Talanta 214 (2020) 120854

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fast, sensitive and selective determination of herbicide glyphosate in water samples with a White Light Reflectance Spectroscopy immunosensor

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ARTICLE INFO

Keywords:
Glyphosate
White light reflectance spectroscopy
Label-free immunosensor
Drinking water

ABSTRACT

An optical immunosensor based on White Light Reflectance Spectroscopy is described for the determination of the herbicide glyphosate in drinking water samples. The biosensor allows for the label-free real-time monitoring of biomolecular interactions taking place onto a SiO_2/Si chip by transforming the shift in the reflected interference spectrum caused by the immunoreaction to effective biomolecular adlayer thickness. Glyphosate determination is accomplished by functionalizing the chip with a protein conjugate of the herbicide followed by a competitive immunoassay format. Prior to the assay, glyphosate derivatization in the calibrators and/or the samples was performed through reaction with succinic anhydride. Under the optimized assay protocol, a detection limit of 10 pg mL^{-1} was achieved. Recovery values ranging from 90.0 to 110% were determined in spiked bottled and tap water samples, demonstrating the accuracy of the method. In addition, the sensor could be regenerated and re-used for at least 14 times without statistically significant effect on the assay sensitivity and accuracy. The excellent analytical performance and short analysis time (approx. 25 min), combined with the small sensor size, should be helpful for the fast on-site determination of glyphosate in drinking water samples.

1. Introduction

Glyphosate (N-phosphonomethyl glycine) is a broad-spectrum non systemic herbicide brought to market for agricultural use in 1974 by Monsanto Company under the trade name Roundup. Glyphosate-based pesticides are typically applied before crops are sown to control weeds and therefore facilitate better growth of crops by eliminating competing plants, an agricultural practice which minimizes the need for soil tillage, thereby reducing soil erosion and carbon emissions [1]. Since its first commercialization, popularity of glyphosate-based herbicides has increased sharply due to the unique mode of action of the active ingredient that involves the inhibition of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase that is part of the biochemical pathway for the formation of aromatic amino acids in plants, making glyphosate a non-selective herbicide active on a very wide range of plant species. The introduction of transgenic glyphosate-resistant crops in 1996, further expanded the use of glyphosate-based herbicides, as almost 90% of all transgenic crops grown worldwide are glyphosate resistant [2].

Despite the fact that, based on its chemical properties, glyphosate is expected to be adsorbed by the soil [3], due to its widespread use, surface and ground water contamination is possible through drift during application or as surface runoff following application [4]. The contamination of surface water creates certain dangers for the aquatic fauna in terms of acute or chronic toxicity that concern also humans as consumers of aquatic species or through consumption of contaminated drinking water [5]. Apart from the acute or chronically toxic effects, glyphosate may demonstrate endocrine disrupting activity [5–7]. Moreover, over the past years there is an ongoing discussion in the scientific community concerning whether glyphosate and/or glyphosate formulations available in the market are carcinogenic. In general, glyphosate is considered by the regulatory authorities and scientific bodies to have no carcinogenic potential [8]. Nevertheless, the International Agency for Cancer Research has recently classified glyphosate as probably carcinogenic to humans (category 2A) [9].

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https://doi.org/10.1016/j.talanta.2020.120854
Received 13 November 2019; Received in revised form 10 February 2020; Accepted 16 February 2020
Available online 17 February 2020
0039-9140/

For all of the above reasons, monitoring of glyphosate in water, food, and feed stuff is considered a necessity and considerable research effort has been devoted to the development of methods for determination of glyphosate. The majority of these methods are based on either gas chromatography (GC) or high performance liquid chromatograph (HPLC) coupled to a variety of detectors, mainly mass spectrometric detectors. The absence of chromophore or fluorophore groups in glyphosate's molecule precludes the direct photometric or fluorometric detection in liquid chromatography methods. Thus, although methods based on ion-exchange chromatography [12] or liquid chromatography with hydrophilic interaction columns [13,14] for the determination of glyphosate without derivatization have been reported in the literature, a pre- or post-column derivatization procedure is usually required to improve both the chromatographic behavior and the detection sensitivity by GC and LC [10]. Most of the aforementioned methods are highly sensitive with limits of detection at the ppb level, but are in most cases tedious since they include numerous steps for the purification and derivatization of the compound. Moreover, they are also bench top-bound and not appropriate for on-site determinations.

In the present work, a label-free sensor is presented based on White Light Reflectance Spectroscopy (WLRS) for the fast, sensitive and selective determination of glyphosate in water samples. The WLRS sensing approach has been already used for the immunochemical determination of high molecular weight analytes [11] as well as for pesticides in wine samples [12] and water [13]. The particular sensor allows for the label-free and real-time monitoring of biomolecular interactions by following the shift of the interference spectrum reflected from a SiO_2/Si chip on the top surface of which the reactions takes place. Glyphosate determination is accomplished by functionalizing the chip with a protein conjugate of the herbicide and probing the surface with an optical reflection probe (Fig. 1a). A competitive immunoassay format follows, in which mixtures of calibrators or samples with a specific antibody against glyphosate were run over the chip, followed by reaction with secondary antibody for signal enhancement (Fig. 1b). Prior to the assay, glyphosate was derivatized with succinic anhydride which enhances the assay sensitivity, as suggested in the literature [14,15]. In addition, due to lack of commercially available protein conjugates for glyphosate, a conjugate with bovine serum albumin has been prepared and evaluated for use as solid-phase reactant in the immunosensor. The cross-reactivity of glyphosate specific antibody towards compounds with structural similarity, namely aminomethylphosphonic acid (the main glyphosate metabolite), gluphosinate (a glyphosate-like naturally occurring broad-spectrum systemic herbicide produced by several species of *Streptomyces* soil bacteria) and the amino acid glycine, as well as against other pesticides was also evaluated. Finally, the accuracy of the determinations performed with the label free optical immunosensor was evaluated through recovery experiments in bottled and tap water samples.

2. Materials and methods

2.1. Reagents

Four-inch Si wafers (< 100 >), purchased from Si-Mat Germany (Kaufering, Germany), were cleaned with acetone and isopropanol and then a 1000-nm thick SiO_2 layer was grown by thermal oxidation at 1100 °C in the clean room facility of the Institute of Nanoscience and Nanotechnology of NCSR "Demokritos". The wafers were diced to fabricate chips of 5 × 15 mm^2. Bovine serum albumin (BSA) and (3-aminopropyl)triethoxysilane (APTES) were from Acros Organics (Geel, Belgium). Glyphosate, gluphosinate, aminomethylphosphonic acid (AMPA) (all in PENTANAL® analytical standard grade) and glycine, as

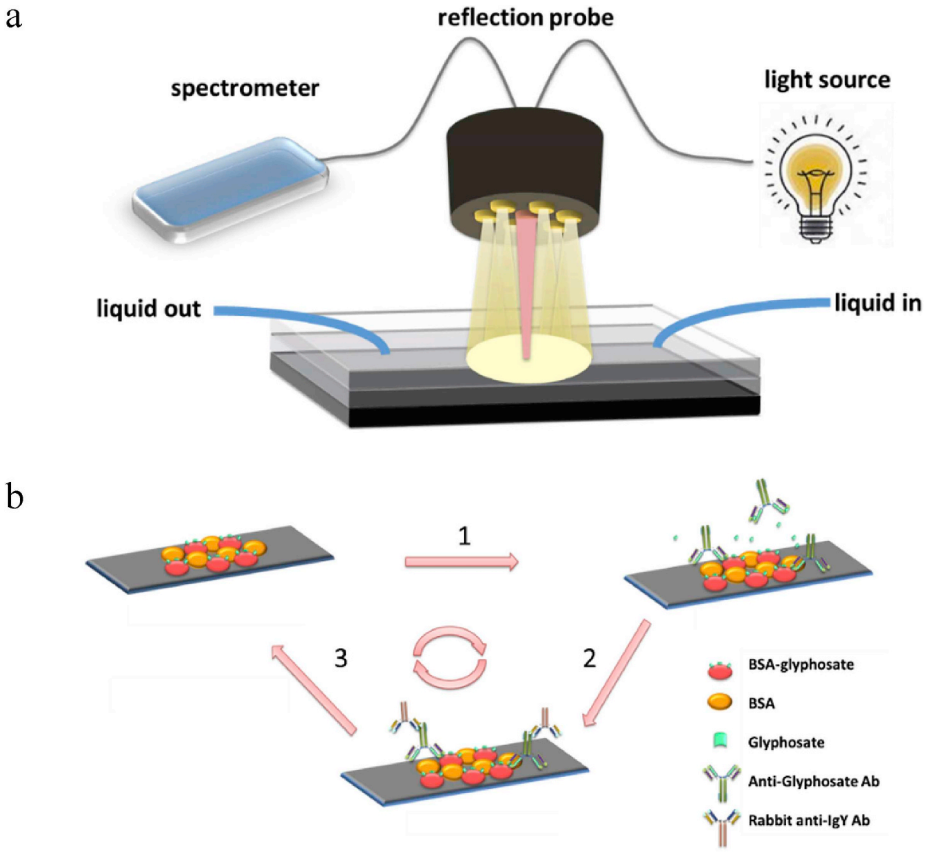


Fig. 1. Schematic of the (a) WLRS set-up and (b) of assay procedure. The assay steps in (b) include: (1) reaction of anti-glyphosate antibody with the glyphosate in the sample and the immobilized onto the sensor glyphosate-protein conjugate, (2) reaction with the rabbit anti-IgY antibody, and (3) regeneration of the chip.

well as 1-ethyl-3-(3-dimethylamonopropyl)carbodiimide (EDC) and succinic anhydride were purchased from Sigma-Aldrich (St. Louis, MO). Chicken anti-glyphosate polyclonal antibody and rabbit anti-chicken IgY antibody (secondary antibody) were from Fitzgerald (Acton, Massachusetts, USA) and Sigma-Aldrich (St. Louis, MO), respectively. The water used throughout the study was doubly distilled.

2.2. Instrumentation

The detection system employed in the present study consists of: a) a visible/near infrared light source, b) a miniaturized spectrophotometer (Maya 2000 Pro, Ocean Optics) with wavelength resolution of 0.25 nm, and c) a reflection probe (AVANTES Inc., Broomfield, CO) which delivers the incident light to the surface through six multi-mode fibers arranged in the periphery of the probe and collects the reflected light through a central fiber which is connected to the spectrophotometer; each one of the seven fibers has a diameter of 400 μm. For the measurements, the chips were assembled with a fluidic compartment (Jobst Technologies GmbH; Freiburg, Germany) composed of a 2-mm thick PMMA cover with milled fluid inlet and outlet to which a 200-μm thick double side adhesive appropriately cut to form the reaction chamber has been applied (Fig. S1a). The dimensions of the reaction chamber were 12 mm × 2.5 mm x 0.2 mm (LxWxH). After assembly with the fluidic compartment, the chip is placed in the docking station and a miniaturized peristaltic pump was employed for the reagents delivery to the chip. Data acquisition was performed by a custom-made software that controlled also the optical set-up. An integration time of 15 ms was set for the spectrometer and the reflectance spectrum was recorded every second. To monitor bioreactions taking place on the sensor, the absolute reflectance spectrum [R(λ)] was calculated using the following equation:

$$R(\lambda) = \frac{S(\lambda) - D(\lambda)}{REF(\lambda) - D(\lambda)} x\ 100$$

where, D(λ) is the dark spectrum received with the light source turned-off, REF(λ) is the reference spectrum, and S(λ) is the reflectance spectrum recorded every second from the chip surface. In Fig. S2a and a dark, a reference, and a characteristic reflectance spectrum are provided. The absolute reflectance spectrum is then fitted in the 450–650 nm spectral range using the Levenberg-Marquart algorithm for a layer stack consisted of water/biomolecular layer/SiO_2/Si and assuming a refractive index value of 1.45 for the biomolecular layer [16]. Thus, the shift of the reflectance spectrum recorded in the course of the biomolecular reaction (Fig. S2b) is converted in real-time by the software to “effective biomolecular adlayer thickness”.

2.3. Calibrator and water samples preparation

A glyphosate stock solution with concentration of 1 mg mL^{-1} was prepared in double distilled water and stored at −20 °C. From this solution, calibrators with concentrations ranging from 0.02 to 10 ng mL^{-1} were prepared in distilled, bottled table or tap water. For the recovery experiments, samples fortified with known concentrations of glyphosate were prepared in bottled table and tap water. The glyphosate present in calibrators and/or samples was derivatized with succinic anhydride following a modification of a published protocol [14,15]. Briefly, a 1 mol L^{-1} solution of succinic anhydride was prepared in DMSO and 20 μL of this solution were added in 1 mL of calibrator solution/sample whose pH value has been adjusted to 8.0 by adding 10 μL of 0.5 M $NaHCO_3$ per mL of calibrator/sample. The mixture was shaken vigorously and left to react for 15 min.

2.4. Preparation of glyphosate conjugate

A glyphosate conjugate with bovine serum albumin (BSA) was prepared by employing carbodiimide chemistry (EDC) to couple glyphosate mainly by its carboxy group to the ε-amine groups of BSA lysine residues using a molar ratio of glyphosate to protein in the reaction mixture of approximately 300. In detail, for the preparation of glyphosate-BSA conjugate 1.5 mg of glyphosate in 500 μL of 0.1 M MES buffer, pH 4.7, were mixed with 200 μL of a 10 mg mL^{-1} BSA solution in the same buffer. To this mixture were added 100 μL of a 10 mg mL^{-1} EDC solution in water, and it was incubated for 2 h at room temperature (RT). The reaction mixture was then extensively dialyzed against 0.1 mol L^{-1} $NaHCO_3$, pH 8.5, 0.15 mol L^{-1} NaCl, 0.05 mg mL^{-1} NaN_3, and stored at 4 °C.

2.5. Preparation of the biochip

The immobilization of biomolecules onto the SiO_2 top layer of the chips requires chemical activation of the surface with a silane layer. Prior to activation, the chips were cleaned/hydrophilized by O_2 plasma treatment for 30 s in a Reactive Ion Etcher at 10 mTorr. Then, they were immersed in a 2% (v/v) aqueous APTES solution for 20 min, washed with distilled water, dried under a nitrogen stream, cured at 120 °C for 20 min, and kept at RT in a desiccator for at least 24 h prior to use. A 50 μg mL^{-1} glyphosate-BSA solution in 0.05 mol L^{-1} carbonate buffer, pH 9.2 (coating buffer) was then deposited in a 3 × 5 mm^2 area at the centre of the APTES-modified chips using the Bio-Odyssey Calligrapher microarray spotter (Bio-Rad Laboratories, Hercules, CA). After spotting, the chips were incubated in controlled humidity (75%) overnight at 4 °C. Then, they were washed with 0.05 mol L^{-1} Tris-HCl buffer, pH 8.9, containing 0.9% (w/v) NaCl (washing solution), and immersed in blocking solution (10 mg mL^{-1} BSA in 0.1 mol L^{-1} $NaHCO_3$, pH 8.5) for 1 h at RT. After rinsing with washing solution and distilled water the chips were dried under a nitrogen stream and used for the assay. The biofunctionalized chips are mentioned hereinafter as biochips.

2.6. Glyphosate assay

The assay procedure is schematically depicted in Fig. 1b. At first, the biochip was equilibrated with assay buffer for 2 min in order to acquire a stable baseline, then 1:1 vol mixtures of derivatized calibrators or water samples with a solution containing 50 μg mL^{-1} of anti-glyphosate specific antibody in assay buffer (0.05 mol L^{-1} Tris-HCl buffer, pH 8.9, containing 0.9% (w/v) NaCl, 0.5% BSA, 0.05% bIgG) were run over the biochip for 15 min (step 1, Fig. 1b) with a flow rate of 30 μL min^{-1} (total sample volume 450 μL). After that, a solution containing 1 μg mL^{-1} of rabbit anti-chicken IgY antibody in assay buffer was run for 10 min at the same flow rate (step 2, Fig. 1b). Finally, the biochip was regenerated by passing a 50 mmol L^{-1} HCl solution for 2 min, followed by equilibration with assay buffer (step 3, Fig. 1b). According to the competitive immunoassay principle followed for the detection of glyphosate, the maximum signal is obtained in absence of analyte, i.e., for the zero calibrator; whereas in presence of analyte due to “competition” between the solid-phase immobilized analyte-conjugate and the analyte in the calibrator/sample for binding to the limited number of free binding sites of the antibody, the signal decreases inversely proportional to the analyte concentration. Thus, the glyphosate calibration curve was created by plotting the effective biomolecular adlayer thickness (signal) obtained for the different calibrators (S_x) as percentage of the zero calibrator's signal (maximum signal; S_0) against the respective analyte concentration in the calibrator solutions in linear/log scale.

3. Results and discussion

3.1. Assay optimization

Several parameters of the WLRS assay have been optimized including the concentration of analyte-protein conjugate used for coating, the concentration of analyte-specific antibody, and the duration of the

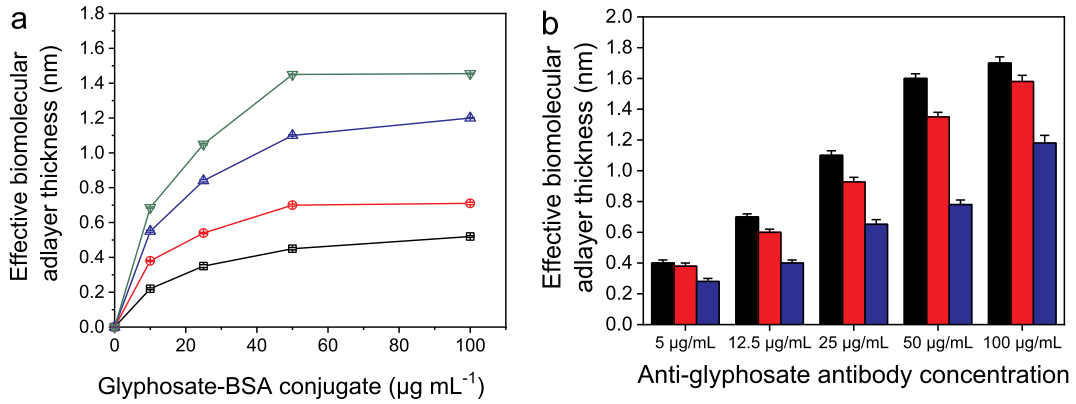


Fig. 2. (a) Effect of glyphosate-BSA conjugate concentration used for spotting on the zero calibrator signal obtained using 5.0 (squares), 12.5 (circles), 25.0 (triangles) or 50.0 μg mL^{-1} (down triangles) solution of anti-glyphosate antibody. (b) Signals corresponding to zero calibrator (black columns) or calibrators containing 0.02 (red columns) and 1.6 ng mL^{-1} of glyphosate (blue columns) obtained from chips coated with 50 μg mL^{-1} glyphosate-BSA conjugate and assayed using anti-glyphosate antibody solutions with concentrations ranging from 5.0 to 100 μg mL^{-1}. In all cases, the anti-glyphosate antibody solution was run for 15 min followed by 10-min reaction with a 1 μg mL^{-1} anti-chicken IgG antibody solution. Each point is the mean value of 3 measurements ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

primary and secondary immunoreaction, since they define both the absolute signal and the assay sensitivity. Thus, the criteria for the optimization of assay parameters are the maximum signal achieved in absence of analyte, i.e., the zero calibrator's signal, and the percent signal drop obtained in presence of calibrators which defines the assay sensitivity.

The zero calibrator signals obtained using different concentrations of glyphosate-BSA conjugate in the coating solution in combination with different anti-glyphosate antibody concentrations are provided in Fig. 2a. As shown, maximum plateau values were obtained when glyphosate-BSA conjugate concentrations equal to or higher than 50 μg mL^{-1} were used for coating with all the anti-glyphosate antibody concentrations tested, and thus this concentration was selected for further experimentation. The optimum anti-glyphosate antibody concentration was selected based on both the zero calibrator signal and the assay sensitivity. As shown in Fig. 2b, the assay sensitivity was significantly improved when the anti-glyphosate antibody concentration was increased from 5.0 to 50 μg mL^{-1}, while higher antibody concentration resulted in sensitivity decrease. Thus, in order to achieve both high zero calibrator signal and assay sensitivity a 50 μg mL^{-1} anti-glyphosate antibody concentration was adopted in the final assay protocol.

The duration of the two immunoreaction steps was also optimized. As shown in Fig. 3a, the signal was continuously increased as the duration of the primary immunoreaction (i.e., the reaction of the pesticide-specific antibody with the immobilized conjugate) increased from 5 to 60 min. Nonetheless, the highest percent signal drop (percent inhibition) for a calibrator containing 0.5 ng mL^{-1} glyphosate was achieved for 15-min reaction with the anti-glyphosate antibody. Regarding the secondary immunoreaction, maximum plateau values were achieved after 10 min of reaction (Fig. S3). Thus, a 15-min reaction with the glyphosate-specific antibody followed by a 10-min reaction with the secondary antibody was adopted in the final immunoassay protocol.

Another critical parameter that affects both the zero calibrator signal and percent inhibition achieved in presence of glyphosate was the flow rate with which the reaction mixture (calibrator/sample and anti-glyphosate antibody) and the secondary antibody was delivered upon the functionalized with the glyphosate-BSA chip surface through the microfluidic compartment. As shown in Fig. 3b, the percent inhibition achieved for a calibrator containing 0.5 ng mL^{-1} glyphosate increased from approximately 45 to 70% as the flow rate was reduced from 100 to 30 μL min^{-1}, while further reduction to 20 μL min^{-1} affected negatively both the zero calibrator signal and percent inhibition obtained for the calibrators probably due to inadequate delivery of the reagents to the biochip surface under this flow conditions. Thus, the flow rate selected for the glyphosate assay protocol was 30 μL min^{-1}.

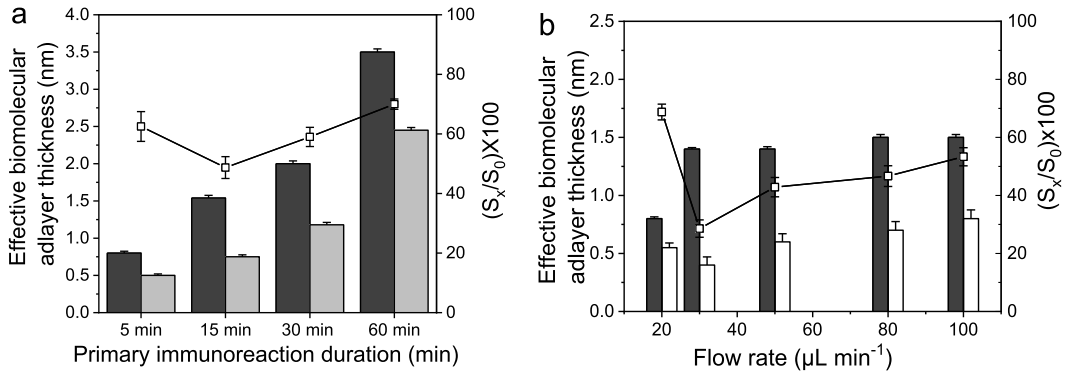
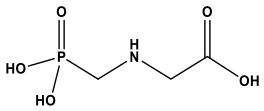
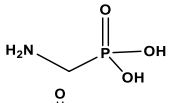
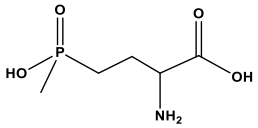
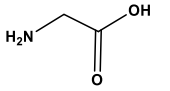


Fig. 3. (a) Effect of reaction time with 50 μg mL^{-1} anti-glyphosate specific antibody on the zero calibrator signal (dark grey columns) and the signal obtained for a calibrator containing 0.5 ng mL^{-1} of glyphosate (light grey columns) (left y-axis) as well as on the percent signal achieved for this calibrator (open squares) with respect to zero calibrator (right y-axis). The reaction with the secondary antibody lasted 10 min. (b) Effect of the flow rate on the zero calibrator signal (black columns) and the signal corresponding to a calibrator containing 0.5 ng mL^{-1} of glyphosate (white columns) (left y-axis) as well as on the percent signal (open squares) achieved for this calibrator with respect to zero calibrator (right y-axis). The duration of the reaction with primary antibody was 15 min and with the secondary antibody 10 min. Each point is the mean value of 3 measurements ± SD.

Table 1
Cross reactivity of structurally similar compounds to glyphosate assay.

Compound	Structure	IC_{50} (ng mL^{-1})	% Cross Reactivity
glyphosate		0.35	100
AMPA		1500	0.02
gluphosinate		10,000	ND < 0.001
glycine		10,000	< 0.001

3.2. Specificity of anti-glyphosate antibody

The specificity of the anti-glyphosate antibody, and in consequence of the assay, against structurally similar compounds such as aminomethylphosphonic acid (AMPA), which is the main metabolite of glyphosate, gluphosinate and glycine was also determined through cross-reactivity experiments. For this purpose, calibrators of each one of the potential cross-reactants were prepared, reacted with succinic anhydride and run in the WLRS sensor using the protocol of the glyphosate assay. Percent cross-reactivity (%CR) was calculated by the following formula: %CR = (IC_{50} target analyte/IC_{50} cross-reactant)x100, where IC_{50} target analyte and IC_{50} cross-reactant are the respective concentrations of glyphosate and the possible cross-reactant which cause 50% inhibition of the zero calibrator signal (see Fig. S4 of Supplementary data). The chemical structures of the compounds checked as cross-reactants and the respective %CR values determined are presented in Table 1.

As shown, only AMPA, the major glyphosate metabolite, exhibits a measurable cross reactivity of 0.02%, whereas the other two compounds did not show any inhibition even in concentrations as high as 10 μg mL^{-1} (Fig. S4). It should be noticed, that for solubility reasons it was not possible to prepare solutions of gluphosinate and glycine with higher concentrations. The assay specificity against other pesticides was also tested. In particular the following substances have been employed: the herbicides paraquat, diquat, atrazine and simazine, the fungicides thiabendazole and imazalil, and the insecticide chlorpyrifos. In all cases no detectable inhibition was observed for any of the substances tested for concentrations up to 10 μg mL^{-1}. These results indicate the high specificity of the anti-glyphosate antibody used in the present study.

3.3. Analytical characteristics of the developed immunosensor

A typical calibration curve for glyphosate obtained with the WLRS sensor with calibrators prepared in distilled water is provided in Fig. 4. Identical calibration curves were obtained with calibrators prepared in bottled or tap water (Fig. S5), thus allowing to directly analysing drinking water samples without any pre-treatment. The assay limit of detection (LOD) was calculated as the concentration corresponding to mean zero calibrator's signal obtained from 10 measurements minus 3 standard deviations (SD), and was 10 pg mL^{-1}. The limit of quantification (LOQ) was determined as the concentration corresponding to mean zero calibrator's signal obtained from 10 measurements minus 6 SD, and was 20 pg mL^{-1} while the assay's working range was up to 10 ng mL^{-1}. It should be noted that the LOQ of the proposed sensor is

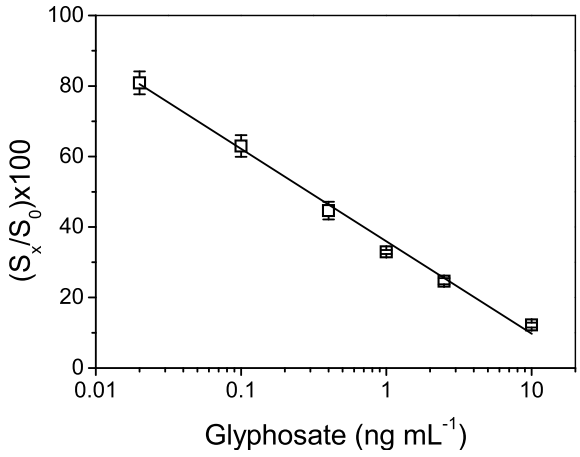


Fig. 4. Typical glyphosate calibration curve. Each point is the mean value of 3 replicates ± SD.

5-times lower than the maximum allowable limit for individual pesticides in drinking water set by EU, which is 100 pg mL^{-1} [17–19]. In addition, in literature there are several reports regarding detection of glyphosate in water samples (both freshwater and groundwater) at concentration much higher than the set maximum allowable limits [20,21].

The assay repeatability was determined using three control samples prepared in bottled water spiked with three different concentration levels of glyphosate. The intra-assay coefficient of variation (CV) was determined by running 3 replicates of each control in the same day while the inter-assay CV was determined by duplicate measurements performed in 5 days over a period of one month. The intra- and inter-assay CV values were less than 10% for the three control samples. The assay's accuracy was evaluated through recovery experiments. For this purpose, tap and two bottled water samples were spiked with 3 different concentrations of glyphosate (0.1, 0.4 and 6.4 ng mL^{-1}). All samples were analysed in triplicate both prior to and after the addition of glyphosate and the percent recovery values were calculated according to the equation: %Recovery = [Amount determined/Amount added] × 100

As shown in Table 2, the recovery values determined ranged between 90.0 and 110% indicating the good accuracy of the immunosensor developed.

3.4. Regeneration capability and re-use of the biosensor

The ability to regenerate and re-use the biochip, i.e., the coated and blocked chip, could reduce considerably the analysis cost, especially when the on-site screening of multiple samples is required. Thus, the following solutions have been evaluated as possible regeneration

Table 2
Recovery values determined using tap and drinking water samples spiked with glyphosate.

Sample	Amount added (ng mL^{-1})	Amount determined (ng mL^{-1})[a]	Recovery %
Tap water	0.10	0.11 ± 0.01	110 ± 9.0
	0.4	0.42 ± 0.02	105 ± 5.0
	6.4	6.1 ± 0.05	95 ± 0.8
Bottled water 1 (Ziria)	0.10	0.12 ± 0.01	120 ± 8.3
	0.4	0.39 ± 0.01	98 ± 2.6
	6.4	6.3 ± 0.08	98 ± 1.3
Bottled water 2 (Vikos)	0.10	0.09 ± 0.01	90 ± 11
	0.4	0.41 ± 0.02	105 ± 4.9
	6.4	6.6 ± 0.06	107 ± 0.9

[a] Each value is the mean of 3 determinations ± SD.

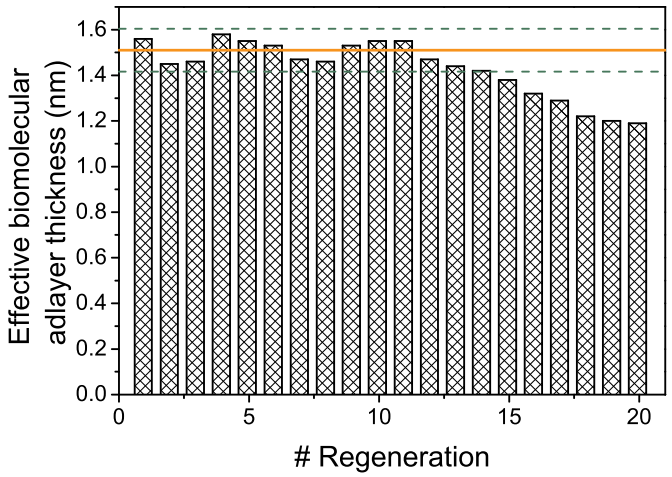


Fig. 5. Zero calibrator values obtained after repetitive assay/regeneration cycles. Solid orange line corresponds to mean value of the 12 first measurements; dashed green lines correspond to mean ± 2SD limits. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reagents: 0.1 mol L^{-1} glycine-HCl buffer, pH 2.5; 50 mmol L^{-1} NaOH solution; 50 mmol L^{-1} HCl solution; 100 mmol L^{-1} HCl solution; and 0.5% (w/v) SDS solution adjusted to pH 1.9 with HCl. Each regeneration buffer was run for 2 min over the chip after assaying the zero calibrator solution (maximum signal), followed by reaction with the respective secondary antibody to determine the percent ratio of anti-glyphosate antibody remaining bound on the surface. As shown in Fig. S6, the solution that removed more efficiently the bound antibodies was HCl with molarity of 50 or 100 mmol L^{-1} (less than 5% of bound antibody molecules remain). Amongst these solutions, the more dilute one, i.e., the 50 mM solution, was selected and the stability of the biochips towards regeneration was assessed through repetitive assay/regeneration cycles. As shown in Fig. 5, at least 12 assay/regeneration cycles could be performed without significantly affecting the signal obtained for the zero calibrator since all values were within the mean value ± 2SD limits.

3.5. Comparison with other optical sensors

Due to the widespread use of glyphosate formulations worldwide there has been a lot of research effort regarding the fabrication of sensors and point-of-need devices for the determination of glyphosate either in water or other matrices. Table 3 summarizes the LODs, working ranges, assays duration, and sample type of glyphosate optical sensors categorized according to the transduction principle.

Regarding determination of glyphosate with label-free optical sensors an SPR biosensor functionalized with an oligopeptide defined from screening of a phage library to bind specifically glyphosate has been reported [22]. The sensor exhibited a LOD of 98 ng mL^{-1} in buffer and moderate cross-reactivity against glycine, thiacloprid, and imidacloprid. A long period fiber grating sensor coated with gold nanoparticles functionalized with cysteamine was also employed for the detection of glyphosate through interaction with the positively charged amino groups of cysteamine causing detectable wavelength shifts of the long period grating bands [23]. The sensor was tested in water providing a LOD of 3.38 ng mL^{-1}.

Optical sensors employing different labelling approaches have been also developed for the determination of glyphosate. A prism coupling hollow-core metal-cladded waveguide has been used to detect chromogenic glyphosate after its incorporation in the hollow core where it acted as guiding medium for high-power wave propagation resulting in excitation of highly sensitive ultrahigh-order modes via small incident

Table 3
Comparison of the analytical characteristics of the proposed optical immunosensor with other biosensors for glyphosate determination.

Sensor type	Transduction principle	LOD (ng mL^{-1})	Dynamic range (ng mL^{-1})	Assay duration	Sample matrix	Ref. #
Proposed sensor	**WLRS**	**0.01**	**0.04–10**	**25 min**	**bottled & tap water**	
Optical label-free	Oligonucleotide functionalized SPR	98	98–845	10 min	buffer	[22]
	LPG-AuNPs-Cys fiber sensor	3.38	84.5–16,900	30 min	water	[23]
Optical labeled	Prism coupling optical waveguide	0.24	0.24–0.74	not mentioned	buffer	[24]
	Fluorescence sensor	0.021	0.05–1	25 min	water, soil	[25]
	Fluorescence intensity based on glyphosate-dsDNA-Au NP conjugates	0.01	0.01–100	2 h	buffer	[26]
	Fluorescence intensity based on glyphosate-dsDNA-FMPs conjugates	0.045	0.17–1700	2 h	buffer	[27]
	Eu^{3+} MIP optical fiber	0.009	0.009–100,000	20 min	buffer	[28]
	Chemiluminesence assay on MIP 96-well plates	46	500–35,000	not mentioned	carrot, spinach	[29]
	Turn-off fluorescence through inhibition of CuO/multiwall carbon nanotubes catalytic activity	0.67	2–10	150 min	water	[30]
	Luminescence quenching of GQDs-AgNPs system	9	30–2000	15 min	peas, lupins	[31]

angle coupling [24]. The sensor could detect glyphosate at concentrations as low as 0.24 ng mL^{-1} (1.42 nM) within several minutes. Another sensor relied on a flow injection system for on-line derivatization and immunochemical detection of glyphosate using a glyphosate-horseradish peroxidase conjugate as label in combination with a fluorescent enzyme substrate [25]. A LOD of 0.021 ng mL^{-1} was achieved for an assay duration of 25 min. Two other reports implemented glyphosate conjugates with DNA-labeled gold [26] or fluorescent magnetic core-shell nanoparticles [27] in a competitive immunoassay format for determination of glyphosate in the samples through measurement of the fluorescence intensity of particles bound onto the antibody or determination of the free DNA probe concentration through UV measurements. The first required confocal laser scanning microscopy while the second UV photometric analysis. The LODs achieved were 0.1 and 0.45 ng mL^{-1}, respectively, the assay duration was 2 h in both cases, and the system could be employed for on-site analysis only in the case of DNA UV signal detection. An optical fiber sensor coated with a molecularly imprinted polymer (MIP) into which a luminescent lanthanide (europium) has been incorporated as a signal transducer has been developed for glyphosate detection at concentrations as low as 0.009 ng mL^{-1} in 20 min [28]. A chemiluminescent MIP sensor was also developed and adjusted on a 96-well plate by replacing the bottom of the wells with a MIP-modified glass substrate in order to use microplate readers for signal acquisition [29]. The sensor provided a detection limit of 46 mg mL^{-1} which is well above the limit for drinking water set by EU. A fluorescence turn-off optical sensor for the determination of glyphosate based on its inhibitory activity of the catalytic properties of CuO/multiwall carbon nanotubes has been also recently reported [30]. The assay required more than 150 min to be completed and involved two centrifugation steps of 10 min each. Another optical sensor for glyphosate was based on the quenching of luminescence signal of graphene quantum dots-cysteine capped silver nanoparticles (GQDs-AgNPs) upon binding of glyphosate [31]. The sensor was tested for glyphosate determination in peas and lupins after QuEChERS sample treatment. The LOD was 9 ng mL^{-1} and the assay time 15 min, not including the sample treatment procedure. From these data, one can conclude, that compared to other reported label-free optical sensors, the proposed one is at least 300-times more sensitive than the more sensitive one, and comparable to the most sensitive labeled optical sensor. Moreover, analysis time is at least comparable to those of the fastest label-free or labeled optical sensors.

4. Conclusions

A label free optical sensor for the fast, sensitive and selective determination of glyphosate in drinking water samples was developed. The sensor was based on the WLRS principle which allowed for the real-time monitoring of immunochemical interactions. The assay developed was highly sensitive, rapid and accurate. Thus, taking into account the excellent analytical performance and short analysis time along with the small sensor size it seems that it can be evolved in a portable device for determination of glyphosate at the point-of-need.

CRediT authorship contribution statement

Eleftheria Stavra: Methodology, Investigation, Formal analysis, Visualization, Writing - original draft. **Panagiota S. Petrou:** Conceptualization, Supervision, Project administration, Writing - review & editing. **Georgios Koukouvinos:** Methodology, Writing - original draft. **Anastasios Economou:** Supervision, Writing - review & editing. **Dimitris Goustouridis:** Resources, Visualization. **Konstantinos Misiakos:** Methodology, Writing - review & editing. **Ioannis Raptis:** Conceptualization, Resources, Visualization, Writing - review & editing. **Sotirios E. Kakabakos:** Conceptualization, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.120854.

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