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Flow Injection Spectrophotometric Determination of Glyphosate Herbicide in Wheat Grains *via* Condensation Reaction with p-Dimethylaminobenzaldehyde

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ABSTRACT

A fast and sensitive method was developed for determination of glyphosate, using flow injection analysis. The analyte was converted to a photoactive product that shows maximum absorbance at 405 nm. Beer's law was obeyed over a range of 0.7–20 μg mL^{-1}. The limit of detection and quantification were found to be 0.20 ± 0.01 and 0.70 ± 0.02 μg mL^{-1} with a high sample throughput of 60 samples h^{-1}. The investigation of interference effect shows that among the compounds studied, only aminomethyl phosphonic acid exhibits a signal enhancing effect when present in higher concentration ratio with the analyte and this can be assumed to be a limitation of the present method. The percentage recoveries and residue of glyphosate in wheat grains samples, were found in the range of 90.0 ± 0.6 % to 93.0 ± 0.6 % and 1.13 ± 0.009 μg g^{-1}, respectively.

KEYWORDS

Glyphosate, photoactive, absorbance, flow injection analysis, residue.

1. Introduction

The active ingredient of Roundup® and about 50 more commercial formulations, glyphosate [N-(phosphonomethyl) glycine], is a broad-spectrum herbicide. It is post emergence, non-selective, contact herbicide and is used to treat crops, water bodies, forests, roadside areas and public and private yards.[1–4] Glyphosate was registered in 1974 and reregistered in 1993 by US EPA.[5] It is most widely used pesticide by volume, thus, it may be a potential contaminant in the environment and its long-term ecological impacts have been under study for decades.[6] Being stable to photodegradation, highly soluble in water, strongly adsorbed to soil particles and only broken down by soil microorganisms, glyphosate has long half-life in the environment (47 days) and is therefore, detectable for a long time in the environmental samples.[5,7]

Though World Health Organization (WHO) has declared glyphosate as non-carcinogenic, still its impacts on the flora and microorganisms are subtle. It has a low mammalian toxicity limits and causes digestive tract irritation, eyes and skin irritation, low blood pressure and respiratory dysfunction in human if orally taken.[1,6] Glyphosate has been listed as one of the US national primary drinking water contaminants with a maximum contaminant level (MCL) of 0.7 mg L^{-1}.[4,8]

In Europe, for any pesticide the drinking water standard has been set at 0.1 μg L^{-1} irrespective of its toxicological profile. United Nations Food and Agricultural Organization (UNFAO) have set a maximum residue limit (MRL) of glyphosate in wheat at 5 mg kg^{-1}, while EU maximum residue level (MRL) for glyphosate in wheat is 10 mg kg^{-1}.[4,9]

Glyphosate is highly polar, insoluble in organic solvents, highly soluble in water and is likely more difficult to be extracted and determined.[10] Across the decades, a variety of analytical techniques, mostly chromatographic, have been developed for determination of glyphosate in various environmental samples. Among these the widely used ones are: gas chromatography (GC),[11] ion chromatography,[12] capillary electrophoresis (CE) with UV-VIS detector,[9] fluorescence,[13] liquid chromatography (LC) with UV-VIS detection,[14] hydrophilic interaction chromatography,[15] differential pulse polarography (DPP),[16] NMR spectroscopy,[1] electrogenerated chemiluminescence (ECL),[17] conductivity detection,[18] ICP-MS,[19] integrated pulse amperometric detection (IPAD) at gold electrode,[20] HPLC with UV or MS detection[1] and with fluorescence detection,[21] nonionic surfactant micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence (LIF) detection,[22] and UV/VIS spectrophotometric method using ninhydrin as chromogenic reagent.[23] One of the online analysis methods for determination of glyphosate is the use of $[Ru(bpy)_3]^{2+}$ ECL,[22] which is very expensive. While all these methods are technically profound and show better results in terms of sensitivity but many of these are tedious, lengthy, complicated, time-consuming and some require special equipment, are expensive and may generate unstable products.

Flow Injection Analysis (FIA) technique is an adaptable and simple mean which is mostly employed for the analysis of various agricultural, clinical and environmental, samples.[24] Determination of a number of herbicides such as 2,4-D, and fluroxypyr in maize and wheat has been reported.[25–27] Similarly, following FIA technique, 2, 4-D, Mecoprop and sulfonylurea were determined in spiked water samples using micellar-enhanced photochemically induced fluorescence (MEPIF) detection.[28–29]

The main objectives of the present study were to develop and apply a simple, easy and rapid method for determination of glyphosate herbicide to support its analysis with the minimum available resources. In this study glyphosate herbicide, was reacted with p-dimethylaminobenzaldehyde (DMAB) in an alkaline medium. A yellow product was formed after acidifying

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the media with hydrochloric acid. The absorbance of the product was measured at 405 nm using FIA technique. The method was successfully applied for determination of residue of glyphosate in wheat grains. Though the proposed method is not comparable in terms of sensitivity to highly sophisticated instrumental methods like HPLC and LC-MS/MS approaches where an LOD and LOQ up to 0.02 and 0.05 μg L^{-1} respectively is achieved but in terms of robustness, sample throughput, solvent consumption and cost of analysis the proposed method is even better than the advanced methods.[30]

The present method is an extension to an analytical method for determination of glyphosate published[2] previously by our research group. That approach has been criticized in a letter to the editor being published recently.[31] The authors emphasized on the interference effect of aminomethyl phosphonic acid (AMPA) which is a biodegradation product of glyphosate. They also pointed out to the low sensitivity of the method. Here it is clarified that both the previous method as well as the present method have low sensitivity compared to highly sophisticated advanced methods such as LC-MS/MS for determination of glyphosate. In the present method the approach of FIA was used to reach better sensitivity which was achieved (LOD 0.2 μg mL^{-1}) and high sample throughput and the effect of AMPA and other possible interfering compounds was investigated and it was found that AMPA has an interference effect (though at higher concentration ratio) in the determination of glyphosate.

2. Experimental

2.1. Chemicals and Reagents

The basic reagents p-dimethylaminobenzaldehyde (Merck-Schuchardt, Hohenbrunn Germany), hydrochloric acid, sodium hydroxide and methanol (Merck, Darmstadt Germany) with reagent grade purity were used. Glyphosate (Reference standard) was obtained from Dr Ehrenstorfer (Augsburg, Germany). Ammonia solution with analytical grade purity was purchased from BDH Laboratory Suppliers Poole, England. Commercial formulation of glyphosate (41 % w/w) was obtained from the local market and used without any further treatment.

2.2. Instruments and Equipment

A UV-visible spectrophotometer (UV-1700 Pharmaspec, Japan) was used for spectrophotometric measurements in batch mode and another one (UNICO, UV-2100 United Products and Instruments Inc. Dayton, NJ, USA) was employed for measurements in online mode. Silicon tubes (1.71 mm, i.d.) were employed for suction of the reagents streams in the peristaltic pump (Model 124869 MP-1 pump Spectra/Chrom Spectrum). The sample solution was injected through a 6-port injection valve (V-450 Upchurch Scientific Inc. USA). The PTFE Tubing (0.8 mm, i.d., Becton Dickson, USA) were used for making the reaction coils and pH was measured using pH meter (model 7020 Kent Industrial Measurement Electronic Instruments Ltd., Chertsey Surrey England).

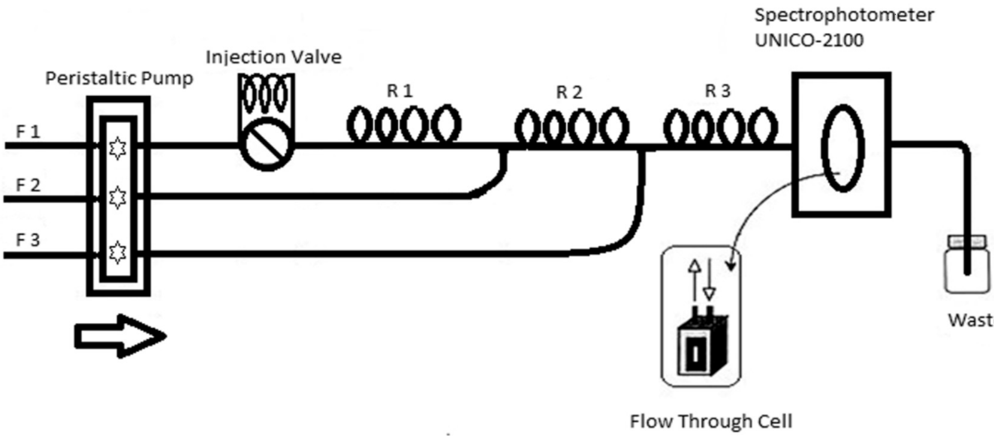
2.3. Analytical Procedures

In a 100 mL volumetric flask, standard solution of glyphosate (1000 μg mL^{-1}) was prepared in distilled water. Appropriate volumes of the stock solution were diluted with distilled water to obtain the working standards. p-Dimethylaminobenzaldehyde was dissolved in methanol to prepare a stock solution of concentration 0.2 mol L^{-1}. Stock solutions, 2.0 mol L^{-1} of sodium hydroxide and 3.0 mol L^{-1} of hydrochloric acid, were also prepared in distilled water.

A three-channel FIA system was assembled for flow injection analysis (Scheme 1). In reagent stream F1, NaOH (0.1 mol L^{-1}) was pumped. An injection valve with 500 μL injection loop was used to inject the sample solution of glyphosate (1.0 μg mL^{-1}) into the F1 stream. The alkaline mixture was then reacted with DMAB (0.016 mol L^{-1}) flowing down in the reagent stream F2. Lastly, the condensation adduct formed was acidified with HCl solution (3.0 mol L^{-1}) flowing in reagent stream F3. The absorbance of the yellow adduct passing through a 1-cm flow through cell was continuously measured at $\lambda_{\max}$ 405 nm.

Samples of wheat grains (10 g each) were individually kept in beakers and different volume of glyphosate solution (10 μg mL^{-1}) with a pH 4.5 was added for adjusting the concentration in the range of 1.0–3.0 μg g^{-1}. In order to achieve uniform mixing, the samples were homogenized by mechanical shaking and reserved for 24 h at room temperature. Extraction of the homogenized wheat grains samples were done for 60 min with 25 mL of NH_4OH (2.0 mol L^{-1}) as per our previously reported method,[2] and percentage recovery was determined by the proposed method, employing both batch and FIA. The recovery investigation was accomplished in triplicate.

In order to study the interference effects of various compounds on the determination of glyphosate, different compounds were screened for their interference effects. The choice of the expected



Scheme 1

Representation of flow injection system for determination of glyphosate.
F-1; Reagent-1(Sodium hydroxide), F-2; Reagent-2 (p-dimethylaminobenzaldehyde), F-3; Reagent-3 (Hydrochloric acid), R-1; Reaction coil-1(Cannizaro's reaction), R-2; Reaction coil-2 (Intermediate formation), R-3; Reaction coil-3 (Acidification and condensation).

interfering compounds was based on similarity in their chemistry to the analyte and/or their possible spraying practice along with glyphosate on the same field or in the nearby fields. A series of 1.0 μg mL^{-1} solutions of all the compounds were prepared and then with 1.0 mL of glyphosate solution, the test compounds were mixed in 1:1, 1:2, 1:3 and 1:4. Each of the mixtures was injected into the flowing stream of reagents under optimized conditions and absorbance measured.

Following the standard sampling plan, wheat grain samples were collected from Malam Jaba, a hilly area of Swat, Khyber Pakhtunkhwa, Pakistan. Glyphosate residues in the samples were determined following the proposed method. For recovery studies, grains samples (10 g each) in triplicates were taken in bottles followed by spiking with a known concentration of standard glyphosate solution. For efficient mixing the bottles were shaken manually and kept for 2 h. Extraction of the analyte glyphosate was done with 25 mL of NH_4OH (2.0 mol L^{-1}). The concentration of extracted herbicide was then evaluated by the proposed method using 1.0 mL of the extract in FI analysis. For residue analysis, optimized conditions of the proposed method were used following standard addition method.

3. Results and Discussion

3.1. Principle of the Method

The polar glyphosate molecule has a secondary amino group and a phosphonate group. It is proposed that the electron rich nitrogen atom of the glyphosate molecule attacks the electron deficient carboxylic carbon of the acid product formed because of Cannizaro's reaction of alkaline p-dimethylaminobenzaldehyde resulting in an unstable intermediate. Condensation takes place in acidic medium and a yellow product is formed which is stable for 5–10 min. The absorbance of the product was scanned at different wavelengths and maximum absorbance was observed at 405 nm (Fig. 1).[32–33]

The reaction was observed to be fast enough at room temperature that it can be adopted to the flow injection system for determination of glyphosate herbicide. Thus, the flow injection system was assembled. The effect of different physical and chemical parameters on the product formation was investigated. Maximum absorbance of the condensation adduct was measured at 405 nm.

3.2. Optimization of Experimental Variables

The effect of concentration of potential reagents such as DMAB, sodium hydroxide, and hydrochloric acid on the formation of the final product was investigated. The absorbance of the product was found to increase from 0.016 to 0.080 mol L^{-1} sodium hydroxide solution and steadily decrease at higher concentration up to 0.2 mol L^{-1}. At concentration beyond optimum, the higher pH of the medium causes the product of Cannizaro's reaction to be unstable and thus the yield of the final product decreases (Fig. 2).

For reaction of alkaline DMAB with glyphosate, the effect of concentration of DMAB was investigated in the concentration range of 0.004 to 0.024 mol L^{-1}. The reaction was observed to start with 0.004 mol L^{-1} solution with a maximum product formation at 0.016 mol L^{-1} of DMAB away from which the signal response steadily decreases. This observation can be attributed to the fact that at higher concentration, the DMAB molecules undergo intermolecular association and thus are unavailable to form the expected product (Fig. 3).

It was observed that acidic medium is required for maximum adduct formation. The concentration of hydrochloric acid was optimized in the range 0.012–0.048 mol L^{-1}. The product formation is maximum at 0.024 mol L^{-1}. Beyond this limit, the absorbance decreases with rise in acid concentration because at lower pH the product is dissociated (Fig. 3).

The effect of temperature on the reaction was investigated using batch analysis and it was found that increasing the temperature, above room temperature, results in dissociation of the adduct and no absorbance signal is observed. Therefore, room temperature was taken optimum for both batch and FI analysis. Physical variables like reaction coil length, flow rate and sample loop volume were also optimized. The effect of reaction coil length R1 was studied from 25–120 cm. Absorbance of the

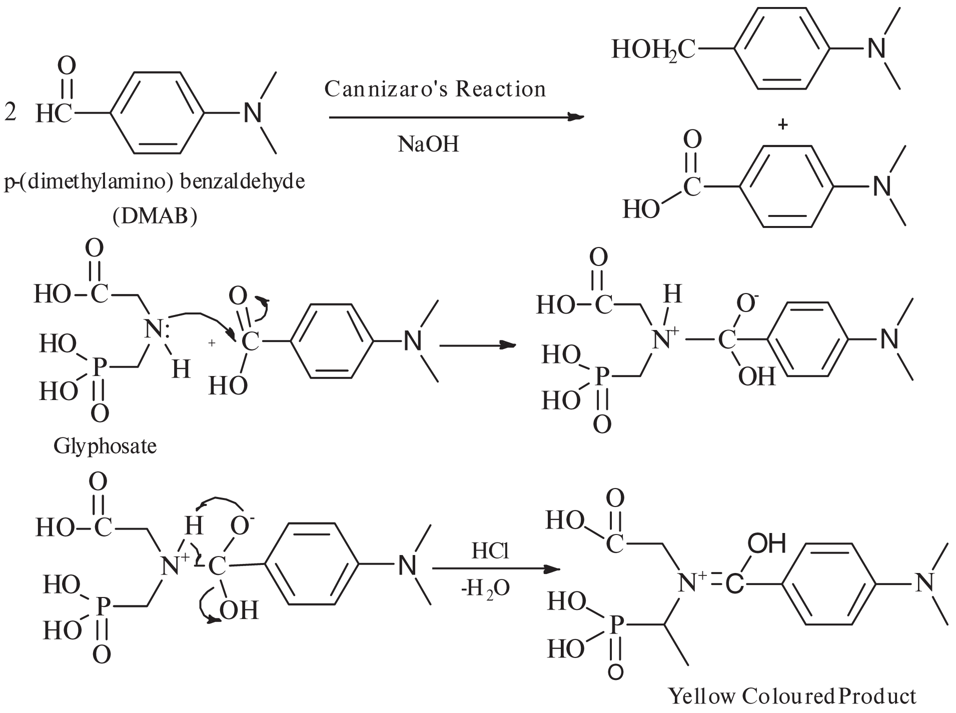


Figure 1 Proposed reaction mechanism for determination of glyphosate.

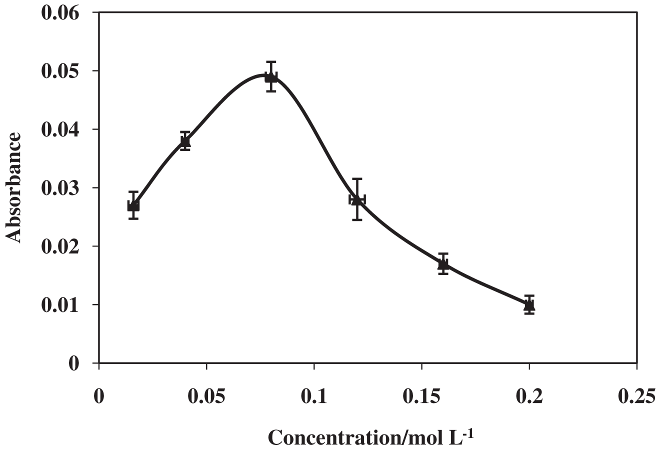


Figure 2 Effect of sodium hydroxide concentration on the reaction (n = 3).

product was found to increase with increase in coil length from 25–110 cm coil length beyond which it drops down. The mixing of the sample with NaOH requires a lengthy channel as it takes more time. In case of reaction coil R2, maximum product formation was observed with 35 cm reaction coil length beyond which a decrease in absorbance indicates the instability of the product in alkaline media. In order to investigate the optimum length of reaction coil R3, required for maximum product yield, the length of the reaction coil was varied and maximum absorbance was found with 60 cm coil length, which shows the optimum acidity limit. The result indicates that a condensation reaction is fast and reproducible in the FI system. (Fig. 4) The average signal response time after sample injection was found to be 30 seconds.

In order to study the effect of flow rate of the FI system on the reaction, flow rate was varied in the range of 0.7 to 5.0 mL min^{-1}. Increase in absorbance was observed with flow rate up to 1.5 mL min^{-1}. Above 1.5 mL min^{-1} absorbance was found to decrease, probably, due to inefficient product formation. A sampling rate of 60 samples h^{-1} was obtained with this flow rate (Fig. 5).

The sample injection volume was optimized in the range of 200–700 μL and maximum signal response was observed with 500 μL, which indicates efficient mixing of the analyte with the NaOH stream. At 700 μL sample volume two peaks near to each other were found to appear having lower intensity, which indicates that mixing with base occurs at the two ends of the sample stream (Fig. 6).

The sufficient time lapse (1 min) between two successive sample injections and the flow rate suggest that the carry-over effect can be estimated to be less than 5 % and usually, if the RSD of an analytical method is less than 10 %, the carryover will not have a substantial effect on the accuracy of the assay when the estimated carryover influence is less than 5 %.[34]

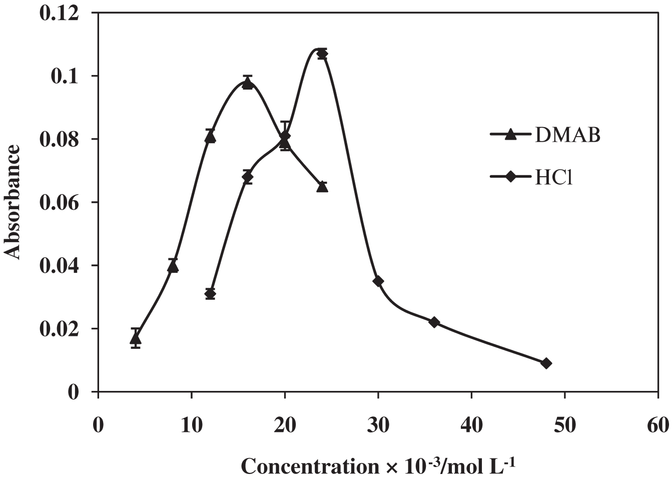


Figure 3 Effect of concentration of p-dimethylaminobenzaldehyde and hydrochloric acid on the reaction (n = 3).

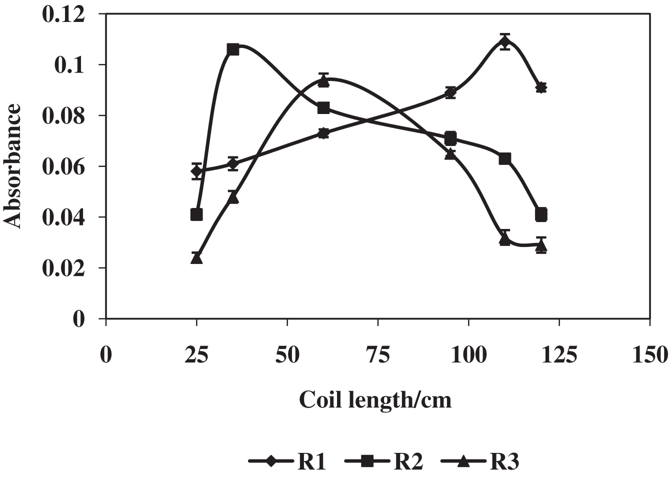


Figure 4 Effect of reaction coil length on reaction (n = 3).

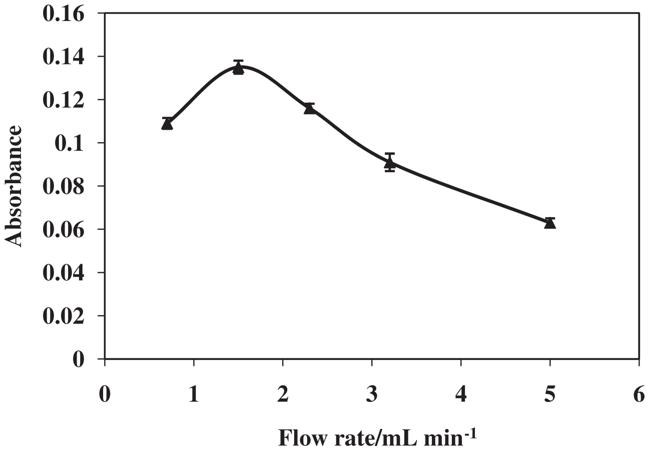


Figure 5 Effect of flow rate on reaction (n = 3).

3.3. Interferences Study

A variety of compounds like aminomethyl phosphonic acid (AMPA), the primary metabolite of glyphosate, urea and several pesticides like isoproturon (IPU), atrazine, metribuzin, terbutryn and methamidophos all having the same -N-H or

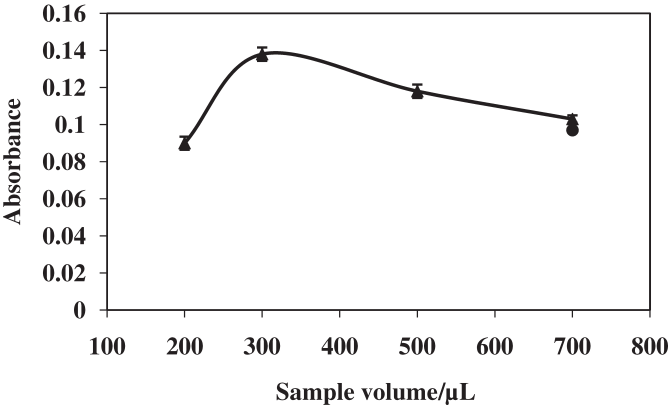


Figure 6 Effect of sample injection volume (n = 3).

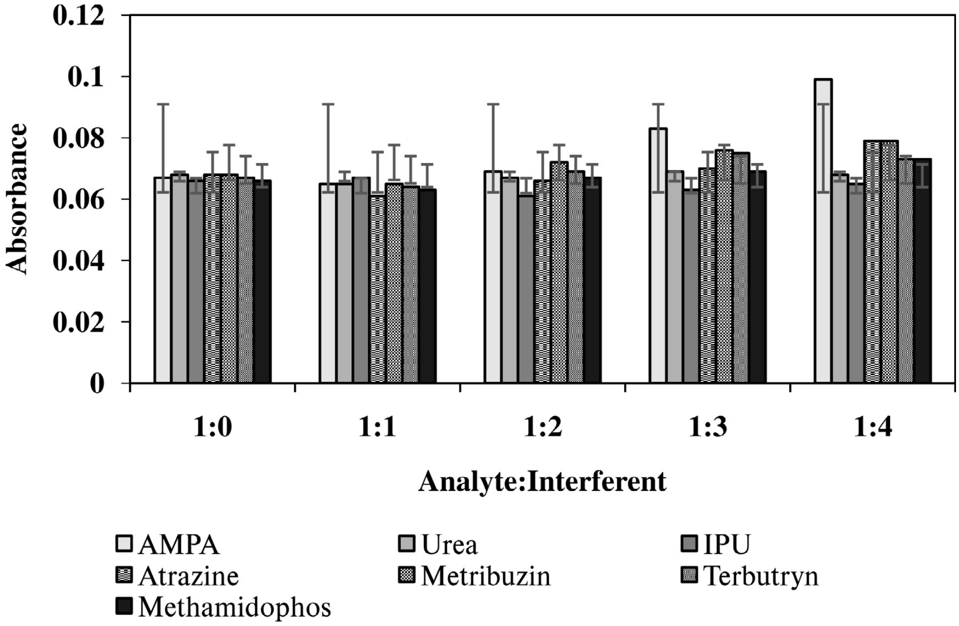


Figure 7 Effect of various possible interfering compounds on the reaction (n = 3).

-N-R functional groups were screened for possible interference. The pesticides tested are sprayed on the same field or on the nearby fields by the farmers in the same season. The effect of concentration of the interferents on absorbance was studied applying the same experimental conditions. The analyte to interferent ratio was varied from 1:0 to 1:4 (Fig. 7). It is evident from the figure that only AMPA has an enhancing effect on the absorbance signal of the analyte while the other compounds have no substantial interference effect. The basic reason for the interference effect of AMPA is similarity of its basic chemical structure and skeleton to that of glyphosate. Though AMPA is the principal degradation product of glyphosate and is assumed to be present with it everywhere but it is clear from Fig. 7 that it shows enhancing effect only at higher concentration of 3–4 times of glyphosate. Also, APMA is reported to be easily biodegraded in soil liberating CO_2. Similarly, glyphosate has been reported to have a high tendency to form complexes with the salt matrix in soil and is retained in a specific sampling area while AMPA is not affected by the salt matrix found in soil. Beside the above-mentioned facts, the interference effect of AMPA on determination of glyphosate cannot be underestimated because glyphosate is not the only source of AMPA and it can also be a metabolite of other aminophosphonate containing complexing agents in detergents.[35,36] Thus the presence of AMPA will affect determination of glyphosate in real environmental samples especially if it is present at high concentration.

Table 1 Optical characteristics of the proposed method

Parameter	Batch	FIA
Beer's law range (µg mL^{-1})	2–15	0.7–20
Molar absorptivity (ε) L mol^{-1} cm^{-1}	2.7×10^3	–
Standard deviation (S)	0.071	0.079
Relative standard deviation (RSD) %	6.02	5.74
Limit of detection (LOD) µg mL^{-1}	0.20 ± 0.06	0.20 ± 0.01
Limit of quantification (LOQ) µg mL^{-1}	0.70 ± 0.10	0.70 ± 0.02
Correlation coefficient	0.988	0.979
Slope	0.0178	0.0126
Intercept	0.0124	0.00
Stability constant (K_f)	4.4×10^5	–
Standard error (Sx-)	0.028	0.032

3.4. Optical and Analytical Characteristics

The optical and analytical characteristics of the proposal method is given in Table 1. The calibration curve was found to be linear in the concentration range of 2.0–15 µg mL^{-1} and 0.7–20 µg mL^{-1} for batch and FI methods, respectively. The molar absorptivity of the resulting complex, which indicates the sensitivity of the method, was found to be 2.7×10^{-3} L mol^{-1} cm^{-1} with batch method. The limit of detection (3S/b) and limit of quantification (10 S/b) was calculated at lower concentration that can be measured with acceptable precision and accuracy and were found to be 0.20 ± 0.06, 0.70 ± 0.10 µg mL^{-1}, and 0.20 ± 0.01, 0.70 ± 0.02 µg mL^{-1} for batch and FIA methods, respectively. The sensitivity of FI analysis is higher than that of batch analysis because in FIA the absorbance of the coloured product is measured just after formation while in case of batch method there is a time lapse between colour development and absorbance measurement. This time lapse involves dilution, transferring the solution to cuvette and adjusting the blank to zero in the instrument. The advantages of the developed FI method are high sample throughput, simplicity and low reagent waste.

3.5. Analysis of Samples

The recovery studies were carried out using spike and recovery method. Different concentrations of standard glyphosate herbicide (1–3 µg g^{-1}) were added to control samples. Glyphosate was extracted with NH_4OH solution following a reported procedure,[2] and analyzed by the proposed method. The recovery

Table 2 Application of the method for the determination of glyphosate in wheat grains.

Method of Analysis	Concentration added /µg g^{-1}	Concentration found /µg g^{-1}	% Recovery ± S.D.
Batch Analysis	1	0.88	88.00 ± 0.25
	2	1.85	92.50 ± 0.42
	3	2.77	92.33 ± 0.36
FI Analysis	1	0.94	94.00 ± 0.63
	2	1.899	94.95 ± 0.66
	3	2.69	89.66 ± 0.65

Table 3 Residue determination in wheat sample by the proposed method.

Total number of samples	No. of samples without residue	Sample LOQ /μg g^{-1}	No. of samples with residue	Method of analysis	Average residue ± S.D. /μg g^{-1}	MRL /μg g^{-1}
62	24	1.08	38	Batch Analysis	1.07 ± 0.022	10.0
				FI Analysis	1.13 ± 0.009	

Table 4 Statistical comparison of the proposed method with a reference method.

No.	Spiking concentration /μg g^{-1}	Concentration found/μg g^{-1}		Tests of significance
		Proposed method	Reference method	
1	5.00	4.65 ± 0.10	5.10 ± 0.09	*F*-test = 3.30 (19)
2	10.00	9.40 ± 0.12	9.60 ± 0.16	*t*-test = 2.1 (4.303)
3	15.00	13.50 ± 0.23	14.20 ± 0.24	

from wheat grains were in the range of 88.00 ± 0.25 % to 92.50 ± 0.42 % for batch method and 89.66 ± 0.65 % to 94.95 ± 0.66 % for FI analysis (Table 2).

Following the optimum physical and chemical conditions, the proposed method was used for residue analysis of wheat grains and the residue level was found to be 1.07 ± 0.22 μg g^{-1} for batch method and 1.13 ± 0.09 μg g^{-1} for FI method (Table 3).

3.6. Statistical Comparison of the Proposed Method with a Reference Method

The proposed method was used for assay of glyphosate in control wheat samples and the results were compared with that of a published method[2] through statistical analysis with respect to precision Student's *t*-test and accuracy using variance ratio *F*-test. Table 4 is a conclusive presentation of all the statistical treatment of the data from the two methods compared. The results of the two methods were plotted and the slope and intercept were found to be 1 and 0, respectively. The results obtained from each of the method show no significant difference regarding the precision and accuracy. It is clear from Table 4 that for 10 μg g^{-1} level, the difference is about 2 % which is minimum and thus it is the recommended spiking level for the proposed instrumental configuration.

4. Conclusions

A simple, fast and sensitive analytical method, based on flow injection analysis has been developed for determination of glyphosate herbicide. The method is based on formation of a yellow condensation adduct upon reaction of glyphosate and alkaline p-dimethylaminobenzaldehyde. The method has low detection limit and high sample throughput. The investigation of interference effect shows that among the compounds studied, only AMPA exhibits a signal-enhancing effect in higher concentration ratio with glyphosate and this is a limiting factor in the applicability of the method to real samples. The method was successfully applied for analysis of wheat grains and recoveries of glyphosate were found in the range of 89.66 ± 0.65 % to 94.95 ± 0.66 %. The proposed method can be used for analysis of samples obtained from areas where the herbicides are sprayed in a haphazard manner without following precautionary and prescribed measures. In comparison to gas chromatographic and liquid chromatographic methods the proposed method is simple and reproducible in terms of number of samples analyzed per unit time, reagents waste and interferences effect.

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