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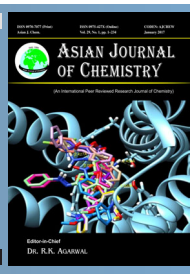

Spectrofluorometric Determination of Glyphosate in Water

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A sensitive, simple and selective spectrofluorometric method was developed for the trace level quantification of glyphosate from water. Due to lack of chromophore in glyphosate, the derivatization was accomplished using 9-fluoroenylmethoxycarbonyl chloride (FMOC-Cl). The derivatization conditions *viz.* pH of buffer, concentration of buffer, FMOC-Cl concentration *etc.* with respect to sensitivity of the analytical method were optimized. Under the optimized derivatization conditions, different concentrations of glyphosate ranging from 0.001-20 μg mL^{-1} were quantified using fluorescence spectrophotometer, HPLC-UV and UV-visible spectrophotometer. In all cases coefficient of determination (R^2) was 0.999. The minimum quantifiable limit using HPLC-UV, UV and fluorescence spectrophotometer were 0.03, 0.15, 0.003 μg mL^{-1}, indicating fluorescence spectrophotometer was the most sensitive technique. The percentage recoveries of glyphosate ranged from 95.2 ± 5.51 to 97.3 ± 6.07 with relative standard deviation (RSD) less than 6.3 %.

Keywords: Derivatization, Fluorescence, FMOC-Cl, Glyphosate, Water.

INTRODUCTION

Glyphosate [N-(phosphonomethyl)-glycine] is a broad-spectrum, non-selective, post-emergence organophosphorous herbicide, which is widely used to control annual and perennial plants [1]. But its widespread use along with high solubility in water and long half-life has lead to controversy regarding environmental contamination and ecological effects [2]. Therefore determination of glyphosate in environmental samples has gained importance making the availability of efficient and sensitive screening methods mandatory.

However, physico-chemical properties of glyphosate like high polarity, low solubility in organic solvents, amphoteric nature, variable acid dissociation constant values (pK_a = 2, 2.6, 5.6 and 10.6) and ease to form complexes with metal ions poses analytical challenge for trace quantification of glyphosate [3]. Several chromatographic methods based upon gas chromatography (GC) and liquid chromatography (LC) have been developed for the analysis of glyphosate from environmental samples but pre-concentration [4] and use of internal standard [5] is necessary to achieve low limit of quantification. The derivatization of glyphosate using FMOC-Cl is the most frequently applied method for the quantification of glyphosate. However, wide range of reaction conditions has been applied with respect to pH, reaction time, reaction temperature and concentration of 9-fluoroenylmethoxycarbonyl chloride (FMOC-Cl) [6] which indicates that the optimum conditions for derivatization are still unclear. Moreover, sophisticated instruments like HPLC-UV [7], HPLC-FLD (fluorescence detector) [8], LC/MS/MS [9,10] and GC [11] are required. Analysis using LC require robust amine column which can tolerate greater pH as silica based columns usually degrade under highly alkaline conditions and show a gradual decrease in efficiency after use of 2 months [12]. Glyphosate detection using spectrophotometer is not widely used because of its poor sensitivity.

Therefore in this study a simple, sensitive, spectrofluorometric method for the trace level quantification of glyphosate was optimized.

EXPERIMENTAL

The analytical standard of glyphosate (99.7 % purity) was purchased from Sigma Aldrich (Mumbai, India). FMOC-Cl and boric acid was supplied by Himedia, Mumbai, India. HPLC grade water, Potassium dihydrogen phosphate, sodium hydroxide, potassium hydroxide, acetonitrile was purchased from Finar Chemicals, Gujrat, India. Diethyl ether was supplied by Molychem (Mumbai, India).

Derivatization procedure: Stock solution (1000 μg mL^{-1}) and working standards of glyphosate were prepared in HPLC grade water. The derivatizing reagent was prepared by dissolving 1 mg of FMOC-Cl in 100 mL acetonitrile. Borate buffer solution (200 mmol L^{-1}) was firstly prepared by dissolving

1.237 g boric acid in 100 mL distilled water and its pH was adjusted to 9 with 200 mmol L^{-1} sodium hydroxide solution. The solutions were stored at 4 °C under dark conditions in polypropylene tubes to avoid adsorption to glass.

In a 15 mL plastic centrifuge tube 3 mL of 0.1 μg mL^{-1} aqueous solution of glyphosate was pipetted followed by the addition of 1.5 mL of borate buffer and 3 mL FMOC-Cl. The solution was then homogenized for 5 min by manual shaking. The derivatization reaction was maintained at 30 °C for 2 h. The excess amount of FMOC-Cl and FMOC-OH was removed by extracting the reaction mixture thrice with diethyl ether (3 × 4 mL) and the two layers were allowed to separate. The upper organic layer was removed while the lower aqueous layer which contained the derivatized product was withdrawn and quantified using fluorescence spectrophotometer, HPLC-UV, UV-visible spectrophotometer.

Instrumentation and conditions: Agilent technologies Carry eclipse fluorescence spectrophotometer equipped with 1 cm quartz cell was used for measurement of fluorescence intensity. Excitation wavelength was set at 268 nm with emission at 313 nm. The slit width of excitation and emission wavelength were 10 nm with 400 V voltage.

Waters 2489 HPLC system was used equipped with 515 HPLC pump and UV/visible detector. Agilent technologies zorbax amine column (5 μm × 4.6 × 250 mm) was used for the separation of glyphosate at 230 nm. The mobile phase composition consisted of 0.05 M KH_2PO_4 (pH adjusted to 6.0 with 7 N KOH) and acetonitrile (0.45:0.55, v/v) at flow rate of 1 mL min^{-1}. Under these operating conditions retention time for glyphosate was found to be 6.82 min.

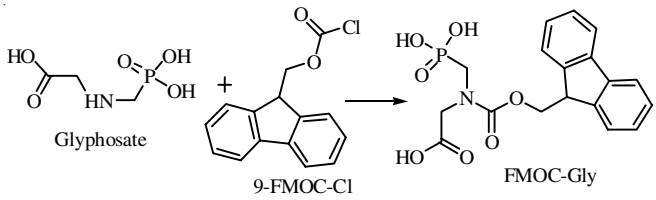
The UV spectra of samples were obtained using JASCO V 530 model spectrophotometer equipped with quartz cells with a path length of 1 cm at λ_{max} = 265 nm. The pH measurements were made on HANNA HI 208 pH meter.

For fluorescence spectrophotometer and UV-visible spectrophotometer the LOD and LOQ values were calculated as 3.3 σ/S and 10 σ/S, respectively, where S is the slope of the calibration curve and σ is standard deviation of blank responses [13]. For HPLC, LOD and LOQ were established at the signal to noise ratio of 3:1 and 10:1, respectively [14].

RESULTS AND DISCUSSION

FMOC-Cl in the reaction media without analyte gave fluorescence intensity of 9.0 (Fig. 1a). Fluorescence intensity of glyphosate in the reaction media without FMOC-Cl was 0.3 (Fig. 1b). However, 0.1 μg mL^{-1} glyphosate standard prepared in distilled water in the presence of FMOC-Cl gave fluorescence intensity of 602 indicating the necessity of derivatizing glyphosate to increase the sensitivity of analytical method (Fig. 1c). In the presence of FMOC-Cl, glyphosate undergo an aminolysis reaction, in which the acyl chloride (FMOC-Cl) reacts with the secondary amine (glyphosate), producing the corresponding fluorescent amide (FMOC-Gly) which showed high fluorescence intensity at λ_{em} = 313 nm with λ_{ex} = 268 nm.

In reaction of glyphosate with FMOC-Cl (**Scheme-I**), derivatization conditions have significant effect on fluorescence intensity and hence these parameters need to be optimized (Table-1).



Scheme-I: Reaction between glyphosate and FMOC-Cl

TABLE-1
PARAMETERS OPTIMIZED FOR THE DERIVATIZATION OF GLYPHOSATE

Parameter	Level
pH of borate buffer	7, 8, 9, 10, 11 and 12
Concentration of buffer (mmol L^{-1})	2, 20, 200 and 300
FMOC-Cl concentration (μg mL^{-1})	1, 5, 10, 100 and 500
Extracting solvent	Pentane, diethyl ether, dichloromethane and dichloromethane: propanol (2:1)
Homogenization time (min)	0, 1, 2, 3, 5 and 10
Incubation temperature (°C)	20, 30, 40 and 50
Reaction time (h)	0, 1, 2, 4 and 6

Effect of pH and concentration of borate buffer: As the reaction between glyphosate and FMOC-Cl results in formation of hydrochloric acid which decrease the pH of reacting media (**Scheme-I**), the reaction was carried out in borate buffer to maintain the consistency of the pH in the reaction medium. Reaction was monitored at pH 7-12 to study the influence of pH on the reaction. Derivatization of glyphosate did not occur at pH 7 and intensity of the derivatized

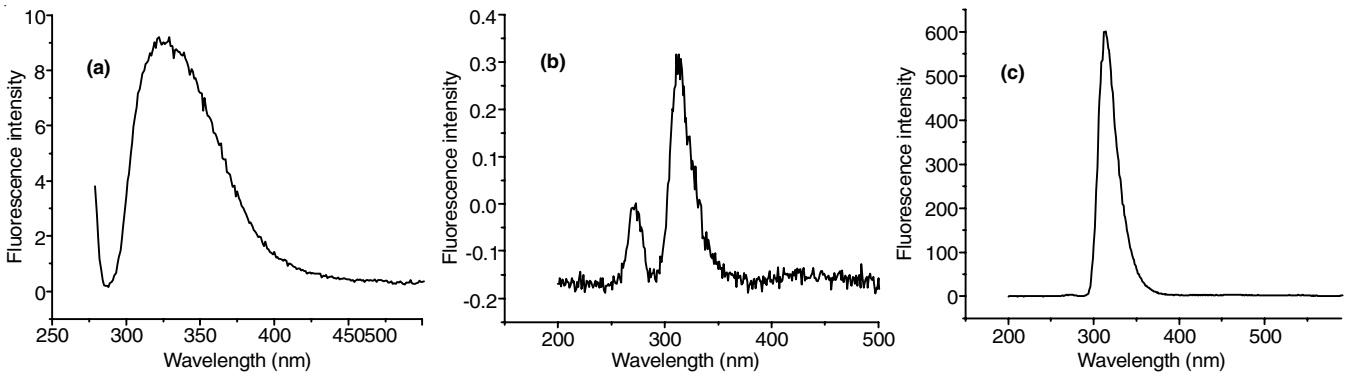


Fig. 1. Fluorescence emission spectrum (a) FMOC-Cl without glyphosate (b) Glyphosate without FMOC-Cl (c) Glyphosate containing FMOC-Cl

product gradually increased with increasing buffer pH (pH 8). In the pH range of 9-10, the intensity of the derivatized product remained almost constant. However, with further increase in pH (pH 11-12) of the reaction medium, the fluorescence intensity decreased, probably because of the hydrolysis of FMOC-Cl (Fig. 2a). Therefore, pH 9 was selected as an optimum pH for derivatization.

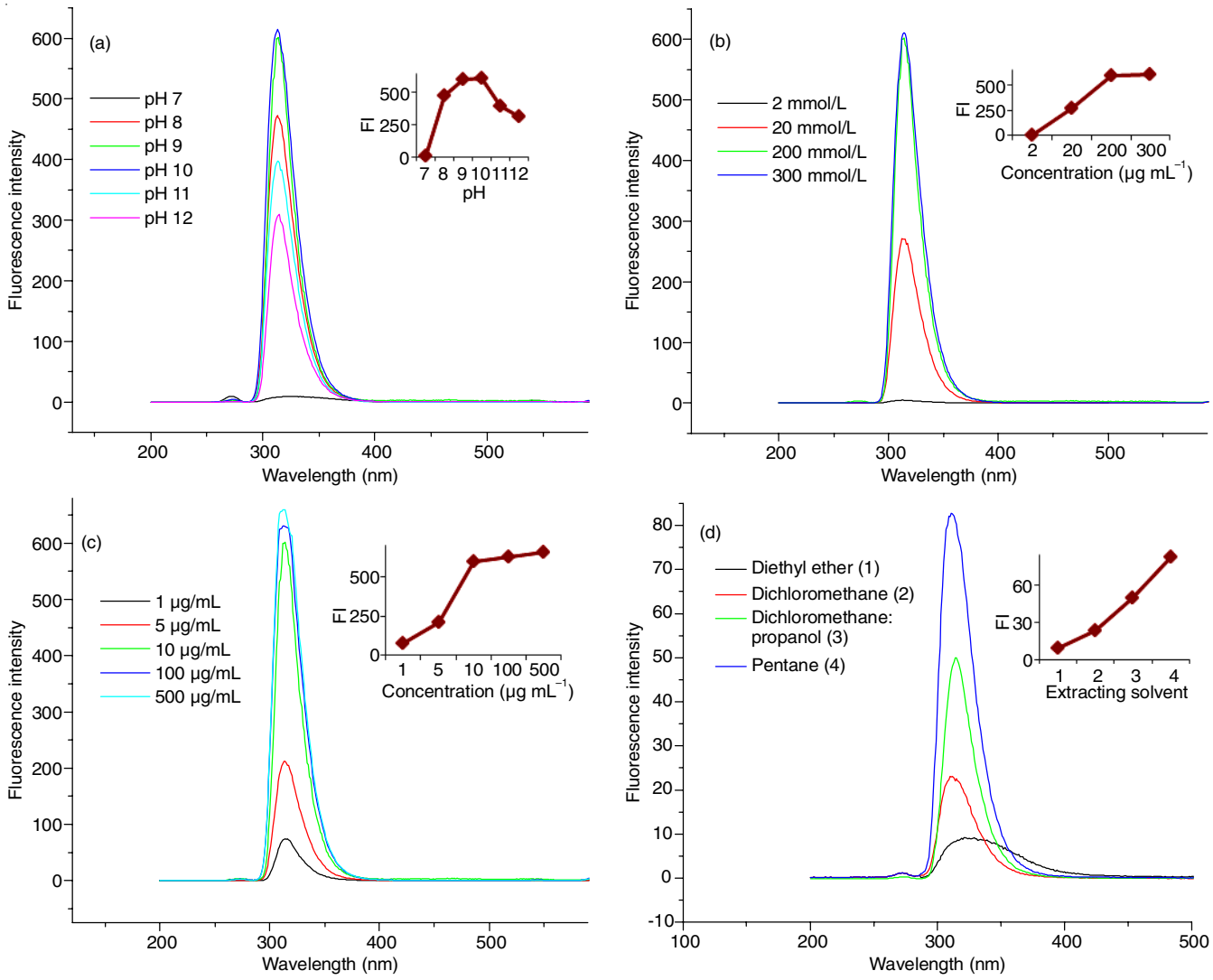
To optimize the concentration of borate buffer for derivatization, concentration of borate buffer (pH 9) was varied in the range of 2-300 mmol L^{-1}. Derivatization reaction did not occur at lower concentration of buffer (2 mmol L^{-1}) and fluorescence intensity increased with increase in concentration of borate buffer. Maximum fluorescence intensity was observed at 200 mmol L^{-1} thereby favouring the process of derivatization. Beyond the optimum concentration of borate buffer no significant increase in the fluorescence intensity of the product was observed (Fig. 2b).

Effect of FMOC-Cl concentration: In order to explore the effect of concentration of FMOC-Cl on the reaction, glyphosate was derivatized with varying concentration of FMOC-Cl. Concentration of 1 and 5 µg mL^{-1} gave a low fluorescence intensity but fluorescence intensity increased with increase in concentration of FMOC-Cl. Concentration of 10 µg mL^{-1} resulted in highest fluorescence intensity (Fig. 2c). No significant increase in fluorescence intensity was observed with further increase in concentration of FMOC-Cl.

However, the excess of FMOC-Cl in the reaction medium reacted with water to form FMOC-OH and interfered with the detection of glyphosate. To avoid this interference solvents such as pentane, diethyl ether, dichloromethane and dichloromethane:propanol (2:1) were used for the extraction of excess of FMOC-Cl and FMOC-OH from blank. Results revealed that 3 successive washings of diethyl ether resulted in maximum removal of excess of FMOC-Cl and FMOC-OH (Fig. 2d).

Effect of the homogenization time: To ensure maximum interaction of analyte with derivatizing reagent the homogenization time varied from 0-10 min. Homogenization for 5 min resulted in maximum fluorescence intensity of glyphosate. Further increase in homogenization time did not increased the fluorescence intensity (Fig. 2e).

Effect of incubation temperature: The effect of incubation temperature (20-50 °C) on the reaction was investigated. It was found that with the increase in temperature from 20 to 30 °C, the fluorescence intensity increased However, beyond the optimum temperature (30 °C) the fluorescence intensity decreased probably due to the favoured reaction between FMOC-Cl and water to form FMOC-OH (Fig. 2f).



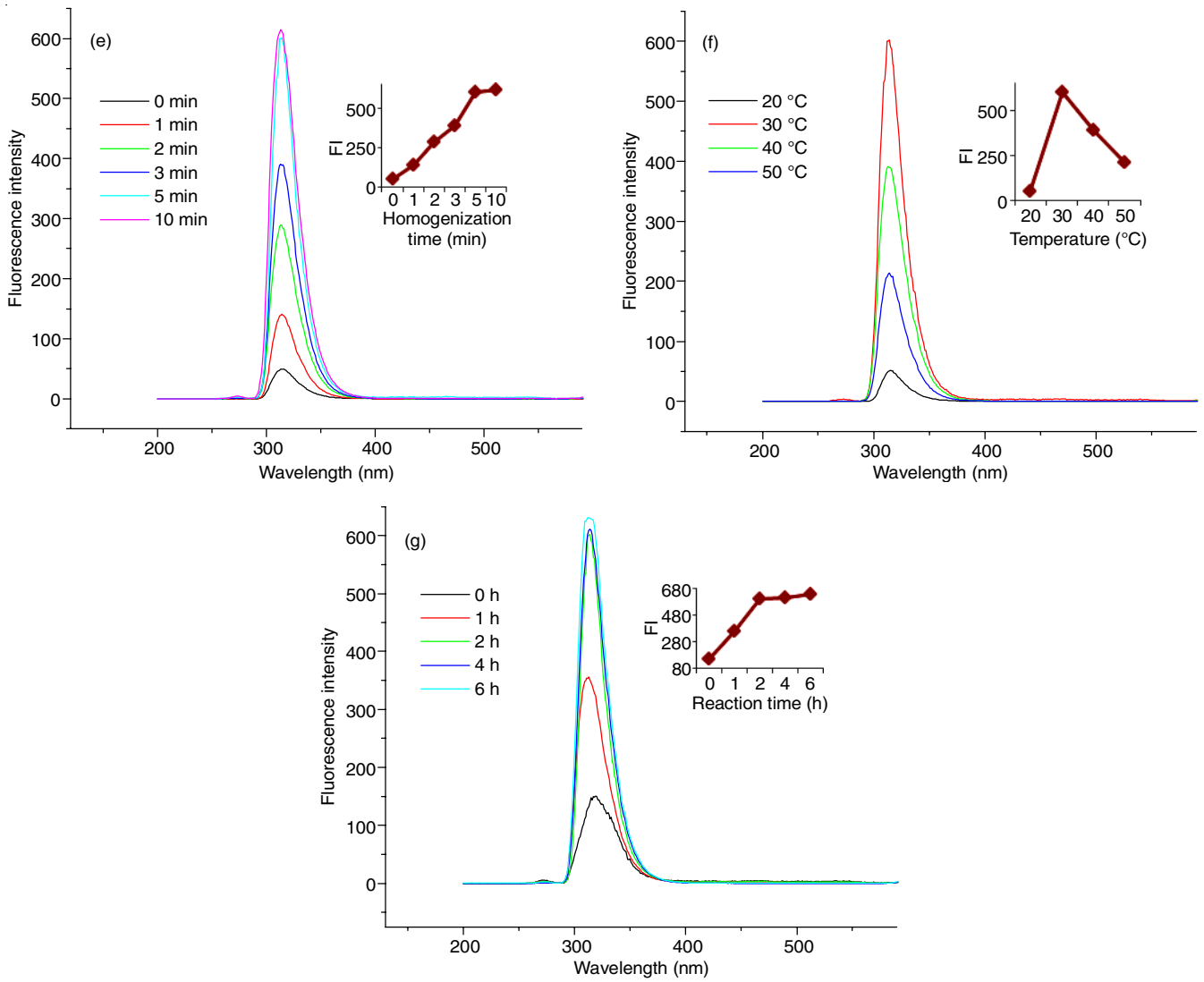


Fig. 2. Effect of derivatization parameters on fluorescence intensity (a) pH (b) concentration of borate buffer (c) FMOC-Cl concentration (d) washing solvent (e) homogenization time (f) incubation temperature (g) reaction time. Inset: Plot of fluorescence intensity (FI) *versus* derivatization parameters

Effect of the reaction time: The reaction time is definitive to ensure derivatization because this time period ensures the replacement of chlorine of FMOC-Cl by the glyphosate molecule. Therefore, in order to examine the effect of reaction time on fluorescence intensity reaction was carried out for 0-6 h at 30 °C. It was found that reaction time of 2 h resulted in highest fluorescence intensity and further increase in reaction time did not improved the fluorescence intensity (Fig. 2g).

It was observed that maximum fluorescence intensity of FMOC-Gly derivatized product in distilled water was obtained using FMOC-Cl (10 μg mL^{-1}) in borate buffer (200 mmol L^{-1}; pH 9) with homogenization for 5 min and incubation for 2 h at 30 °C.

Stability of derivatized product: The stability of FMOC-Gly derivatized product at 4 °C and room temperature was investigated. The product was stable for 7 days and 3 days at 4 °C and room temperature, respectively since no significant decrease in fluorescence intensity was observed. However, with further increase in time, fluorescence intensity decreased and showed declination of fluorescence intensity upto 70 % and 90 % in 30 days at 4 °C and room temperature, respectively (Fig. 3).

Method validation: Under the optimized derivatization conditions, different concentrations of glyphosate ranging from 0.001-20 μg mL^{-1} were prepared and quantified using fluorescence spectrophotometer, HPLC-UV and UV-visible spectrophotometer. In all cases R^2 was 0.999. The LOD of glyphosate using UV-visible spectrophotometer, HPLC-UV and fluorescence spectrophotometer were 0.05, 0.01 and 0.001 μg mL^{-1}, respectively (Table-2). The results indicated that among the three techniques, fluorescence spectrophotometer was 10 and 50 fold more sensitive for the quantification of glyphosate from water than HPLC-UV and UV-visible spectrophotometer, respectively.

The proposed fluorescence spectrophotometric method was applied for the determination of glyphosate from spiked distilled water samples (0.003, 0.005, 0.01 and 0.03 μg mL^{-1}). It was observed that the average percentage recovery of glyphosate ranged from 95.2 ± 5.51 to 97.3 ± 6.07 with relative standard deviation (RSD) less than 6.3 % (Table-3).

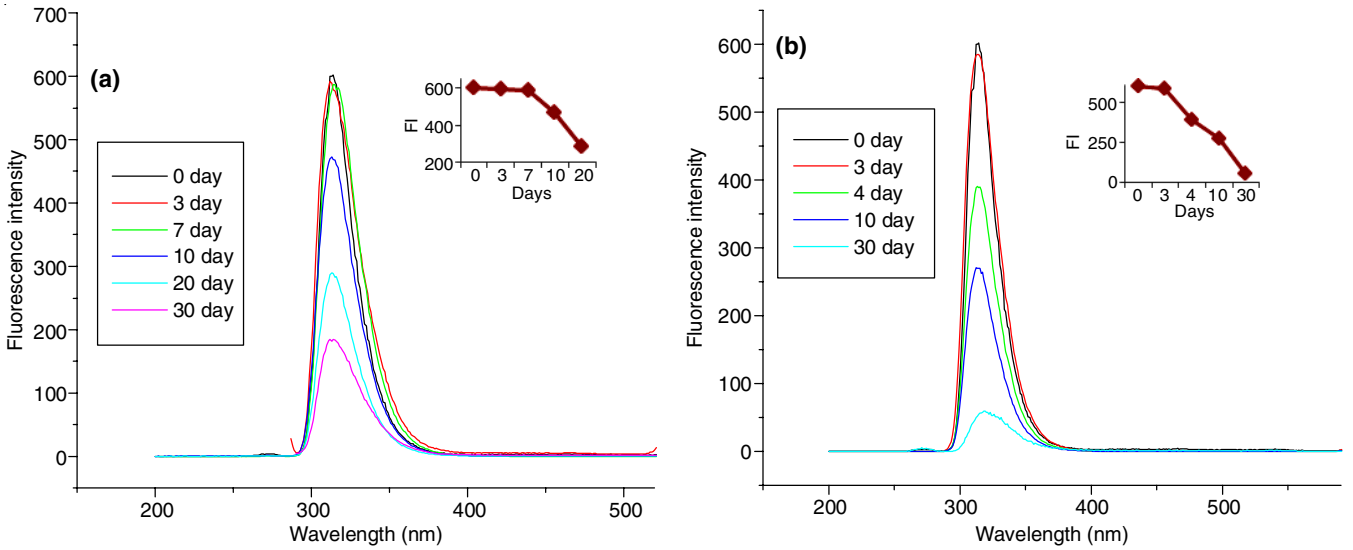


Fig. 3. Stability of FMOC-Gly (a) 4 °C (b) Room temperature. Inset: Plot of fluorescence intensity (FI) *versus* days

TABLE-2
QUANTIFICATION OF GLYPHOSATE USING DIFFERENT TECHNIQUES

Technique	Linearity range (μg mL^{-1})	Regression equation	R^2	LOQ (μg mL^{-1})	LOD (μg mL^{-1})
UV-visible spectrophotometer	0.1-20	y = 0.047x + 0.002	0.999	0.150	0.050
HPLC-UV	0.03-10	y = 15552x + 394.2	0.999	0.030	0.010
Fluorescence spectrophotometer	0.003-0.1	y = 5900x + 0.795	0.999	0.003	0.001

TABLE-3
PERCENTAGE RECOVERY OF GLYPHOSATE IN SPIKED WATER SAMPLES

Amount added (μg mL^{-1})	Recovery (%)	RSD (%)
0.003	95.2 ± 5.51[a]	5.79
0.005	96.6 ± 4.45	4.61
0.010	97.0 ± 5.51	5.68
0.030	97.3 ± 6.07	6.24

[a]Each value is mean recovery percent ± SD (standard deviation) of three values.

Conclusion

The proposed spectrofluorometric method is simple, accurate, sensitive which does not require any preconcentration of the sample and tedious extraction procedure. The lower detection limit of fluorescence spectrophotometer in comparison to HPLC-UV and UV-visible spectrophotometer demonstrated the sensitivity of the technique for the micro quantitative assay of glyphosate in water samples.

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