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DETECTION AND ASSESSMENT OF SURFACE WATER POLLUTANTS USING IMMUNOANALYTICAL AND BIOCHEMICAL METHODS

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1. INTRODUCTION AND OBJECTIVES

A significant portion of the agricultural crop production sector operate as open-system technologies. These systems are in constant interaction with their surrounding environment, allowing substances used in the technology and produced within the crop production area to be released as pollutants into environmental elements.

In industrial agriculture, the most common form of pest control is chemical crop protection, where pest control is achieved by applying pesticides, which, in turn, serves as a source of environmental pollution. The two most significant types of organic microcontaminants from agricultural technologies are pesticides used against crop pests and mycotoxins produced by certain plant pathogenic fungi. The latter are examples of compounds produced within the agrotechnology system. These organic microcontaminants can spread in the environment after their release through drift, surface runoff, or infiltration. As a result, the pollutants and their degradation products can reach surface and groundwater, contaminate the habitats of aquatic organisms and the water resources of other living beings, including humans.

Mycotoxins are secondary metabolites secreted by some pathogenic microscopic fungi. They occur in crops, food, and feed, and their effects on living organisms can be genotoxicity, cytotoxicity, mutagenicity, teratogenicity, or even carcinogenicity (CIMBALO et al. 2020). Among mycotoxins, we selected zearalenone (ZON) as our target compound, produced by species of the *Fusarium* genus, widespread in the Americas, Asia, and Europe. Its most well-known impact is its hormone-modulating effect, hence it is also called a mycoestrogen. In recent years, it has been shown to emerge as a surface water contaminant (along with other mycotoxins) possibly originated from leaching or sometimes from the biosynthesis of mycotoxins in water as well (RUSSELL and PATERSON 2003). Although ZON has low water solubility, it has been detected in various water samples in Europe, Brazil, and the USA.

The herbicide active ingredient glyphosate we studied has been the most widely used pesticide to date (SZÉKÁCS and DARVAS 2018). It is applied to control over 150 weed species in monoculture-based agricultural systems, as well as in forestry, urban environments, and aquatic cultures (KANISSERY

et al. 2019). Its water solubility is exceptionally high among pesticides, contributing to its presence as a surface water contaminant. Its mode of action is based on inhibiting a key enzyme in the shikimic acid pathway, which is essential for the biosynthesis of aromatic amino acids. This pathway exists only in plants, bacteria, and fungi, thus glyphosate is expected to be of low acute toxicity to animals (APARICIO et al. 2013). Despite this, glyphosate and its primary degradation product (aminomethylphosphonic acid, AMPA) have been shown to exert ecotoxicological effects on both terrestrial and aquatic organisms. Studies suggest that environmental occurrence of the active ingredient and AMPA may be linked to potential human health effects associated with low-dose exposure (VAN BRUGGEN et al. 2018).

Organic microcontaminants of agricultural origin appear not only in environmental matrices (soil, surface water, groundwater, or air) but also in plants, crops, and processed foods. Therefore, it is crucial to have access to determination methods capable of detecting these target compounds at very low concentrations in various samples. This is particularly important for pesticide residues and even more for mycotoxins, as they can exert toxic effects even at very low doses. Therefore, their legally permissible levels (maximum residue limits) are very low (0.1 μg/l for pesticide residues in drinking water, 0.002–0.50 mg/kg for mycotoxins in food and feed, 0.01–0.20 mg/kg for pesticide residues in food).

For the instrumental analytical determination of glyphosate and ZON, chromatographic methods are most commonly used. However, these methods are expensive, time-consuming, and often require a sample preparation step. Immunoanalytical methods can be good alternatives to these methods, ensuring selectivity through antibodies that specifically recognize the target compound.

The most widespread form of immunoanalytical methods is the classical enzyme-linked immunosorbent assay (ELISA), which is used for the quantitative determination of both target compounds (glyphosate and ZON). In ELISA, the binding of the recognizing antibody and the target compound is labeled with an enzyme, which catalyzes a measurable color reaction after the addition of its substrate and/or a chromophore. Instead of a chromophore, a luminophore or fluorophore can also be used, where the product of the enzymatic reaction is detected by luminescence or fluorescence. Using these more sensitive detection methods can lower the detection limit within the same immunoanalytical measurement process.

We were the first to demonstrate that the herbicide active ingredient glyphosate, described above, inhibits the binding of αvβ3-integrin to its RGD recognition sequence (SZEKACS et al. 2018). As the active ingredient is a commonly found environmental contaminant, potentially presenting a new exposure route for living organisms, we used a modified receptor inhibition ELISA test during my work to examine this newly identified effect across a broader range of integrin proteins and potential inhibitors (compounds structurally similar to glyphosate).

Integrins are common transmembrane, bidirectional signaling and adhesion receptor proteins in vertebrates, composed of α- and β-subunits. Their primary function is to maintain connection with the extracellular matrix (ECM) surrounding connective tissue cells. Through specific binding sites on their extracellular segments, integrins can recognize certain ECM proteins (receptor–ligand interaction), transmitting information between the cell and its environment upon binding or adhesion (MORSE et al. 2014). Through this activity, they regulate cellular processes (e.g., cell proliferation, migration, differentiation), cell–cell interactions, cellular development, and certain pathological processes. The 24 integrins found in the human body are divided into four groups: those recognizing leukocytes, collagen, laminin, and the RGD (arginine-glycine-aspartic acid) sequence. RGD-specific integrins play a crucial role in a represent a wide range of processes including cell adhesion, proliferation, migration, and angiogenesis, making them frequent targets in medical diagnostics, imaging, and experimental drug treatments. Ligands containing the RGD sequence are also studied in cancer research because certain integrins are expressed more in various types of cancer (CHENG and YI 2019). Among the RGD-specific integrins we examined, αvβ3 and α5β1 are receptors for vitronectin and fibronectin proteins, respectively, which are commonly expressed in the body (e.g., on endothelial cells, lymphocytes, macrophages). αIIbβ3 is primarily expressed, in addition to giant bone marrow cells and certain tumor cells, in platelets (SCHAFFNER and DARD 2003) where it plays an essential role in the blood clotting process as a fibrinogen receptor. Integrins also participate in numerous processes, the disruption of which can lead to disease. This includes severe conditions associated with the imbalance of programmed cell death (apoptosis), potentially resulting in malignant carcinogenic changes.

During my work, my research institution was the consortium leader of project Aquafluosense (NVKP_16-1-2016-0049), within which a modular sensor and instrument family was developed to facilitate complex in situ water quality assessment. This instrument family is based on induced direct and immunofluorescence, as well as optical and laser plasma spectroscopic analysis. In line with this project, my objectives were as follows:

1. To develop an enzyme-labeled fluorescent immunoassay (ELFIA) that, using the fluorimeter module prototype developed in the project, is suitable for the in situ detection and quantitative determination of zearalenone (ZON) in water samples with sensitivity meeting regulatory requirements. The work included investigating the inherent fluorescence of the target compound, as well as examining the applicability of the developed ELFIA method on surface water samples, and determining potential matrix effects and cross-reactions.
2. To develop an ELFIA method for the detection and determination of glyphosate content in water samples. This included defining the analytical parameters of the method, examining its applicability to environmental and biological samples other than water (soil and plant tissue), and investigating potential matrix effects and cross-reactions.
3. To apply a modified enzyme-labeled immunoassay method to investigate potential inhibitory effects of glyphosate, its degradation products, and other related compounds on the ligand-binding capability of integrins recognizing the arginine-glycine-aspartic acid (RGD) oligo-sequence and other integrins (receptors). This task also included, among others, the description of the concentration dependence of the inhibitory activity on integrins.

2. MATERIALS AND METHODS

During the development of enzyme-labeled fluorescent immunoassay (ELFIA) methods for the quantitative determination of zearalenone (ZON) mycotoxin and glyphosate herbicide, the underlying principle of the methods was the same. In such phase-heterogeneous competitive immunoassay methods, the binding sites of the antibody specific to the target compound are competed for between target compound in surface-bound and in dissolved forms. Therefore, the assays were performed on microtiter plates sensitized with a compound (analog) chemically similar to the target compound. Minor structural modifications were necessary to attach the ZON or glyphosate analog to the surface using carrier proteins during microtiter plate sensitization. After the competition step, unbound antibodies were washed away, and surface-bound target-specific antibodies were incubated with a secondary antibody conjugated with a marker enzyme. The enzymatic reaction, upon addition of the substrate and chromophore (which in our case also had fluorophore properties), produced an analytical signal measurable by detection of absorbance and fluorescence.

Prior to the development of the ZON ELFIA, we investigated the detection potential of the induced autofluorescence of the target compound for quantitative determination. For this purpose, we recorded autofluorescence spectrum of ZON in the excitation and emission wavelength range of 250–830 nm and 270–830 nm, respectively. The optimal excitation and emission wavelength pair determined by the spectrum map was used to establish the calibration curve of the autofluorescence of ZON in the concentration range of 0.6–2000 ng/ml, and to determine the limit of detection (LOD). ZON concentrations corresponding to the calibration curve points were prepared in phosphate-buffered saline solution.

Subsequently, to optimize the ELFIA method, we performed a static titration with two antisera to select the one more sensitive to the target compound. The microtiter plates were sensitized with 5 μg/ml ZON-BSA, and the sera were applied in dilutions ranging from 1:50 to 1:12200. We then set the appropriate concentration-dilution pair for the sensitizing compound and the more sensitive serum by cross-titration.

With the optimized system, we determined the points of the ZON calibration curve prepared in buffer solution in the concentration range of 0.004 pg/ml–

2 µg/ml, recording the enzymatic reaction product (resorufin) detected by both absorbance and fluorescence. Detection by absorbance and fluorescence was performed at 576 nm and 593 nm wavelengths, respectively.
Cross-reactivities were examined with structurally similar compounds to ZON. The compounds tested were α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, and β-zearalanol (Figure 1).

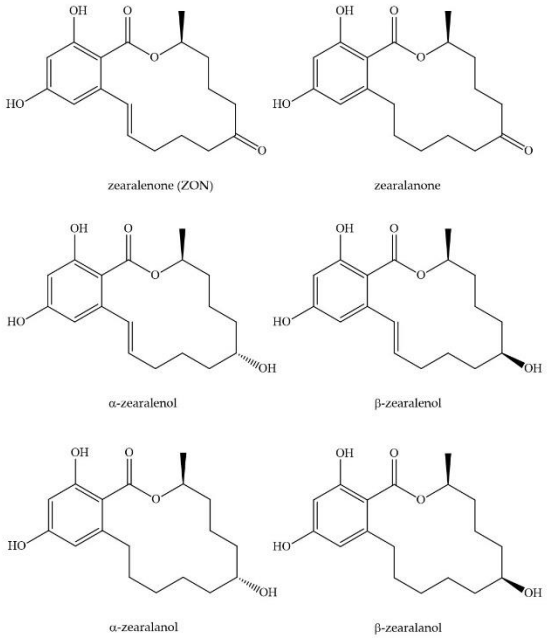

Figure 1: The chemical structure of compounds involved in the cross-reactivity assessment of the enzyme-labeled fluorescent immunoassay (ELFIA) for quantitative detection of zearalenone.

To determine matrix effects, the calibration curve points obtained in buffer solution were also recorded in three surface water samples obtained from Lake Velencei, Lake Balaton, and River Danube near Budapest. The IC_{50} values of the calibration curves in the surface water samples were then compared with the IC_{50} value of the calibration curve obtained in buffer solution.

Prior to the ELFIA measurement process for glyphosate, a single-step sample preparation reaction required. During this process, glyphosate molecules in the buffer solution and environmental samples (for matrix effect determination) were N-acylated to match the structure of the immunogen (derivatization).

Subsequently, as described at the beginning of the section, the calibration curve points for the target compound were recorded in buffer solution between 0 and 400 ng/ml. At the end of the measurement, the results were recorded using detection by both absorbance and fluorescence. The detection wavelengths were similar to those in the ZON ELFIA method, 576 nm and 593 nm for absorbance and for fluorescence, respectively.
For glyphosate, cross-reactivities were examined with structurally similar compounds such as AMPA, glycine, iminodiacetic acid, N-(phosphonomethyl)iminodiacetic acid, N-acetylglycine, and sarcosine (Figure 2), as well as with phosphate ions.

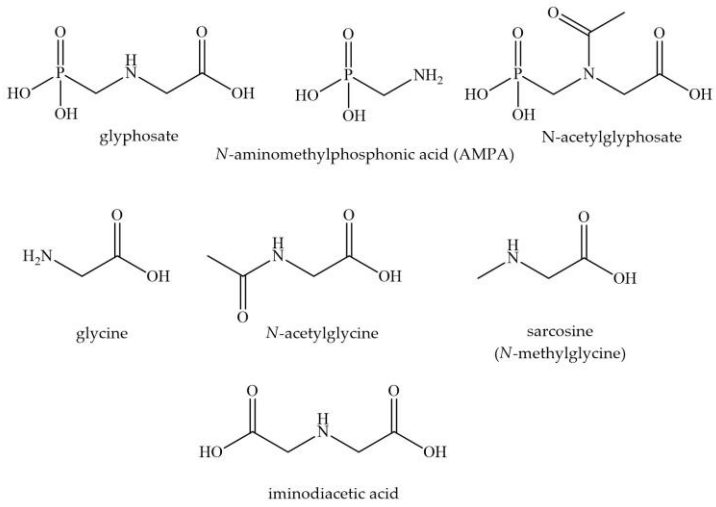

Figure 2. The chemical structure of compounds involved in the cross-reactivity assessment of the enzyme-labeled fluorescent immunoassay (ELFIA) for quantitative detection of glyphosate.

Matrix effects were studied with six different surface water samples, and in this case, also with soil samples, as well as leaf and root samples from sunflower (*Helianthus annuus*). Soil and plant tissue samples were prepared by homogenizing 40 mg of the lyophilized sample in 1 ml of buffer solution, centrifuging, and then collecting the liquid phase. Calibration curve points, which were also taken in the buffer solution, were recorded in the prepared sample extracts and surface water samples, and the IC_{50} values were compared. If necessary, the samples were diluted with buffer solution at a ratio of 1:10 or 1:100.

The inhibitory effects of glyphosate on various integrins was investigated with several structural analogs of glyphosate. This cross-reactivity study included, in addition to the pesticide active ingredient (in the form of its isopropylamine salt) the primary degradation product AMPA, as well as glycine, sarcosine, N-acetylglycine, N-acetylglyphosate, and iminodiacetic acid. In the modified receptor inhibition ELISA system, the concentrations examined were 0.138, 1.1, 5.5, 11, and 22 mM in all cases.

During the work, we constructed four different integrin-binding receptor immunoassay systems based partly on our previous results (SZEKACS et al. 2018) and partly on the scientific literature (KAPP et al. 2017). These systems allowed us to determine the inhibitory effect on the connection between integrin and its ligand (either a complete ECM protein or the RGD recognition sequence) in the following cases:

- αVβ3 binding to the RGD sequence,
- αVβ3 binding to vitronectin (ECM protein),
- α5β1 binding to the RGD sequence,
- αIIbβ3 binding to fibrinogen (ECM protein).

As a supplementary study, we also determined the inhibitory effect of glyphosate on the formation of the αVβ3–SARS-CoV-2 spike protein interaction.

The schematic structure of the systems is shown in Figure 3.

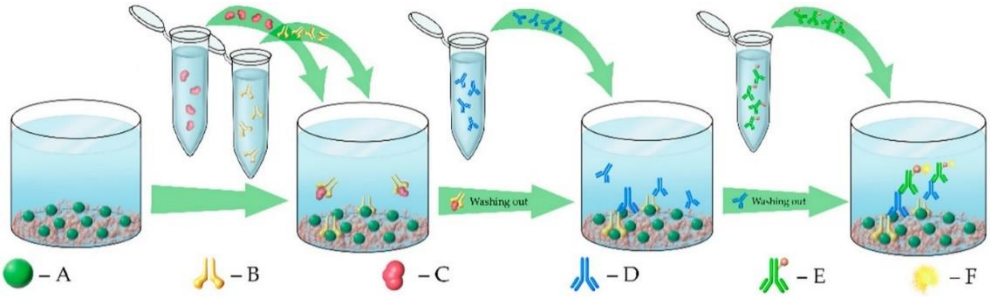

Figure 3. Schematic illustration of the enzyme-linked immunosorbent assay (ELISA). The wall of each well of a 96-well microplate was coated with a macromolecule showing high affinity to a given integrin (an RGD sequence-based synthetic polymer or ECM protein, e.g., vitronectin, fibrinogen or S-protein (the SARS-CoV-2 spike protein receptor binding domain)) and the remaining free surface was blocked with bovine serum albumin (BSA) (A). The corresponding integrin (B) was allowed to interact with the surface-immobilized macromolecules in the presence or absence of the ligands (glyphosate and its related compounds) at various concentrations ranging from 0 to 22 mM (C) that could modulate this binding process. The integrin molecules bound to the coating macromolecules were detected by an integrin-specific primary antibody (D), followed by an IgG-specific secondary antibody conjugated to a tracer enzyme (horseradish peroxidase, HRP) (E) and a colorimetric reaction of HRP using hydrogen peroxide as a substrate and 3,3',5,5'-tetramethlybenzidine (TMB) as a chromophore (F). If the ligand inhibited binding of the integrin molecules to the coating macromolecules, further steps of the ELISA process were blocked, leading to no color signal formation.

The microtiter plates were sensitized with a synthetic biopolymer containing the RGD recognition sequence or an ECM protein, and then any remaining free binding sites were blocked with bovine serum albumin. In the next step, the given integrin interacted with its surface-bound ligand, depending on the concentration of the inhibitor present in the system. The bound integrin molecules were identified using integrin-specific antibodies produced in mice, followed by labeling with secondary antibodies specific to mice and conjugated with horseradish peroxidase enzyme. If glyphosate or its related compounds inhibited the binding of the integrin to the surface macromolecule (RGD-containing synthetic biopolymer or ECM protein), the subsequent steps in the system did not proceed, and no color signal was formed. After adding the enzyme substrate and chromophore, the enzymatic reaction was stopped with sulfuric acid following appropriate color development, and the signal was detected by measuring absorbance at 492 nm.

3. RESULTS AND DISCUSSION

By obtaining the autofluorescence spectrum map of zearalenone (ZON) through scanning measurements, we identified the optimal excitation and detection wavelengths as 280 nm (excitation) and 520 nm (emission). Using these wavelengths, the intensity of the emitted light in aqueous ZON-solution samples showed a sigmoidal (logistic) relationship as a function of ZON concentration. Based on the fitted sigmoidal curve, the limit of detection for autofluorescence measurement was determined to be 11.5 μg/ml. This value is too high for the practical application of mycotoxin measurement, thus necessitating the development of the ELFIA system.

Based on the results of the static serum titrations, the more sensitive serum was selected for cross-titration, and it was determined that the ZON-analog–carrier protein conjugate should be applied at a concentration of 1 μg/ml, and the serum specific to the target compound should be used at a dilution factor of 1:1000 for subsequent measurements. For the calibration points of ZON concentrations ranging from 0.004 pg/ml to 2 μg/ml obtained in buffer solution, the limit of detection of the method was determined to be 0.25 ng/ml for absorbance detection. This is 3.4 times lower than the detection limit of a similar colorimetric ELISA method using 1,2-phenylenediamine hydrochloride as a chromophore. For fluorescence detection, the limit of detection was 0.09 ng/ml, making the method 2.8 times more sensitive for fluorescence detection.

Among structurally related compounds included in the cross-reactivity studies (α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, and β-zearalanol), the highest relative cross-reactivity (CR%) was observed for zearalanone in both detection methods (25.7 ± 3.6 CR% for absorbance and 26.7 ± 3.4 CR% for fluorescence), followed closely by β-zearalenol. The lowest relative cross-reactivity was observed for β-zearalanol (1.1 ± 0.2 CR% for absorbance and 0.9 ± 0.1 CR% for fluorescence). The results indicate that the sensitivity of the antibodies is enhanced by the presence of an unsaturated bond in the resorcyclic lactone structure, the spatial arrangement of the hydroxyl group may influence the antigen recognition process, and the antibodies have lower affinity for the reduced (hydroxy) metabolite derivatives.

As for matrix effects, no statistical difference was found between the IC_{50} values of the calibration curves obtained in surface water samples and that obtained in buffer solution ($p > 0.05$).

In our developed ELFIA method for the quantitative determination of glyphosate, the specific antibodies exhibit a strong affinity for the modified structured molecule (glyphosate-hapten) bound to a carrier protein on the surface of the plate. Therefore, the glyphosate molecules to be measured were converted to a structure similar to the hapten molecule using a one-step (N-acylation) derivatization process before measurement. The method proved to be adequate in terms of antibody selectivity.

Based on the curve fitted to points with glyphosate concentrations ranging from 0 to 400 ng/ml in buffer solution, the limit of detection of the developed method was 0.22 ng/ml with detection of absorbance. For detection of fluorescence, the limit of detection was found to be 0.09 ng/ml, making the method 2.4 times more sensitive with detection of fluorescence.

Among the compounds included in the cross-reactivity study i.e, AMPA, N-(phosphonomethyl) iminodiacetic acid, iminodiacetic acid, glycine, N-acetyl-glycine, and sarcosine, the highest cross-reactivity was found for N-(phosphonomethyl) iminodiacetic acid, where the system detected 0.89 ng/ml at a nominal concentration of 1650 ng/ml, resulting in a cross-reactivity (CR%) value of 0.018%. The results indicate that structurally similar compounds affected the measurements only at extremely high concentrations, which are not environmentally relevant. Phosphate ions can interfere with glyphosate determination from surface water, soil, food, or plant tissues. Our findings show that cross-reactivity in the method is very low, but it may need to be considered in certain cases.

We assessed matrix effects of the method in surface water samples, as well as soil and plant tissue samples. Similarly to the ZON ELFIA method (see above), the IC_{50} values of calibration curves obtained in various samples were compared to that of the calibration curves obtained in buffer solution. The IC_{50} value from the buffer test was 12.5 ± 0.4 ng/ml, while for water samples, it ranged between 11.9 and 12.3 ng/ml with variations between 0.3 and 0.6 ng/ml. No statistical difference was found between these values in surface water samples ($p > 0.05$).

As for soil samples, a slight matrix effect was observed, but statistically significant differences were not present at a 1:100 dilution ($p > 0.05$). For plant

tissue samples, the matrix effect could be eliminated with a 1:10 dilution. In this case, the IC_{50} values for leaf and root extracts were 12.5 ± 0.3 ng/ml and 11.8 ± 0.3 ng/ml, respectively, which did not statistically differ from the IC_{50} value of the calibration curve obtained in buffer (12.5 ± 0.4 ng/ml) ($p > 0.05$).

In our modified receptor inhibition ELISA systems, complete inhibition of αVβ3 integrin with binding to the RGD sequence was achieved by glyphosate and also by AMPA, with a lower IC_{50} value (0.46 ± 0.08 and 0.14 ± 0.02 mg/ml, respectively). All structural analogs exhibited inhibitory effects in this system, but even the strongest, N-acetylglycine, did not reach 50% inhibition at concentrations up to 22 mM. Glyphosate showed a greater inhibitory effect on αVβ3 integrin with binding to vitronection, while the effect of AMPA was less pronounced compared to the inhibition observed with the RGD sequence. At a concentration of 22 mM (2.44 mg/ml), glyphosate and AMPA resulted in 44.8 ± 3.6% and 75.3 ± 2.1% inhibition, respectively. In this system, the IC_{50} for the inhibitory effect of AMPA was 1.5 ± 0.4 mM (0.17 ± 0.04 mg/ml). The inhibitory effects of N-acetylglyphosate, N-acetylglycine, and iminodiacetic acid were stronger in the RGD-based system.

Glyphosate, which had the strongest inhibitory effect on αVβ3, only achieved 35.6 ± 4.4% inhibition of αVβ3–SARS-CoV-2 spike protein binding at the highest concentration tested. Consequently, the spike protein was not used further as a sensitizer in other systems.

All structural analogs exerted some degree of inhibition on α5β1 with binding to the RGD sequence, with N-acetylglycine achieving complete inhibition at a concentration of 22 mM (2.58 mg/ml) and an IC_{50} value of 5.8 ± 0.9 mM (0.68 ± 0.11 mg/ml). The effect of glyphosate on α5β1 with binding to the RGD sequence was 3.7 times lower, and that of AMPA was 17.5 times lower, compared to their effects on αVβ3 binding to RGD.

Among the compounds tested, only glyphosate inhibited αIIbβ3 binding to fibrinogen, achieving only 40.2 ± 2.6% inhibition at the highest concentration of 22 mM (3.72 mg/ml). None of the other compounds exhibited inhibitory effects on this system, and both AMPA and N-acetylglycine had activation effects.

The detailed results of the studies are presented in Table I.

Table I. IC_{50} values and maximal inhibitory effects on integrins by the compounds studied (glyphosate and its structural analogs)

ELISA	IC_{50} value (**mg/ml** and mM)[1] (maximal inhibition % obtained)			
Integrin Surface antigen	αVβ3 RGD	αVβ3 vitronectin	α5β1 RGD	αIIbβ3 fibrinogen
Inhibitor				
glyphosate	**0.46±0.08** 2.7±0.5 (100.0±3.9%)	>3.72 >22 (44.8±3.6%)	**1.64±0.54** 9.7±3.2 (85.7±2.1%)	>3.72 >22 (40.2±2.6%)
aminomethyl-phosphonic acid (AMPA)	**0.14±0.02** 1.3±0.2 (95.9±2.2%)	**0.17±0.04** 1.5±0.4 (75.3±2.1%)	>2.44 >22 (44.8+2.6%)	>2.44 >22 (activation)[2]
N-acetyl-glyphosate	>4.64 >22 (40.6±2.2%)	>4.64 >22 (47.5±1.2%)	>4.64 >22 (15.0±2.0%)	>4.64 >22 (n. d.[3])
glycine	>1.65 >22 (39.5±6.6%)	>1.65 >22 (39.5±2.4%)	>1.65 >22 (22.1±3.3%)	>1.65 >22 (n.d.)
N-acetyl-glycine	>2.58 >22 (46.8±3.7%)	**1.08±0.12** 9.2±1.0 (58.5±0.8%)	**0.68±0.11** 5.8±0.9 (98.9±2.3%)	>2.58 >22 (activation)
sarcosine	>1.96 >22 (35.0±1.2%)	>1.96 >22 (37.9±2.3%)	>1.96 >22 (13.4±2.1%)	>1.96 >22 (n.d.)
iminodiacetic acid	>2.93 >22 (24.6±7.9%)	**1.53±0.20** 11.5±1.5 (52.4±4.2%)	>2.93 > 22 (8.0±2.0%)	>2.93 >22 (n.d.)

[1] The IC_{50} value is the concentration at which the tested substance achieves 50% of its maximum inhibitory effect. [2]In cases of activation, we found an enhancing effect instead of inhibition, which was reflected in an absorbance signal higher than that of the negative control. [3] No effect was detected.

4. CONCLUSIONS AND RECOMMENDATIONS

1. ELFIA method development for quantitative determination of ZON mycotoxin. As part of the Aquafluosense project, we successfully developed a modular instrument system capable of determining certain water quality parameters based on fluorescence. The developed competitive ELFIA method, which relies on an immobilized ZON-analog antigen, significantly increased the method's sensitivity for quantifying ZON. This improvement allowed us to achieve a limit of detection that was 2.8 times lower compared to colorimetric determination. The limit of detection with fluorescence detection was 0.09 ng/ml, whereas it was 0.25 ng/ml with traditional colorimetric ELISA. The colorimetric determination using resorufin also resulted in a lower limit of detection than the classic OPD method (LOD_{OPD} = 0.85 ng/ml). This enables more effective monitoring of agricultural pollutants in surface waters at lower concentrations.

Our measurement results were validated using high-performance liquid chromatography (HPLC) and total internal reflection ellipsometry (TIRE) immunosensor techniques. Although the sensor technology provided a detection limit an order of magnitude lower than our developed immunofluorescence method, the latter's major advantage is the ability to perform in situ measurements using the developed measurement module prototype. Additionally, using a 96-well microtiter plate during measurements allows for the simultaneous determination of 25 samples in three parallel measurements with a seven-point calibration curve. The results of matrix effect studies indicate that the developed method is suitable for the quantitative determination of ZON content in undiluted surface water samples within the concentration range of 0–400 ng/ml.

2. ELFIA method development for quantitative determination of glyphosate pesticide active ingredient. The development of the competitive ELFIA method based on an immobilized glyphosate-analog antigen, reported here, also represents the application potential of the established method and instrument family made possible within the Aquafluosense project. In the method, the limit of detection (0.09 ng/ml) achieved through measurement of the analytical signal by fluorescence is 2.4 times lower than that achieved with colorimetric detection (0.22 ng/ml). The glyphosate-specific antibodies used

in the tests were inhibited neither by AMPA (the main breakdown product of glyphosate), nor by other structurally similar compounds. The limit of detection achieved through fluorescence allows for the determination of glyphosate at a concentration of 0.1 ng/ml, which is the maximum allowable level for individual pesticide residues in drinking water within the European Union. The developed immunoassay enables cost-effective detection of the target compound under in situ conditions with low limits of detection and high sensitivity. Similarly to the immunoassay developed for ZON measurement, the use of a 96-well microtiter plate allows for the parallel measurement of 25 samples at once. The developed method is suitable for the quantitative determination of glyphosate content in undiluted surface water samples within the concentration range of 0–100 ng/ml, as well as in plant tissue and soil samples with 1:10 and 1:100 dilutions. Moreover, the greatest advantage of the newly developed modular instrument system and our two ELFIA methods is their capability to simultaneously determine multiple water pollutants.

3. **A modified receptor inhibition ELISA**. In our receptor inhibition tests, we investigated in a broader context the inhibitory effect of the herbicide active ingredient glyphosate on the αvβ3 integrin, which we first identified. We included two additional integrins that recognize the RGD sequence, as well as compounds and degradation products related to the active ingredient. The inhibition tests were conducted in a heterologous phase receptor binding immunoassay format, and our conclusions based on the results are as follows: similarly to the previously determined complete inhibition, where glyphosate fully blocked the binding of the αvβ3 integrin to its sensitizing macromolecule, we observed comparable effects but only in certain cases. Besides glyphosate, AMPA also fully inhibited the binding of αvβ3 to the RGD sequence, and N-acetylglycine similarly fully inhibited the binding of α5β1 to an RGD-containing synthetic polymer. Some degradation products of glyphosate caused only partial inhibition (8.0–58.5%), while the inhibitory capability of the other compounds was proven negligible. In addition to the full inhibition of αvβ3 binding to the RGD sequence (95.9 ± 2.2%) and strong inhibition of its binding to vitronectin (75.3 ± 2.1%), AMPA actually enhanced the binding of αIIbβ3 to the ECM protein fibrinogen, which could be explained by its Ca^{2+}-binding ability. The same phenomenon was observed with N-acetylglycine. Although it inhibited the binding of αvβ3 to the RGD sequence by 46.8 ± 3.7%, its binding to vitronectin by 58.5 ± 0.8%, and the

binding of α5β1 to the RGD sequence by 98.9 ± 2.3%, at the same time it (N-acetylglycine) had a strong activating effect on the binding of αIIbβ3. Therefore, the inhibitory effect of glyphosate and its primary degradation product AMPA appears to be specific to αvβ3 integrin, which plays a key role in regulating the process of angiogenesis, such as during tumor growth. It is hypothesized that the presence of the phosphonomethyl group is necessary for the inhibition.

5. NEW SCIENTIFIC RESULTS

1. A novel enzyme-labeled fluorescent immunoassay (ELFIA) method has been developed for the detection and quantification of the mycotoxin zearalenone. Using polyclonal anti-ZON–CONA rabbit serum, the method showed a sigmoidal concentration dependence of the analytical signal, with an IC_{50} value of 2.20 ± 0.31 ng/ml and a limit of detection (LOD) of 0.09 ng/ml. The upper limit of the measurement range is 400 ng/ml. Cross-reactivity studies were conducted with α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, and β-zearalanol derivatives. Among these, the lowest cross-reactivity was observed with β-zearalanol (1.1 ± 0.2 CR%), and the highest was with zearalanone (26.7 ± 3.4 CR%) using the target compound-specific rabbit serum employed in the study. No matrix effect was detected in surface water samples, indicating that the method is suitable for determining zearalenone content in surface water samples without the need for sample preparation.

2. A novel enzyme-labeled fluorescent immunoassay (ELFIA) method has been developed for the detection and quantification of the herbicide active ingredient glyphosate. Using polyclonal anti-glyphosate-analog–HSA rabbit serum, the method showed a sigmoidal concentration dependence of the analytical signal, with an IC_{50} value of 12.5 ± 0.4 ng/ml and a limit of detection of 0.09 ng/ml. The upper limit of the measurement range is 100 ng/ml. Cross-reactivity studies were conducted with compounds such as aminomethylphosphonic acid (AMPA), N-(phosphonomethyl)iminodiacetic acid (PMIDA), iminodiacetic acid, sarcosine, glycine, and N-acetylglycine. Among these, the highest cross-reactivity was observed with PMIDA at 6700 and 1650 ng/ml, with CR% values of 0.013 and 0.018, respectively. No matrix effect was detected in surface water samples. A slight matrix effect was observed in plant tissue and soil samples, which was eliminated by diluting the samples at 1:10 for plant tissues and 1:100 for soil samples. The method is suitable for determining glyphosate content in surface water, soil, and plant tissue samples.

3. In an enzyme-linked immunoassay system suitable for studying integrin ligand-binding capacity, we tested the inhibitory effects of the herbicide active

ingredient glyphosate and its analog compounds on αvβ3, α5β1, and αIIbβ3 integrins. Through examination of structurally analogous compounds, we demonstrated that the presence of the phosphonomethyl group is essential for the inhibitory effect on αvβ3 integrin binding to the RGD sequence. In this case, the inhibitory effects of glyphosate and aminomethylphosphonic acid (AMPA) were similar. When binding to vitronectin by the integrin the inhibitory effect of glyphosate occurred to be decreased, while the effect of AMPA did not change significantly, and the effects of N-acetylglycine and iminodiacetic acid increased. In terms of binding to the RGD sequence, the inhibitory effect of glyphosate was found to be more specific compared to AMPA for α5β1 integrin, with N-acetylglycine achieving complete inhibition. Only glyphosate exerted an inhibitory effect on the binding of αIIbβ3 integrin to fibrinogen, while activation was detected in the cases of AMPA and N-acetylglycine.

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7. LIST OF PUBLICATIONS

1. Scientific journal articles

a) Articles in journals with impact factors (IF)

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Takács, E., **Gémes, B.**, Szendrei, F., Keszei, Cs., Barócsi, A., Lenk, S., Domján, L., Mörtl, M., Székács, A. (2022) Utilization of a novel immunofluorescence instrument prototype for the determination of the herbicide glyphosate. *Molecules*, **27** (19): 6514. (Q1, IF 4.710, doi: 10.3390/molecules27196514)

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b) Articles in journals without impact factors (IF)

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2. Conference publications

a) In Hungarian (full article)

b) In Hungarian (abstract)

Gémes, B., Takács, E., Barócsi, A., Kocsányi, L., Domján, L., Szarvas, G., Nabok, A., Székács, A. (2020). Felszíni vizek zearalenone-tartalmának meghatározása immunfluoreszcencia és teljes belső visszaverődéses ellipszometria alapú módszerekkel. *Ökotoxikológia*, **2** (2), p. 12.

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Gémes, B., Takács, E., Nabok, A., Mörtl, M., Székács, A. (2021). Development of an Immunofluorescence Assay Module for Determination of several agricultural pollutants in water. In: Jakab, G., Csengeri, E. (Szerk.): *Minden csepp számít: IV. Víztudományi nemzetközi konferencia = Every drop counts: IVth International Conference on Water Science*. 4-9. pp.

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d) In foreign language (abstract)

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