Direct Sensitive Spectrophotometric Determination of Glyphosate by Using Ninhydrin as a Chromogenic Reagent in Formulations and Environmental Water Samples

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A simple, sensitive, and rapid spectrophotometric method for the assay of glyphosate (=*N*-(phosphonomethyl)glycine) in various formulations (*Roundup*, *Excel Mera 71*) and environmental water samples is described. The method is based on the reaction of glyphosate with ninhydrin in presence of sodium molybdate in neutral aqueous medium at 100° to give a *Ruhemann*'s purple product having the VIS absorption maximum at 570 nm (*Scheme*, *Fig. 1*). The product is stable for 10 h. *Beer*'s law is obeyed in the concentration range of 0.1–3.5 μg ml^{-1}. The molar absorptivity and *Sandell*'s sensitivity are $3.2816 \cdot 10^4$ l mol^{-1} cm^{-1} and 5.2 ng cm^{-2}, respectively (*Table 1*). The method is highly reproducible, as confirmed by the relative-standard-deviation (r.s.d.) values (1.70%; *Table 2*). Recovery studies establish that this method is accurate (*Table 5*) and can be successfully employed for the determination of glyphosate.

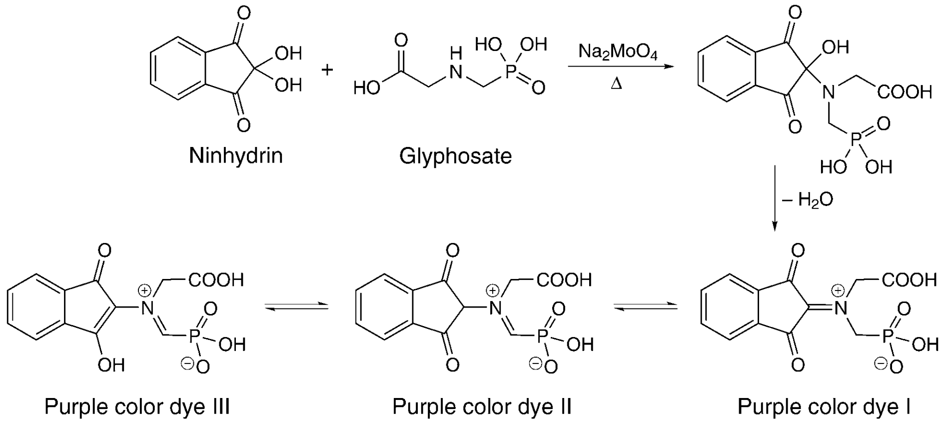
Introduction. – Glyphosate (*Roundup*) is one of the world's most popular nonselective effective herbicide used primarily for perennial weed control and killing weeds. It was discovered by Dr. *John E. Franz* of Monsanto in 1971 and released commercially in 1974. Starting in 1983, *Roundup* became the first pesticide with worldwide sales of over 1 billion dollars. It is known as a 'postemergence' herbicide, because it can not be used until the weed species has sprouted or emerged.

Glyphosate, an organophosphorus compound, is often used as the active ingredient in mixed herbicides. It is a phosphonoglycine that does not inhibit cholinesterase activity. Generally it is said to have very low toxicity, but several *in vivo* and *in vitro* studies on animals have revealed its teratogenic, mutagenic [1], carcinogenic effect [2], and its impact on environment and on aquatic life is becoming more pertinent day by day. Contamination of ground water is due to its high affinity towards water (solubility is 12 ppt at 250°). These features prompted us to find an effective analytical method for the quantification of glyphosate.

A literature survey reveals that so far, very few methods have been developed for the quantitative estimation of the presence of glyphosate, *viz*., gas-chromatography [3–5], GC/MS [6][7], liquid-chromatography [8], HPLC [9], electrophoresis [10] and current oscillopolarographic titration [11]. We now report the first direct, simple, rapid, and accurate spectrophotometric method for the determination of glyphosate residues thus meeting an ever increasing demand for the analysis of glyphosate in water samples. The proposed method involves the reaction of glyphosate with ninhydrin (=2,2-dihydroxy-1*H*-indene-1,3(2*H*)-dione) as a chromogenic reagent, in aqueous neutral medium and in the presence of sodium molybdate as a catalyst, yielding *Ruhemann*'s purple product.

Results and Discussions. – *Spectral Characteristics of the Colored Product.* A *Ruhemann*'s purple product with a maximum VIS absorption at 570 nm was formed when glyphosate was allowed to react with ninhydrin in the presence of sodium molybdate in aqueous neutral medium at 100° (*Scheme*). The colorless reagent blank had a practically negligible absorption at 570 nm. Absorption spectra of the colored product against the corresponding reagent blank are shown in *Fig. 1.*

Scheme. *Proposed Reaction Path of Glyphosate with Ninhydrin in the Presence of Sodium Molybdate*



Optimization and Reagent Concentration. Various concentration and volume ranges for all the reagents in H_2O were studied in detail. It was found that 5% (*w*/*v*) ninhydrin in the range 1.0–2.0 ml and 5% (*w*/*v*) sodium molybdate in the range 1.0–3.0 ml were necessary to obtain a stable *Ruhemann*'s purple product with maximum absorption at 570 nm. Hence, 1.0 ml of aqueous ninhydrin solution and 1.0 ml of aqueous sodium molybdate solution were selected to produce maximum color intensity. There was no appreciable change in the absorbance or color of the product if the order of addition of reactants was varied. Dilution of the reaction mixture was tried with EtOH, acetone, and H_2O where the latter gave the best result with maximum color intensity and stability.

Quantification. Beer's law is obeyed over the glyphosate concentration range of 0.1–3.5 μg ml^{-1}. A calibrated graph was constructed by using values obtained with solutions containing varying amounts of analyte and specified amounts of reagents, against colorless reagent blank. The limit of quantification (LOQ) was determined by taking the ratio of the standard deviation (σ) of the blank with respect to H_2O and the slope of the calibration curve (s) multiplied by a factor 10. The limit of detection (LOD) was determined by taking the ratio of the standard deviation (σ) of the blank with respect to H_2O and the slope of the calibration curve (s) multiplied by a factor 3.3. The upper limit of the *Beer-Lambert* range was determined by a series of concentrated solutions, and the lower limit of the *Beer-Lambert* range was determined by a

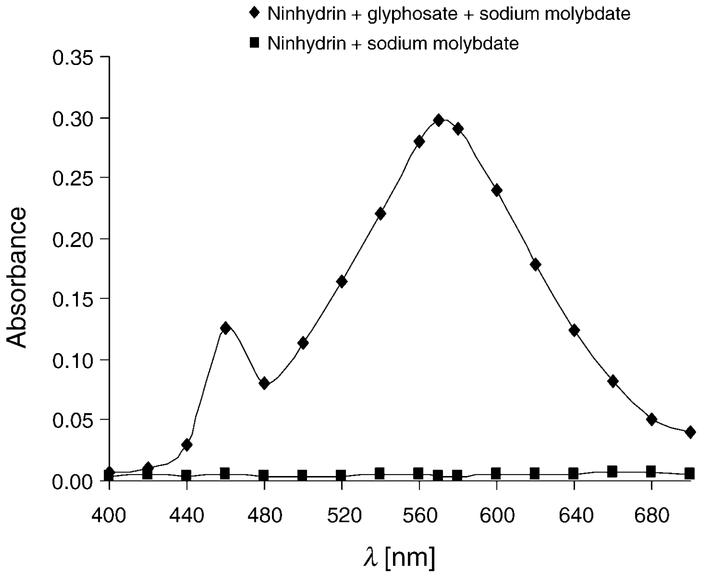


Fig. 1. *Absorption spectra of both reagent blank and reaction product of glyphosate and ninhydrin in the presence of sodium molybdate.* Final concentration of glyphosate = 1.75 μg ml^{-1}.

Table 1. *Optical Characteristics of Glyphosate in the Presence of Ninhydrin as Chromogenic Reagent and Sodium Molybdate as Catalyst*

Optical characteristics	Ninhydrin/molybdate method
Color	*Ruhemann*'s purple
λ_{max} [nm]	570
Stability [h]	10
Beer's-law range [μg ml^{-1}]	0.1–3.5
Molar absorptivity [l mol^{-1} cm^{-1}]	$3.2816 \cdot 10^4$
Sandell's sensitivity [ng cm^{-2}]	5.2
Limit of detection [μg ml^{-1}]	0.04
Limit of quantification [μg ml^{-1}]	0.11
Regression[a]) slope *b*	0.1976
Regression intercept *a*	0.0046
Correlation coefficient *r*	0.998
Relative standard deviation [%][b])	1.74

[a]) Regression curve: $y = ax + b$, where x is the concentration of glyphosate (in μg ml^{-1}) and y is the absorbance of the colored product. [b]) Mean value of seven determinations.

series of diluted solutions at the value of λ_{max}. Beyond this limit, the correlation results were affected. Hence, the measurements were excluded above and below these limits to keep the relationship linear. The *Beer*'s-law plot and analytical parameters are given in *Fig. 2* and *Table 1*, respectively.

Method Validation. Accuracy and Precision. The accuracy of our method was established by recovery studies of glyphosate at three levels (within the calibrated graph).

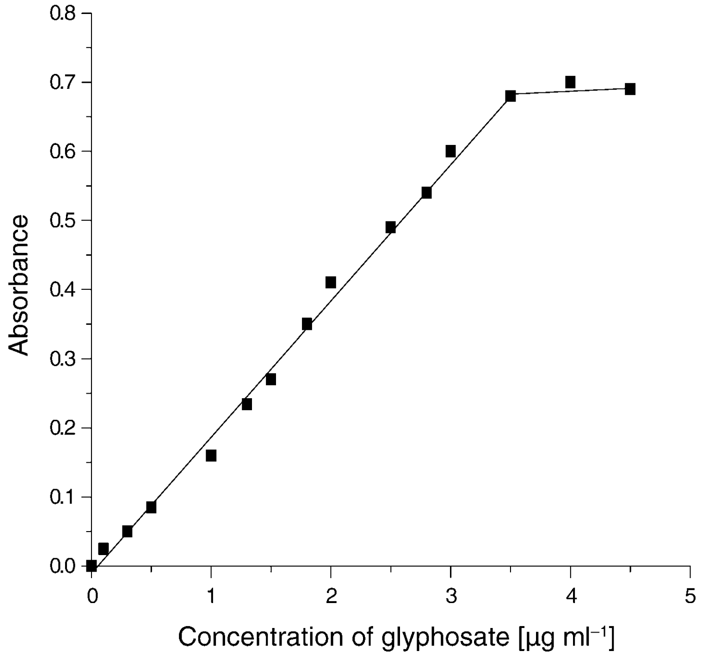


Fig. 2. Beer*'s-law plot of glyphosate with ninhydrin and sodium molybdate*

The precision was ascertained by calculating the relative standard deviation (r.s.d.) of seven replicate determinations at three levels. The range of error (%) and r.s.d. (%) values which were less than 2% are indicative of a good accuracy and precision of the method (*Table 2*).

Table 2. *Evaluation of Accuracy and Precision of the Glyphosate Quantification by the Proposed Method* (glyphosate concentration 10, 17.5, and 30.0 µg per 10 ml)

Glyphosate taken [µg ml^{-1}]	Glyphosate found [µg ml^{-1}][a])	Range [µg ml^{-1}]	Standard deviation [µg ml^{-1}][a])	Relative standard deviation (r.s.d.) [%][a])	Range of error [%]
1.0	1.005	0.062	0.020	1.944	1.942
1.75	1.736	0.057	0.020	1.175	1.174
3.0	3.029	0.164	0.060	1.983	1.982

[a]) Mean value of seven determinations.

Proposed Reaction Mechanism. The secondary-amine moiety of glyphosate reacts with ninhydrin in the presence of sodium molybdate as catalyst under elimination of a H_2O molecule to give products I and III forming *Ruhemann*'s purple [12] in aqueous neutral medium. The tentative mechanism is as shown in the *Scheme.*

Stability of the Colored Product. The purple product obtained from glyphosate by the proposed method was studied at different temperatures. The results indicate that the absorbance values of the color product remain constant in the temperature range of 20–40°. At higher temperature, the absorbance values decrease indicating the dissociation of the product. To obtain color at the initial stage after addition of all the reagents, it was mandatory to heat on a boiling water bath for *ca.* 3–5 min. After cooling to room temperature, the colored product can sustain a temperature up to 40°. *Ruhemann*'s purple product formed under the optimized conditions was stable up to 10 h at room temperature.

Effect of Foreign Species. The suitability of the method for the application to water samples was studied by studying the influence of various cations, anions, and other pesticide species in solutions containing 1.75 μg ml^{-1} glyphosate. The results are given in *Table 3.* Except for carbofuron and propoxur, other pesticides such as parathion ethyl, malathion, fenitrothion, carbaryl, 2,4-D, parathion methyl, phenol, *o*-cresol, and 4-nitrophenol did not interfere seriously. The tolerance limits given in *Table 3* are the concentrations of diverse species that cause ±2% error in the determination of glyphosate by our method.

The results also showed that a large number of metallic and nonmetallic ions did not interfere. However, $Fe^{2+}$and Cu^{2+} had strong interference which, when present up to 100 μg ml^{-1} could be masked by using 450 μg ml^{-1} of EDTA (= ethylenediaminetetraacetic acid = *N*,*N'*-ethane-1,2-diylbis[*N*-carboxymethylglycine]) solution.

Applications: Determination of Glyphosate in Commercial Formulations. The method was applied to determine the glyphosate content in different glyphosate formulations. Two different commercial formulations, *Roundup* 41% SL (soluble liquid) and ammonium salt of glyphosate 71% SG (soluble granule), were diluted with H_2O (within the *Beer*'s-law range 0.1–3.5 μg ml^{-1}) and analyzed by the recommended procedure. The results are represented in *Table 4.* Statistical data showed that this method can be effectively employed for the determination of glyphosate in various commercial formulations.

Recovery of Glyphosate from Enriched Water Samples. A known amount of glyphosate was added to different water samples, *viz.*, tap water, pond water, and river water. These spiked water samples were evaporated to dryness, and the residues obtained were dissolved in distilled H_2O to give a known volume. With these solutions, recovery of glyphosate was carried out by the recommended procedure. Data are shown in *Table 5.*

Determination of Glyphosate in Runoff Water. Runoff water from nearby different fields where glyphosate had been sprayed was collected in two different 100-ml bottles. These two samples were filtered and extracted separately with 100 ml (4 × 25) of CH_2Cl_2 [13]. The extracts were evaporated under reduced pressure on a water bath (*ca.* 50°), the residues dissolved in a 10-ml calibrated flask each, and the volume completed to 10 ml with H_2O. Suitable aliquots were analyzed by the proposed method. Data are shown in *Table 6.*

Conclusions. – From the literature, it is known that no direct spectrophotometric method for the quantitative estimation of glyphosate has been reported so far. We now propose for the first time *a sensitive and direct spectrophotometric method for the quantification of one of the top 10 herbicides* (glyphosate) *in the world.* This method is economically viable, simple, does not need an extraction procedure, and can efficiently be used for the analysis of the glyphosate in environmental water samples

Table 3. *Effect of Natural Water Contaminants and Other Pesticides on the Determination of Glyphosate Residues.* The concentration of glyphosate is 17.5 μg per 10 ml.

Foreign species	Tolerance limit [μg ml^{-1}] a)
Parathion, malathion, fenitrothion, carbaryl, 2,4-D, parathion methyl	2500
Phenol, *o*-cresol, 4-nitrophenol	1300
Na^+, K^+, Ca^{2+}, Al^{3+}, Mg^{2+}, Cl^-	700
Mn^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, EDTA tartarate, Cd^{2+}	450
PO_4^{3-}, SO_4^{2-}, NO_2^{2-}, CO_3^{2-}, citrate, I^-	300
Cu^{2+}, Fe^{2+} b)	100

a) Tolerance limit which caused up to ±2% error of recovery. b) Can be masked up to 100 μg ml^{-1} by the addition of 450 μg ml^{-1} of EDTA.

Table 4. *Determination of Glyphosate Active Ingredient in Commercial Formulations* (41% soluble liquid (SL) and 71% soluble granule (SG))

Roundup 41% SL (IPA salt)a)			*Excel Mera 71* b) (ammonium salt of 71% SG)		
amount added [μg ml^{-1}]	amount found [μg ml^{-1}]	recovery [%] (r.s.d.)c)	amount added [μg ml^{-1}]	amount found [μg ml^{-1}]	recovery [%] (r.s.d.)c)
1.0	0.93	93.00 ± 1.02	1.0	0.92	92.18 ± 1.44
1.5	1.41	93.89 ± 1.10	1.5	1.35	89.84 ± 0.75
2.0	1.81	90.56 ± 0.99	2.0	1.80	90.04 ± 0.98
2.5	2.31	92.35 ± 1.25	2.5	2.30	92.16 ± 1.24
3.0	2.81	93.55 ± 1.19	3.0	2.76	91.86 ± 1.38
3.5	3.27	93.46 ± 1.42	3.5	3.25	92.91 ± 1.11

a) Manufactured and marketed by *Monsanto India Ltd.*, Mumbai, India. b) Manufactured and marketed by *Excel Crop Care Ltd.*, Mumbai, India. c) Mean value of seven determinations.

Table 5. *Average Recovery of Glyphosate from Enriched Water Samples by Using Proposed Method*

Glyphosate added [μg ml^{-1}]	Tap watera)		Pond waterb)		River waterc)	
	glyphosate found [μg ml^{-1}]	recovery [%] (r.s.d.)d)	glyphosate found [μg ml^{-1}]	recovery [%] (r.s.d.)d)	glyphosate found [μg ml^{-1}]	recovery [%] (r.s.d.)d)
0.5	0.48	95.80 ± 0.06	0.47	94.21 ± 0.70	0.45	89.50 ± 0.06
1.25	1.13	90.50 ± 0.80	1.21	96.81 ± 0.30	1.16	92.56 ± 0.80
2.0	1.77	96.25 ± 0.1	1.86	93.19 ± 1.06	1.86	93.01 ± 0.10
2.5	2.24	92.30 ± 0.89	2.28	91.29 ± 1.45	2.34	93.43 ± 0.89
3.0	2.75	91.50 ± 0.72	2.89	96.49 ± 0.76	2.79	92.90 ± 0.59
3.5	3.23	92.40 ± 1.04	3.21	91.83 ± 0.91	3.27	93.56 ± 1.01

a) Collected (100 ml) from Manasagangotri, University of Mysore, India. b) Collected (100 ml) from Kukkarahalli, Mysore District, Karnataka, India. c) Collected (100 ml) from Kaveri River, Mandya District, Karnataka, India. d) Mean value of seven determinations.

Table 6. *Determination of Glyphosate Residual Level in Runoff Water*

	Glyphosate originally found by proposed method [µg/10ml]	Glyphosate added [µg/10ml]	Total glyphosate found [µg/10ml]	recovery [%] (r.s.d.)[a])
Field water[b])	8.1	10.0	17.7	96.10 ± 1.02
	8.6	17.5	25.4	95.80 ± 0.91
	8.5	25.0	35.0	96.27 ± 0.89
Field water[c])	11.5	10.0	21.1	96.00 ± 1.01
	11.0	17.5	27.5	94.56 ± 1.29
	10.9	25.0	34.8	95.61 ± 1.27

[a]) Mean value of seven determinations. [b]) Collected from Elawala irrigated land, Mysore District, Karnataka, India; sample size 100 ml. [c]) Collected from Hinkle irrigated land, Mysore District, Karnataka, India; sample size 100 ml.

and in commercial formulations. Even though alternative analytical techniques like HPLC or GC/MS exist, they are costly and may involve multiple steps. Our method can efficiently be performed even in an ordinary laboratory by using a simple spectrophotometric instrument and novel proposed chromogenic reagents.

Using ninhydrin as a chromogenic reagent for the determination of glyphosate is very suitable since it is H_2O-soluble, noncarcinogenic, and eco-friendly in nature. The purple product formed is highly stable at room temperature (10 h) which makes the method more practicable. The optical characteristics established that the results are appreciably reproducible and reliable which was confirmed by precision and accuracy results. Thus, the proposed method can efficiently be employed for the determination of glyphosate in different commercial formulations and environment water samples.

One of the authors (*B. B. L.*) thanks Mysore University for granting permission to carry this research.

Experimental Part

General. All the chemicals used were of AR grade. Solns. were prepared with double distilled H_2O throughout the experiment. Class-A glassware treated with KOH/EtOH were used to avoid the apparent binding of glyphosate to glass [14]. VIS Spectroscopy: *Systronics* Spectrophometer; model *106*, with 10-mm matched quartz cells.

Standard Glyphosate Solution. A stock soln. of 500 µg ml^{-1} of glyphosate (96% purity; *Ponali*, Bombay) was prepared by dissolving 50 mg in H_2O in a 100-ml volumetric flask and diluted to 100 ml with H_2O. From this stock soln., a 100 µg ml^{-1} soln. was prepared by appropriate dilution with H_2O.

Aqueous Ninhydrin Solution. A 5% (*w*/*v*) soln. was prepared by dissolving 5 g of ninhydrin (= 2,2-dihydroxy-1*H*-indene-1,3(2*H*)-dione; *Merck*, Germany) in H_2O in a 100-ml volumetric flask and diluting to 100 ml with H_2O.

Sodium Molybdate Solution. A 5% (*w*/*v*) soln. was prepared by dissolving 5 g of sodium molybdate (*Merck*, Germany) in H_2O in a 100-ml volumetric flask and diluting to 100 ml with H_2O.

Sample Preparations. a) A 100 μg ml^{-1} *Roundup* working soln. (glyphosate 41% soluble liquid; *Monsanto India Ltd.*, Mumbai, India) was prepared by dissolving 24.40 mg in H_2O in a 100-ml volumetric flask and diluting to 100 ml with H_2O. Suitable aliquots were analyzed for the estimation of glyphosate by the proposed method.

b) A 100 μg ml^{-1} *Excel Mera 71* working soln. (glyphosate 71% *w*/*w* soluble granule; *Excel Crop Care Ltd.*, Mumbai, India) was prepared by dissolving 14.08 mg in H_2O in a 100-ml volumetric flask and diluting to 100 ml with H_2O. Suitable aliquots were analyzed for the estimation of glyphosate by the proposed method.

c) Tap water, pond water, and river water were collected (100 ml each) from Manasagangotri, Kukkarahalli, and Kaveri River, resp. To these samples, known amounts of glyphosate lying within the *Beer*'s-law range (1–35 μg) were added. Recovery studies were then performed by the proposed method.

d) Two runoff-water samples (100 ml each) were collected in polyethene bottles from nearby fields (Elawala and Hinkle rural area, Mysore) where glyphosate was known to be used. These two samples were extracted with CH_2Cl_2 and used for the determination of glyphosate residue by the proposed method.

Recommended Procedure for the Preparation of the Calibration Curve. A soln. containing 1.0–35 μg (*Beer*'s-law range) of glyphosate was transferred into a series of 10-ml volumetric flasks to which 1.0 ml of 5% ninhydrin soln. and 1.0 ml of 5% sodium molybdate soln. were added. The mixture was kept on a boiling water bath for 3–5 min. The *Ruhemann*'s purple dye formed was cooled to r.t. and diluted with H_2O. The absorbance of the purple dye was measured at 570 nm against the corresponding reagent blank, and a calibration graph was constructed.

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Received June 8, 2006