Competitive Quenching Fluorescence Immunoassay for Chlorophenols Based on Laser-Induced Fluorescence Detection in Microdroplets

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An improved biomonitoring system for the analysis of 2,4,6-trichlorophenol (TCP) in urine samples has been developed. The principle of the biosensor device is the detection of laser-induced fluorescence (LIF) in single microdroplets by a homogeneous quenching fluorescence immunoassay (QFIA). The competitive immunoassay occurs in microdroplets (d = 58.4 µm) produced by a piezoelectric generator system with 10-µm-diameter orifice. A continuous Ar ion laser (488 nm) excites the fluorescent tracer; its fluorescence is detected by a spectrometer attached to a 512 × 512 cooled, charge-coupled device camera. Fluorescence is quenched by specific binding of TCP polyclonal antibodies to the fluorescent tracer (hapten A–fluorescein); the quenching effect is diminished by the presence of the analyte. Thus, an increase in the signal is produced in a positive dose-dependent manner when TCP is present in the sample.

In 10 mM PBS buffer, the IC₅₀ of the LIF-microdroplet QFIA is 0.45 µg L⁻¹ reaching a LOD of 0.04 µg L⁻¹. The QFIA with the same reagents performed in microtiter plate format achieved a LOD of 0.36 µg L⁻¹ in buffer solution. Performance in human urine was similar to that observed in the buffer. A LOD of 1.6 µL⁻¹, with a dynamic range between 4 and 149.5 µg L⁻¹ in urine, was obtained without any sample treatment other than dilution with the assay buffer. The detectability achieved is sufficient for occupational exposure risk assessment.

Fluorescent detection methods have led to major improvements in bioanalytical applications because of their extraordinary sensitivity and selectivity. One of the most exciting aspects of fluorescence technologies is their ability to support decreasing sample sizes down to the single-molecule detection level, which in turn provides the opportunity for miniaturization and high-throughput screening. Fluorescence immunoassays (fluorimunoassay, FIA) employ a fluorescent signal for analyte detection. Homogeneous FIAs are separation-free, making automation easy and permitting simple adaptation to existing instrumentation. A variety of homogeneous FIA systems have been developed based on detection schemes such as fluorescence polarization, fluorescence energy transfer, time-resolved fluorescence, and fluorescence quenching or enhancement (see recent reviews). Fluorimunoassays have become a common clinical chemistry procedure for the analysis of a wide range of analytes, such as drugs, hormones, and proteins. They have also found application in environmental analysis for the determination of pesticide contaminants in water and their metabolites in urine samples.

Direct quenching FIAs (QFIAs) are assays in which the antibody-binding reaction significantly decreases the fluorescence signal of the labeled antigen due to changes in the microenvironment around the fluorophore. The direct quenching approach has

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proven to be suitable only for haptenys of small size, because the antibody binding site lies sufficiently close to the label to influence its signal. Competitive direct QFIAbs based on the fluorescence quenching of a labeled hapten upon binding its antibody (Ab) have been developed for the detection of gentamycin18 and cortisol19 in serum, atrazine,20 2,4-dichlorophenoxyacetic acid, 21 and pro-

More recently, fluoroimmunoassays have been combined with laser-induced fluorescence (LIF) technology to make use of sensitive optical microscope and charge-coupled device (CCD) systems.22–28 Hence, by use of modern lasers and CCD cameras it is possible to construct compact and highly sensitive detection systems. Laser excitation allows significant reduction of the illuminated sample volume, minimizing the background interferences resulting from Raman and Rayleigh scattering and from impurity fluorescence. This results in an improved fluorescence-to-background signal-to-noise ratio, making detection at single-molecule levels feasible.2,27,28 LIF detection of single fluorescent molecules in solution has been performed in flowing streams, in sheath flow cuvettes, and in capillaries.29,30 One of the main drawbacks of these flowing techniques is that the optically defined probe volume is smaller than the flow cell itself, so that many of the molecules may not be probed by the laser. As a result of these limitations, research has been focused on alternative detection schemes, including levitated27,31–34 and falling35–38 microdroplets.

The microdroplet approach to single-molecule detection in liquids is unique in several aspects. The probe volume is defined, in this case by the droplet instead of by the laser beam, which means that all fluorophore molecules can be interrogated. Small detection volumes afforded by micrometer-sized droplets minimize background fluorescence. Contact of the sample to container walls is avoided, eliminating scattering from walls and possible binding of reagents to the wall. In addition, spontaneous emission in spherical microdroplets is significantly enhanced due to cavity resonances that increase optical emissions significantly.

Previously we reported the preparation of specific antibodies and development of an enzyme immunoassay for the detection of 2,4,6-trichlorophenol (TCP; see Figure 1 for chemical structure) in urine samples39,40 with the aim of providing high-throughput screening tools for biological monitoring. TCP is one of the five chlorophenols classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans.41 Contamination by chlorophenols is widely spread (i.e., soil,42 groundwater and surface waters,43 drinking water,44 etc.) due to their extensive use as fungicides, insecticides, wood, leather and textiles preservatives, and bleaching agents in the pulp and paper industries. Technical grade chlorophenol products have been shown to contain polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzo furans (PCDFs) as impurities. Significant concentration levels of chlorophenols (2–5 μg L−1) have been found in the urine of a great part of the population45,46 as a result of the exposure to chlorophenols or other organochlorinated substances such as phenoxy herbicides, hexachlorocyclohexanes, and chlorobenzenes.47–49 The main routes of

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**Figure 1.** Chemical structures of the target analyte (TCP), hapten A and the fluorescein tracer (hapten A–fluorescein, AF).
exposure for the general population are the contaminated environment, domestic preservatives, and edible products.\(^{45-48,50}\) Urinary excretion of TCP from occupationally exposed people is higher (up to 30 μg L\(^{-1}\)), and it has been reported to occur in workers of plants producing chlorinated pesticides,\(^{47,50}\) sawmills,\(^{51}\) waste incinerators,\(^{52}\) etc.

The TCP enzyme-linked immunosorbent assay (ELISA) requires sample pretreatment to allow urine analysis with sufficient detectability for biomonitoring of occupationally exposed persons.\(^{53}\) In this paper, we report the improvement in a TCP assay by developing a LIF-microdroplet QFIA. This novel biodetection system combines the selectivity of immunoassays and the sensitivity of laser-induced fluorescence detection in microdroplets. It has been evaluated for water and urine analysis.

**EXPERIMENTAL SECTION**

### Chemical Reagents

The synthesis of 3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid (hapten A, Figure 1) has already been evaluated for water and urine analysis.\(^{54}\) Other chemical reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Fluoresceinamine isomer I (5-aminofluorescein) and other chemical reagents were obtained from Sigma Chemical Co. The hapten A–fluorescein conjugate (see Figure 1 for chemical structure) was prepared as follows.

**Hapten A–Fluorescein Conjugate (AF).** The hapten A (19.37 mg, 0.1 mmol) and the fluoresceinamine isomer I (25 mg, 0.1 mmol) were dissolved in anhydrous THF (5 mL) containing 6% DMF. After adding N,N′-disproplycarbodiimide (DIC; 22.8 μL, 0.15 mmol) the mixture was stirred for 16 h at room temperature under an argon atmosphere. The reaction was followed by TLC until a yellow spot (R\(_f\) = 0.27) appeared using 0.25-mm precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) using toluene/ethyl acetate/acetic acid (5:5:1) as the mobile phase. The solvent was evaporated to dryness under reduced pressure, and the residue was then dissolved in ethyl acetate and purified on 0.5-mm silica gel 60 F254 plates with the same mobile phase. The yellow band with R\(_f\) = 0.73 obtained after four runs was extracted with ethyl acetate/methanol (1:1) and the extract evaporated to dryness to obtain an orange solid corresponding to the AF conjugate (22.58 mg, 37.7% yield), identified according to its spectroscopic data on a Varian Unity-300 (Varian Inc., Palo Alto, CA) spectrometer (300 MHz for 1H and 75 MHz for 13C): 1H NMR (300 MHz, CD\(_3\)OD) \(\delta\) 2.68 (t, J = 8.4 Hz, 2H, CH\(_2\)CONH), 3.32 (t, J = 8.4 Hz, 2H, PhCH\(_2\)), 6.55 (dd, J\(_{1,2}\) = 9 Hz, J\(_{2,3}\) = 2.4 Hz, 2H, FNH\(_2\)), 6.67 (d, J = 2.4 Hz, 2H, FNH\(_2\)), 6.68 (d, J = 9 Hz, 2H, FNH\(_2\)), 7.14 (d, J = 8.4 Hz, 1H, FNH\(_2\)), 7.38 (s, 1H, \(\text{CH}_2\)) meta, hapten A), 7.85 (dd, J\(_{1,2}\) = 8.4 Hz, J\(_{2,3}\) = 1.9 Hz, 1H, FNH\(_2\)), 8.3 (d, J = 1.8 Hz, 1H, FNH\(_2\)); 13C NMR (75 MHz, CD\(_3\)OD) \(\delta\) 28.53 (t), 35.81 (t), 103.6 (d), 111.9 (s), 114.4 (d), 116.7 (d), 121.8 (s), 124.7 (s), 125.4 (s) [this signal probably corresponds to two carbon atoms (C-2 and C-4 at the hapten fragment moiety)], 126.3 (d), 127.5 (d), 129.3 (d), 130.2 (s), 130.5 (d), 137.2 (s), 141.7 (s), 147.0 (s), 150.6 (s), 154.7 (s), 159.9 (s), 171.5 (s), 173 (s).

**Immunoreagents.** Unless otherwise indicated, all solutions of the standards and the immunoreagents were prepared in 10 mM PBS (10 mM phosphate buffer, 0.8% saline solution, pH 7.5). The preparation of the polyclonal antibodies (Ab43, Ab44, Ab45) against TCP using hapten A conjugated to keyhole limpet hemocyanin (KLH) has been described before.\(^{55}\) The IgG fraction of the antisera was isolated by precipitation with saturated ammonium sulfate solution. After dialysis against PBS, aliquots were stored at −20 °C. The antibody concentration was determined spectrophotometrically at 280 nm, assuming that the absorbance of 1 mg mL\(^{-1}\) antibody solution is 1.35. Stock solutions of hapten A–fluorescein conjugate and TCP were prepared in methanol. All working solutions were prepared in phosphate buffer (PBS 10 mM, pH 7.5).

**Urine Samples.** A sample was prepared by pooling urine from different healthy individuals (24 h void). Prior to the assay, the pooled urine was centrifuged at 5000 rpm for 10 min and pH was adjusted to the buffer pH 7.5. Subsequent dilutions used in the experiments were prepared using PBS.

**LIF-Microdroplet QFIA.** Instrument Experimental Setup. Laser-induced fluorescence in microdroplets was measured using the instrument setup presented in Figure 2. Microdroplets were generated by a TSI model 3450 vibrating orifice aerosol generator (orifice diameter 10 μm). Droplets were illuminated by a laser beam (continuous Ar ion laser, Coherent Innova 70, λ = 488 nm). A holographic laser band-pass filter (Kaiser Optical Systems Inc.) was used to eliminate unwanted plasma lines from the laser source and to transmit only the laser line at 488 nm. The fluorescence was collected by a Mutoyo microscope objective lens (NA of 0.55) and focused onto the entrance slit of the imaging spectrometer (SpectraPro-150, Acton Research Corp.). Spectra were recorded with a thermoelctroly cooled camera (TEA/CCD-521-TK1, Princeton Instruments Inc.) with a 512 × 512 pixel CCD detector. The camera was controlled by a ST130 controller (Princeton Instruments Inc.). A 488-nm holographic Raman notch filter (Kaiser Optical Systems Inc.) was placed before the slit of the spectrometer to block elastically scattered laser radiation.

**General Methods.** The fluorescence emission from microdroplets of AF was determined in standard PBS solutions (laser excitation wavelength at 488 nm, emission wavelength of 520 nm). For antibody quenching studies, a standard PBS solution of AF (final concentration 10 nM) was mixed with different concentrations of Ab solution in PBS (Ab43, Ab44, Ab45). The reaction mixture was incubated at room temperature for 55 min. For kinetics estimation, the fluorescence emission was measured every 5 min. Prebleed (preimmune) Ab was used as a control for the specificity of the quenching effect. For data presentation, the maximum fluorescence intensity (at 520 nm) measured in the presence of Ab (I\(_{Ab}\)) was divided by the maximum fluorescence intensity corresponding to AF emission (I\(_{AF}\)) in the absence of Ab.

**Competitive Procedure.** Solutions of TCP standards (10⁻⁵ to 10⁻¹¹ M), AF (10 nM, final concentration), and Ab43 (2.5 μg mL⁻¹, final concentration) were mixed and incubated for 45 min at room
RESULTS AND DISCUSSION

The feasibility of using a homologous (immunizing hapten is the same as the competitor hapten) direct enzyme immunoassay to determine TCP has already been demonstrated in previous work. Thus, the same approach was initially planned to develop a fluoroimmunoassay by coupling hapten A to a fluorescent label. For maximum sensitivity, the fluorophore should have a high molar extinction coefficient, high quantum yield, and large Stokes shift. Fluorescent dyes from the fluorescein family (e.g., fluorescein, rhodamine, Texas Red, etc.) have relatively high molar extinction coefficients (60 000—100 000 M⁻¹ cm⁻¹) and fluorescence quantum yields greater than 85%. In particular, fluorescein has an effective excitation wavelength range of about 488—495 nm, closely matching the emission of an Ar laser (488 nm). Frequently, fluoresceinamine is used for labeling haptens containing a COOH group, employing dicyclohexylcarbodiimide (DCC) as an activating agent to form the amide bond. In our case, hapten A was coupled to fluoresceinamine using DIC in THF containing 6% DMF. This activating agent offers the advantage of allowing elimination of the nonreacted DIC and the diisopropylurea byproduct while the solvent evaporates, facilitating subsequent purification steps of the product, AF. The %content of DMF was needed to increase fluoresceinamine solubility in the media. However, a higher concentration inhibited the establishment of the amide bond favoring the formation of the corresponding hapten—acylurea derivative.

**LIF Detection of AF in Microdroplets.** Microdroplet size and shape have very important effects on fluorescence detection. For a fixed orifice diameter, these parameters are determined by the piezoelectric frequency of the droplet generator and the liquid flow rate. In these experiments, a frequency of 40 kHz was used with a liquid flow rate of 0.25 mL min⁻¹ (4.16 × 10⁻³ cm³ s⁻¹). According to a simple relationship between these parameters, the diameter of the generated droplet was 58.4 μm and its volume was 104 pL. With a shutter exposure time of 1 s, 40 000 droplets passed the laser beam, and hence, the detected volume was 4.2 μL. Microdroplets of hapten A—fluorescein conjugate (AF) in PBS buffer were generated and the laser-induced fluorescence was measured using the instrumental setup described in the Experimental Section. Figure 3A compares the fluorescence emission spectra obtained from droplets of the AF conjugate and from droplets of the dye molecule fluoresceinamine (FNH₂) used for labeling. As expected, the maximum emission for both molecules is at ~520 nm (close to the 488-nm laser excitation). The fluorescence intensity of both molecules is very dependent on pH of the solution, showing enhanced intensity in basic conditions (data not shown). The assay media provided by the PBS buffer (pH 7.5) showed suitable fluorescence intensity.

A linearly dependent fluorescence intensity on AF concentration was observed for both laser powers used in this study (see Figure 3B). The regression coefficients are 0.996 and 0.998 for laser power 60 and 120 mW, respectively. Transit times were of the order of 100 μs, leading to a very short interaction time between excitation source and fluorophore, consequently avoiding photobleaching. The absolute detection limit of the fluorescein conjugate in the microdroplets is ~5 × 10⁻¹⁰ M (0.5 nM) corresponding to 5.2 × 10⁻²⁰ mol of AF per microdroplet, while...
The detection limit was 2 in a cuvette (2 mL) or in a microwell (200 μL), respectively. Measurements were made after 20 min of immunoreaction. The decreased fluorescence intensity is presented as a ratio between the fluorescence intensity of AF in the presence of Ab (IAb) and the fluorescence intensity of AF in the absence of Ab (IAF). The three Abs used (Ab43, Ab44, Ab45) were raised against hapten A–KLH conjugate. The preimmune serum was used as control of the nonspecific binding.

**Figure 4. Quenching effect on the fluorescence of the AF conjugate upon Ab binding.** The AF and the Ab concentration were 10 nM and 10 μg mL⁻¹, respectively. Measurements were made after 20 min of immunoreaction. The decreased fluorescence intensity is presented as a ratio between the fluorescence intensity of AF in the presence of Ab (IAb) and the fluorescence intensity of AF in the absence of Ab (IAF). The three Abs used (Ab43, Ab44, Ab45) were raised against hapten A–KLH conjugate. The preimmune serum was used as control of the nonspecific binding.

in a cuvette (2 mL) or in a microwell (200 μL) the absolute detection limit was 2 × 10⁻¹² and 2 × 10⁻¹³ mol, respectively, which demonstrates one of advantages of the microdroplet approach allowing detection close to single-molecule levels. A concentration of AF of 10 nM was chosen for further studies as a compromise between the goal of maximizing the signal-to-background ratio (to facilitate observation of the antibody quenching) and the recognition of the fact that low AF concentrations would provide better detectabilities in the competitive assays.

**Evaluation of the Quenching Effect Produced by the Ab on the AF Fluorescence.** It is well known that an immunochimical reaction between a fluorescent-labeled antigen and its specific antibody can cause a decrease in the fluorescence of the labeled antigen, whereas the free fraction retains its full signal. The effect of the antibody-binding reaction on the fluorescent properties of the AF conjugate was tested on a noncompetitive LIF-microdroplet QFIA for three different Abs specific for TCP (Ab43, Ab44, Ab45). Figure 4 shows the quenching effect observed after 20 min of immunoreaction time between AF (10 nM) and the three Abs (10 μg mL⁻¹). A preimmune serum, used as a negative control, did not show fluorescence quenching, demonstrating the specificity of the observed effect using Ab43–45. The inhibition of the fluorescence signal was highest for Ab43, followed by Ab45, and is lowest for Ab44. A similar correlation (Ab43 > Ab45 > Ab44) was found regarding the Ab affinity for A-HRP on the direct ELISA previously reported. Higher Ab affinity means stronger binding, resulting in larger changes in the microenvironment of the fluorescent label, which is expressed in a stronger quenching effect. Hence, the Ab with the strongest quenching effect (Ab43) was chosen for further study.

The kinetics and the fluorescence emission spectra of the AF fluorescence quenching in microdrops using different concentrations of Ab43 was investigated in order to ascertain the time needed for maximum quenching and to determine the most suitable Ab concentration. It was observed that, at high Ab concentrations (15 μg mL⁻¹), a steady-state level was reached in ~15 min, while with lower Ab concentrations, more time (30–40 min) was needed to reach equilibrium (see Figure 5A). Thus, an incubation time of 45 min was selected to ensure complete reaction of the Ab with AF although complete quenching of the signal was not accomplished even at very high Ab concentrations. Thus, in a situation where all binding sites of the antibody are saturated with AF (i.e., Ab concentration 10 μg mL⁻¹), a maximum 70% quenching of the AF fluorescence signal was accomplished (see Figure 5B). This is in agreement with other direct quenching fluorimunnoassays with small haptens where the fluorescence of the tracer is only partially quenched upon Ab binding. In fact, complete quenching (100%) has only been reported on biotin–avidin systems characterized by even a stronger binding than the Ag–Ab; it has been attributed to the very tight association that brings avidin to the surface of the biotin–fluorophore conjugate. It can be anticipated that a hapten with a shorter spacer arm on the fluorescent label would have given rise to a more efficient quenching due to closer proximity of the fluorophore to the Ab in the Ab–Ag complex. Unfortunately, all efforts to synthesize the respective fluoroscein tracer using 3-(3-hydroxy-2,4,6-trichlorophenyl)acetic acid have failed. For the competitive QFIA, an Ab concentration of 2.5 μg mL⁻¹ was selected, producing a signal around 80% of the complete saturation (80% of the maximum quenching). We must also mention that the antibody solutions themselves did not show a significant fluorescence background (see Figure 5B insert).

**Competitive LIF-Microdroplet QFIA.** The AF tracer competes with the analyte (TCP) for a limited number of binding sites.
At low analyte concentrations, the AF conjugate was preferentially bound to the Ab and the fluorescence of the label was quenched. Higher analyte concentrations lead to a decreased occupation of the Ab binding sites by the AF conjugate and an increase of the fluorescence intensity. The standard curve was measured; it is presented in Figure 6. The curve shows an IC50 of 2.3 nM (0.45 μg L⁻¹) and a LOD of 0.2 nM (0.04 μg L⁻¹). The LOD of an immunoreagent is often determined by the relative affinity of the Ab for the analyte and the competing hapten–label conjugate. However, it also depends on other parameters.

A comparison between the QFIA performed in a 96-well plate, the LIF-microdroplet QFIA, and a conventional microplate ELISA, using the same immunoreagents and assay buffer, is presented in Table 1. It can be observed that both microplate immunoassays (heterogeneous ELISA and homogeneous QFIA) have a very similar LOD and IC50 despite the different labels (A–HRP and AF). According to the literature, conventional fluorophores give fluorescence emission spectra of 10 nM AF (1) and a mixture of 10 nM AF and Ab43 at varying concentrations made in PBS: 0.6 (2), 2.5 (3), and 15 μg mL⁻¹ (4). The emission in (5) corresponds to the absence of AF.

The LOD of an immunoassay is often determined by the relative affinity of the Ab for the analyte and the competing hapten–label conjugate. However, it also depends on other parameters. Nonspecific adsorption of the immunoreagents or certain nonpolar analytes to the microwell walls can be a problem, along with greater background fluorescence that is found in the microdroplet format. Background interferences due to Raman and Rayleigh scattering and impurity fluorescence are minimized when small microdroplet volumes are used. In addition, the excitation source in the plate reader had a wavelength of 485 nm (bandwidth of 20 nm full width half-maximum, fwhm), whereas in the LIF-microdroplet QFIA we used a CCD with low readout noise. The microdroplet format ensured that all fluorophores in the sample were exposed to the laser illumination, permitting detection limits to be extended to very low concentrations of fluorophore. This could be achieved in a microplate format, in principle, if the laser beam were expanded to illuminate an entire well, at the expense of much greater elastic light scattering at the laser wavelength. Although the notch filter had a high rejection ratio for the laser wavelength, some background would be expected to interfere with the measurements. It should be noted that the microdroplet detection scheme was arranged so that the fluorescence was observed in the plane of polarization of the incident laser beam at an angle of 90°, leading to an additional reduction in elastic scattering as described by the Mie theory of light scattering. Finally, the microdroplet approach allowed detection to be performed in a volume much smaller than the volume of the standard microwells (200 mL) resulting in lower LOD. Thus, in absolute mass, the LIF-microdroplet QFIA LOD is 1.68 × 10⁻⁴ ng while for the microplate QFIA it is 7.2 × 10⁻² ng. The small absolute volume and mass that can be detected with the micro-
Table 1. Features of the Immunochemical Techniques Developed for TCP Analysis

<table>
<thead>
<tr>
<th>ELISA</th>
<th>microplate</th>
<th>LIF-microdroplet</th>
</tr>
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<tbody>
<tr>
<td>IC_{50} (μg L(^{-1}))</td>
<td>2.74</td>
<td>4.2</td>
</tr>
<tr>
<td>LOD (μg L(^{-1}))</td>
<td>0.2 (1 nM)</td>
<td>0.36 (1.8 nM)</td>
</tr>
<tr>
<td>LOD (ng)</td>
<td>4 x 10(^{-2}) (2 x 10(^{-13}) mol)</td>
<td>7.2 x 10(^{-2}) (3.7 x 10(^{-13}) mol)</td>
</tr>
<tr>
<td>tracer(^a)</td>
<td>A-HRP</td>
<td>A-fluorescein</td>
</tr>
<tr>
<td>procedure</td>
<td>multiple wash and incubation steps</td>
<td>homogeneous assay</td>
</tr>
<tr>
<td>excitation</td>
<td>485 nm (20-nm band)</td>
<td></td>
</tr>
<tr>
<td>volume, μL</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>488 nm</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>488 nm</td>
<td>0.04 (0.2 nM)</td>
<td></td>
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<tr>
<td>4.2</td>
<td></td>
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\(^a\) From Galve et al.\(^{40}\) \(^b\) Hapten A is 3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid.

LIF-Microdroplet QFIA for Analysis of Urine Samples.

The application of homogeneous fluorescence immunoassays to the measurement of analytes in untreated biological materials is usually complicated due to the presence of endogenous fluorescent compounds and other species that quench the fluorescence of the labeled probe. In general, interferences from endogenous substances may be minimized by sample dilution or blank correction of the sample.\(^56\) The detection of TCP in urine could be used for biological monitoring of occupational exposure to chlorophenols and other organohalogenated compounds. Therefore, we evaluated the background fluorescence signal of the urine in the conditions of our LIF-microdroplet QFIA (see Figure 7A). With laser excitation at 488 nm, the urine has a fluorescence emission in the region of 520–545 nm that decreases with urine dilution. The emission of the AF tracer in diluted urine does not suffer any shift and the only effect observed is the increased fluorescence intensity of AF due to the presence of matrix background. However, the AF spectrum of 50-fold diluted urine is almost identical to the AF in PBS.

Urine components can affect not only the fluorescence background of the sample but also the Ab–Ag interaction. The interference produced by the urine matrix in the competitive immunoassay in a TCP ELISA has been demonstrated in earlier work.\(^39\) In the present case, we investigated the effect of interference on the immunoassay by comparing the fluorescence quenching produced in urine diluted 10, 20, and 50 times and in PBS buffer. The urine matrix interfered with the Ab–AF interaction, resulting in a very small quenching effect that could be eliminated by dilution (see Figure 7B). A 50-fold PBS dilution of the urine gave rise to a fluorescence quenching effect similar to that in the assay buffer, and thus it was used to prepare a standard curve. As seen in Figure 6, the curves prepared in PBS buffer and in 50 times diluted urine are almost identical. The IC\(_{50}\) is 2.14 nM (0.42 μg L\(^{-1}\)), and the calculated LOD is 0.2 nM (0.03 μg L\(^{-1}\)) with a dynamic range between 0.41 and 15.1 nM (0.08–2.99 μg L\(^{-1}\)). Taking into account the dilution factor applied to the urine, the LOD of the analytical method is 1.6 μg L\(^{-1}\), which is sufficient for occupational exposure assessment studies.

CONCLUSIONS

A LIF-microdroplet QFIA has been developed that has proven to be fast, highly sensitive, and simple to perform (no separation steps are needed). It has been demonstrated that this approach offers better detection limits than those of microplate immunoassays. The results reported here indicate that urine samples can be directly analyzed for TCP, without any sample treatment and with enough detectability to perform biological monitoring and risk assessment of exposure to chlorophenols and other organohalogenated compounds.

nochlorinated substances that are excreted as TCP in urine. It is worth noting that contamination of the environment and edible products by chlorophenols have been considered to be one of the main sources of exposure to dioxins.\(^\text{57}\) Similarly, recent investigations have demonstrated that chlorophenols are readily converted to polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/ Fs) by in vitro biochemical-catalyzed oxidation reactions.\(^\text{58}\) The technology presented here is promising regarding development of high-throughput screening and miniaturized biosensor devices. Significant improvement in precision and reproducibility could be achieved by better droplet generation (for example, using ink-jet printer micropiezoe technology) and charged microdroplet focusing by electrostatic fields, resulting in better control of the volume and droplet stability. Future work will be dedicated toward smaller volume assay development and its application to miniaturized devices (microchip). Similarly, other features such as precision, accuracy, and specificity of the assay will be assessed in the new configuration. This work is the first application of a steady-state QFIA with LIF-microdroplet detection system to human urine samples, demonstrating the potential of this analytical approach for bioassays.

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