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Development of a non-labeled immunosensor for the herbicide trifluralin *via* optical waveguide lightmode spectroscopic detection

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Abstract

A highly sensitive immunosensor using optical waveguide lightmode spectroscopy (OWLS) was developed for the detection of the herbicide trifluralin. OWLS as an *in situ* and label free method of detection, based on the measurement of the diffraction of a linearly polarized laser beam (He–Ne laser, 632.8 nm) on a diffraction grating in a thin waveguide layer (SiO_2–TiO_2), offered means to produce immunosensors utilizing immobilized antibodies raised against trifluralin allowing a non-competitive biosensor, or immobilized trifluralin conjugate allowing a competitive biosensor for this analyte. Immobilization of molecules sensitizing the sensor was undertaken on amino silanized waveguide surfaces in a two-step procedure using glutaraldehyde. Within the immobilized antibody (Ab) based immunosensor the signal measured was proportional to the trifluralin content in the samples, but the method allowed detection of trifluralin only above 100 ng ml^{-1} due to the small molecular size of the antigen (Ag). In the immobilized antigen based immunosensor, a trifluralin–bovine serum albumin (BSA) conjugate was covalently linked to the waveguide surface. During measurements the standard solutions and samples were mixed in 1:1 ratio with antiserum, containing constant amounts of antibodies. The amount of free antibodies bound to the surface was inversely proportional to the trifluralin content of the solutions measured. The immobilized antigen based method allowed detection of trifluralin in the concentration range of 2×10^{-7} to 3×10^{-5} ng ml^{-1}. Results of trifluralin determinations were compared to those obtained in parallel enzyme-linked immunosorbent assay (ELISA) tests and in gas chromatorgraphic-mass spectrometric (GC-MS) analyses, and indicated an increase of six orders of magnitude in the limit of detection (LOD).
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Keywords: Immunosensor; OWLS; Immunoassay; ELISA; GC-MS; SPME; Trifluralin; Detection in surface water

1. Introduction

Acting as a potent microtubule inhibitor, the dinitroaniline herbicide trifluralin has been used in agricultural applications as a selective, pre-emergence herbicide in various plant cultures including grain crops, vegetables, fruits and nuts since the 1960s. In recent years, the compound is a subject to increasing toxicological and environmental concerns, e.g. to cause various physiological changes and endocrine effects in animal studies including liver parameters [1,2], kidney damage [3], decreased fetal weight and size and increased number of miscarriages [3], serum parameters [4], moderate mitogenic effects [5] and T-lymphocyte

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defects of blastogenesis [5,6] and allergies (e.g. allergic dermatitis). As a result, trifluralin has been listed on the European Union list as an endocrine disrupter [7]. Moreover, a recent report lists trifluralin among the top-ranking individual pesticides in correlation between childhood cancer and pesticide exposure [8].

In addition to its mammalian toxicity, concerns have been raised regarding the environmental fate and effects of trifluralin. Although it is of low water solubility, and is rapidly decomposing in aqueous environments [9], it has been reported as a water pollutant [10,11], and due to its strong absorption to soil particles [9,12], it is a persistent soil contaminant [9,12,13]. Because of its low mobility in soil, it is claimed not to leach to surface waters [14–16], while other sources consider it leachable [13,17]. It is readily volatilized to air [9] and reported as an air pollutant [18]. In water and particularly in soil, it is subjected to microbial degradation resulting in an extremely complex fate in soil [19]. Its ecotoxicologic effects have been evaluated [20], and the compound has been found to be toxic to fish and other aquatic organisms [21,22]. Due to its unfavorable toxicological features, trifluralin is no longer registered in Sweden, and was banned in Denmark in 1998.

Trifluralin is most commonly detected using instrumental methods, e.g. UV spectrometry [23], liquid chromatography [24–27] more recently including HPLC-mass spectrometry (MS) [28] or gas chromatography (GC) [29–44], capillary electrophoresis [26] or in a recent report, solution-state nuclear magnetic resonance spectroscopy (high resolution-magic angle spinning NMR, HR-MAS NMR) [45]. Common sample preparation methods for instrumental analysis include SPE [27,33,39,42,43], SPME [41,44] and SFE [27,46], but direct sample introduction (DSI) has also been reported [47]. An attempt to develop an enzyme-linked immunosorbent assay (ELISA) method has been reported in the literature, but it had a detection range too poor for practical applications [48]. For soil analysis, other reports also claim immunoassays as impractical [38]. We previously developed an ELISA system for this analyte allowing detection of trifluralin at concentration levels ranging from 0.2 to 60 ng ml^{-1} [49], yet increased assay sensitivity was expected to be achieved if immunoreagents of the above ELISA system are applied in optical immunosensor formats.

Optical immunosensors have proven their utility in pesticide monitoring [50–53]. Optical waveguide lightmode spectroscopy (OWLS) is an optosensor technique using evanescent field for the *in situ* and label free study of surface processes at molecular levels [51,54–57]. OWLS technique is based on the precise measurement of the resonance angle of polarized laser light, diffracted by a grating and incoupled into a thin waveguide. Such incoupling resonance occurs at very precise angles depending on the optical parameters of the sensor chips and the complex refractive index of the covering sample medium (Fig. 1). The intensity of the incoupled light guided within the waveguide layer by multiple internal reflection is detected by photodiodes. The refractive index, determined from the resonance incoupling angle detected at high accuracy, allows determination of layer thickness and coverage (or mass) of the adsorbed or bound material with ultra high sensitivity. The limit of detection (LOD) of the technique is a few pg mm^{-2}. The method allows construction of both chemosensors and biosensors, and therefore, can be applied for direct sensing of various kinds of biomolecules by immobilizing bioactive substances on the waveguide surface specific for the molecule of interest. In order to use grating coupler sensors in a flow-injection analyzer (FIA) system and to make the formed biospecific layer regenerable, the molecules need to be immobilized on the sensor surface by covalent attachment. Thus, immunoreactants (antibodies (Ab) or antigens (Ag)) covalently linked to the surface can be utilized in the analytical technique. OWLS immunosensors can be formed, e.g. by monomolecular bioresponsive coating, which consists of immobilized antibody molecules against the corresponding antigen and *vice versa*.

2. Experimental

2.1. Chemicals

Immunoreagents (immunogen, coating antigens, antisera) were prepared as described earlier [49]. Chemicals were purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA), unless otherwise stated. Trifluralin, benfluralin, ethalfluralin and 2,6-dinitro-4-trifluoromethyl-1-chlorobenzene were obtained from Budapesti Vegyimûvek Rt.

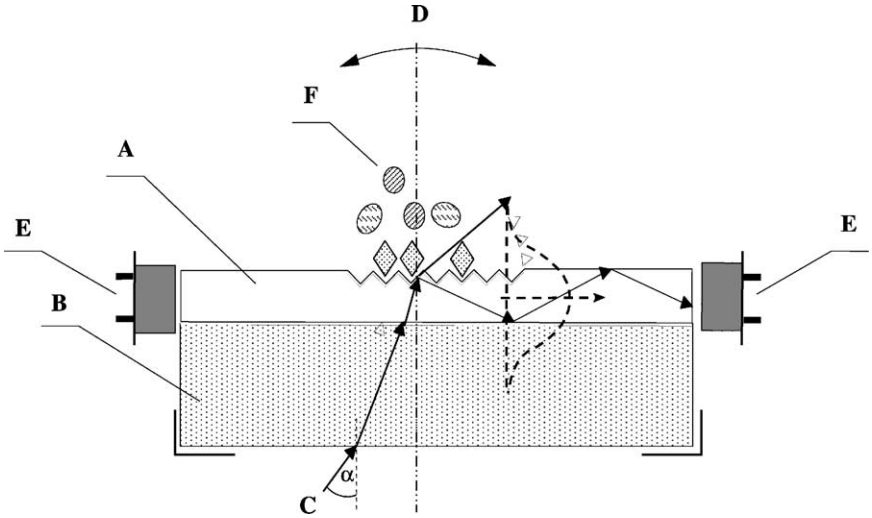

Fig. 1. The schematic arrangement of an optical waveguide lightmode spectroscopy immunosensor. The sensor is constructed of an optical waveguide layer (A) on top of a glass support (B) and of a fine optical grating made in the waveguide. When polarized laser beam (C) (typically He–Ne laser with 632.8 nm wavelength) reaches the grating, it is diffracted. The angle of diffraction (D) depends not only on the optical parameters of the sensor, but also on the refractive index of the cover medium. The waveguide is gradually rotated around it rotation axis (D), and when the diffracted beam is incoupled into the waveguide, it is propagating toward the edge of the sensor through multiple internal reflections. The intensity of the incoupled light is measured with a photodiode (E). The light intensity vs. incoupling angle (α) follows a sharp, resonant peak profile. Measuring the incoupling angle vs. time, one can follow refractive index changes in real time. The change of the effective refractive index due to the presence/association/dissociation of the analyte molecules (F) at the sensor surface results in a shift in the incoupling resonance angle. Association/dissociation of molecules on the surface of the sensor chip can be followed with high precision without labeling and in real time by continuously measuring the change in the incoupling angle.

(Hungary). γ-Aminopropyltriethoxysilane (APTS) was obtained from Fluka (Neu-Ulm, Germany). Isopropalin and pendimethalin were kindly provided by the Soil Conservation and Plant Hygiene Service (SCPHS, Hungary). Solvents purchased from Merck (Darmstadt, Germany) were of analytical grade.

2.2. Instruments

OWLS measurements were undertaken by using integrated optical waveguide sensors (chips) type of OW2400 (MicroVacuum, Budapest, Hungary). The sensors were read out with an OWLS100 instrument controlled by the software BioSense 1.1 (MicroVacuum). All the experiments were performed in flow-injection analyzer system. The sensors were put into the holder of the OWLS100 fixed with a flow-through cell (cuvette) and the instrument was connected to the FIA system containing a peristaltic pump (Minipuls3, Gilson, Middletown, WI, USA) and an injector (Rheodyne, Rohnert Park, CA, USA) equipped with an injector loop at a volume of 200 μl. A flow rate of 195 μl min^{-1} was maintained during the experiments.

ELISAs were carried out in high capacity 96-well microplates (Nunc, Roskilde, Denmark, No. #442404), and were read in an iEMS microplate reader (LabSystems, Helsinki, Finland). GC-MS analyses were carried out on a Saturn 2000 workstation (Varian, Walnut Creek, CA, USA).

2.3. OWLS

OWLS experiments were carried out using amino functionalized waveguide sensors. To introduce amino groups onto the surface before experiments, a series of sensors were silanized by dipping them into 10% γ-aminopropyltriethoxysilane in distilled water and were heat treated [58]. The modified sensors were stocked in distilled water until further applications.

Covalent coupling of antibodies raised against trifluralin in the case of non-competitive measurement

and trifluralin–bovine serum albumin (BSA) conjugate when measuring in a competitive way (through binding inhibition by trifluralin), was performed in the flow-through cell just before the measurements. During sensitization amino groups were first activated by injecting 200 μl of 2.5% glutaraldehyde in distilled water into the system. The surface was washed with distilled water then the medium was changed to 16.6 mM Michaelis phosphate buffer (pH 7.4). After washing, in the case of the non-competitive method, surfaces were treated with polyclonal sera at dilutions of 1:2000 and 1:6000, while in the case of the competitive method, trifluralin–BSA conjugate was applied to the surfaces at concentrations of 1 or 10 $\mu g\,ml^{-1}$. Immobilization was carried out by injecting 200 μl of the bioactive agents in 16.6 mM Michaelis buffer. After thorough washing, glutaraldehyde groups remained on the surface were blocked with 10 mM lysine. At last a washing cycle with 200 μl of 10 mM HCl was performed, and the sensors were ready for measurements. After each measuring cycle, when molecules were selectively bound during immunochemical reactions onto the immobilized immune complex, the surfaces were washed by injecting 200 μl of 10 mM HCl, thus regenerating the sensor. Sensor responses during the process of sensitization of the waveguide surfaces for the competitive (immobilized antigen) method are depicted in Fig. 2.

2.4. Enzyme-linked immunosorbent assay

ELISA determinations were performed in 96-well microplates based on a solid phase immunoassay principle [49]. Microplates were coated by incubating at 4 °C overnight with 100 μl per well of 1 $\mu g\,ml^{-1}$ of a trifluralin–BSA conjugate as coating antigen [49] in 0.1 M carbonate buffer (pH 9.6). After washing with 0.01 M phosphate-buffered saline (PBS) (0.8% NaCl), pH 7.4 buffer, plates were blocked by incubation at 38 °C for 1 h with 150 μl per well of blocking agent (1% gelatin in PBS, pH 7.4). After washing the microplate wells with PBS containing 0.2% Tween 20 (PBST 0.2) in a manual plate washing device, 50 μl per well of standards or samples and 50 μl per well of antiserum diluted in PBS containing 0.2% Tween 20 (PBST 0.05) were added, and plates were

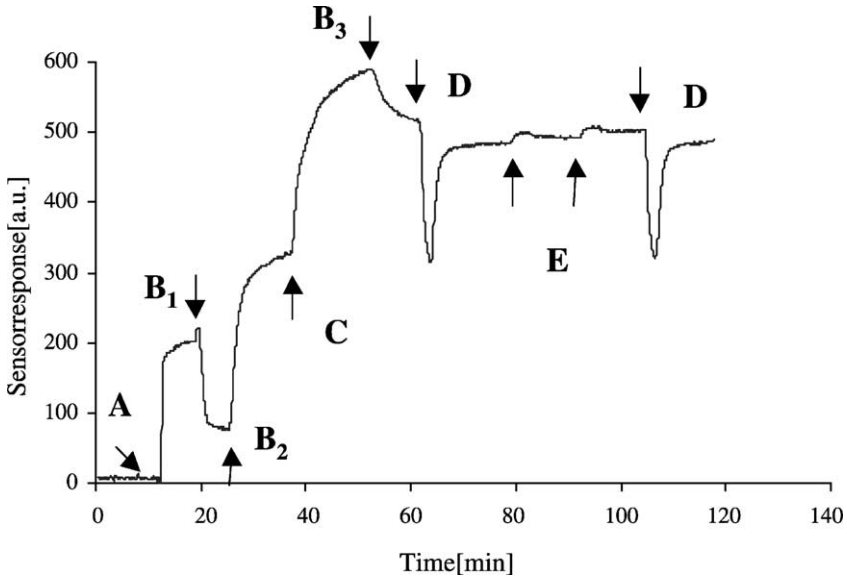

Fig. 2. Sensitization of the surface of the OWLS immunosensor. The surface is coated with glutaraldehyde (at 2.5% concentration) (A), rinsed with water (B_1) and with buffer (B_2), the sensitizing trifluralin–BSA conjugate is immobilized (C), rinsed with buffer (B_3), washed with aqueous hydrochloric acid (D), blocked with 10 mM lysine (E), and washed again with aqueous hydrochloric acid. Notice that the optical refractive index of the medium increases at the first rinse with buffer (B_2) resulting in signal increase, while it remains unchanged at the second rinse (B_3), and signal drops due to removal of weakly bound conjugate.

incubated at 38 °C for 60 min. After an additional wash with PBST 0.2, 100 μl per well of goat anti-rabbit IgG–HRP conjugate (Bio-Rad Laboratories, Hercules, CA, USA) at a dilution of 1:12,000 in PBST 0.05 was added, and incubated for 60 min as before. After a final washing step with PBST 0.2, 200 μl per well of the substrate solution (1.2 mM H_2O_2 with 3 mM 1,2-phenylenediamine (OPD) in 200 mM potassium dihydrogen citrate buffer, pH 3.8) were added. Upon sufficient color development (after 10–60 min) the enzymatic reaction was stopped by the addition of 50 μl per well of 4N H_2SO_4. Color intensities in the wells (endpoint mode) were read at 492 nm.

Standard curves from the raw data were calculated using a four-parameter (sigmoid) equation. The LOD was defined as the analyte concentration, causing a decrease of three standard deviations from the blank standard absorbance (a mean of at least three replicates).

2.5. Detection of trifluralin by GC-MS

Trifluralin was detected in water upon SPE sample preparation using gas chromatography with a mass spectrometric detector (GC-MS). GC-MS analyses were carried out by injecting the above isooctanic solutions. GC-MS conditions were as follows: fused-silica column CP-Sil 8 CB, 0.25 μm film thickness, 30 m × 0.25 mm i.d.; injection mode splitless; injection volume 5 μl; injector temperature programmed from 60 °C (held for 0.5 min) to 260 °C at a rate of 200 °C min^{-1}, and the final temperature was held for 5 min; column temperature programmed from 70 °C (held for 0.5 min) to 100 °C at a rate of 60 °C min^{-1} and then to 240 °C at a rate of 10 °C min^{-1} and the final temperature was held for 20 min. Helium was used as carrier gas, pressure 0.097 MPa; ionization current 350 μA; electron energy 70 eV. The iontrap was scanning in EI-mode from 40 to 650 u. The selected ions for quantitation of trifluralin were 264 and 306 u.

2.6. Sample preparation

Water samples for analysis included distilled water, tap water and various surface water samples (water from the River Danube and surface, lake and river water samples collected throughout Hungary). In the scope of a national monitoring program, 120 surface water samples were received annually from the SCPHS (Hungary). Water samples were subjected the OWLS and ELISA detection without any purification. For OWLS, water samples were diluted with Michaelis buffer to reach measurement range. For ELISA determinations, water samples were used without dilution, their pH was set to 7.4 (surface water samples were slightly alkaline, their pH ranged from 8.1 to 9.1), standard dilution series of trifluralin between concentrations of 3.13×10^{-4} and 2×10^4 ng ml^{-1} were prepared (in 1:20 dilution steps) in the neutralized water samples, and trifluralin content was detected in competitive ELISA.

For GC-MS analyses, water samples were spiked with trifluralin at concentrations between 0.25 and 25 ng ml^{-1}, and were subjected to solid phase extraction (SPE). Thus, water samples were filtered to remove floating particles, were stirred for 1 min and let stand for 10 min. Carboprep-90 columns (500 mg, 6 ml, Restek, Bellefonte, PA, USA) were placed on a vacuum suction manifold, and were conditioned by slowly passing through them against reduced pressure 5 ml of a mixture of dichloromethane–methanol (8:2), 2 ml of methanol and then 15 ml of a 10 g l^{-1} solution of ascorbic acid in distilled water. Then, 1000 ml of each water sample were loaded onto the conditioned Carboprep-90 columns, and were passed through with a flow rate of 10–15 ml min^{-1}. The columns were rinsed with 7 ml of distilled water, remained under suction against air for 10 min to reach air dryness, were rinsed with 1 ml of a solution of methanol and distilled water (1:1), and again remained under air suction for 10 min. Non-acidic contaminants concentrated on the column were eluted with 1 ml of methanol, followed by 6 ml of a mixture of dichloromethane–methanol (8:2) using a low flow rate of 2 ml min^{-1}. Each combined eluate was concentrated under nitrogen to approximately 0.2 ml, 2 ml of isooctane was added, and the solutions were concentrated to 1 ml of final volume.

3. Results and discussion

3.1. Detection of trifluralin by non-competitive method with OWLS

In the immobilized antibody based OWLS system, immunoglobulins from polyclonal anti-trifluralin

antisera were immobilized on the sensor surface, and the binding of trifluralin was detected at a later step in a non-competitive manner. To study the effect of dilution ratio of the antiserum used for sensitization, surfaces were treated with sera at dilutions of 1:2000 and 1:6000, respectively, and for measurement trifluralin standards at concentrations ranging from 10^{-3} to 10^{4} ng ml^{-1} were used. Sensor responses in both dilutions were obtained first only at 100 ng ml^{-1} trifluralin concentration. Increasing the concentration of the analyte, responses increased proportionally up to 10^{4} ng ml^{-1}. Although sensors treated with antiserum at a dilution of 1:6000 gave relatively higher signals in the range of 100–1000 ng ml^{-1} analyte concentrations, responses were very unstable and above 1000 ng ml^{-1} hardly increased at all. In the case of sensors prepared with antiserum at dilution of 1:2000, the linear range of the measurement, where stable and relatively reliable responses could be obtained, was in the range of 500–10 000 ng ml^{-1} of trifluralin concentration. A typical calibration curve obtained for this analyte concentration range is depicted on Fig. 3. From these data it is apparent that the limit of detection that this method allowed for trifluralin is not sufficiently low for investigation of environmental samples. Therefore, to get a lower LOD, a competitive method was also investigated.

3.2. Detection of trifluralin by competitive method with OWLS

The analyte concentration range detectable by the OWLS immunosensor, and therefore achievable LODs by the system are highly dependent on which component of the immunocomplex is immobilizing on the OWLS chip surface. Depending on which immunocomponent binding step we are determining, higher or lower changes in molecular mass of the complex can be achieved. Consequently, the immobilized antigen format (when binding of an immunoglobulin of molecular weight (MW) 160 000 takes place to a small analyte) is expected to result in improved LOD

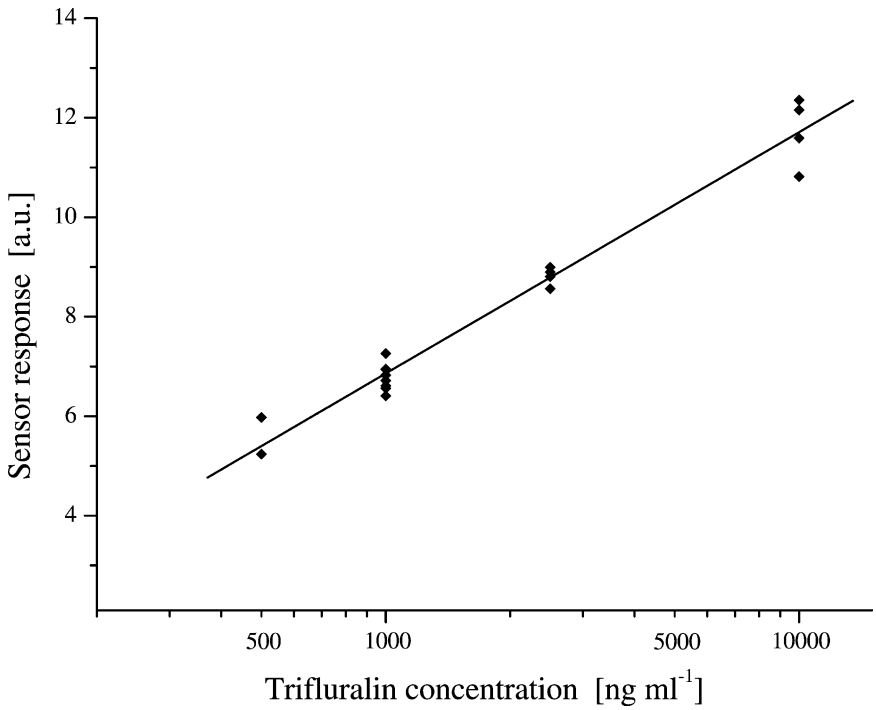

Fig. 3. Calibration of the sensor response and the concentration of the analyte trifluralin in the medium. The sensor surface is sensitized with antiserum diluted 1:2000. Relative mass (mass per area) on the surface of the waveguide layer is in linear correlation with the concentration of the analyte in the non-competitive immunosensor system.

relative to the immobilized antigen format. In the immobilized antigen based OWLS system, trifluralin antigens (trifluralin–BSA conjugates) were immobilized on the sensor surface, and the binding of anti-trifluralin antibodies was detected at a later step. Trifluralin content in the sample could inhibit binding of the antibodies to the sensitized surface in a competitive manner. Alternatively, trifluralin and anti-trifluralin antiserum could be preincubated prior to being injected to the sensor surface, and then detection was carried out as a binding inhibition test, resulting in a further decreased LOD relative to the competitive method.

To study the effect of the concentration of the coating antigen (trifluralin–BSA conjugate) immobilized, sensor surfaces were treated with such conjugates at different concentrations ranging between 1 and 10 $\mu g\,ml^{-1}$. In order to compare antibody binding capacity of the sensors, polyclonal sera at dilutions of 1:6000 and 1:3000 were applied to these sensor surfaces, and corresponding OWLS signals were measured. Sensor responses given for different dilutions of antiserum by sensor surfaces sensitized with a trifluralin–BSA conjugate at concentrations of 1 and 10 $\mu g\,ml^{-1}$ indicated that sensors sensitized with 10 $\mu g\,ml^{-1}$ of the trifluralin conjugate produced higher responses and provided more stable signals. Thus, this latter concentration was used for further experiments.

3.3. The effect of antiserum dilution

In the case of the binding inhibition test method, standards and samples were mixed with polyclonal antiserum, were preincubated for a certain period and the mixture was injected into the OWLS system. During determination, trifluralin content of the solution is measured while trifluralin–BSA conjugates immobilized on the surface bind to (or compete) for available antibodies in the mixture. This is a rather sensitive equilibrium, therefore the choice of the appropriate analysis parameters is very important. Because increasing trifluralin concentrations in the samples result in larger decreases in the assay signal, one of the parameters of key importance is the antibody concentration in the antiserum. To find the adequate antibody concentration, polyclonal antiserum raised against trifluralin [49] at dilutions between 1:500 and 1:6000 was measured. Fig. 4 depicts sensor responses obtained for different antiserum dilutions. Comparing the signals, antiserum dilution of 1:3000 and 1:2000 can be considered suitable for measurements. At these dilutions responses given by the sensor are high enough for evaluations, yet the method is still sufficiently sensitive to detect small amounts of trifluralin.

A standard calibration curve of the resulting OWLS (method parameters: trifluralin–BSA conjugate concentration 10 $\mu g\,ml^{-1}$, antiserum dilution 1:2000) is depicted in Fig. 5. The IC_{50} value for trifluralin with the OWLS sensor was calculated to be 1.053×10^{-6} ($\pm 0.52 \times 10^{-6}$) $ng\,ml^{-1}$. This value is six orders of magnitude lower than that detected with ELISA (see below). It has to be noted that this outstanding improvement in the detection range corresponds to both methods performed in distilled water. Both signal stability requirements and possible matrix effects in real samples are expected to moderate the range of detectable trifluralin concentration of the OWLS sensor on real analytical samples. Yet, the major shift in the LOD of the assay is clearly due to the immunosensor principle, because other parameters determined by antiserum quality appear to be very similar. Standard curves by OWLS and ELISA are almost identical in shape (slopes: 0.91 and 0.88, respectively), and the cross-reactivity (CR) pattern of the antiserum determined for close structural analogs of trifluralin are very close to each other in the two systems. Table 1 lists CR values for four dinitroaniline fungicides (benfluralin, ethalfluralin, isoporopalin and pendimethalin) and for four related intermediates (2,6-dinitro-4-trifluoromethylaniline, 2,6-dinitro-4-trifluoromethyl-1-chlorobenzene, 2,6-dinitroaniline and aniline), and percentage cross-reactivities are within 4% difference for each compound in both immunoanalytical systems.

Thus, the immobilized antigen format OWLS system appears to allow detection of the target analyte in the femtomolar range (3.14×10^{-15} M). Such remarkably low LOD is rare but not unprecedented among various immunoanalytical methods: similar LOD's have been seen for proteins [59,60] or microbial toxins [61]. Thus, hepatitis B surface antigen (MW 2000) was detected using a difference interferometer affinity sensor at 2×10^{-13} M [59], avidin (MW 68 000) and BSA (MW 68 000) were detected by a thin film waveguide fluoroimmunosensor with an

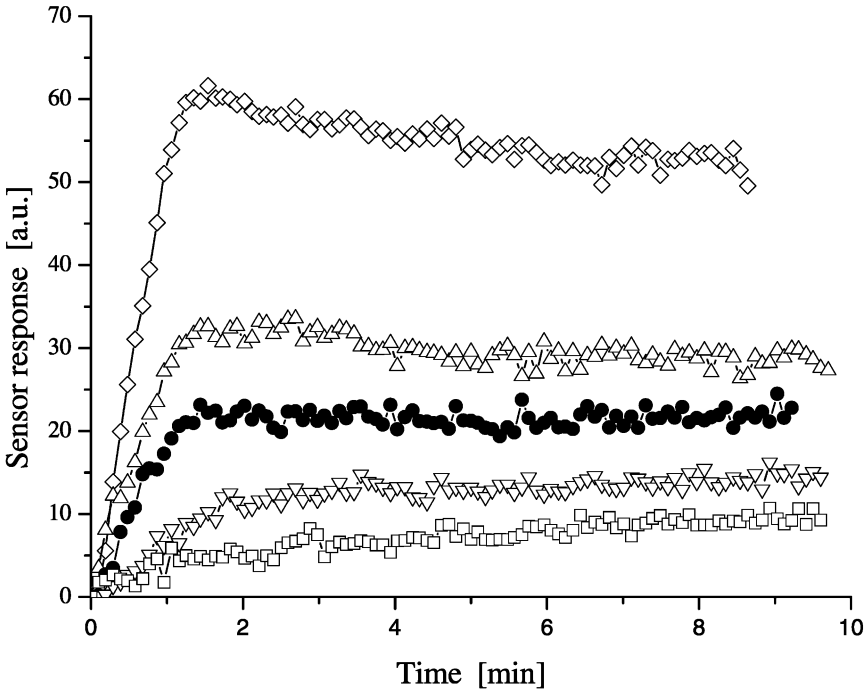

Fig. 4. The choice of optimal antiserum dilution used in the competitive OWLS immunosensor at antiserum dilutions of 1:500 (◇), 1:1500 (△), 1:2000 (●), 1:3000 (▽) and 1:6000 (□). Sensor surfaces were sensitized with the coating antigen (trifluralin–BSA) at a concentration of 10 $\mu g\,ml^{-1}$. At antiserum dilution of 1:2000 the sensor provides a stable and detectable signal without loss of sensitivity.

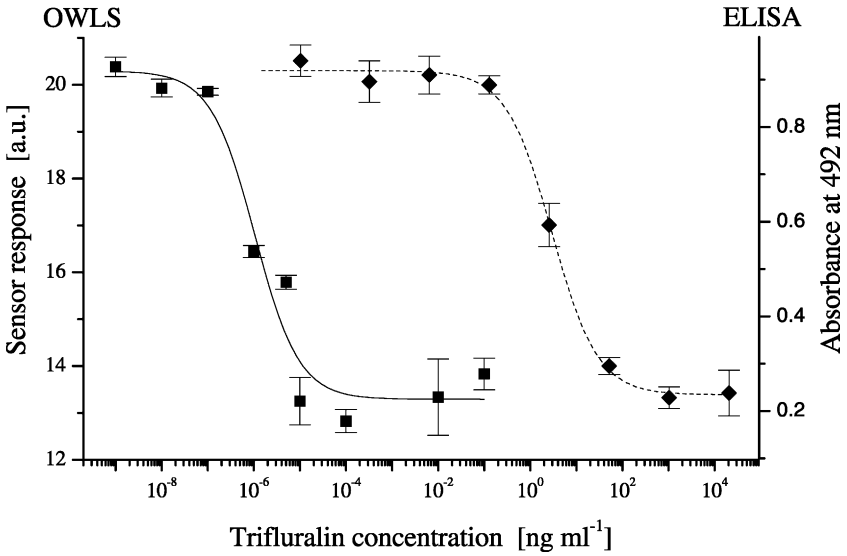

Fig. 5. Standard inhibition curves of the immobilized antigen based, competitive OWLS immunosensor (■) and ELISA (◆) by trifluralin. The two standard curves appear to be very similar in shape (slopes: 0.91 and 0.88, respectively) with a six orders of magnitude shift (IC_{50} values: $(1.053 \pm 0.52)\ 10^{-6}$ and $2.87 \pm 0.39\,ng\,ml^{-1}$, respectively).

Table 1
Cross-reactivities of dinitroaniline herbicides, haptens and various synthetic intermediates in the competitive OWLS immunosensor

Compound	IC_{50} (ng ml^{-1})	CR[a] (%)	CR[b] (%)
Trifluralin	6.10×10^{-7}	100	100
Ethalfluralin	8.88×10^{-6}	6.9	2.9
Benfluralin	1.75×10^{-5}	3.4	5.2
Isopropalin	2.35×10^{-4}	0.26	<0.1
2,6-Dinitro-4-trifluoromethylaniline	6.90×10^{-4}	0.09	0.18
Pendimethalin	5.80×10^{-3}	>0.01	≪0.1
2,6-Dinitro-4-trifluoromethyl-1-chlorobenzene	$>10^{-2}$	≫0.01	≪0.1
2,6-Dinitroaniline	$>10^{-2}$	≫0.01	≪0.1
Aniline	$>10^{-2}$	≫0.01	≪0.1

[a] Relative cross-reactivity defined as a percentage obtained by calculating the ratio of the IC_{50} value of the reference compound (trifluralin) to that of the given compound in the immunoanalytical system.

[b] Data from the literature [49].

LOD of 10^{-15} M [60], and toxins from *Clostridium botulinum* (MW 150 000) and *Vibrio cholerae* (MW 84 000) were detected using a ganglioside–liposome based sandwich immunoassay with LOD of 10^{-13} and 2×10^{-16} M, respectively [61]. Such outstanding LODs can be due to extremely high amplification factor due to liposomes [61] or extremely high dependence of the incoupling angle of the optical immunosensor from the molecular masses on the sensor surface. This allows detection of antigen–antibody interactions in real time at almost molecular level: the number of interacting molecules on the surface analyzed is in the range of 10^4.

3.4. Detection of trifluralin by ELISA

The range of detectable analyte concentration of the ELISA method was characterized with the assay midpoint, IC_{50} value (analyte concentration causing a 50% decrease in the assay signal as compared to the uninhibited signal) and LOD of the assay. The IC_{50} value of the optimized immunoassay (the coating antigen and the antiserum applied at a concentration of 1 μg ml^{-1} and at a dilution of 1:2000, respectively), was measured to be 2.87 ± 0.39 ng ml^{-1} (Fig. 5) with an apparent LOD value of 0.8 ng ml^{-1} in water. This is statistically identical to the previously reported IC_{50} value (3.94 ± 1.59 ng ml^{-1}) [49]. Standard calibration curves obtained with crude antiserum in ELISA and OWLS tests were very similar in shape (see above).

3.5. Validation of OWLS results by ELISA and GC-MS

In order to verify the accuracy of the OWLS determination, trifluralin content was detected both by ELISA and by GC-MS in distilled water samples spiked with trifluralin at concentrations up to 25 ng ml^{-1}. GC-MS analyses were carried out following SPE on a graphitized carbon column and delivery 5 μl of the eluate to the gas chromatograph with temperature programmed injection. The masses of the fragment ions chosen from trifluralin mass spectra for quantification were 264 and 306 amu. A calibration curve for the quantitative determination of trifluralin was established based on peak areas of the target fragment ions in spiked solutions in distilled water. The chosen GC column and temperature program allowed a peak retention time (R_t) of 10.86 min. (The corresponding R_t value for isothermic injection at 230 °C was 9.41 min.) Trifluralin concentrations detected at spike levels in isooctane between 0.5 and 200 ng ml^{-1} eluent offered excellent regression characteristics ($r^2 = 0.9998$) and indicated the minimal detectable amount (MDA) in the assay being 0.005 ng. (Using isothermic injection at 230 °C, the corresponding MDA was 0.01 ng.) Subjecting distilled water samples spiked at corresponding aqueous concentration levels of 0.5–200 ng l^{-1} to SPE indicated that the recovery of the SPE procedure ranged between 78 and 84% depending on spike concentrations. (At spike concentration in distilled water corresponding to the double of the theoretical LOD based on LDA in isooctane, 0.01 ng ml^{-1}, the recovery was measured to be 78.3 ± 8.1%.)

Based on earlier findings [49] spike levels in distilled water for validatory purposes of the OWLS determinations using both GC-MS and ELISA analyses were set between 1 and 10 ng ml^{-1}. (An additional spike level at 25 ng ml^{-1} was also prepared, knowing that at concentrations above 10 ng ml^{-1} ELISA tends to overestimate trifluralin levels [49].) While GC-MS analyses were carried out as seen above, ELISA was performed on the spiked distilled water samples without sample preparation.

Table 2
Detection of trifluralin in spiked and field surface water samples by OWLS formats, ELISA and GC-MS

Estimated (spike) concentration (ng ml^{-1})	Concentration by non-competitive OWLS (ng ml^{-1})	Concentration by competitive OWLS (ng ml^{-1})	Concentration by ELISA (ng ml^{-1})	Concentration by GC-MS (ng ml^{-1})
0	<100	<0.0001	<0.02	<0.01
2.5	<100	1.81 ± 0.30	1.79 ± 0.38	2.42 ± 0.08
5	<100	5.43 ± 0.30	4.16 ± 1.16	4.32 ± 1.01
25	<100	36.3 ± 1.11	34.1 ± 4.01	24.6 ± 3.76
–[a]	<100	3.08 ± 1.02	1.29 ± 0.53	1.94 ± 0.53

[a] Field sample with trifluralin content. *Source*: Main Eastern Irrigation Channel (Keleti Főcsatorna), Hungary, sampled at 18 July 2001.

In addition to spiked distilled water samples, trifluralin was also monitored by GC-MS following SPE sample preparation in numerous surface water samples collected at various regions of Hungary in a 2-year campaign. Trifluralin contamination was seen in a single occasion out of 238 surface water samples analyzed. This sample, found to contain 1.94 ng ml^{-1} trifluralin, was subjected to both OWLS and ELISA analysis, and was included in the set of validatory samples. Trifluralin concentrations detected in spiked distilled water samples and in a surface water sample (collected from the Main Eastern Irrigation Channel (Keleti Főcsatorna) in Hungary, unspiked) by GC-MS, ELISA and OWLS are listed in Table 2. Trifluralin concentrations in given samples were calculated by cross-validation (leave-one-out method): concentrations for each point were calculated from regression lines on the basis of the other points. OWLS and ELISA determinations were carried out on parallel aliquots of the same distilled water samples used for GC-MS, and concentrations were determined on the basis of the standard curve established in assay buffer, as described above. As seen above, LOD of the non-competitive OWLS sensor was insufficient to detect trifluralin at concentrations below 100 ng ml^{-1}, while the competitive OWLS sensor allowed a detection range of 2×10^{-7} to 3×10^{-5} ng ml^{-1}. Therefore, samples were diluted with buffer solution prior to competitive (or binding inhibition) OWLS detection. Trifluralin concentrations detected by the indirect OWLS sensor well correlated with those detected by ELISA and GC-MS methods ($r^2 = 0.998$ and 0.9975, respectively): concentrations between 2.5 and 5 ng ml^{-1} showed good agreement in all three methods, while both OWLS and ELISA overestimated trifluralin content at 25 ng ml^{-1}, as compared to GC-MS. The regression slope of the correlation between the OWLS and GC-MS methods is therefore lower (0.66), while that between the OWLS and ELISA method (0.962) is very close to 1.

4. Conclusions

A non-competitive (immobilized antibody-based) and a competitive or binding inhibition (immobilized antigen-based) OWLS immunosensor was developed and optimized for the detection of trifluralin. The non-competitive method appeared to be less sensitive, and produced analyte concentration related sensor response above 100 ng ml^{-1} concentration of trifluralin. In contrast, the competitive OWLS immunosensor offered major improvements in assay LOD relative to a previously developed ELISA system [49]. This prominent difference between the measuring range of the non-competitive and competitive methods in not surprising in light of the fact that the OWLS signal is related to relative masses bound to the waveguide surface: binding of a small analyte (MW 335.3) to an immobilized antibody (MW 150 000–900 000) does not produce a great shift in the coverage (mass per area) on the waveguide. In contrast, binding of an antibody to an immobilized antigen conjugate (MW 69 300) results in more extensive changes in the response. The OWLS sensor was optimized for major method parameters, such as sensitizing antigen concentration (10 μg ml^{-1}) and antiserum dilution (1:2000), and offered a standard curve for trifluralin in the analyte concentration range of 2×10^{-7} to 3×10^{-5} ng ml^{-1} in water. Consequently, the authors have applied for a patent for the OWLS immunosensor and its applications [62]. The two immunoana-

lytical systems, competitive OWLS and ELISA were found to be similar in several assay characteristics e.g. shape of the standard curve and cross-reactivity of the antiserum. The outstanding detectable analyte concentration range ($IC_{50} = 1.05 \pm 0.52 \times 10^{-6}$ ng ml^{-1}) of the competitive OWLS sensor in water is expected to be limited by other factors (signal stability and matrix effects in real samples), and although this LOD should be considered as a methodological limit because the antigen–antibody interaction is presently monitored without the possible interfering effects of the medium, the results illustrate the perspective of the present OWLS technique in the detection of trifluralin: if the LOD in real samples is two or even three orders magnitude higher that the reported value in buffer, the method allows 100- or 1000-fold dilution of the sample prior to analysis, and that should dilute matrix effects out.

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