ENZYME-LINKED FLUORESCENT IMMUNOASSAY FOR MONITORING THE HERBICIDE ACTIVE INGREDIENT GLYPHOSATE IN WATER

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Abstract
Within a modular water quality assessment fluorimeter instrument family, a newly developed enzyme-linked fluorescent immunoassay has been utilized for the quantitative analytical measurement of the herbicide active ingredient glyphosate in surface water. The developed 96-well microplate-based competitive immunoassay with fluorescence detection provides a 2.5-fold lower limit of detection (LOD = 0.09 ng/mL) in the investigated concentration range of glyphosate (0–100 ng/mL) compared to the detection of visual absorbance signals. Additionally, fluorescence detection resulted in a wider dynamic range for glyphosate measurement. Matrix effect was not observed for the undiluted surface water samples, and cross-reaction was not detected between glyphosate and its main metabolite (N-aminomethylphosphonic acid) and structurally similar compounds. The method allows rapid monitoring of glyphosate as a ubiquitous water contaminant of agricultural origin that can affect, due to its global use, both aquatic and terrestrial ecosystems.

Introduction
Various organic micropollutants (e.g., pesticides) have been identified as emerging water pollutants that can exert possible adverse effects on the ecological environments. Due to the intensive use of various plant protection products as still major tools in agrochemical crop protection, continuous monitoring of pesticide residues is required in surface waters [1]. Glyphosate (N-(phosphonomethyl) glycine) is one of the most widely and globally used herbicide active ingredients. Due to the outstandingly high application rate (globally around 800 thousand tons per year, in the European Union 30-9600 tons per year varying among different member states), glyphosate has become a ubiquitous water contaminant [2,3]. The environmental fate of glyphosate in water and in soil is highly affected by various abiotic and biotic factors (e.g., physical and chemical characteristics of the matrix, environmental conditions, pH, composition and stability of the microbial communities capable to facilitate decomposition). The degradation or dissipation of glyphosate can be much slower in the presence of various metal ions (e.g., Al, Mn Fe) in soil due to the substantial complex formation capacity of glyphosate [4]. As a result of its high water solubility, the appearance of glyphosate is a globally observed phenomenon in surface waters [2]. The occurrence of glyphosate as an agrochemical residue was proved in different agricultural products (e.g., soybean, maize, and certain fruits and vegetables), food (e.g., bread, cereal products, honey, olive oil, fruit juices, alcoholic beverages, beer, wine), and biological samples as well (e.g., the urine of livestock and humans) [5,6].

Recently, various quantitative analytical procedures (e.g., spectrophotometric and chromatographic methods) have been developed for the determination of glyphosate [7–10] in different natural samples, but most of these techniques are expensive, time-consuming, and need specialized instrumentation. Within Project Aquafluosense [11], the aim was to develop a new modular induced fluorescence-based instrument family as a water analysis system for *in situ* assessment and monitoring of quality of natural and artificial waters. The aim of the present study was to develop an enzyme-linked fluorescent immunoassay (ELFIA) module prototype within the above-mentioned instrument family for the quantitative detection and monitoring of glyphosate.

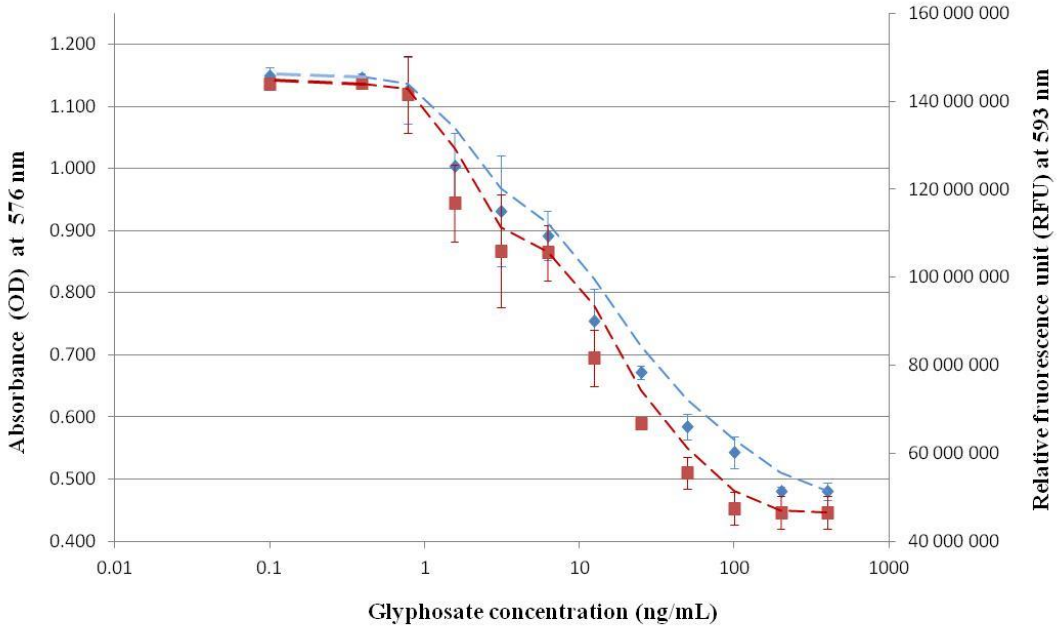
Experimental

The immunofluorescence module of the prototype developed within Project Aquafluosense is suitable for the *in situ* quantification of organic micropollutants by the use of immunoanalytical procedures. The development of the instrumentation (e.g., optics, sample holder, and detector electronics) was conducted by consortium partners at the Budapest University of Technology and Economics (Budapest, Hungary) and at Optimal Optik Ltd. (Budapest, Hungary), as described by Gémes *et al.* [12]. During the measurement, the samples were illuminated by an LED light source, and fluorescence emission was detected by a dichroic beam path with silicon photodiodes. The applied competitive ELFIA is based on a phase-heterologous competition and interaction between the immobilized hapten analogue conjugate on the surface of the 96-well microplate and the analyte in the samples. During the immunoassays, the glyphosate analogue haptenic compound prepared by the Institute of Isotopes Co. Ltd. (Budapest, Hungary), and the commercially available N-(phosphonolmethyl)iminodiacetic acid (PMIDA) was reacted with succinic anhydride and conjugated to human serum albumin (HSA). After dialization and lyophilization, the conjugate was used for the production of the coated microplate as the solid phase in this assay. During the analysis 100 µL of standard/control/sample and 40 µL of the derivatization mixture were homogenized, then 100 µL of borate buffer was added, and a 20-minute incubation was applied. The microplates were coated with glyphosate analogue hapten conjugate (0.5 µg/mL), and the unbound conjugate was washed off with washing buffer. After the derivatization, 100 µL chicken polyclonal anti-glyphosate (0.4 µg/mL), as a primary antibody and 20 µL of derivatized standard/control/sample were added to each well, then microplates coated with glyphosate-analogue-HSA conjugate were incubated (at room temperature, 2 h), then the wells were washed with 250 µL of diluted washing buffer, and finally 100 µL of tracer (0.5 µg/mL) goat anti-chicken IgY–HRP, as a secondary antibody was added and incubated (room temperature, 30 min). After washing off the unbound secondary antibodies, 100 µL/well of working solution (1:50:50 v/v mixture of ADHP, peroxide, and enhancer) ensured by QuantaRed Enhanced Chemifluorescent HRP Substrate Kit was added. After a 5-minute incubation, the enzymatic activity was stopped by 10 µL of QuantaRed Stop Solution, and the absorbance of the final product resorufin was detected at 576 nm by a SpectraMax iD3 Multi-Mode Microplate Reader. Fluorescence was measured at 593 nm by the immunofluorescence module developed within Project Aquafluosense [13, 14]. The applicability and the matrix effect were examined in surface water samples of different origins based on a comparison of the calibration curves obtained. Surface water samples were collected from various Hungarian natural and artificial water bodies and their catchment areas (e.g., River Danube, Lake Balaton, Visegrád Trout Lake). The accuracy and specificity of the immunoassay were also tested. Statistical analyses of the standard curves were performed by the comparison of IC_{50} values with the use of the one-way ANOVA method followed by *post hoc* Tukey tests (with a significance level of 0.05).

Results and discussion

A hapten-homologous competitive immunoassay was developed in a heterologous phase format with the use of colorimetric substrates with chromophores for fluorescence and visual detection, and was optimized for the detection of glyphosate in surface water. During the immunoassay on the solid surface of the microplate, a protein conjugate of glyphosate was immobilized to react with the glyphosate-specific antibody resulting in immunocomplex formation, which is competitively inhibited by glyphosate present in the sample. During the measurements, a derivatization step was needed to reach the highest utilization of the antibody avidity and affinity. Based on the calibration curves and LODs determined using both absorbance and fluorescence signals, the two modes of detection were compared (Figure 1). The developed ELFIA for the detection of glyphosate was characterized with LOD values of 0.22 and 0.09 ng/mL, for visual absorbance and fluorescence, respectively. The detection with induced fluorescence resulted in 2.5-fold lower LOD values compared to the visual absorbance, moreover, a wider and steeper dynamic range was provided as well, but the better LOD seems to be as a result of the very low standard deviation (SD) of the maximal signal, rather than the observed accuracy at points of partial inhibition by glyphosate.

Figure 1. Competitive indirect calibration curves of the immunoassay with a detection of absorbance at a wavelength of 576 nm in optical density (OD) units (blue) or fluorescence at a wavelength of 593 nm in relative fluorescence units (RFU) (red) for glyphosate in assay buffer [14].



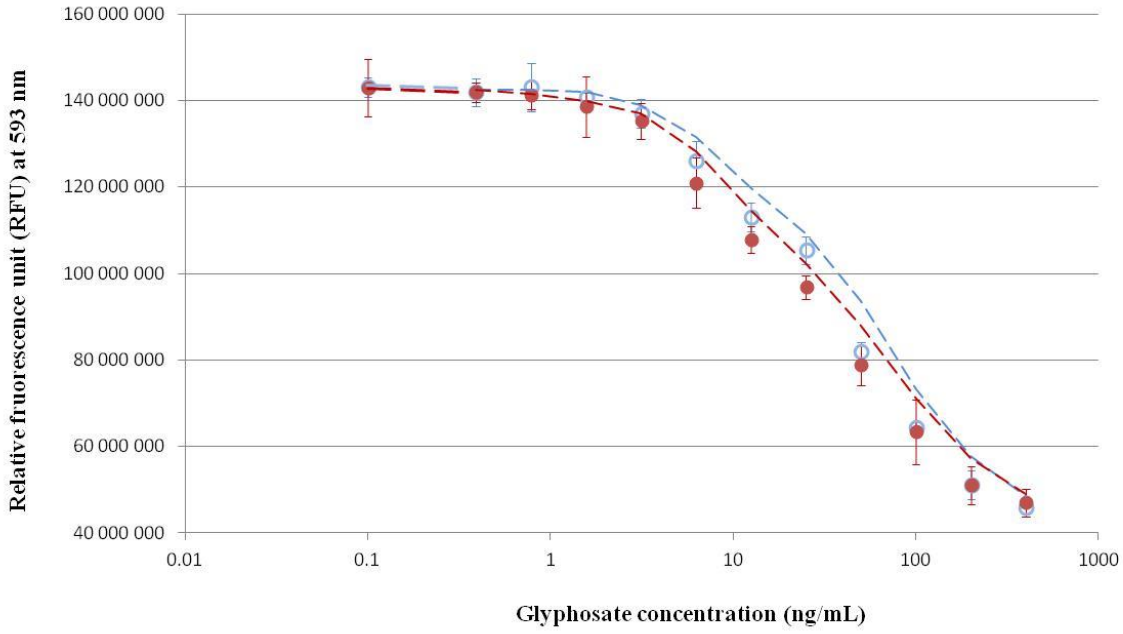
The developed ELFIA method as a part of the modular device system designed by Project Aquafluosense for *in situ* measurements of various parameters characterizing the water quality of natural and artificial water bodies. However, the coating and the blocking steps of the microplate were performed under laboratory conditions before performing the immunoassay. During the coating step, a glyphosate analogue conjugated to HSA was immobilized onto the solid surface of the microplate by passive adsorption, while with the application of the optimal conjugate concentration, the desorption was negligible and did not affect the reliability of the assay. Stabilization of the coated and blocked plates was carried out with the use of 300 µL/well of a 2% aqueous saccharose solution. The duration of the total immunoassay is approximately

3 h (20 min – derivatization, 2 h – incubation with the sample and the first antibody, with washing steps, 30 min – incubation with the second antibody, 2–10 min – signal development) for the measurement of 25 parallel samples in the 96-well microplate format, but the microplates were needed to be the covered during the process to prevent evaporation. Due to the reversibility of HAS adsorption at low pH, the repeated use of the microplates is potentially allowed, however, reuse of the microplates is not common in ELISA/ELFIA protocols.

The results of the determination of the intra-assay and inter-assay SDs indicated that the reproducibility, reliability, and accuracy of the assays is not optimal towards the lower plateau of the typical sigmoid calibration curve, thus the SD of the measurements is higher near the LOD than at the IC_{50} level, which affects the precision of the glyphosate detection. At the lower concentrations, the coefficient of variability (CV) is 25.1 and 15.0 for intra- and inter-assay, respectively, while near the IC_{50} (10.75±9.07 ng/mL), the CV values are 8.7 and 7.7, respectively. Based on the results, structurally similar compounds can affect the results of the measurements only at environmentally not relevant extremely high concentrations, while the detection of glyphosate in soil, surface water, and plant tissue samples can be disturbed by phosphate ions, therefore the inhibition of the glyphosate-specific antibody by phosphate was also needed during the measurements.

During the investigation of the matrix effects in surface water samples, the matrix effect was evaluated by the statistical comparison of IC_{50} values of the calibration curves of ELFIA measurements at the concentration range of 0–100 ng/mL of glyphosate. No matrix effect was observed in the surface water samples (Figure 2), and ELFIA proved to be capable for the accurate detection of glyphosate below the official limit (0.1 ng/mL) of pesticide residues (residues of individual pesticide active ingredients and their metabolites) in drinking water in the European Union [15].

Figure 2. Competitive indirect calibration curves of the immunoassay with a detection of fluorescence at a wavelength of 593 nm in relative fluorescence units (RFU) for glyphosate in assay buffer (blue) and in undiluted surface water samples from River Danube (red) [14]



Conclusion

Enhanced sensitivity with a lower LOD was proved for the developed competitive ELFIA method compared to the measurement of the absorbance in the colorimetric assays. The applied glyphosate-specific antibody was not inhibited by AMPA and the other structurally similar compounds in the immunoassay. Fluorescent detection provides a suitable quantitative method for the detection of glyphosate in surface and tap waters below the maximum allowed limit for pesticide active ingredients [15], thus the ELFIA system performed in the immunofluorescence module of the novel, induced fluorimetry-based instrument prototype serves as a more cost-effective, *in situ*, highly sensitive tool of environmental monitoring for glyphosate in surface water.

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