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Highly sensitive fluorescence quantification of picloram using immunorecognition liposome

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ABSTRACT

Picloram is a widely used chlorinated herbicide, which is quite persistent and mobile in soil and water with adverse health and environmental risks. A simple and efficient method with high sensitivity and good selectivity was developed in this work to analyze picloram. The aldehyde group functionalized quartz glass plate was used to catch picloram by Schiff base reaction, and reacted with the liposomes-labeled antibody. The fluorescein isothiocyanate (FITC) solution was encapsulated in the liposomes. After being released from the liposomes, the fluorescence of FITC was measured by a fluorimeter. It was found that the fluorescence intensity is linearly correlated to the logarithm of picloram concentration, ranging from 1.0×10^{-4} to 100 ng mL^{-1}, with a detection limit of 1.0×10^{-5} ng mL^{-1}. Picloram concentration in real wastewater samples were accurately measured by the proposed method and HPLC, the results of the two methods were approximately the same. The proposed method showed high sensitivity and good selectivity, and could be an efficient tool for picloram quantitative analysis.

1. Introduction

Picloram (4-amino-3,5,6-trichloro-2-pyridincarboxylic acid) is a widely used chlorinated herbicide for wood plants, wheat, barley, and wide range of broadleaf weeds [1]. However, picloram is suspected to be an endocrine disruptor, which adversely affects the environment and threatens human health. High levels of the herbicide can damage human central nervous system and reproductive system or cause other health problems [2]. Picloram is quite persistent and mobile in soil and water [3,4]. It can remain active for nearly one year in suitable soil environment, and can easily leach into subsurface and ground water. The maximum contaminant level of picloram in drinking water set by USEPA is 500 ng mL^{-1}. Therefore, it is important to have sensitive and quantitative analysis method for picloram to meet the evolving requirements of social life and analysis technique.

Many analysis methods for picloram have been reported, including gas/liquid chromatography with electron capture detector and capillary electrophoresis/mass spectrometry [1,5,6], radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), and electrochemical methods [7–9]. Most of these methods, however, have limitations such as large instruments, radiation hazards, low sensitivity, poor selectivity or high complexity. Fluorescence analysis techniques have attracted great interests because of its operational simplicity, high sensitivity, and broad adaptability. Fluorescence staining [10], fluorescence in situ hybridization (FISH) [11], quantum dot [12] and fluorescence sensor [13] have found extensive applications recently. However, there are few reports about quantitative analysis of picloram using fluorescence analysis technique [14–16].

Early researches of picloram fluorescence analysis have mainly focused on the emitted fluorescence of picloram in strong acid condition, but with poor detection efficiency [14,15]. Aoyagi et al. [17] developed a method to directly detect the fluorescence from FITC labeled on protein A on the glass by a fluorimeter, which is different from a flow injection fluorescence system that requires a special flow-cell with optical fibers joined a fluorimeter. Although this strategy is convenient, its sensitivity relies on the abundance of fluorescence quantum. To address the problem, liposomes were used in the proposed study. Liposomes are spherical phospholipid bilayer vesicles containing a void aqueous volume and can entrap almost any water-soluble marker molecule within the interior of those lipid vesicles [18]. Liposomes are excellent carriers and can enhance the intensity of marker. Thousands of marker molecules can be entrapped in liposomes for signal amplification. To something detection, comparing with in the absence of liposomes, the detection limit obtained varying degree improvement in the pres-

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ence of liposomes [19]. They have been applied in immunoassays and DNA detections [19–23].

This study developed a sensitive method for fluorescence analysis of picloram. To reduce the interference of the scattering light from solid substrates, a quartz glass plate was used. The aldehyde group functionalized quartz glass plate was used to catch picloram molecules by Schiff base reaction, and immunoreacted with the anti-picloram-IgG labeled liposomes. The resulting glass plate was placed into the detection cell. After the liposomes lysis, the fluorescence from the released FITC was measured by a fluorimeter. The quantity of picloram is proportional to the fluorescence intensity. Fluorescence analysis promised a high sensitivity, and the specific antigen–antibody interaction provided a good selectivity. Comparing with the other picloram detection methods, this strategy is relative simple and had a lower detection limit [1,5–9,14]. A detection limit of 5 ng mL^{-1} [9] was the lowest detection limit in previous works. We herein report that a lower detection limit of 20,000-fold could be obtained by this proposed strategy. To the best of our knowledge, this proposed picloram analysis method has not been reported yet.

2. Materials and methods

2.1. Reagents and apparatus

The analytical standards of picloram, quinclorac, triclopyr, and lontrel were purchased from J&K Chemical Ltd. (Beijing, China). Fluorescein isothiocyanate (FITC), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were purchased from Sigma and used as received. Cholesterol, glutaraldehyde, 3-aminopropyltriethoxysilane (APTES), and all the other chemicals were of analytical grade or the highest purity commercially available, and were used as received. All solutions were prepared in deionized water of 18 MΩ purified from a Milli-Q purification system. The anti-picloram-IgG was self-prepared as described in our previous work [9].

The fluorescence excitation and emission spectra as well as the halide response spectra were analyzed using a Perkin-Elmer LS-55 spectrofluorimeter (Waltham, MA). Both the excitation and emission slits were set at 5 nm and controlled by a computer data processing unit. The light source was a pulsed Xe lamp. The quartz glass plates were 8 × 44 mm. All fluorescence measurements were made at ambient temperature of 25 °C. The osmolality of solution used in this study was measured by a Fiske 210 Micro-Sample osmometer (Advanced Instruments, Norwood, MA). Zetasizer Nano ZEN3600 (Malvern Instruments, Malvern, UK) was used to determine the particle size of liposomes. Agilent 1100 high performance liquid chromatograph (Agilent Technologies, Santa Clara, CA) was used to determine the concentration of picloram in wastewater.

2.2. Functionalizing glass plate

The quartz glass plates was soaked in piranha solution (mixture of H_2SO_4 and H_2O_2 with the volume ratio of 3:2) at 60 °C for 30 min, and rinsed with water. Then, they were soaked for 20 min in H_2O_2, $NH_3{\cdot}H_2O$ and water mixture (volume ratio 1:1:2) at 70 °C, and then rinsed with water and methanol for three times, respectively. To modify the surface of glass plates with amino groups, they were incubated in APTMS and methanol solution (volume ratio 1:1.5) at 37 °C for 6 h. Next, they were rinsed with methanol and water to remove the weak adsorptive, and dried in the air. At this point, the plates were immersed in 2.5% glutaraldehyde for 3 h and then washed to yield the functionalized glass plates. The aldehyde group modified on the glass plate surface would have a Schiff base reaction with the amino group of picloram.

2.3. Encapsulant preparation

FITC was dissolved in a little dimethylsulfoxide. And then, it was slowly added into a phosphate buffer solution (PBS, pH 7.4, 67 mM) with stirring to yield a 70 mM FITC solution. The final osmolality was 759 mOsmol kg^{-1}. To keep the liposomes intact, all of the other buffers used for liposome preparation were prepared with an osmolality of up to 50–100 mOsmol kg^{-1} higher than the encapsulant osmolality.

2.4. Liposome preparation

The liposomes were prepared according to the previous literatures [17,18,24,25] with some modifications. The lipid mixture consisted of DPPC, cholesterol, DPPG, and DPPE (10:10:1:0.4 in molar ratio). The total lipid mixture was dissolved in 6 mL of chloroform/isopropyl ether/methanol solution (volume ratio 6:6:1). After sonicating the mixture at 45 °C under N_2 for 1 min, 1 mL of FITC was added, and sonication was continued for 3 min. Then, the organic solvent was removed using a vacuum rotary evaporator. The last two steps were repeated once. An orange jelly of liposomes was obtained. The liposomes were incubated at 45 °C for 30 min before passing through a 0.2-μm polycarbonate filter 20 times to produce a homogeneous suspension of uniform size. The liposomes were centrifugated for 10 min at 10,000 rpm for four times to remove the unencapsulated dye or trace organic solvent from the liposome preparation. The prepared liposomes were stored in 5 mL of PBS solution (pH 7.4, 0.47 M) at 4 °C in the dark.

2.5. Conjugating antibody to liposomes

Conjugating the anti-picloram-IgG to liposomes was done via glutaraldehyde coupling. First, the liposome suspension was added into 3 mL of 2.5% glutaraldehyde solution to react for 40 min. They were dialyzed in PBS solution (pH 7.4, 0.47 M) overnight at 4 °C to remove the excess glutaraldehyde. Next, 3 mg of anti-picloram-IgG was added in the dialysis product under gentle agitation at 4 °C in the dark for 2 h. To block excess aldehyde group on the liposome surface, 1 mL of 0.4 M glycine–NaOH (pH 7.4) was added and incubated overnight at 4 °C in the dark. Finally, the resulting suspension was centrifugated for 10 min at 8000 rpm for four times and the precipitates were collected.

2.6. Measurement procedure

The glass plate was placed into a 5-mL centrifuge tube, incubated in 2 mL of various concentrations of picloram solution at 37 °C for a certain time, and followed by washing with PBS (pH 7.4, 67 mM). Then, it was incubated in 2 mL of 5% skim milk to block the non-specific binding sites on plate surface. After washing with PBS, a certain volume of liposomes-labeled antibody was added onto the plate surface for immunoreaction at 37 °C for a certain time. Before fluorescence analysis, the plate was washed with PBS, and the top of non-reaction zone was inset a circular latex plate. The circular latex plate could keep the glass plate vertical and at the proper position in the detection cell. The detection cell contained 2.5 mL of PBS (pH 7.4, 67 mM). After lysis with the methanol solution of Triton X-100, the released FITC from the liposomes was measured by the spectrofluorimeter. The excitation and emission wavelengths were 490 nm and 520 nm, respectively. The excitation and emission silts were both set at 5 nm. The process was shown in Fig. 1.

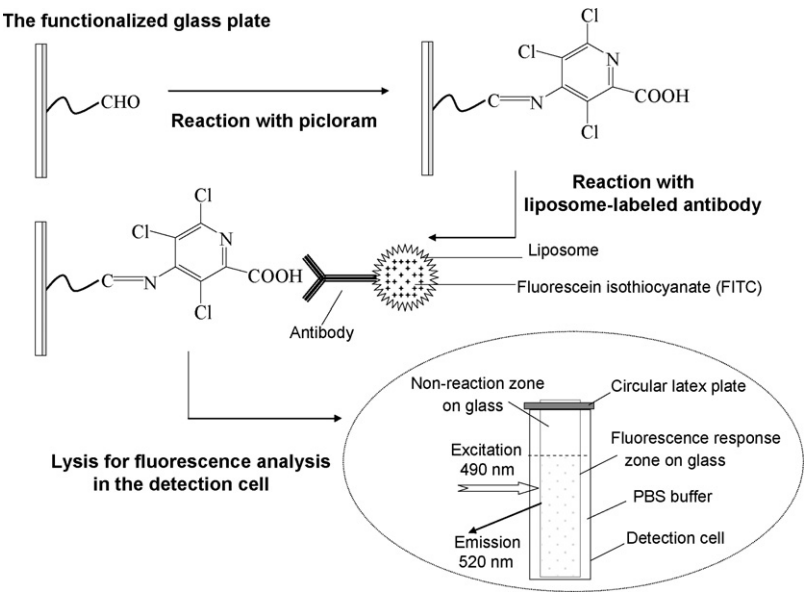

Fig. 1. Sketch map of picloram measurement process.

2.7. Sample treatment and detection

The wastewater samples used in this study were obtained from a municipal wastewater treatment plant in Changsha, China. First, they were centrifuged at 10,000 rpm for 5 min, and then filtered. Next the pH value of supernatants was adjusted to 7.4 with the phosphate buffer solution. The resulting wastewater samples were spiked with certain concentration of picloram that was dissolved in 3% MeOH-PBS (pH 7.4). After that, fluorescence measurement was performed. At the same time, the same samples were filtrated via the 0.2-μm polycarbonate filter and analyzed by high performance liquid chromatography (HPLC) for comparison. The mobile phase consisted of an isocratic mixture of water and acetonitrile (40:60, v/v), with the aqueous phase containing 0.1% (m/v) H_3PO_4. Under a flow rate of 1.2 mL min^{-1}, the concentration of picloram was detected by ultraviolet spectrophotometer at 220 nm [1].

3. Result and discussion

3.1. Characterization of liposomes

Liposomes have good biological adaptation and stability. They can maintain bioactivity of a biomolecule complex for a reasonably long period. The fluorescence marker was encapsulated in liposomes. This formation would reduce the ambient interference. The particle-size analysis shows that the liposomes have a mean diameter of 342 nm. To obtain good reproducibility, it is very important to make the size of liposomes as uniform as possible. Extrusion of the liposome preparations through polycarbonate filters reduced the size heterogeneity. The total volume of the liposomes (2.1×10^{-11} μL) was calculated from the diameter. The entrapped volume (1.9×10^{-11} μL) was calculated from an inner diameter of 334 nm (assuming bilayer thickness of 4 nm). The encapsulation efficiency is 3.2%, assuming that the concentration of encapsulated FITC equal to the original solution used, and by comparing the fluorescence of lysed liposomes to that of standard FITC solutions. The characteristics of the liposomes are summarized in Table 1. It was also proved that these liposomes offered adequate fluorescence quantum in subsequent assays.

Table 1
Characteristics of the liposomes.

Experiment parameter	Experiment result
Mean diameter (nm)	342
Entrapped volume of liposome (μL)	1.9×10^{-11}
FITC concentration (mM)	70
Encapsulation efficiency (%)	3.2

3.2. Fluorescence response

To test the feasibility of this strategy, fluorescence response experiment was performed. The excitation and emission wavelength was 490 nm and 520 nm respectively. The excitation and emission slits were both set at 5 nm. The fluorescence response was displayed in Fig. 2. The result showed that this strategy for picloram detection was realizable.

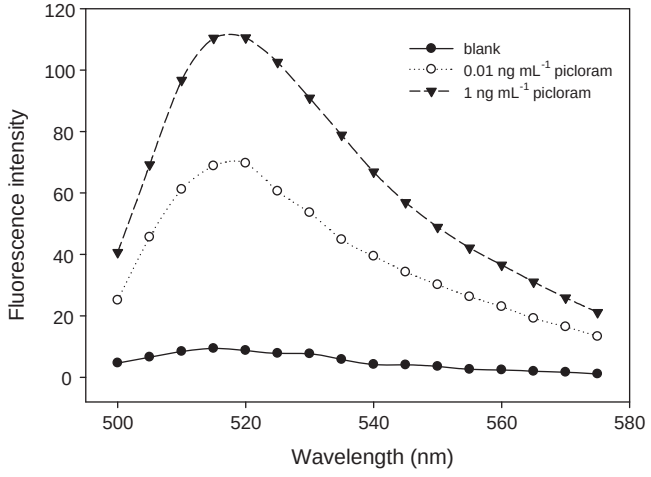

Fig. 2. Fluorescence response of this strategy to 0.01 ng mL^{-1} and 1 ng mL^{-1} picloram. Using 1:10 diluted liposomes-labeled antibody, and a methanolic solution of Triton X-100 as lysis reagent, and providing adequate conditions for thorough reaction.

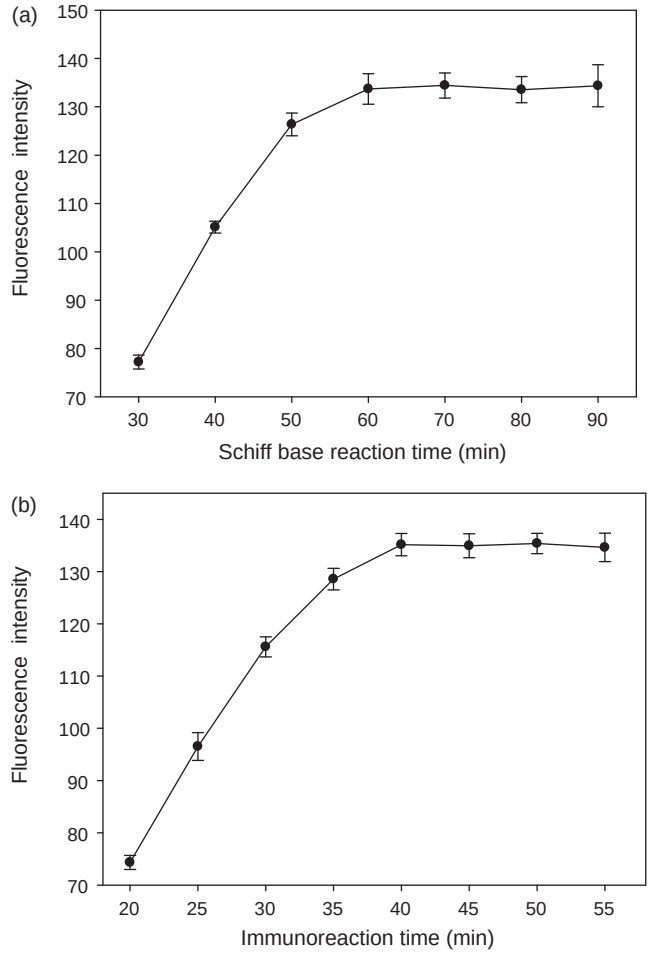

Fig. 3. Effect of incubation time in (a) catching picloram molecules process and (b) immunorecognition reaction process. Using 10 ng mL^{-1} picloram, 1:10 diluted liposomes-labeled antibody, and a methanolic solution of Triton X-100 as lysis reagent, and providing adequate conditions for thorough reaction.

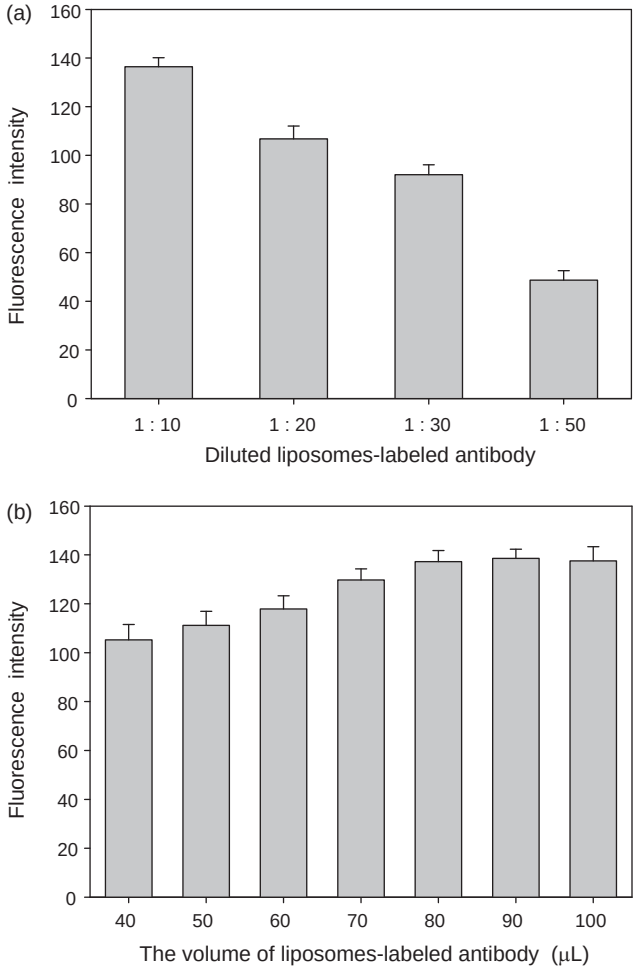

Fig. 4. Effect of various dilution rates of liposomes-labeled antibody and dosage. (a) Optimizing the dilution rates of liposomes-labeled antibody and (b) optimizing the dosage of the optimal diluted liposomes-labeled antibody.

3.3. Optimizing experiment conditions

A series of experiments was performed to optimize the conditions with maximum signal intensity. The effect of incubation time, dilution rate of liposomes-labeled antibody, volume of liposomes-labeled antibody, volume of lysis reagent, and lysis time before measurement were investigated.

Incubation time is an important factor to ensure the adequacy of a contact reaction. Two incubation steps were carried out before fluorescence analysis. One was the process of catching picloram molecules using Schiff base reaction on the functionalized glass plate surface, and the other was the immunorecognition reaction between picloram and its antibody. As shown in Fig. 3, the signal intensity increased with the incubation time, and then reached a plateau. Therefore, 60 min and 40 min were used respectively in the subsequent measurement.

Various dilution rates of liposomes-labeled antibody and volumes of liposomes-labeled antibody were investigated. 100 μL of liposomes-labeled antibody with different dilutions (1:10, 1:20, 1:30, 1:50) was added on the glass plate of 30-mm length reaction zone. From Fig. 4a, the highest fluorescence signal was obtained at the dilution rate of 1:10. Thus, 1:10 diluted liposomes-labeled antibody was chosen as the optimal dilution rate and was further optimized for its dosage in each analysis. The fluorescence signal increased with the volume of the diluted liposomes-labeled antibody, and reached the maximum at 80 μL (Fig. 4b).

In addition, a methanolic solution of Triton X-100 was used as the lysis reagent. Its dosage and the lysis time before measurement was optimized. The optimal lysis occurred at 130 μL of 0.2% (v/v) Triton-X 100 in 20% (v/v) methanol–water solution within 8 min.

3.4. Picloram quantitative analysis

Under optimal experimental conditions, picloram quantitative analysis was carried out. Fig. 5 shows the calibration of fluorescence signal and the concentrations of picloram. The fluorescence intensity was linearly related to the logarithm of picloram concentration C (ng mL^{-1}), ranging from 1.0×10^{-4} to 100 ng mL^{-1} with the following regression equation.

$$\text{Fluorescence intensity} = (22.287 \pm 0.704) \times \log C + (119.041 \pm 1.638) \quad (1)$$

The correlation coefficient is 0.996. Each point of the calibration was done in triplicates, and the average relative standard deviation was 4.36%, which confirmed the precision of the fluorescence analysis method. The detection limit was 1.0×10^{-5} ng mL^{-1}, displaying a lower detection limit than the results previously reported [1,5–9,14].

Table 2
Picloram concentration in wastewater sample determined by fluorescence analysis strategy and HPLC.

Samples	Picloram concentration ($ng\,mL^{-1}$)		Relative signal deviation (%)
	The fluorescence analysis strategy[a]	HPLC[b]	
1	12.84 ± 0.47	13.26 ± 0.41	3.17 ± 0.03
2	1.72 ± 0.08	1.66 ± 0.09	3.61 ± 0.05
3	40.72 ± 1.64	40.24 ± 1.87	1.20 ± 0.04

[a] An average of three replicate measurement.
[b] An average of two replicate measurement.

Table 3
Comparison of the two methods in operation.

Step	Method	
	The fluorescence analysis strategy	HPLC
Filtration process via the 0.2-μm polycarbonate filter/min	None	10
Capturing analyte and labeling process/min	140	None
Preheating equipment	5	15
Detection process/min	8	20
Equipment washing/min	None	30
Total time cost/min	153	75

3.5. Interference

The proposed method had good selectivity, which was attributed to the following reasons. First, no matter what was attracted on the glass plate, only picloram could trigger the fluorescence signal response due to immune specificity between picloram and its antibody, other substances could not form the joint structure to complete the signal response process. And in samples detection process, the functionalized quartz glass plate reacted with wastewater samples and then was taken out to wash. If there were something (e.g. heavy metal ion, i.e. Fe, Hg, Cd, Ca, etc.) might interfere the fluorescence signal, they had been washed or chelated with functional groups in the plate before they could reacted to the liposomes-labeled antibody. Finally, the fluorescence marker encapsulated in liposomes was protected before lysis. To evaluate the selectivity, some possible interfering substances with similar structure, such as quinclorac, lontrel, triclopyr and pyridine, were examined under the same condition as for picloram determination. Measurements were carried out by adding $500\,ng\,mL^{-1}$ various substrate in PBS (67 mM, pH 7.4). Compared with the fluorescence intensity to $1\,ng\,mL^{-1}$ picloram, the relative fluorescence intensity were 2.76% for quinclorac, 1.67% for lontrel, 4.52% for triclopyr and 3.87% for pyridine. It can be inferred that these substances cause negligible interference. Additionally, heavy metal ion (Fe, Hg, Cd, Ca, Cu, and Mn) interference was also tested. The concentration of $1\,ng\,mL^{-1}$ picloram respectively containing Fe^{2+} ($500\,ng\,mL^{-1}$), Hg^{2+} ($100\,ng\,mL^{-1}$), Cd^{2+} ($400\,ng\,mL^{-1}$), Ca^{2+} ($500\,ng\,mL^{-1}$), Cu^{2+} ($500\,ng\,mL^{-1}$), and Mn^{2+} ($300\,ng\,mL^{-1}$) was measured. Comparing with the fluorescence intensity to $1\,ng\,mL^{-1}$ picloram without the metal ions, the almost same but slightly lower results were obtained. Among these results, the maximum reduction of fluorescence intensity reached 2.27% by Ca^{2+}.

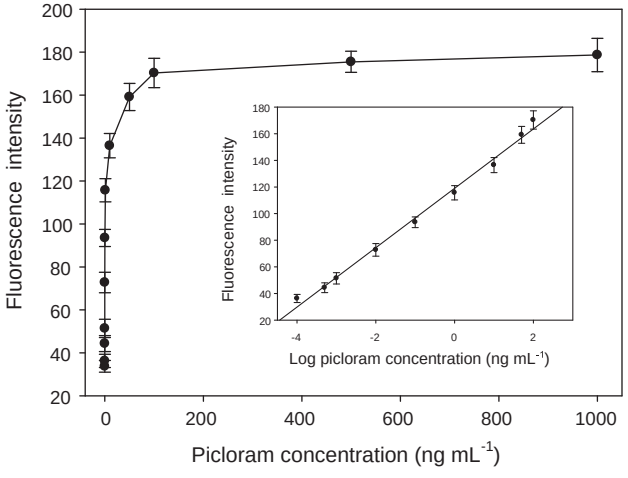

Fig. 5. Fluorescence intensity vs. picloram concentration between 1.0×10^{-5} and $100\,ng\,mL^{-1}$ in PBS (2.5 mL, 67 mM, pH 7.4). The vertical bars designate the standard deviation of three replicate tests. (Inset) Calibration plot of fluorescence intensity vs. logarithm of picloram concentration between 1.0×10^{-4} and $100\,ng\,mL^{-1}$.

3.6. Application in real sample

Picloram concentration of three wastewater samples were determined by the proposed fluorescence method and HPLC, the results of the two methods were approximately the same, as shown in Table 2. It could be concluded from the results that the two methods displayed a good correlation. In addition, the comparison of the proposed method and HPLC method in operation was shown in Table 3. Although the total time of the proposed method was longer than the HPLC method, the actual machine detection time of the former was far below that of the latter. Furthermore, comparing with the limitation of HPLC method in application, e.g. large instrument, relatively expensive cost, and tedious pretreatments, the method possessed certain advantages. Consequently, the proposed method offered a simple, fast and sensitive method for picloram quantitative analysis with high accuracy and good specificity.

4. Conclusion

This work developed a simple and sensitive strategy for picloram analysis. The functionalized glass plate was successively reacted with picloram and liposomes-labeled anti-picloram-IgG. The FITC solution was encapsulated in the liposomes. The fluorescence intensity of the released FITC was measured after the liposomes were lysed by the methanol solution of Triton X-100. The fluorescence intensity was linearly related to the logarithm of picloram concentration, ranging from 1.0×10^{-4} to $100\,ng\,mL^{-1}$, with a lower detection limit of $1.0 \times 10^{-5}\,ng\,mL^{-1}$. This method showed much higher sensitivity than the other approaches previously reported. In the determination of picloram in real wastewater,

the detection results of the proposed method and the parallel HPLC method were very close, while the proposed method was simpler, more convenient, and more sensitive.

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