DETERMINATION OF THE MYCOTOXIN ZEARALENONE IN WATER BY IMMUNOFLUORESCENCE AND TOTAL INTERNAL REFLECTION ELLIPSOMETRY METHODS

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Abstract

In the scope of project Aquafluosense developing prototypes of fluorescence-based instrumentation for *in situ* measurement of several characteristic parameters of water quality, an immunofluorescent method have been developed for the detection of several environmental xenobiotics, including mycotoxin zearalenone (ZON). ZON, produced by several plant pathogenic *Fusarium* species, has recently been identified as an emerging pollutant in surface water, presenting a hazard to aquatic ecosystems. Due to its physico-chemical properties, detection of ZON at low concentration in surface water is a challenging task. The 96-well microplate-based fluorescent instrument is capable to detect ZON in the concentration range of 0.4–400 ng mL^{-1}. The sensitivity and accuracy of the analytical methods has been demonstrated by comparative assessment with detection by total internal reflection ellipsometry.

Introduction

Through the exhaustion of our global water reserves, water has become an environmental asset of key ecological importance. Water protection is a strategic issue worldwide and is one of the priorities of the European Commission as well. Thus, the EU Water Framework Directive, as one of the key EU policy measures, aims to reach a good status both chemical and ecological, for water bodies in Europe [1]. Within efforts to water conservation, water quality assessment underwent vast advancements driven by emerging water quality problems at a global scale, and by the appearance of novel technologies that allow parallel measurement of water quality parameters at increasing sensitivities.

Project Aquafluosense (NVKP_16-1-2016-0049) [2] aims to develop a new water analysis system for natural and artificial waters, allowing complex, systematic and for main parameters (chlorophyll-a content, chemical and biochemical oxygen demand, total organic carbon, polyaromatic hydrocarbon and certain agricultural pollutants content) *in situ* fluorescence-based assessment and monitoring of water quality, by developing a modular instrument family that can be individually configured for target tasks at each monitoring point.

Mycotoxin zearalenone (ZON) is a well-known food and feed contaminant with serious risk on both livestock and human health. Its occurrence as environmental contaminant is surface water is quite new discovery investigated only in the last decade [3]. The wide occurrence requires effective monitoring systems and analytical technologies. Traditional chromatographic separation e.g., high-performance liquid chromatography (HPLC) [4], thin layer

chromatography (TLC) [5] and liquid or gas chromatography coupled with mass spectroscopy (LC or GC MS) [6] are time consuming technologies requiring special instrumentation. In contrast, immunoanalytical methods allow cost-effective and rapid monitoring [7]. Enzyme-linked fluorescent immunoassays (ELFIA) are simply a variation of colorimetric enzyme-linked immunosorbent assay (ELISA), where the label enzyme converts a substrate into a reaction product fluorescent upon excitation by light of a particular wavelength. In comparison to the colorimetric ELISA, fluorescent immunoassays are more sensitive by widening the dynamic range of the assay and by measuring absorbance and fluorescence in different ways [8]. The fluorescence-based modular water analysis system developed in the frame of the project Aquafluosense utilizes this advantage of fluorescence in water monitoring. Innovative approaches in environmental analysis are sensor technologies that exhibit great sensitivity and specificity. The method of total internal reflection ellipsometry (TIRE) in conjunction with competitive and non-competitive direct label-free immunoassay was successfully applied for detection of low molecular weight ZON [9], thus this study reports comparative assessment of immunfluorescent methods and TIRE sensor technique for determination of ZON.

Experimental

The ZON standard, goat anti-rabbit IgG–HRP (horseradish peroxidase) conjugate as secondary antibody and salts for buffers were purchased from Sigma-Aldrich Inc. (St. Louise, MO, USA). Zearalenone-6'-carboxymethyloxime-bovine serum albumin conjugate (ZON-BSA), immunization in rabbit and serum collection, purification of rabbit anti-ZON IgG as primary antibody were prepared based on literature [10].

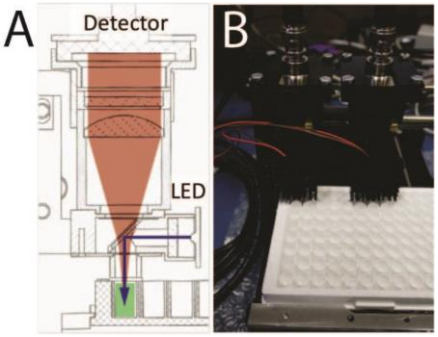

Figure 1. Schematic view of the ELISA plate compatible sensor head (A). Photo of the ELISA plate compatible instrument (B).

Immunoassays were carried out in high capacity 96-well microplates (Nunc, Roskilde, Denmark) for colorimetric assay and in low profile 96-well (white wells for increased fluorescence) PCR plates (Bio-Rad Laboratories, Hercules, CA, USA) with self-designed holder printed by 3D printer by Budapest University of Technology and Economics for ELFIA. QuantaRed Enhanced Chemifluorescent HRP Substrate Kit was used as last step in immunoassays (Thermo Fisher Scientific Inc., Waltham, MA USA). The kit contains 10-acetyl-3,7-dihydroxyphenoxazine (ADHP), a non-fluorescent compound that is dehydrogenated (oxidized) by HRP to resorufin, a highly fluorescent reaction product. Also, resorufin can be measured on a colorimetric plate reader. Absorbances were read by SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) at 576 nm wavelength. Relative fluorescent signs were determined by the prototype (Fig 1. A,B) equipped with CREE XPEBGR-L1-0000-00F01 LED (520-535 nm min-max dominant wavelength) as light source and FF01-593/40-25 output filter (peak: 593 nm, width: 40 nm) developed in Aquafluosense project [11].

In an indirect competitive ELISA format, plates were coated with 1 µg/ml bovine serum albumin-ZON conjugate (BSA-ZON) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH=9,6) overnight at 4°C. Blocking was carried out with 1% gelatine in phosphate buffer saline without Tween20 (137 mM NaCl, 2,7 KCl, 10 mM $Na_2HPO_4x2H_2O$, pH=7.4) at 37°C for 1.5 hours. After 4 times washing 50 µl/well of both ZON dilution series and antiserum (rabbit, dilution 1:1000) were added and incubated at 37°C for 1 hour. After washing 100 µl/well goat anti-rabbit-HRP (dilution 1:7500) were added and incubated for 1 hour at 37°C. The unbound fraction of the labeled secondary antibody was washed out with PBS, and QuantaRed Enhanced Chemifluorescent HRP Substrate Kit was used as substrate to get detectable fluorescence product and color. The process was ended with a stop solution supplied in the fluorescence kit. As this immunoassay is a competitive format, the higher the ZON concentration in the sample, the lower analytical signal is recorded. Assays were performed in triplicates.

Results and discussion

Indirect competitive ELISAs were performed to determine ZON calibration curve and the limit of detection (LOD) was found to be 0.4 ng/ml for ZON, and the detection range was investigated in a concentrations series of 0.004 pg/ml – 2 µg/ml ZON. After the colorimetric assay the liquid phase was transferred with 8-channel pipette to low profile 96-well PCR plate where fluorescence was determined. ZON at concentration of 2000 ng/ml triggered complete inhibition (no free primary antibody remained to connect to BSA-ZON conjugate coated on to the surface of the plate, thus the immunocomplex between the coating antigen and the primary antibody could not be formed), it was applied as background in determination of relative fluorescence sign. Calibration curves and LODs were determined for absorbance and fluorescence (Figure 2.). For comparable representation relative analytical signs are presented.

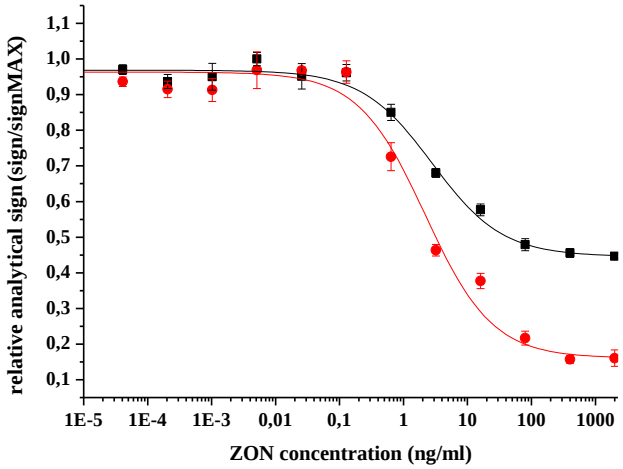

Figure 2. Competitive indirect calibration curves for zearalenone (ZON) determined by absorbance (■) and fluorescence (●).

Different LOD values were calculated for the two analytical signal, LOD = 0.6 and 0.4 ng/ml for visual absorbance and fluorescence detection, respectively. Determination by fluorescence provided a wider and steeper dynamic range, thus ELFIA proved to be a more sensitive method than the corresponding ELISA. Parameters of the sigmoid curves are presented in Table 1. For a colorimetric assay, application of QuantaRed Enhanced Chemifluorescent HRP Substrate Kit with HRP enzyme reaction provided lower LOD than o-phenylenediamine dihydrochloride (OPD) as chromofore (LOD for OPD=0.85 ng/ml).

Table 1. The correlation equation and parameters of sigmoidal calibration curves performed by absorbance and fluorescence detection for zearalenon determination.

Equation for fitting:

$$y = A_2 + (A_1-A_2)/(1 + (x/x_0)^p)^*$$

Adjusted R^2: 0.99037 (absorbance)
0.98804 (fluorescence)

		Value±standard deviation
Absorbance	A_1	0.97±0.01
	A_2	0.45±0.01
	x_0	2.81±0,48
	p	0.79±0.12
Fluorescence	A_1	0.96±0.02
	A_2	0.16±0.03
	x_0	2.20±0.53
	p	0.83±0.19

* A_1: upper platau, A_2: lower platau, x_0: IC_{50}, 50% inhibition, p: power.

Results determined by ELFIA were compared to corresponding results in sensor technology total internal reflection ellipsometry (TIRE) [9]. The sensor surfaces were prepared by a thermal evaporation of layers of chromium (Cr) – 3 nm thick and gold (Au) – 25 nm on standard microscopic glass slides. The Au-surface was modified with mercaptoethyl sodium sulfonate to enhance the negative surface change. For competitive immunoassay, zearalenone-6'-carboxymethyloxime-ovalbumin conjugate (ZON-OVA) was electrostatically immobilized on the Au-surface via a polyallylamine hydrochloride layer. In order to block all the remaining binding sites, an additional adsorption of OVA was carried out. Then a mixture of ZON-specific antiserum and solutions of free ZON (0.01 ng/ml – 10 µg/ml) were injected. The mixtures were pre-incubated for 5 min before injecting (Figure 4). The LOD value for TIRE was 0.01 ng/ml.

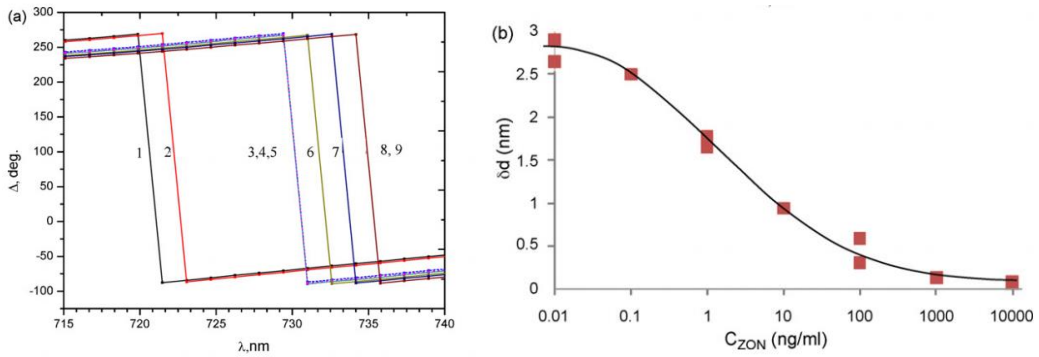

Figure 4. Competitive immunoassay for zearalenone carried out by detection via total internal reflection ellipsometry (TIRE). (a) A typical set of Δ(λ) spectra measured on bare Au surface (1), polyallylamine hydrochloride (2) ZON-OVA conjugate (3), OVA (4), Ab-ZON of from pre-incubated mixtures containing ZON: 100 ng/ml (5), 10 ng/ml (6), 1 ng/ml (7) and 0.1 ng/ml (8). (b) Changes in the adsorbed layer thickness versus the concentration of ZON (in the mixture with Ab-ZON) obtained by fitting the TIRE data.

Conclusion

Within project Aquafluosense, successful development provides a modular instrumentation setup for fluorescence-based determination of several characteristic parameters of water quality. Application of fluorescence, as an analytical signal in an enzyme-linked immunoassay format results in a method of improved sensitivity with a lower LOD value than in the

colorimetric assay. This benefit allows determination of lower pollutant concentrations in surface water, which contributes to a more effective monitoring technique. Although a sensor technology, total internal reflection ellipsometry provides orders of magnitude lower LOD than the immunofluorescent method developed, it is not appropriate for *in situ* determination due to its special laboratory instrumentation need and its limit in number of samples measureable simultaneously. In contrast, the 96-well microplate format in the immunofluorescent determination allows an assay capacity of 25 samples in parallel in triplicates (with standard curves of 7 calibration points).

Acknowledgements

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