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Zearalenone-selective biomimetic-based sensor system and its validation for real samples' analysis

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Aim. The paper presents the calibration and validation of a recent biomimetic-based fluorescent sensor system for the analysis of zearalenone (ZON) contamination in cereals. **Methods.** ZON-specific biomimetic sensing elements in a form of molecularly imprinted polymer (MIP) membrane were prepared by the *in-situ* polymerization method using cyclododecyl-2,4-dihydroxybenzoate as a dummy template molecule and ethylene glycol methacrylate phosphate (EGMP) as a functional monomer. The detection is based on the fluorescence of ZON selectively adsorbed on the MIP membrane surface, which is registered by the spectrofluorimeter. **Results.** A biomimetic-based sensor system was calibrated for zearalenone analysis in cereal food products. The working range of the sensor system as well as its detection limit and the selectivity were examined. The calibrated biomimetic-based sensor system was successfully validated using maize and wheat flour with mean recoveries about 109 %. **Conclusions.** Calibration and validation studies of the novel ZON-selective biomimetic-based sensor system were successfully performed in cooperation with SE Ukrmetrteststandard. The standardized biomimetic-based fluorescent sensor system holds great potential to provide reliable prevention instrument against ZON contamination in cereals-based food products and feeding stuff.

K e y w o r d s: zearalenone, molecularly imprinted polymer membranes, biomimetic sensor system

Introduction

Annually, fungal diseases cause 1.6 million deaths among the farm animals due to the animal feed contamination with mycotoxins [1]. As a result, annual economic losses in developing countries caused by the impact of mycotoxins on the livestock industry are more than 5 billion $ [2]. Forages can be contaminated with varying mycotoxins, toxic secondary metabolism compounds produced by microscopic fungal species such as *Fusarium* spp. [3], *Penicillium* spp. [4] and *Aspergillus* spp. [5]. However, among all mycotoxins, the most threatening farmers all over the globe is zearalenone (ZON) because of its toxicity and wide distribution in forages [6]. Zearalenone produced by *Fusarium* spp. (*F. graminearum*, *F. culmorum*, *F. cerealis*) is distributed worldwide and naturally found in cereals crops, particularly in maize [7]. According to the results obtained in 2020, the most prevalent mycotoxins globally were *Fusarium* mycotoxins, and 43 % of the samples tested were positive on zearalenone [8].

Consumption of feed contaminated with ZON can cause deleterious effects in farm animals such as pigs and chicken. Thus, zearalenone shows strong estrogenic effect [9] due to its structural similarity to 17-β-estradiol [10]. The estrogenic-like effect of ZON results in sterility, feminization of male animals, decrease in the survival of embryos, leading to significant economic losses among farmers [7]. Moreover, ZON was found to exhibit hepatotoxic [11], hematotoxic [7], immunotoxic [12], and genotoxic [13] effects. It is known that ZON is heat stable (up to 150 °C) and does not degrade during feed processing [14]. More importantly, there is no effective antidote for this toxin [15]. So, the primary way to prevent zearalenone exposure is the ZON control in the cereal-based food and forages.

Until now, HPLC (high-performance liquid chromatography) with fluorescent detection has been considered as the gold standard for mycotoxins monitoring in food and feed [16]. Nevertheless, this method requires expensive instrumentation, extensive sample preparation, and well-trained personnel [17]. Therefore, varying analytical methods were developed for ZON detection, i.e., enzyme-linked immunosorbent assays (ELISAs) [18], biosensor methods (DNA-sensors [19], immunosensors [17]). Since most of the new methods are based on the application of natural biomolecules, that are quite unstable in extreme environments, this leads to the problems with commercialization of such devices and requires new approaches.

Among the great variety of novel biosensors, biomimetic-based sensors exhibit excellent potential as a modern, simple, rapid, and robust analytical technique. Molecularly imprinted polymers (MIPs) are used as sensing elements for such sensors. There are varying techniques to synthesize MIPs, but the main principle remains. MIPs are generally obtained after co-polymerizing an analyte with the monomer mixture containing mainly functional and cross-linking monomers [20]. After polymerization, a template (analyte) is removed, leaving selective binding cavities behind that are physically and chemically complementary to an analyte [21]. Thus, selective binding cavities in MIPs structure mimic the

active sites of natural receptors and antibodies. MIPs offer a number of benefits, including strong sensitivity, selectivity, and stability. Moreover, the process of their synthesis is inexpensive and straightforward.

To date, MIPs in a form of membranes were synthesized for mycotoxins [22] and other toxic compounds by our group [23]. It was shown that free-standing MIP membranes could be successfully integrated with biosensor devices due to their capability of generation sensor responses that can be quickly and easily registered. So, considering our previous research, we created a novel, simple sensor system capable of ZON selective recognition using MIP membrane as its selective element. Therefore, the current work is aimed at validation of the recent biomimetic-based sensor system as for the reliable analysis of mycoestrogen zearalenone in real cereal samples in cooperation with SE Ukrmetrteststandard.

Materials and methods

Materials. Mycotoxins: zearalenone (ZON), aflatoxin B1 (AFB1), ochratoxin A (OchA) were purchased from Sigma-Aldrich (St. Louis, USA). Resorcinol or m-dihydroxybenzene was purchased by TCI (Japan). Ethylene glycol methacrylate phosphate (EGMP) was obtained from ABCR GmbH & Co (Karlsruhe, Germany). Polyethylene glycol MM 20,000 (PEG 20,000) and tri(ethylene glycol) dimethacrylate were purchased from Sigma-Aldrich (St. Louis, USA). Oligourethaneacrylate (OUA) was obtained as described [24]. Cyclododecyl-2,4-dihydroxybenzoate (CDHB) was synthesized according to a previously described method [25] and kindly provided by Dr. I.Ya. Dubey. All the other reagents were obtained from Sigma-Aldrich (USA) and used without additional purification.

Samples. Real flour (wheat and maize) samples were purchased from a local retail store (Kyiv, Ukraine).

Synthesis of CDHB-imprinted and non-imprinted polymer membranes for zearalenone recognition. Molecularly imprinted polymer (MIP) membranes capable of zearalenone recognition were synthesized by taking 5 mg of a dummy template — cyclododecyl-2,4-dihydroxybenzoate (CDHB) [25] and 6 mg of functional monomer — ethylene glycol methacrylate phosphate (EGMP). CDHB was used instead of zearalenone in order to make the procedure safe and avoid pseudo-positive results [26]. Next, 180 mg of the main components of the polymeric network — a mixture of triethyleneglycoldimethacrylate and oligourethaneacrylate MM 2600 (TGDMA/OUA 85/15 ratio) were added. Subsequently, porogens 100 mg of dimethylformamide and 30 mg of polyethylene glycol MM 20 000 were added. Finally, 1 mg of the polymerization initiator — 2,2′-dimethoxy-2-phenylacetone was added immediately before polymerization. The procedure of non-imprinted polymer membrane (NIP-membrane) synthesis was similar except for CDHB addition during the polymer mixture preparation.

Finally, MIP and NIP membranes were prepared by an *in-situ* polymerization method. The prepared mixtures of monomers were polymerized between two glass slides. Along the perimeter of the glass slides, 60-μm polytetrafluoroethylene spacers regulating membranes' thickness were placed. The polymerization procedure was initiated by UV light (λ=365 nm, intensity 3.4 Wm^{-2}) and

conducted for 30 min. The dummy template (CDHB) as well as unpolymerized monomers were removed by Soxhlet extraction with ethanol for 8 hours. Additionally, the polyethylene glycol was removed by washing both MIP and NIP membranes in the distilled water at 80 °C for another 8 hours. Finally, the MIP membranes and corresponding NIP membranes were dried at room temperature and used for ZON detection.

Analytical procedure. The 1 × 2 cm MIP and NIP membrane samples were incubated in 10 mL of a sample solution containing varying amounts of zearalenone in 20 mM Na-phosphate buffer (pH 7) with 10 % of acetonitrile. After 10 h incubation, the sensor's response was examined by measuring the fluorescence of zearalenone which was bound by the synthetic receptor sites in the MIP membrane structure. The sensor responses were registered after UV-irradiation of the membranes' samples fixed in a holder for membranes and solid samples using Fluoromax_PLUS_PR928P spectrofluorimeter (Horiba, Japan) directly at the membranes' surface. Zearalenone fluorescence detection was obtained with excitation at 320 nm wavelength (bandwidth 1 nm) and emission detection at 465 nm wavelength (measurement range 380–540 nm, bandwidth 1 nm). The fluorescent sensor responses measured from the membranes' surface were plotted as a function of ZON concentration in the samples.

Real samples preparation and extraction procedure. To prepare real samples, 1 g of flour samples was mixed with 10 mL 80:20 v/v acetonitrile:H_2O solution and shaken for 10 min using Vortex laboratory shaker (Fisher Scientific, Germany). Then, the solution was filtered using Whatman paper №1. The extraction procedure was repeated. Additionally, the collected extracts were centrifuged for 10 min at 10,000 g. Finally, flour extracts were diluted at 1:10 with the buffer solution (20 mM Na-phosphate buffer (pH 7.0) containing 10 % of acetonitrile) and spiked with different concentrations of ZON. Then, the analysis was conducted, as indicated in the general analytical procedure. The concentration of ZON in the solid flour sample was calculated as described in our previous work [22].

Statistical analysis. All measurements were made ten times, and the data were expressed as means. The obtained results were statistically processed using the computer program Microsoft Excel. The data of mean, standard deviation, relative standard deviation were determined.

Results

All the newly-developed analytical methods, including biosensor-based ones require the standardization of the analytical procedure. Therefore, a reliable calibration graph for the created fluorescent sensor system based on MIP membranes was obtained at the first stage of investigation. We examined the ability of MIP and NIP membranes to bind zearalenone in laboratory conditions using standard solutions with varying ZON concentrations. For this purpose, MIP and NIP membranes, synthesized using EGMP as the functional monomer, were incubated in aqueous solutions with known concentrations of ZON (from 1 to 50 ppm). Since zearalenone shows the natural ability to fluorescence, ZON molecules adsorbed on the MIP membrane surfaces were detected fluorometrically. Fluorescent sensor

signals registered from the MIP membrane surface are directly proportional to ZON concentration in the analyzed samples. Fig. 1 presents the typical calibration graph for the biomimetic sensor system based on MIP membranes for zearalenone detection.

According to significant differences between MIP and NIP membrane fluorescence intensities, it is clear that zearalenone binds to the MIP membrane due to the formation of artificial receptor sites in their structure. It also indicates that the imprinting procedure using a dummy template was successful.

The linearity of the calibration graph was assessed using determination coefficients (R^2). For the MIP membrane calibration curve, R^2=0.952 corresponds to the generally accepted values for this parameter, indicating good linearity. As one can see from the linear calibration graph (Fig. 1), the proposed biomimetic-based sensor system demonstrates a wide linear dynamic range from 1 to 50 ppm.

The limit of detection (LOD) was calculated as 1 ppm, based on the signal-to-noise ratio equal to 3 (3σ). In Ukraine, the maximum permissible levels of ZON in cereals food commodities is between 20-100 ppm (in accordance with the order of the Ministry of Health of Ukraine «On approval of the State hygiene rules and norms "Regulation of maximum levels of certain contaminants in food "» (№ 368 of 13.05.2013)). So the LOD of the proposed biomimetic-based sensor system was well below the regulated level for zearalenone in food products and animal feed.

Selectivity is one more critical parameter needed to be evaluated along with the linear dynamic range and LOD of the sensor device. Therefore, cross-reactivity of the proposed sensor system was tested in an adsorption experiment using close structural analogues of zearalenone, including its metabolite — zearalenol (ZOL), 17-β-estradiol, bisphenol A and resorcinol. Moreover, considering that cereal

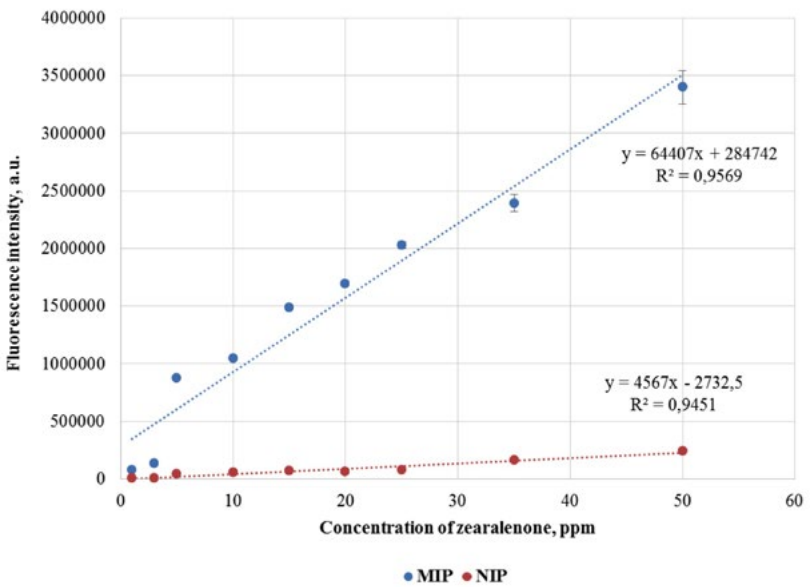

Fig. 1. Calibration plots of the biomimetic-based fluorescent sensor system for zearalenone detection. Fluorescence of MIP and NIP polymeric membranes after incubation in solutions with different concentrations of ZON in 20 mM Na-phosphate buffer (pH 7.0) containing 10 % of acetonitrile. Spectrofluorimeter Fluoromax_PLUS_PR928P (λ ex = 320 nm, λ em = 465 nm). Error bar = SE, n = 10.

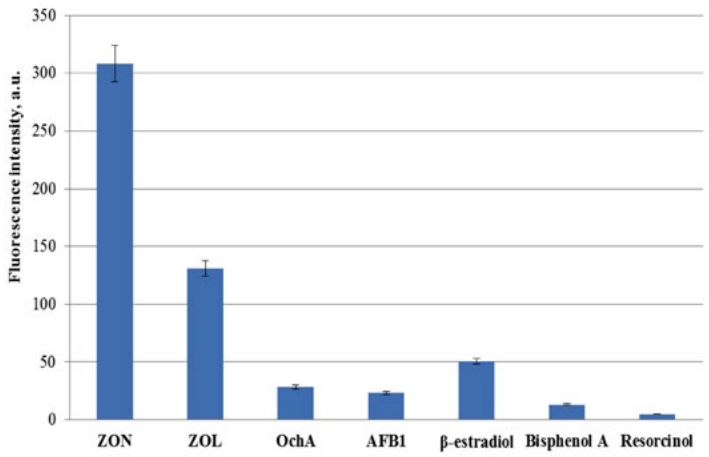

Fig. 2. Selectivity of the MIP membrane-based fluorescent sensor system for zearalenone detection. Fluorescence of EGMP-based MIP membranes after incubation in 10 ppm solutions of zearalenone (ZON), zearalenole (ZOL), ochratoxin A (OhA), aflatoxin B1 (AFB1), β-estradiol, bisphenol A and resorcinol (20 mM Na-phosphate buffer (pH 7.0) containing 10 % of acetonitrile was used for the measurements). Spectrofluorimeter PerkinElmer LS 55 (λ ex = 320 nm, λ em = 465 nm). Error bar — SE, n=5.

food can be contaminated with several mycotoxins, aflatoxin B1 (AFB1) and ochratoxin A (OchA) were added to this experiment.

As shown in Fig. 2, the synthesized MIP membranes demonstrate high selectivity towards ZON along with low cross-reactivity to its structural analogues and other mycotoxins. Therefore, high selectivity of the developed sensor towards the analyte of interest (zearalenone) was clearly proven.

Finally, the calibrated biomimetic-based sensor system was validated using different real sample matrixes. In this case, cereal extracts from wheat and maize flour samples produced by the biggest Ukrainian manufactures were used for the investigation. The concentration of ZON in an analyzed sample was calculated using the calibration graph obtained earlier. The obtained results are summarized in Table 2.

Table. **2. Determination of Zearalenone in real samples.**

Sample, No	Added, ppm	Found, ppm[a]	RSD, %[b]	R, %[c]
1 (wheat flour, Zernari LLC, Oleksandriia, Ukraine)	1	1,5 ± 0,24	12,05	150
2 (wheat flour, "Khutorok", Zmiiv, Ukraine)	5	4,8 ± 0,5	10,46	96
3 (wheat flour, TOB EuroMill, Boryspil, Ukraine)	10	10,5 ± 0,73	7,03	105
4 (maize flour, "Lavka Tradytsiy", Lviv, Ukraine)	25	23,7 ± 1,8	7,77	94,7
5 (maize flour, Alta Vista KO LLC, Kyiv, Ukraine)	50	50,1 ± 0,53	1,07	100,1

[a] Results are expressed as the mean ± SD (n=15)
[b] Relative standard deviation (RSD) is calculated using the following equation: RSD = 100 %×SD/mean
[c] Recovery (R) = (detected concentration/spiked concentration) × 100 %

The obtained results demonstrate that the proposed sensor system for the determination of zearalenone has good reproducibility (R average = 109 %). In addition, the accuracy of the analytical method in units of relative standard deviation (RSD < 20 %) was determined and found to be satisfactory and meets generally accepted standards [27].

As compared to the traditional analytical methods for ZON analysis in food products and feeding stuff, the proposed assay has numerous advantages. Firstly, a small amount of the analyzed sample (1 g) is needed. Secondly, the procedure of the sample preparation and extraction (up to 30 minutes) is fast and simple. Finally, the obtained results indicate that applying the MIP membrane-based sensor system towards ZON analysis was performed in a reliable and reproducible manner.

Conclusions

The biomimetic-based sensor system for zearalenone analysis based on EGMP-containing MIP membrane was developed and further investigated in calibration and validation studies. The calibrated sensor system demonstrated a wide linear dynamic range (1–50 ppm) and a low absolute detection limit (1 ppm). In addition, the MIP membrane-based sensor system was found to be highly selective towards zearalenone. The proposed sensor system was successfully applied for ZON analysis in maize and wheat food matrixes with mean recoveries of 109 % and RSD mean of 7.7 %. The sensor system based on MIP membranes provides fast, sensitive, simple zearalenone analysis with high accuracy and reproducibility in real flour samples. In cooperation with SE Ukrmetrteststandard, a full range of metrological studies was conducted, and the method of zearalenone detection using calibrated sensor systems based on MIP membranes was standardized.

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Зеараленон-селективна сенсорна система на основі біоміметиків та її валідація для аналізу реальних зразків

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Мета. У статті представлені результати калібрування та валідації нової флуоресцентної сенсорної системи на основі біоміметиків для аналізу зеараленону (ЗОН) у зернових культурах. **Методи.** ЗОН-селективні біоміметики були отримані у формі молекулярно-імпринтованих (МІП) мембран методом полімерізації in-situ з використанням циклодецил-2,4-дигідробензоату та этиленгликольметакрилатфосфату, як матричної молекули та функціонального мономеру, відповідно. Принцип детекції заснований на реєстрації флуоресценції ЗОН, селективно адсорбованого на поверхні МІП мембрани, за допомогою спектрофлуориметра. **Результати.** Сенсорна система на основі біоміметиків була відкалібрована для визначення зеараленону в харчових продуктах. Було досліджено робочий діапазон сенсорної системи, а також межу визначення та селективність. Розроблена сенсорна система була успішно використана для аналізу зразків кукурудзяного та пшеничного борошна, а ступінь відповідності методу (R) становив 109%. **Висновки.** Спільно з Державним Підприємством "Укрметртестстандарт" проведено калібрування та валідація нової ЗОН-селективної сенсорної системи на основі біоміметиків. Стандартизована сенсорна система може розглядатися як надійний метод запобігання забрудненню зеараленоном харчових продуктів та кормів на основі зернових.

Ключові слова: зеараленон, молекулярно-імпринтовані полімерні мембрани, сенсорна система на основі біоміметиків.

Зеараленон-селективная сенсорная система на основе биомеметиков и ее валидация для анализа реальных образцов

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Цель. В статье представлены результаты калибровки и валидации разработанной флуоресцентной сенсорной системы на основе биомиметиков для анализа содержания зеараленона (ЗОН) в зерновых. **Методы.** ЗОН-селективные биомиметики были получены в форме молекулярно-импринтированных (МИП) мембран методом полимеризации in-situ с использованием циклододецил-2,4-дигидробензоата и этилен-гликольметакрилатфосфата, как матричной молекулы и функционального мономера, соответственно. Принцип детекции основан на регистрации флуоресценции ЗОН, селективно адсорбированного на поверхности МИП мембраны, с помощью спектрофлуориметра. **Результаты.** Сенсорная система на основе биомиметиков была прокалибрована для определения содержания зеараленона в пищевых продуктах. Были исследованы рабочий диапазон сенсорной системы, а также ее предел обнаружения и селективность. Разработанная сенсорная система была успешно использована для анализа образцов кукурузной и пшеничной муки, а степень соответствия результатов (R) составляла 109%. **Выводы.** Совместно с Государственным Предприятием "Укрметртестстандарт" проведены исследования по калибровке и валидации новой ЗОН-селективной сенсорнорй системы на основе биомиметиков. Стандартизованная сенсорная система может рассматриваться, как надежный метод предотвращения контаминации пищевых продуктов и кормов на основе зерновых зеараленоном.

Ключевые слова: зеараленон, молекулярно-импринтированные полимерные мембраны, сенсорная система на основе биомиметиков

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