), 75 (b), 50 (c) ng/mL. The concentration of antibody–MNP conjugate was 25 µg/mL (n=3).

Table 2. Limits of ZEN detection by the developed MNP–ELISA for different concentrations of ZEN–HRP conjugate.

ZEN–HRP, ng/mL	IC_{10}, ng/mL
50	0.2
75	0.04
100	0.06

Using the same immunoreagents, MNP–ELISA with conventional colorimetric detection was implemented. The detection limit calculated from the obtained competitive curve (Fig. 5) was 1.5 ng/mL. An almost 40-fold gain in the assay sensitivity is attributed to the chemiluminescent detection of the label. A significant decrease in the detection limit after application of chemuliminescence reaction for the measurement of enzymatic activity of peroxidase conjugates (instead of chromogenic substrate oxidation) was described earlier – for example, for ochratoxin A immunodetection [23].

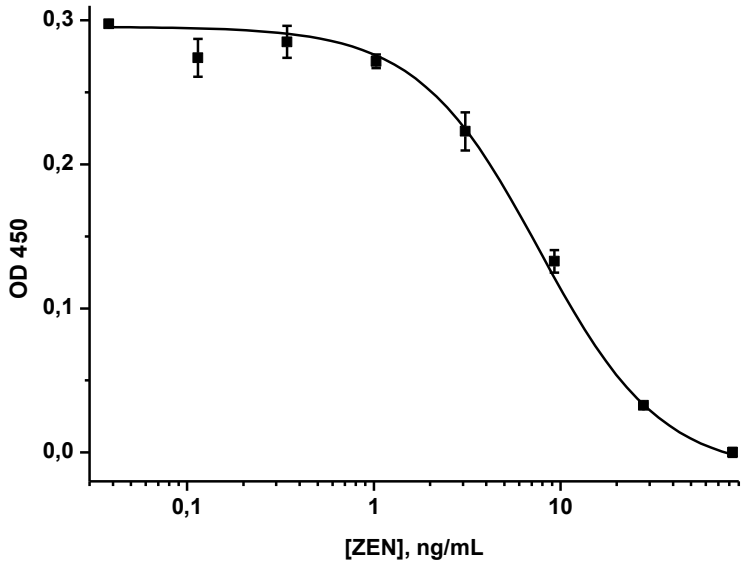

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Figure 5. Competitive curve for the MNP–ELISA of ZEN with colorimetric detection. Concentrations of ZEN–HRP and antibody–MNP conjugates were 20 ng/mL and 25 μg/mL, respectively (n=3).

During the development of MNP–ELISA, a comparison of this format with the traditional direct microplate assay was also performed with the chemiluminescent detection of the enzyme label. In this scheme of the ELISA, free analytes and ZEN–HRP conjugate in solution competitively interacted with specific antibodies immobilized in the microplate wells. The detection limit of zearalenone was 0.4 ng/mL, and the assay duration was 1.5 h. Therefore, the application of magnetic nanoparticles provided a 3-fold acceleration of total assaying time, as compared with common heterogeneous analysis, and a 10-fold increase of its sensitivity. The final characteristics of different ELISAs are presented in Table 3.

Table 3. Analytical characteristics of ELISAs of ZEN.

ELISA format	IC_{10}, ng/mL	Time of the analysis, min
Chemiluminescent MNP–ELISA	0.04	30
Colorimetric MNP–ELISA	1.5	30
Direct chemiluminescent ELISA	0.4	90

Detection of ZEN in plant extracts

The developed analytical system was tested for ZEN detection in a plant matrix. For this purpose, a grinded wheat sample (determined to be free of mycotoxin by HPLC analysis) was treated by a water-organic extraction mixture. Then, the prepared extract was spiked with various concentrations of ZEN and used in the MNP–EILSA,

with under 10% final content of methanol in the reaction medium. The extraction solution without a matrix was also spiked and used in control experiments.

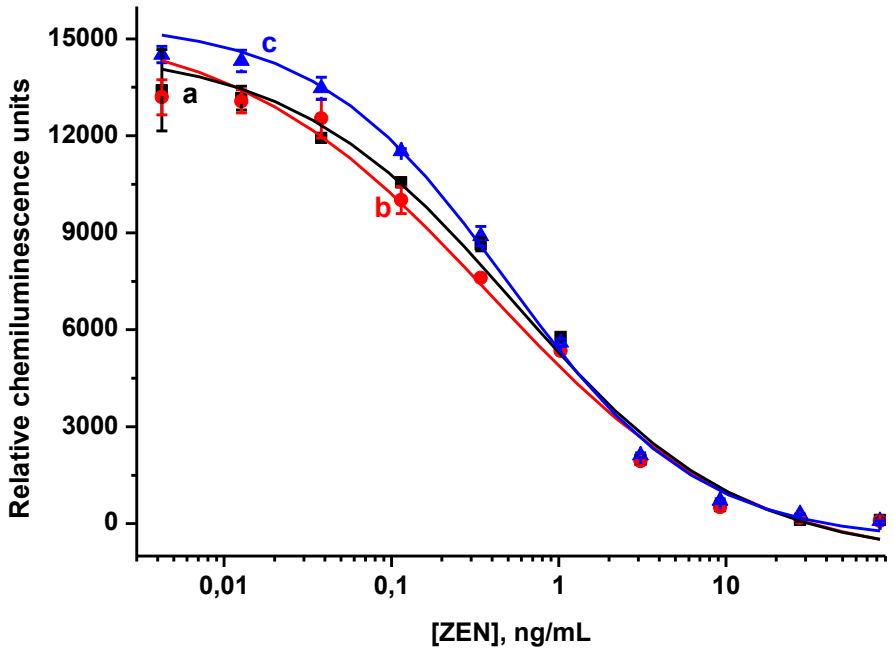

Figure 6. Competitive curves of zearalenone for MNP–ELISA in PBST-BSA (a), extraction solution (b), and wheat extract (c).

Fig. 6 shows the competitive curves of ZEN in standard conditions, an extraction solution and a plant extract. The detection limit and working range for ZEN detection did not reliably differ for all three samples. Neither the organic component from the extraction solution nor the biological matrix of the probe influenced the immune interactions; there was no shifting in the amplitude of the analytical signal. The detection limit of zearalenone in the extract was 0.04 ng/mL; it accords to 0.24 ng of ZEN per g of wheat. Taking into account that the maximum permitted levels of ZEN in different food stuffs are 50–200 ng/g (Commission Regulation [EC] No. 1881/2016), the developed assay can be recommended as a sensitive and rapid method for controlling zearalenone contamination.

CONCLUSION

A pseudo-homogeneous MNP–ELISA, with chemiluminescent detection for the rapid and sensitive determination of mycotoxin zearalenone, was developed. When compared with conventional microplate chemiluminescent ELISA, the detection limit of 0.04 ng/mL and total assay time of 30 min demonstrated a 10-fold gain in the sensitivity and 3-fold reduction in the duration. The developed approach is suitable for ZEN detection in real plant samples (with the detection limit of 0.24 ng/g). The

universal nature of the proposed approach allows its consideration as an effective tool for the rapid testing of various contaminants.

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