Use of Buffers in Spectrophotometric Determination of *N*-Phosphonomethylglycine by the Ninhydrin Colour Reaction

[1]Lami A. Nnamonu*, [2]Nnadozie N. Nkpa

Centre for Agrochemical Technology, Department of Chemistry, University of Agriculture, Makurdi Nigeria.

Abstract: *The aim of this work was to develop a spectrophotometric method of glyphosate assay that is consistent and reproducible over a wide range of concentrations. The use of two buffer systems 4M CH₃COOLi pH 5.2/Hydrindantin/DMSO and 0.2M sodium citrate buffer pH 5.0/Ascorbic acid/DMSO which have not been used before for ninhydrin quantification of glyphosate is hereby reported. The absorbance of the resulting purple derivative was measured at 570 nm with molar absorptivity of 5.799×10^4 and 1.975×10^4 $L\ mol^{-1}\ cm^{-1}$. Linear relationships between concentration and absorbance were observed over Beer's law range from 0.3 to 4.5µg/ml. For the citrate system, $R^2 = 0.982$, SD = 0.3744, N= 9 and P < 0.0001; while $R^2 = 0.984$, SD = 1.0956, N= 8 and P < 0.0001 for the acetate system. The citrate buffer/ascorbic acid system was employed successfully in adsorption studies of glyphosate unto kaolin and starch. Limits of detection and quantification were 0.6159 and 0.699 µg/ml respectively for citrate buffer and 1.802 and 5.5465 µg/ml respectively for the lithium acetate buffer system. The method was applied to quantification of glyphosate adsorption to kaolin and starch, where the citrate buffer gave R^2 values of 0.803 (Freundlich) and 0.964 (Langmuir) for kaolin.*
Keywords: *Buffer, Glyphosate, Ninhydrin, Spectrophotometric determination*

I. Introduction

N-Phosphonomethyl glycine (glyphosate or NPMG) is the world's most popular broad-spectrum, non-selective, systemic herbicide used for post-emergence control of annual and perennial weeds. Its impact on the environment is becoming more significant by the day [1, 2]. The same properties that make it very effective also make simple methods for its determination and quantification, especially at residue levels, difficult to establish. Its polar nature and high water solubility make extraction difficult and restrict the options for using standard derivation techniques often employed for gas chromatographic (GC) analysis [3]. The absence of a chromophore or fluorophore in NPMG also necessitates derivation techniques for the determination of glyphosate residues by liquid chromatography. The use of chromatographic methods and emerging techniques in glyphosate analysis has been reviewed [4]. These methods involve the use of sophisticated and expensive equipment, requiring lengthy clean up procedures, giving less than ideal recoveries. In spite of numerous published methods, it has been noted that the analysis of glyphosate at residue levels has tested the patience of many experienced analysts [5]. The increasing load of literature on methods of analysis of glyphosate is indicative that a robust, reliable method is still being sought.

A method of analysis that can be used in simple laboratories, the type in developing countries, is the spectrophotometric method. The reaction of glyphosate with carbon disulphide to convert the amine group into dithiocarbamic acid has been used as basis of a spectrophotometric determination of glyphosate [6]. The dithiocarbamate group was used as chelating group for reaction with transition metal ion, Cu (II). The resultant yellow complex was measured at 435 nm. Another UV-VIS spectrophotometric method of glyphosate analysis based on the transformation of amino group of glyphosate in aqueous acetonitrile to the corresponding dithiocarbamate derivative has been reported [7]. The derivative is reacted with copper (I) perchlorate to form a yellowish green colored complex with absorbance measured at 392 nm. Spectrophotometric determination of glyphosate using ninhydrin has been done in neutral aqueous medium in the presence of sodium molybdate at 100°C to give a Ruhemann's purple adduct [2]. However, at the concentrations investigated in this work, the Ruhemann's purple adduct formed decomposed (as evidenced by fading of the rich purple colour) on standing for a few minutes at 18°C while measurements were going on, thereby giving inconsistent results. We wanted therefore, to make the ninhydrin assay reproducible over a wide range of concentrations.

The similarity of NPMG and its principal metabolite, aminomethyl phosphonic acid (AMPA) to naturally occurring amino acids and amino sugars contributes to the difficulty in determining residues of these compounds in crops and animal products. In this paper, this similarity is exploited and the buffers used in amino acid analysis are employed in the spectrophotometric determination of glyphosate. Buffers were utilized because it was reported that buffers and the presence of reduced ninhydrin are essential for the performance of reproducible and sensitive assays of amino acids [8, 9] The use of two buffers 4M CH₃COOLi pH 5.2/Hydrindantin/DMSO and 0.2M sodium citrate buffer pH 5.0/Ascorbic acid/DMSO in the reaction of ninhydrin with glyphosate was explored and the Ruhemann's purple product obtained (stabilized by the buffers)

was isolated and confirmed by ^{1}H-NMR. This method was used in quantifying glyphosate adsorbed to kaolin and starch.

II.   Materials and methods

II.1 Materials

N-phosphonomethyl glycine 96%, ninhydrin, lithium hydroxide monohydrate, anhydrous citric acid, sodium citrate dihydrate, hydrindantin dehydrate, corn starch and kaolin were obtained from Sigma Aldrich, UK and used as received. All reagents were of analytical grade purity. Absorbance at 570 nm was measured on a Cary 50 Bio PCB 150 Water Peltier system UV-visible spectrophotometer. Plastic vials were used for the experiments to obviate the binding of glyphosate to glass. All solutions were prepared in high purity water that was obtained from a Milli-Q water system (Millipore, Billerica, MA, USA). ^{1}H-NMR was taken on a Bruker 400. FTIR was run on Perkin Elmer Spectrum 100 spectrophotometer (PIKE Technologies 16077).

II.2 Buffers
II.2.1 Lithium acetate buffer.

 M lithium acetate buffer pH 5.2 was prepared with 168 g (4 mol) lithium hydroxide monohydrate and 293 ml glacial acetic acid [10]. The alkali was weighed in the fume hood (to avoid breathing in the dust), poured into 200 ml high purity water in a 500 ml conical flask and placed on a vortex mixer. When it was about half dissolved, the acetic acid was added. After vigorous effervescence subsided and the solution cooled to about $25^{o}C$, it was poured into a 1000ml volumetric flask, properly rinsed in and water added close to the mark. The flask was corked, filtered through a sintered glass funnel and the pH taken. When pH was lower than 5.2, it was adjusted with $LiOH.H_2O$ (1g gave a 0.01 rise in pH units). When it was higher than 5.2, pH was adjusted by adding glacial acetic acid (1 ml acid equal to a 0.01 rise in pH unit). The solution was made to the mark.

II.2.2 *Citrate buffer*.

 0.2 M citrate buffer pH 5.5 was prepared by sonicating 3.412g (17.76 mmol) anhydrous citric acid and 0.6588 g (2.24 mmol) sodium citrate dihydrate, $C_6H_5O_7Na_3 .2H_2O$, in 60 ml high purity milli-Q water in a 100ml volumetric flask. 2.0 cm^3 of 12.5 M NaOH was added and the solution made up to the mark [11].

II.3 Ninhydrin reagents
II.3.1 Ninhydrin Reagent I.

This reagent solution was prepared by adding 1.6 g (8.99 mmol) ninhydrin with vigorous stirring to 60 ml DMSO in a 250 ml round bottom flask. 0.24 g (0.745 mmol) hydrindantin was added into the deep vortex created by the stirrer (for fast dissolution) and stirring continued for 5 minutes till it was completely dissolved. 20 ml lithium acetate buffer pH 5.2 was then added and stirred for another 3 minutes [9, 10]. The golden-yellow mixture was poured into an amber-coloured bottle and stored under nitrogen in the refrigerator.

II.3.2 Ninhydrin Reagent II.

This reagent was prepared by a modification of the method of Yokoyama and Hiramatsu [12] that used ascorbic acid/ninhydrin/methyl cellosolve in the determination of glutamic acid. In this work, methyl cellosolve was replaced by DMSO as advocated by Moore [10]. Into 30 ml of DMSO in a 100 ml conical flask was added 0.25 g (1.4 mmol) ninhydrin with stirring. 7.5 mg (0.043 mmol) ascorbic acid was then added and stirring continued for another 3 minutes to ensure complete dissolution. The light–yellow reagent was corked and stored in an amber-coloured bottle under nitrogen in the refrigerator.

II.4 Stock solutions

20 mg *N*-phosphonomethyl glycine was weighed and sonicated in 20 ml water to give a 1000 μg/ml $(5.917x10^{-3}M)$ stock solution. 1ml of this solution was diluted to volume in a 100ml volumetric flask, to give a 10 μg/ml solution of glyphosate. Working calibration solutions of 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5 and 0.3 μg/ml were made by serially diluting this solution. The concentration of glyphosate in these solutions was determined using ninhydrin in the proposed buffer systems and calibration curves plotted.

II.5 Derivatization with ninhydrin reagents

Into 2 ml glyphosate sample solution in plastic screw cap bottles was added 1ml of the ninhydrin reagent I. To use ninhydrin reagent II, 2ml of the reagent was added to 1ml of glyphosate sample and 2 ml citrate buffer pH 5.5. The vials were immersed to a depth of about 2 inches in a boiling water bath for 30 mins. The rate of heat applied to the bath was sufficient to bring the bath back up to 99 - 100°C within two minutes after insertion of a full rack of 20 vials. The vials containing the Ruhemann's purple derivative were then cooled rapidly under running water and the absorbance measured at 570 nm.
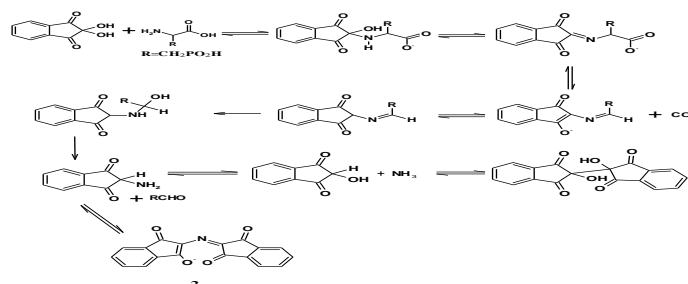
II.6 Adsorption to starch and kaolin

Adsorption experiments were done using the batch equilibration method [13]. 2 ml water was added into 250 mg sorbent (i.e. starch or kaolin) samples in capped plastic vials. 5 ml of standard herbicide solutions with concentrations ranging from 0.05 mmol to 0.7 mmol were added. These were equilibrated by shaking on a mechanical shaker (250 rpm, shaking amplitude 12.5 mm, orbital) for 24 hrs at room temperature (18°C). After equilibration, the suspensions were centrifuged (4500 rpm for 10 mins) and 3ml of the supernatant was drawn for analysis of glyphosate, using the proposed buffers.

III. Results and discussion

III.1 Mechanism

The reaction of ninhydrin with glyphosate is likely similar to that of the alpha-amino acids [14], where nucleophilic type displacement of an OH group of ninhydrin by the amine group of glyphosate takes place. This is followed by decarboxylation, hydrolysis (to eliminate phosphoric acid) and the addition of a second ninhydrin molecule to form the Ruhemann's purple adduct (2). The proposed mechanism is shown in Scheme 1:

Scheme 1 Mechanism of reaction of Ninhydrin and *N*-Phosphonomethylglycine

^{1}H-NMR of the isolated ninhydrin-glyphosate adduct showed one peak at δ7.65. Structure 2 as written should give two peaks (δ7.65 and δ8.00) of equal intensity due to the aromatic protons of the two non-equivalent indanetrione rings. However, resonance stabilization of the negative charge of (2) renders the two benzene rings equivalent, thereby resulting in the absence of the second peak expected at δ8.0, similar to previously reported observation [15]. IR (cm^{-1}): 3445, 3094, 1702, 1150-1180.

III.2. Analysis

Using the buffers at optimum conditions, a linear dependence of absorbance on concentration was observed over Beer's law range from 0.3 to 4.5 μg/ml with a molar absorptivity of 5.799×10^4 L mol^{-1} cm^{-1} for the citrate and 1.975×10^4 L mol^{-1} cm^{-1} for the acetate buffer system. The calibration curves are presented in Figs. 1 and 2.

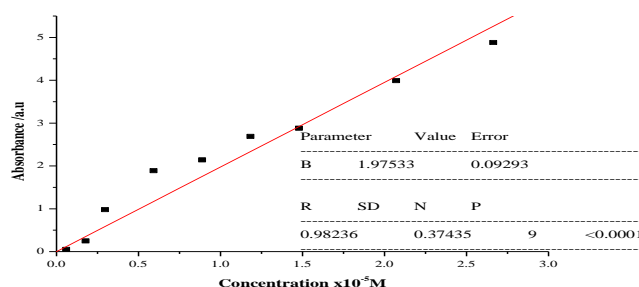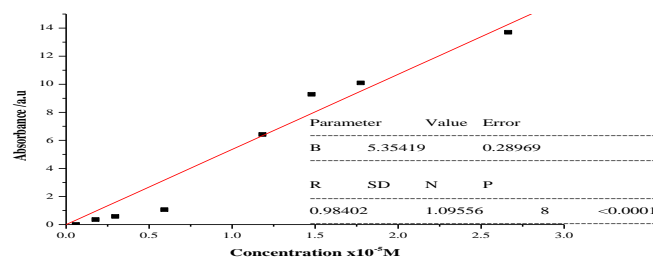Fig 1 Acetate Buffer Calibration Curve

Fig 2 Citrate Buffer Calibration Curve

The limit of detection (LOD) was estimated by dividing the relative standard deviation (RSD) of the blank with respect to water by the slope of the calibration curve (m) and multiplying by a factor of 3.0. The limit of quantification (LOQ) was calculated by dividing the standard deviation (RSD) of the blank with respect to water by the slope of the calibration curve (m) and multiplying by 10. The limit of detection, limit of quantification, correlation coefficient (R), RSD and slope were calculated and summarized in Table 1.

Table 1 Quantification Parameters

Parameter	Values	
	CH_3COOLi buffer system	Citrate buffer system
Molar absorptivity (ε) L mol^{-1} cm^{-1}	1.975×10^4	5.799×10^4
RSD	0.3744	1.0956
Correlation Coefficient (R)	0.982	0.984
Slope	1.9753	5.799
Limit of Detection (μg/ml)	1.802	0.6159
Limit of Quantification (μg/ml)	5.5465	0.699

It is noteworthy that the citrate buffer /ascorbic acid system gave higher R^2 values in addition to the fact that it is economically more viable, similar to earlier reported results [12]. The cost of hydrindantin is about 100 times higher than that of ascorbic acid. Also, about $10 - 20$ times more hydrindantin than ascorbic acid is required to prepare the same volume of ninhydrin reagent.

II.3. Application

The method of buffers was applied in quantification of glyphosate in adsorption studies of glyphosate unto corn starch and kaolin. Aliquots of the extract were analysed by the proposed method giving stable Ruhemann's purple adduct at the ambient temperature.

Glyphosate adsorption isotherm data have been obtained using the batch technique, where various concentrations of herbicide solution were added directly to aqueous suspensions of the adsorbent. After equilibration, the concentration of pesticide remaining in solution was determined in the centrifuged supernatant.

The amount of NPMG adsorbed, q_e (mg/g) was calculated on the basis of a mass balance principle according to Eq.1:

$$q_e = (C_o - C_e) * V / w \qquad \qquad ...1$$

where C_o and C_e are initial and equilibrium concentrations (mg/L) of herbicide in the aqueous phase, V is the aliquot volume (L) of aqueous solution taken and w is the mass of adsorbent (g).

The Langmuir isotherm is mathematically expressed as follows:

$$1/q_e = 1/ K_L.b \, (1/C_e) + 1/K_L \qquad \qquad ...2$$

Where K_L is the maximum adsorption at the monolayer (mg g^{-1}), C_e is the equilibrium amount of glyphosate (mg/L) in the aqueous phase, q_e is the amount of herbicide adsorbed per unit mass of adsorbent , b is the Langmuir constant related to the affinity of binding sites (mg g^{-1}) for the sorbate molecules, and is a measure of the energy of adsorption.

A plot of $1/q_e$ against $1/C_e$ was used to calculate the Langmuir constants K_L and b.

The widely used empirical Freundlich equation based on sorption on a heterogeneous surface is given by:

$$\log q_e = \log K_F + (1/n)\log C_e \qquad \qquad ...3$$

where K_F and n are Freundlich constants indicating sorption capacity (mg g^{-1}) and intensity respectively. K_F is a measure of the amount of pesticide sorbed for an equilibrium concentration of 1 mg/L. The constant, n, is a measure of the intensity of adsorption and reflects the degree to which adsorption is a function of the concentration. K_F and n_F were determined (using the least squares method) from a linear plot of $\log q_e$ against $\log C_e$.

The n_F value can be considered as an index of site energy distribution (i.e., the higher the n_F values, the less heterogeneous the sorption sites) [16]. Also $1 \le n_F \le 10$ has been interpreted as indicative of favourable adsorption [17].

The regression coefficient, the calculated Langmuir constants K_L and b, and the Freundlich constants K_F and n_F, are shown in Table 2.

Table 2 Derived Langmuir and Freundlich Isotherm Constants

Adsorbent	Langmuir			Freundlich		
	K_L	b	R^2	K_F	n	R^2
Kaolin	6.711	0.160	0.964	2.355	2.132	0.803
Starch	0.837	2.609	0.722	1.905	2.882	0.591

IV.   Conclusion

A spectrophotometric method of determination of *N*-phosphonomethyl glycine using ninhydrin, where the Ruhemann purple adduct is stable enough to allow measurements over a wide range of concentrations has been developed. The method, which uses two buffer systems (lithium acetate and sodium citrate) was applied to quantifiaction of the herbicide adsorbed unto kaolin and starch giving good results. It could be extended to detection and quantification of glyphosate in environmental samples.

V. Acknowledgement

References

[1]   T. V. Nedelkoska, G. K. C. Low, High-Performance Liquid Chromatographic determination of glyphosate in water and plant material after pre-column derivatization with 9-fluorenylmethyl chloroformate, *Anal. Chim. Acta., 511, 2004, 145–153.*

[2]   B. L. Bhaskara, P. Nagaraja, Direct sensitive spectrophotometric determination of glyphosate by using ninhydrin as a chromogenic reagent in formulations and environmental water samples, *Helvetica Chimica Acta, 8(11), 2006, 2686–269.*

[3]   P. L. Alferness, Y. Iwata, Determination of glyphosate and aminomethyl phosphonic acid in soil, plant and animal matrices and water by Capillary Glass Chromatography with mass-selective detection *J. Agric. Food Chem. 42, 1994, 2751-2759.*

[4]   C. D. Stalikas, C. N. Konidari, Analytical methods to determine phosphonic and amino acid group-containing pesticides, *J. Chromatography, 907, 2001, 1-19.*

[5]   FAO/WHO, Food and Agriculture Organization of the United Nations, Joint FAO/WHO Meeting on Pesticide Residues (FAO Plant Production and Protection Paper 78). *Pesticide residues in food — 1986, Evaluations — 1986, Part I — Residues.* 1986, Rome.

[6]   M. R. Jan, J. Shah, M. Mohammed and B. Ara, Glyphosate herbicide residue determination in samples of environmental importance using spectrophotometric method, *Journal of Hazardous Materials 169, 2009, 742–745.*

[7]   D. K. Sharma, R. Kashyap and N. Kumar, Spectroscopic method for determination of glyphosate in relation to its environmental and toxicological, *Arch. Environ. Sc. 6, 2012, 42-49.*

[8]   A. C. Beckwith, J. W. Paulis and S. Wall, Direct estimation of lysine in corn meals by the ninhydrin color reaction, *J. Agric. Food Chem., 23 (2), 1975, 194-196.*

[9]   S. Moore and W. H. Stein, A modified ninhydrin reagent for photometric determination of amino acids and related compounds, *J. Biol. Chem., 211, 1954, 907.*

[10]   S. Moore, Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. *J. Biol. Chem., 243, 1968, 6281-6283.*

[11]   S. Moore and W. H. Stein, Photometric ninhydrin method for use in chromatography of amino acids, *J. Biol. Chem., 176, 1948, 367-388.*

[12]   S. Yokohama, J-I, Hiramatsu, A modified ninhydrin reagent using ascorbic acid instead of KCN, *J. of Bioscience & Engineering, 95(2), 2003, 204-205.*

[13]   M. Cruz-Guzmn, R. Celis, M. C. Hermosin, W. C. Koskinen and J. Cornejo Adsorption of pesticides from water by functionalized organobentonites, *J. Agric. Food Chem., 53 (19), 2005, 7502.*

[14]   ] M. Friedman, Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences, *J. Agric. Food Chem., 52 (3), 2004, 385-406.*

[15]   D. C. Wigfield, G. W. Buchanan and S. Croteau, On Ruhemann's purple, *58(3), 1980, 201-205.*

[16]   B. Xing, B. Sorption of naphthalene and phenanthrene by non-amended soils and humic acids *J. Environ. Qual.* 26:1264–1270 *Environ. Pollut.* 111, 2001, 303–309.

[17]   F. E. Okieimen, F. I. Ojokoh and R. A. Wuana, Preparation and evaluation of activated carbon from rice husk and rubber seed shell *ChemClass J., 2004, 191-196.*