On-line determination of 3,5,6-trichloro-2-Pyridinol in human urine samples by Surface Plasmon Resonance immunosensing

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Abstract

An immunochemical method for the analysis of chlorpyrifos major urinary metabolite: 3,5,6-trichloro-2-Pyridinol (TCP) is developed using a Surface Plasmon Resonance (SPR)-based biosensor. The stability of the assay was assessed by linking covalently the analyte derivative over a gold-thin modified sensor surface. For optimization of analyte derivative immobilization, sensor chips were activated via alkanethiol monolayers with terminal amine or carboxylic groups. Binding inhibition tests were performed in untreated urine samples and compared to those obtained in distilled water and PBS used as control. In all cases, similar detection limits, in the micrograms per litre level ($0.1\text{--}0.24\ \mu\text{g L}^{-1}$), were attained for TCP assays independently of the dilution buffer. Reproducibility of measurements was studied throughout more than 130 regeneration cycles, which allowed the repeated use of the same immunosensor surface without significant variation of the SPR signal. All measurements were developed in real-time in only

10 minutes, using a SPR portable system, the device could be applied as a valuable analytical method to both environmental screening and clinic diagnostics.

Keywords: TCP; Chlorpyrifos; SPR immunosensor; On-line determination; Urine Biomarkers; Clinic Diagnostics

1. Introduction

Chlorpyrifos is an organophosphorus pesticide that has been widely used in agricultural and urban applications due to their relatively low persistence in the environment [1]. Its broad range of insecticidal activity has permitted the effective control of pests on agricultural crops, households and farms. Chlorpyrifos enters the environment primarily from its application as insecticide on fruit and vegetable crops, lawns, domestic dwellings, commercial establishments and farm buildings. It can also be released through direct use on pet, cattle, sheep and turkeys parasitic extermination. Once in the environment, chlorpyrifos can be either tightly adsorbed by soil or partitioned from the water column to sediments if released to water. Although chlorpyrifos degrades rapidly in the environment by a combination of photolysis, microbial degradation and chemical hydrolysis, low levels may persist for long periods of time after being applied either inside or outside the home [2].

Exposure to chlorpyrifos by breathing or ingesting may affect the central nervous system as well as the cardiovascular and respiratory functions, since it acts as a cholinesterase-inhibiting compound. The toxic effect in humans ranges from numbness, incoordination and dizziness to convulsions, respiratory depression and death depending on the amount and length of exposure. Children may be particularly susceptible to chlorpyrifos harmful effects by dermal exposure or inhalation because of laying or crawling on recently sprayed areas [3, 4].

Chlorpyrifos is changed by the liver into less toxic forms of the compound and almost completely eliminated through the kidneys. The major metabolite that can be found in urine is 3,5,6-trichloro-2-pyridinol (TCP), wherein it can be detected during several days after exposure. TCP is also the metabolic product of other structurally related compounds such as methyl chlorpyrifos and triclopyr. However, the main cause of TCP urinary levels is the direct exposure to chlorpyrifos [4]. TCP does not inhibit cholinesterase and it is not mutagenic. Therefore, it could be considered as a valuable biomarker to estimate the total pesticide uptake via ingestion, inhalation and dermal exposure.

Evaluations of the putative relationship between adverse neurobehavioral effects in infants and perinatal exposure to chlorpyrifos has already been reported by measuring TCP in urine-soaked diapers samples [5]. Likewise, recent studies on body burden levels found in children after chlorpyrifos indoor applications have provide information on the release and movement of chlorpyrifos by testing urinary TCP excretion [6].

To protect the public health and the environment from the toxic effects of chlorpyrifos, governments and environmental regulatory agencies have developed regulations that involve restrictions on emissions (Decision No 2451/2001/EC) and recommendations on chlorpyrifos levels in drinking water [7]. For instance, chlorpyrifos is considered a hazardous substance and is included in a list of 33 priority substances by the European legislation. The Environmental Protection Agency (EPA) has limited the amount of chlorpyrifos in water for children to a maximum of 0.03 mg/L for a period of 10 days. In like manner, the Food and Drug Administration (FDA) has set tolerances for chlorpyrifos for agricultural products ranging from 0.05 to 15 parts chlorpyrifos per million parts of food (0.05–15 ppm).

Determinations of chlorpyrifos and TCP have been currently developed by a number of analytical methods based on gas-chromatography (GC) detection [8]. Applicability of these

methods to biological and environmental matrices requires preparation of samples through complicated procedures that include solvent extraction and several cleanup steps prior to GC analysis. The need of reducing the time and cost of analysis has allowed the rapid development of immunoassay-based methods to determine the presence of chlorpyrifos and other environmental or clinically relevant compounds in food, water or body samples. In the case of TCP, only a few immunochemical methods have been previously described for quantitative determinations in real samples. Enzyme-linked immunosorbent assays (ELISA) have been carried out for TCP evaluation in environmental water samples, food and urine [9] and also in dust and soil samples via commercial assay testing kits [10-12]. However, on-line quantification of TCP by means of biosensor techniques has not been reported before.

The objective of this work is to develop a simple, rapid and cost-effective method that may assess human exposure to chlorpyrifos by measuring TCP in human urine samples. With this aim, different immunoassay formats were carried out to allow the most sensitive detection of the target analyte in a reversible and reproducible manner. The absence of matrix effect was evaluated by comparing TCP calibration curves in untreated urine samples with those used as control. This study reports on considerable improvement to a previously chlorpyrifos SPR immunoassay [13] and demonstrates the capability of our portable immunosensor for application in either environmental or medical diagnostics.

2.1. Chemicals

2.1. Chemicals and immunoreagents

The immunoreagents used were all produced and characterized at the Centro de Investigación e Innovación en Bioingeniería (Universidad Politécnica de Valencia, Spain). The synthesis of TCP hapten TS1; the preparation of the BSA-TS1 conjugate; and the production of anti-TCP monoclonal antibody (MAb) LIB-MC2, have been previously described in other papers [14, 15]. TCP derivative was prepared by introduction of alkyl chain spacers, ending in a carboxylic

acid, in two sites of the pyridyl ring. The covalent linkage to BSA was accomplished using the modified ester method. Finally, the procedure of producing monoclonal antibodies to chlorpyrifos involved: (1) immunization of 8-10 weeks-old female mice; (2) cell fusion of mouse spleen lymphocytes with myeloma cells; (3) selection of high-affinity antibody-secreting hybridoma clones, which were expanded and cryopreserved in liquid nitrogen; and (4) purification of monoclonal antibodies and storage at 4°C as ammonium sulphate precipitates. The affinity and specificity of the monoclonal antibody produced against TCP was characterized using either antibody-coated or conjugate-coated ELISA formats.

Standards of TCP were supplied by the Centro de Investigación e Innovación en Bioingeniería. Analytical standards were prepared as stock solutions of 1 mM in dry dimethylformamide (DMF) and stored at -20°C. Working standards were prepared daily by serial dilutions in PBST (PBS: 10 mM phosphate-buffered saline solution, pH 7.35, containing 0.05% of the surfactant Tween- 20).

Common chemicals for the immobilization procedure were purchased from Sigma-Aldrich: mercaptoundecanoic acid; N-Hydroxysuccinimide (NHS) and 1-ethyl-3-(3 dimethyl-amino-propyl) carbodiimide hydrochloride (EDC); S-Acetylthioglycolic acid N-hydroxysuccinimide ester (SATA); 3-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) and hydroxylamine hydrochloride. Ethanolamine hydrochloride used as blocking agent was provided by Acros Organics. Tween 20 (T20) was obtained from Quantum Appligene and the organic solvents utilized for the gold-coated film cleaning: Ethanol and Acetone were supplied by Merck. The additional compounds Potassium chloride, Sodium chloride, di-Sodium Hydrogen Phosphate and Potassium di-Hydrogen Phosphate, used for the preparation of 1x PBS 10 mM, were provided by Panreac.

2.2. SPR instrumentation and sensor chip preparation

TCP determination was carried out using the β -SPR sensor system commercialised by the company SENSIA S.L. (Spain). The configuration of the SPR device has been reported in previous papers [15-16] (Fig.1). The resonant condition is achieved in the Kretschmann configuration by means of a prism coupler structure. A laser beam emitting at 670 nm from a 3 mW laser diode source is divided into two equal beams to allow two-channelled simultaneous measurements onto the gold-coated film used as sensing surface. For monitoring binding events in real time, SPR measurements are performed at a fixed angle of incidence. Variations of the refractive index corresponding to shifts in mass at the sensing surface are detected as changes in the reflected light intensity by a multielement photodiode. Finally, the SPR signal is amplified and converted to a digital signal.

For SPR measurements, microscope cover glass slides (10 x 10 x 0.15 mm) (TAAB Ltd, England) coated with 50 nm gold were used as sensor chips. The sensing surface preparation involved a cleaning procedure including the use of organic solvents and dipping in a freshly prepared piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$, 3:1). The cleaning treatment is completed with the rinsing and ultrasonication of the sensor chip in distilled water. Sensor chips are placed into the biosensor system by coupling the gold film side to the flow cells and the subsequent adhering of the prism via a matching oil with the same refractive index ($n = 1.52$).

2.3. Liquid handling system

The flow delivery system, including two flow cells (300 nL), a peristaltic pump and two diaphragm pumps for loading the sample, is integrated into the β -SPR platform. A precise volume of sample (220 μL) is pumped over the SPR sensor chip at a constant speed whilst a continuous flow of buffer or distilled water is delivered onto the sensor surface. The complete assay cycle takes 15-20 minutes, depending on the flow-rate of regeneration cycle. Samples

containing the analyte are injected at $30 \mu\text{L min}^{-1}$ and regeneration solutions are flowed either at $30 \mu\text{L min}^{-1}$ or $45 \mu\text{L min}^{-1}$. The flow-rate selected for the immunoassay performance assures both the sensitivity and low time of response of the analysis [16].

2.4. Immobilization formats

The immobilization of biomolecules was accomplished via different immobilization formats that involve the formation of self-assembled monolayers (SAMs) [17]. First, amine groups of the recognition element to carboxylic terminal groups of the alkanethiol monolayer through a carbodiimide linkage. The other approach consisted on the covalent attachment between amine groups of both the analyte derivative and the thiol layer via a sulphur modification of the immobilized element. The biological layer thus formed may assure the reproducibility of measurements and the reusability of the sensor surface throughout a large number of assay cycles.

The formation of carboxylic terminated SAMs was achieved flowing mercaptoundecaonic acid at 0.05 mM in ethanol over the gold sensing layer. The activation of the alkanethiol carboxylic groups to a stable intermediate (N-hydroxysuccinimide ester) was accomplished using a mixed solution of EDC/NHS (0.2/0.05M in distilled water) [18]. At this state, the modified sensor surface is easily available for the amine groups of the BSA-hapten conjugate used as recognition element. The second immobilization strategy required the successive injection of cystamine 0.05 mM in distilled water and MBS 0.25mM in PBST in order to create a surface monolayer capable of binding to thiolate groups. MBS acts as a heterobifunctional crosslinking reagent which can couple initially to primary amine groups and react afterwards with compounds containing sulphydryl groups [19]. The thioacetylation of the hapten carrier protein was performed by pre-incubating a mixed solution of BSA-TS1 $5 \mu\text{g mL}^{-1}$ and SATA $0.25 \mu\text{M}$ for

30 minutes, followed by the subsequent addition of hydroxylamine 0.1 M during a second incubation step (1 hour) so that thiol groups can be deprotected.

Undesirable effects due to non-specific binding and formation of conjugate multilayers were counterbalanced by using a solution of ethanolamine 1M, pH 8.5 as blocking agent in either carboxylic or amine terminated monolayers .

2.5. SPR immunoassay protocols

TCP evaluation in either distilled water, PBST or urine samples was performed by using inhibition binding assays. In this format, samples containing different analyte concentrations and a fixed antibody dilution are flowed over the hapten derivative coated surface [20]. After a short incubation step, only the antibody that remained free in the mixture may couple the immobilized conjugate. SPR detection of TCP in mixed dilutions is based on the inhibition of the antibody binding to the immobilized hapten by the presence of analyte. Therefore, higher analyte concentrations will yield diminishing biosensor responses.

Serial dilutions of TCP ($0.002\text{--}184\text{ }\mu\text{g L}^{-1}$), were prepared in PBST 1x, distilled water or human urine from a stock solution in DMF of 0.2 g L^{-1} , and mixed with a fixed concentration of LIB-MC2 monoclonal antibody (1:1; v/v). Before injection, final dilutions were incubated for 10 minutes at room temperature. SPR response to the binding between antibody in solution and the immobilized hapten is monitored in real-time. To achieve a reusable surface, antibody-hapten associations were disrupted by regenerating the biosensing layer with 0.1 M HCl. Standard calibration curves were obtained by averaging three individual curves. Verification of the selectivity of the assay was confirmed by measuring the effect of non-specific antibodies on the immobilized hapten.

2.6. Measurements in urine samples

Human urine samples were collected from a healthy adult volunteer over 2 days. Samples did not need to be reconstituted by diluting urine concentration. This solution was fortified with TCP at different concentrations to perform binding inhibition tests in urine. From a 0.2 g L⁻¹ TCP stock solution, urine samples under study were spiked with TCP at 0.002, 0.018, 0.184, 1.84, 18.44 and 184.43 µg L⁻¹.

2.7. Data analysis

The reliability of the generated surface to reproduce measurements was assured determining seven analyte-antibody samples for each concentration of TCP standard. Normalized standard curves were obtained by plotting reflectance (V) against the logarithm of chlorpyrifos concentration. The experimental data were fitted to a four-parameter logistic equation:

$$y = \{D + (A-D)/[1 + (x/C)^B]\}$$

where A is the asymptotic maximum (maximum SPR signal in absence of analyte, A_{max}), B is the curve slope at the inflection point (related to the analyte concentration giving 50% inhibition of A_{max}: C, I₅₀) and D is the asymptotic minimum (background signal). The standard curves were obtained by averaging three individual standard curves and normalized by expressing the SPR signal (SPR_{signal}) of each standard point as the percentage of the maximum response [100 x (SPR_{signal}/SPR_{signal,max})].

3. Results

3.1. Assay sensitivity

3.1.1. Characterization of hapten-derivative immobilization

SAMs technology provides a simple and reliable method of coupling a biological sensing element to the surface of a transducer since it assures both the biological activity and well orientation of the immobilized molecule. The affinity of sulfur-containing compounds for gold surfaces allows the spontaneous self-assembly of the alkanethiol monolayer through stable Au-S bonds. One of the main advantages of SAMs is the possibility of varying the alkyl chain ending to generate different interface layers between the gold metal surface and the immobilized element. To enable feasible measurements of TCP in human urine samples, the covalent attachment of the hapten derivative BSA-TS1 to the sensor surface was accomplished by using two immobilization strategies that vary depending on the monolayer terminal group (amine or carboxylic). For carboxylic terminated thiols, the ideal immobilization procedure involves the formation of a carbodiimide bond that couples amine groups of the hapten carrier conjugate to carboxylic acids of the monolayer, as previously described in other papers. On the other hand, monolayers ending in amine groups were activated by a heterobifunctional crosslinking between alkanethiol amines and the conjugate sulphydryl groups obtained after thiolating their primary amines. This method avoids the potential formation of multilayers described when cross-linking a protein to amine groups of an alkanethiol monolayer with glutaraldehyde [21].

Once formed both immunosurfaces were exposed to antibody-analyte mixtures in order to determine the most sensitive approach. Calibration graphs were obtained using a fixed amount of antibody and seven successive dilutions of the TCP standard. The seven TCP concentrations ranged from 0.002 to 184.43 $\mu\text{g L}^{-1}$ whilst the selected antibody dilution was 1 $\mu\text{g mL}^{-1}$ for the carboxylic monolayer and two-folded more concentrated (2 $\mu\text{g mL}^{-1}$) for the amine ending surface in order to obtain the same SPR signal. However, the immunosensor specific response was not affected since all measurements were normalized with respect to the blank signal. Binding inhibition tests were performed independently for each immobilization format by

injecting in triplicate the blank sample, containing the antibody in absence of analyte, and the seven antibody-analyte mixtures.

The sensitivity was estimated by comparing the limit of detection, determined as the analyte concentration providing a 10% decrease of the blank signal, and I_{50} values of both assays. Detection limits of $0.26 \mu\text{g L}^{-1}$ and $1.16 \mu\text{g L}^{-1}$ were obtained for carboxylic and amine terminated thiols monolayers, respectively. I_{50} values were also calculated from the standard calibration curves, showing similar results for both immobilization formats (2.06 - $2.92 \mu\text{g L}^{-1}$).

According to these criteria, TCP was detected more sensitively when using the immobilization procedure based on the carboxylic ending thiols (Fig. 3) with the additional advantage of involving less expense of MAb. Therefore, the carbodiimide-based immobilization format was chosen for the optimization of the immunoassay.

3.1.2. Optimization of immunochemical conditions

Since the ionic strength of the assay buffer may affect the immunoassay performance, immunochemical determinations of TCP were carried out by using different buffer conditions. To evaluate the effect of salt concentration, TCP samples were prepared in distilled water and PBS containing 0.05% of Tween 20 detergent. For TCP measurements, analyte standards were diluted in its corresponding buffer in the 0.002 to $184.43 \mu\text{g L}^{-1}$ range, mixed with $1 \mu\text{g mL}^{-1}$ LIB-MC2 MAb, incubated for 10 minutes and flowed over the immobilized conjugate, as described above. Calibration graphs obtained after immunoreaction showed similar sensitivity values regardless of the assay buffer used for TCP dilution. Detection limits of $0.13 \mu\text{g L}^{-1}$ and $0.24 \mu\text{g L}^{-1}$ were attained for distilled water and PBST samples respectively, whilst I_{50} values ranged from $1.84 \mu\text{g L}^{-1}$ in distilled water to $2.09 \mu\text{g L}^{-1}$ in PBST assay buffer (Fig. 4a). The

only remarkable difference was found in the assay variability, which showed lower relative standard deviations when using PBST as dilution buffer, as shown in Table 2. Therefore, PBST was chosen as the optimal TCP solvent to provide more robust and stable immunoassays.

Under the optimum conditions, the optimization of TCP immunoassay involves: the immobilization of hapten derivative BSA-TS1 at a concentration of $10\ \mu\text{g mL}^{-1}$ over a carboxylic terminated monolayer; the use of LIB-MC2 monoclonal antibody at a concentration of $1\ \mu\text{g mL}^{-1}$ in assay buffer (PBST) and serial dilutions of TCP standards in PBST. All measurements, from the formation of the alkanethiol monolayer to immunoreactions, are monitored in real-time under a continuous flow of $1\times$ PBS, pH 7.35, with 0.05% Tween 20 in the SPR device.

3.2. Performance of SPR measurements in urine samples

Exposure to chlorpyrifos may occur in many places due to its wide range of applications, from indoor uses to agricultural pest control. Studies on chlorpyrifos metabolism, transformation and excretion are needed to assess health risks after breathing, eating, drinking or dermal exposure to the substance. Since TCP is the major chlorpyrifos metabolite excreted by the kidneys, the quantitative determination of TCP in urine may represent chlorpyrifos equivalent absorbed-doses [10]. Therefore, urine samples appear to be good candidates to allow screening of population exposed to chlorpyrifos.

With this aim, untreated urine samples were fortified with several TCP concentrations ranging from 0.002 to $184.43\ \mu\text{g L}^{-1}$, like in the optimized immunoassay. Binding inhibition tests were performed in triplicate by flowing over the immobilized hapten mixed solutions of spiked urine samples and MAb. To examine the influence of variations of ionic strength on urine samples, monoclonal antibodies were prepared in $1\times$ PBST and $2\times$ PBST (Fig. 4.b).

The SPR response to hapten-antibody interactions increased as the concentration of buffer diminished. A significant drop of SPR signal was observed for samples prepared in 2x PBST with regard to 1x PBST mixtures, as shown in Figure 5. To assure clear differentiation between diminishing analyte concentrations, the MAb concentration needs to be maintained within a minimum allowable signal for the blank (analyte-free) sample. Therefore, higher MAb dilutions would be required when using 2x PBST as antibody solvent. The assay sensitivity was not affected and TCP was detected with similar sensitivity in 2x PBST ($0.09 \mu\text{g L}^{-1}$) and 1x PBST MAb dilutions ($0.1 \mu\text{g L}^{-1}$).

A slight difference in I_{50} values was also found between the two buffers evaluated ($0.56\text{-}1.5 \mu\text{g L}^{-1}$). The improvement in sensitivity observed in TCP urine spiked samples when compared to TCP dilutions in PBST could be due to the stability provided by urine salt composition. With respect to assay variability, mean RSD values of 10.79 and 5.27% were obtained for 2x PBST and 1x PBST calibration curves, respectively. As similar detection limits and intra-assay variation were attained independently of the assay formats, $1\times$ PBST was selected for the performance of TCP immunoassays.

Fig.5. SPR simultaneous response to antibody-hapten interactions using 1x PBS and 2x PBS as MAb solvents in cell 1 (black) and cell 2 (red) and TCP samples in human urine. Injections of antibody-analyte mixtures at different concentrations are followed by the corresponding regeneration cycle.

An important validation parameter is recovery, the ability of an assay to accurately quantitate the amount of analyte in individual urine samples. To validate the procedure, several samples containing unknown concentrations of TCP prepared by a different operator were tested. Table 3 shows a good correlation between data with recovery rates within the 70-120% rate, in accordance with AOAC recommendations.

Although SPR technology has been previously used for analyte measurements in urine, this is the first SPR-based biosensor to allow TCP determinations in urine samples. In addition this assay did not require complicated pre-treatment procedures by diluting or compensating urine raw samples. Calibration curves with low reproducibility have been reported when using high dilutions of urine [22]. To date only successful immunochemical determination were possible by using ELISA rapid assay methods described by Shackelford and Chuang [10,11].

3.3. Reproducibility of the assay

In contrast with classical ELISA formats, the repeated use of the sensor surface coated with the covalently immobilized hapten derivative is a crucial point to assess the efficiency of biosensor-based assays. Likewise, the reproducibility of measurements assures the feasibility and robustness of the method for TCP detection in urine samples

To evaluate the binding capacity of the immobilized hapten-protein conjugate upon regeneration, antigen/antibody complexes were disrupted with 0.1M HCl flowing at $45 \mu\text{L min}^{-1}$. Under these regeneration conditions, the stability of the surface was studied with regard to both regenerability and antigenic integrity of the immobilized hapten. The antigen support appeared to be reusable throughout the whole duration of the assay involving 130 regeneration cycles.

The stability after regeneration was measured from the beginning to the end of the analysis by measuring the immunosensor response to antigen-antibody interactions. The interaction signal remained practically inalterd showing a slight variation of 4.54 % over the whole assay. In addition, the use of urine buffered samples did not affect the SPR signal significantly.

Consequently, the use of 0.1M HCl at the selected speed provided the efficient desorption of the immunoreagent reagent without degrading the immobilized hapten.

The method was validated by the comparison between assays performed in different days. With this purpose, calibration curves were repeated on 2 days following the optimum assay conditions. Binding inhibitions tests were carried out in triplicate of seven different concentrations of chlorpyrifos in the 0.0035-350.59 $\mu\text{g L}^{-1}$ range, as described above. Intra-assay variations were calculated from the analysis of three replicates of each TCP concentration on a single day. The coefficients of variation of intra and inter assays did not exceed 4.48 % and 3.5 % respectively, thus indicating the good reproducibility of the assay.

4. Conclusions

This work describes a novel on-line immunoanalytical method for the evaluation of chlorpyrifos major metabolite from its primary via of elimination. The immunoassay format allows a highly sensitive detection of TCP in human urine without the need of previous clean-up and preparation of samples.

In order to assure the stability and binding capacity of the covalently attached hapten derivative, two immobilization methods have been developed through the formation of alkanethiol monolayers ending in either amine or carboxylic groups. The amine functionalized surfaces permitted TCP determination at relatively low levels (1.16 $\mu\text{g L}^{-1}$). However, TCP was detected more sensitively when using gold modified substrates (0.26 $\mu\text{g L}^{-1}$) with carboxylic terminated-thiols. Therefore, all analyte measurements were carried out via hapten derivative immobilization over carboxylic-activated sensor surfaces.

The SPR immunoassay conditions were optimized by using different TCP buffer dilutions. The lowest detection limit was attained for TCP standard solutions in PBST buffer ($0.24 \mu\text{g L}^{-1}$). These results reached comparable levels and even were quite below previously reported immunochemical techniques. Furthermore, the reusability of our method was demonstrated through the complete regeneration of the biosensing surface along 130 assay cycles. The immunosensor response deviation from the beginning to the end of the analysis was negligible, showing the stability of the activated surface for withstanding the regeneration conditions.

Finally, TCP detection was performed in human urine samples in order to demonstrate the potential application as a biological screening method of urinary biomarkers. The comparison between TCP limits of detection in urine and assay buffer showed similar sensitivity values. Standard calibration curves in human urine and PBST solutions yield I_{50} values ranging from 1.5 to $2.09 \mu\text{g L}^{-1}$ whilst a LOD of $0.1 \mu\text{g L}^{-1}$ was obtained for TCP urinary determinations.

Our SPR direct detection approach to TCP quantification in urinary samples could be easily transferred to other environmentally relevant substances, drug residues, veterinary products or exposure markers susceptible to be found in body fluids (blood, urine). The simplicity, low time of response and real-time determination are inherent advantages over chromatographic and immunochemical-based methods. The validation of the system by correlation with a reference method as well as the assessment of different population groups to chlorpyrifos exposure through TCP urinary determination will considerably improve the current assay.

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Figures

a)

b)

Fig. 1. Schematic set up of the β -SPR sensor system from SENSIA, S.L., including sensor, optics, electronics and flow delivery system: a) scheme of the SPR device and b) SENSIA β -SPR platform.

Fig. 2. SPR response to the immobilization of BSA-TS1 using amine or carboxylic thiol formats. The scheme shows the whole process since the activation of the thiol monolayer till the injection of the blocking agent ethanolamine.

Fig. 3. Standard calibration curves for amine and carboxylic immobilization formats. Measurements were done in triplicate.

Fig. 4. a) Standard calibration curves for TCP using distilled water and PBS as TCP solvents.

b) Standard calibration curves for TCP spiked urine samples using PBS 1x and PBS 2x as MAb solvents.

Fig.5. SPR simultaneous response to antibody-hapten interactions using 1x PBS and 2x PBS as MAb solvents in cell 1 (black) and cell 2 (red) and TCP samples in human urine. Injections of antibody-analyte mixtures at different concentrations are followed by the corresponding regeneration cycle.

Table 1

Influence of immobilization formats based on amine and carboxylic SAMs on the standard curves for TCP in spiked samples.

	TCP		
	I_{50} ($\mu\text{g L}^{-1}$) Mean $\pm$ SD	LOD ($\mu\text{g L}^{-1}$) Mean $\pm$ SD	I_{80-20} ($\mu\text{g L}^{-1}$)
Amine monolayer	$2.92 \pm 0,10$	$1.16 \pm 0,11$	1.65-5.96
Carboxylic monolayer	$2.06 \pm 0,13$	$0.26 \pm 0,04$	0.56-7.91

Table 2

Effect of the ionic strength on the assay variability and sensitivity calculated in TCP calibration curves.

	TCP solvent	
<i>Inter-assay variability RSD (%)</i>		
TCP concentration ($\mu\text{g L}^{-1}$)	Distilled water	PBST
0	6.93	5.09
0.002	2.94	1.88
0.018	8.17	1.88
0.184	10.3	3.31
1.844	15.8	2.13
18.44	17.1	6.78
184.43	17.1	10.3
Mean	11.18	4.48
<i>I₅₀ ($\mu\text{g L}^{-1}$)</i>		
Mean $\pm$ SD	1.84 $\pm$ 0.61	2.09 $\pm$ 0.13
RSD (%)	33.15	6.22
<i>LOD ($\mu\text{g L}^{-1}$)</i>		
Mean $\pm$ SD	0.19 $\pm$ 0.2	0.24 $\pm$ 0.04
RSD (%)	105.2	16.6

Table 3. Recovery of TCP from spiked blind samples

	TCP added, $\mu\text{g L}^{-1}$	TCP recovered, $\mu\text{g L}^{-1}$	Recovery (%)	SD, $\mu\text{g L}^{-1}$	CV (%)
Blind sample	0.515	0.052	100.58	0.017	32
	5.15	0.484	92.427	0.109	22
	0.051	5.843	113.4	0.404	7