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A simplified approach to the determination of *N*-nitroso glyphosate in technical glyphosate using HPLC with post-derivatization and colorimetric detection

Manuela Kim, Jorge Stripeikis, Fernando Iñón, Mabel Tudino *

Laboratorio de Análisis de Trazas, Departamento de Química Inorgánica, Analítica y Química Física, INQUIMAE, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina

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Abstract

A simple and sensitive HPLC post-derivatization method with colorimetric detection has been developed for the determination of *N*-nitroso glyphosate in samples of technical glyphosate. Separation of the analyte was accomplished using an anionic exchange resin (2.50 mm × 4.00 mm i.d., 15 μm particle size, functional group: quaternary ammonium salt) with Na_2SO_4 0.0075 M (pH 11.5) (flow rate: 1.0 mL min^{-1}) as mobile phase. After separation, the eluate was derivatized with a colorimetric reagent containing sulfanilamide 0.3% (w/v), [*N*-(1-naphtil)ethilendiamine] 0.03% (w/v) and HCl 4.5 M in a thermostatized bath at 95 °C. Detection was performed at 546 nm. All stages of the analytical procedure were optimized taking into account the concept of analytical minimalism: less operation times and costs; lower sample, reagents and energy consumption and minimal waste. The limit of detection ($k = 3$) calculated for 10 blank replicates was 0.04 mg L^{-1} (0.8 mg kg^{-1}) in the solid sample which is lower than the maximum tolerable accepted by the Food and Agriculture Organization of the United Nations.

Keywords: *N*-nitrosoglyphosate; Glyphosate; HPLC with post-derivatization

1. Introduction

Glyphosate *N*-(phosphonomethyl)glycine is one of the most common herbicides used principally for the elimination of parasites plants of alimentary products such as corn, soya, citrus, potatoes, coffee, peanuts, pineapple, etc. [1].

Every pesticide formulation is a toxic mixture of active ingredients, by-products of the method of synthesis and a great variety of additives such as solvents, surfactants, etc. Even though, there are regulations for the manufacturing and commercialization, producers are not obliged to list the inert ingredients as these ingredients are considered secrets of the formulation. However, there are maximum tolerable values for certain impurities derived from the method of synthesis that need to be accomplished at the moment of commercializing the product.

Amongst the impurities of commercial glyphosate, *N*-nitrosoglyphosate (NNG) is of great concern due to its toxicity. Since 2000 [2,3] the maximum concentration of NNG accepted for the Food and Agricultural Organization of the United Nations (FAO) is 1 mg kg^{-1}, 10 times lower than that established during the 1980s. However, the accepted methodology for NNG determination – proposed by Monsanto Agricultural Company in 1986 [4] – has not been modified or improved in order to easily reach the new limit of quantification imposed by regulations.

The Monsanto method consists of the dissolution of the solid sample in a solution of NaOH and its injection in a high performance liquid chromatographer (HPLC) packed with an anionic exchange resin. Elution is performed with monobasic ammonium phosphate as mobile phase. Thus, NNG is separated from the other compounds present in the dissolved sample. After separation, the eluate reacts with a strong acid in order to obtain NNG desnitrosation. The nitrosil ion generated reacts with [*N*-(1-naphtil)ethilendiamine] (NED) and sulfanilamide (SD) giving an azo red compound which absorption is measured spectrophotometrically [4].

The objections to the methodology described above are mainly related to the instrumentation and the operative conditions of the post-column derivatization. As a matter of fact, the

* Corresponding author. Tel.: +54 1145763360; fax: +54 1145763341.
E-mail address: tudino@qi.fcen.uba.ar (M. Tudino).

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performance of the method barely reaches the limit of detection (LOD) imposed by FAO in 2000. This can be easily viewed considering that, even in absence of dilution and/or physical dispersion in the HPLC column and the detector, the absorbance values measured in the liquid extract can be hardly discriminated from the instrumental noise (absorbance value about 0.002 units).

In the present work, the operative variables involving NNG desnitrosation followed by colorimetric quantification using Griess reagent were carefully studied. The optimized system was then on-line coupled to the HPLC as a module of spectrophotometric detection. A LOD of 0.8 mg kg^{-1} of NNG was obtained, which is lower than the maximum tolerable level accepted by regulations [2].

2. Experimental

2.1. Chemicals

- *N*-nitrosoglyphosate aniline salt 97% (w/v) (EPP Ltd. Pentlands Science Park, Midloth, UK). Double deionised water (DIW) obtained with a MilliQ system (Millipore Corp., Bedford, MA, USA). Sulfanilamide (SD), NED, sulfamic acid, fuming HCl 37% (w/w), H_3PO_4 85% (w/w), HBr 38% (w/w), Na_2SO_4 anhydrous, H_2SO_4 98% (w/w), buffer pH 4.00 ± 0.02 (20 °C), all of analytical and purchased from Merck KgaA. NaOH (s) and buffer pH 7.00 ± 0.02 (20 °C) of analytical grade (Anedra, Buenos Aires, Argentina).
- Stock standard solutions of 1.000 g L^{-1} of NNG and diluted standards were prepared with DIW. The colorimetric reagent (CR) was prepared by mixing a solution of SD 5% (w/v) in HCl 20% (w/v) and NED 0.5% (w/v) in DIW with concentrated HCl (15:15:70). A solution of sulfamic acid 1% (w/v) was employed for cleaning up all the glassware.

2.2. Preparation of technical glyphosate solid samples

A well known amount of the solid sample (approximately 2.5 g) was extracted in 20 mL of sulfuric acid 0.1 M with vigorous and constant agitation on a rotatory plate for 10 min with a frequency of 250 min^{-1}. The supernatant solution was transferred onto a round bottom flask of 25 mL. The remanent solid was filtered through a 0.22 μm membrane. Then, it was washed with no more than 2 mL of sulfuric acid 0.1 mol L^{-1} and the solution was brought up to volume with DIW.

2.3. Preparation of solutions for the standard addition curve

Different amounts of the NNG standard solution were added to 2.5 g of the different samples. Afterwards, they were treated as described above. The final concentration of NNG added to the samples ranged between 0.1 and 1.0 mg L^{-1} in the final solution.

2.4. Apparatus

Water bath with thermostatizer (Haake E8 Fisons), UV–vis HP 8453 Diode Array Spectrophotometer, Thermostatized cell for Diode Array HP 8453, UV–vis Shimadzu Model SPD-10AVvp Detector, equiped with a flow cell of 8 μL, optical path 10 mm, Shimadzu Chromatopac C-R6A Registrator, Wheaton Void filtration equipment, with filter membranes of 0.22 μm, Shaker with rotatory plate Edmund Buhler, Model K-M2, Teflon tubing of 0.38 mm i.d. and 8 m length, ice bath, Microprocessor pH meter BT-500 (Boeco, Germany). Programmable High Pressure Bomb Waters Model 590, Programmable High Pressure Bomb Thermo Separation Products, Spectrosystem P2000, Injection Valve Rheodyne, Analytical Column Dionex Model AS4A, 4 mm × 250 mm, 15 μm of particle size, functional group: quaternary ammonium salt-, Column Guard Dionex AG4A, 4 mm × 50 mm, 15 μm of particle size, functional group: quaternary ammonium salt.

2.5. Procedure

The HPLC was coupled to the optimized post-derivatization system shown in Fig. 1. A sample volume of 250 μL was injected through the Rheodyne valve into the HPLC equipped with the

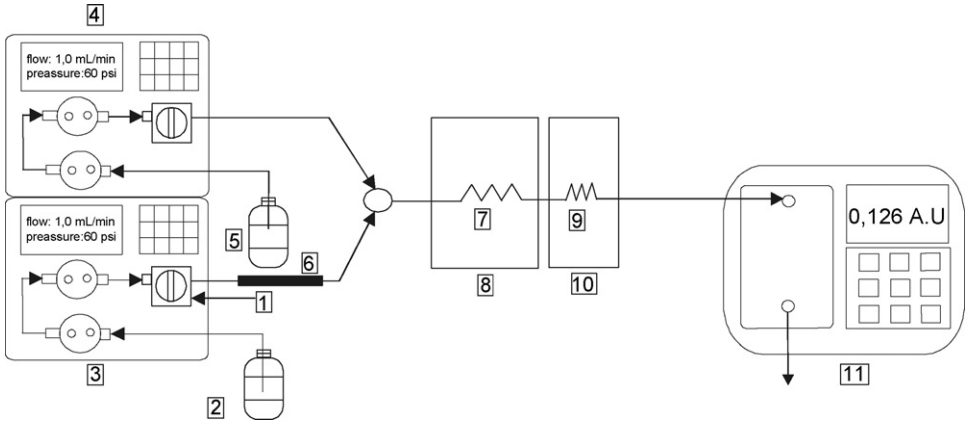

Fig. 1. (1) Sample introduction; (2) carrier solution: Na_2SO_4 0.0075 mol L^{-1} (pH 11.5), flow rate: 1.0 mL min^{-1}; (3, 4) HPLC bombs; (5) CR solution, [HCl]: 4.5 mol L^{-1}, [NED]: 0.03% (w/v), [Sulfanilamide]: 0.3% (w/v), flow rate: 1.1 mL min^{-1}; (6) anionic exchange column; (7) heating/reaction coil, length: 8 m; (8) heating bath, temperature: 95 °C; (9) cooling coil, length: 1.5 m; (10) cooling bath, temperature: 0 °C; (11) detector, wavelength (λ): 546 nm.

Table 1
Measurement strategies for the optimization of colour development

Experience	Reagents order		
	1°	2°	3°
A	A + S + N	s	
B	A + N	s	S
C	A + S	s	N

A: Mineral acid (H_3PO_4); S: sulfanilamide (0.1% (w/v) in 10% (w/v) H_3PO_4); N: NED (0.1% (w/v) in DIW); s: denotes the analyte (NNG or NO_2^-), temperature: 70 °C, holding time in the thermostatized bath before measurements: 3 min.

anionic exchange resin detailed in Section 2.2. After separation with Na_2SO_4 0.0075 mol L^{-1} (pH 11.5) as mobile phase, the eluate was derivatized with CR as described under chemicals. Temperature was kept at 95 °C and the holding time inside the thermostatized bath was 30 s. Details on flow rates of samples and reagents, reactor lengths and tubing radius are given in Fig. 1. Detection was performed at 546 nm with the spectrophotometric module of the HPLC equipped with a flow cell of small volume (8 μL).

3. Results and discussion

3.1. Influence of the order of addition of the colorimetric reagents on the yield of the reaction

Standard solutions of 1.0 mg L^{-1} of NNG and 0.2 mg L^{-1} of $NaNO_2$ were prepared from proper dilution of the stock standard solutions of each compound. In this way, the efficiency of desnitrosation of NNG by comparison with nitrite behaviour and the order of addition of the colorimetric reagents were tested. SD concentration was 0.1% (w/v) in 10% (w/v) H_3PO_4 and NED concentration was 0.01% (w/v) in DIW [5]. Temperature was kept at 70 °C and the holding time in the thermostatized bath before measurements was 3 min.

Equal volumes of the colorimetric reagent (CR) and the standard solution were added to a test tube. After heating, the temperature of the tube was lowered to 0 °C in an ice bath in order to stop the colorimetric reaction and to keep constant the absorbance value. The different experiments are shown in Table 1.

Desnitrosation of NNG is favoured in strong acid media. Initially, H_3PO_4 was chosen because this acid is generally used in nitrites determination for masking interfering cations [6–8] in real samples. Table 2 shows the absorbance values obtained for nitrite and NNG solutions using the different experimental approaches listed in Table 1. It was observed that no significant differences appear for NNG solutions and that, in the case of the nitrite ion, the order of reagents addition remarkably alters the absorbance values. Note that in case C (Table 1) no colour was developed. This fact can be explained through nitrite behaviour which is able to produce *N*-nitroso compounds with secondary amines (NED), avoiding in this way the formation of the diazo salt of sulphanilamide and thus, the generation of the red copulation product. As no significant differences were observed between A and B absorbance values, experiment A (all reagents mixed together and then added to the sample) was selected due to a simpler and faster operation. This finding makes a great difference with Monsanto's proposal where the reagents addition is sequential.

Table 2
Absorbance values obtained through the measurement strategies shown in Table 1

	Absorbance/experience		
	A	B	C
Nitrite (200 μg L^{-1})	0.230 ± 0.003	0.239 ± 0.005	0.002 ± 0.002
NNG (1000 μg L^{-1})	0.012 ± 0.06	0.012 ± 0.005	0.011 ± 0.004

n = 10, confidence interval 95%.

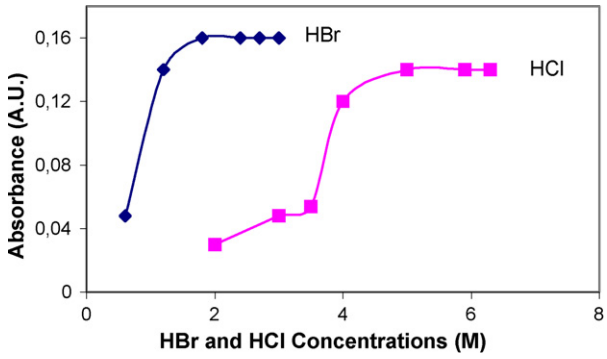

Fig. 2. Influence of proton concentration and acid force on absorbance values: [NNG]: 1.0 mg L^{-1}; incubation temperature: 70 °C, [SD]: 0.1% (w/v); [NED]: 0.01% (w/v); [HCl]: 1–6.5 mol L^{-1}; [HBr]: 0.5–3 mol L^{-1}; incubation time: 3 min.

Regarding the low signals obtained for NNG solutions, they could be related to the efficiency of nitrosyl ion release which is conditioned by the force of the mineral acid and the temperature of incubation.

3.2. Influence of the acid force and the proton concentration on NNG signal

For the study of the influence of proton concentration on the absorbance signal, HCl and HBr [9–14] were tested instead of H_3PO_4 for both, NNG and NO_2^- solutions. Each acid was added to the standard solutions together with CR. CR concentration was kept constant and those of HCl and HBr ranged between 1–6.3 and 0.5–3.0 mol L^{-1}, respectively. The concentration of standards, temperature and holding times before measurement were kept as before. No significant differences in analytical signal were observed for nitrite (data not shown), which confirms that the nature and concentration of the mineral acids influence only the desnitrosation reaction.

The effect of HCl and HBr concentration on NNG analytical signals are shown in Fig. 2. Absorbance values were obtained as the average of four replicates of each one of the experimental conditions assayed (CV% = 5%).

At low acid concentrations, H^+ may be considered as the limitant reagent and the desnitrosation reaction is not complete. Thus, the measured absorbance is low. From 4.5 mol L^{-1} HCl

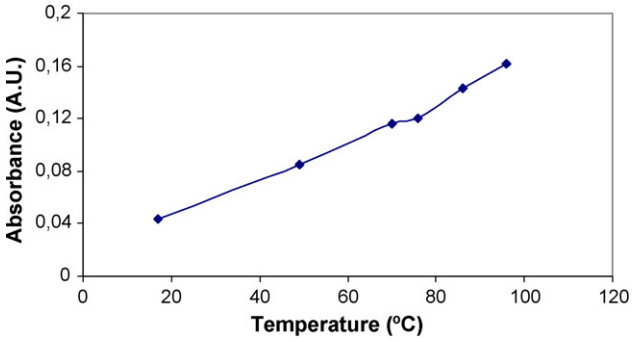

Fig. 3. Effect of temperature on absorbance values: [NNG]: 1.0 mg L^{-1}; [SD]: 0.1% (w/v); [NED]: 0.01% (w/v); [HCl]: 4 mol L^{-1}; incubation time: 3 min.

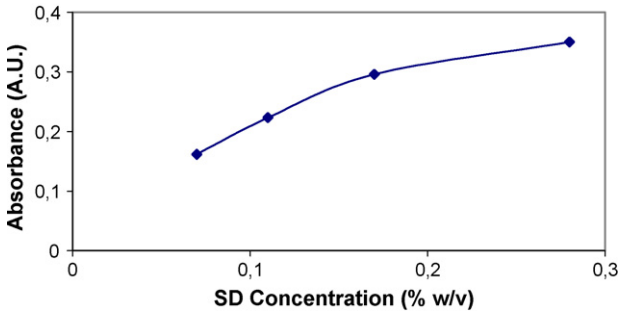

Fig. 4. Influence of SD concentration on absorbance values: [SD]: 0.075–0.3% (w/v) and [NED]: 0.0075–0.03% (w/v), relationship between SD:NED was kept in 1:10.

and 1.2 mol L^{-1} HBr onwards, the acid is no more the limitant reagent and then the signal reaches the stationary state. As a relevant signal increase was observed for HCl and HBr when compared to H_3PO_4 (Table 1), HCl and HBr were found to be more useful for desnitrosation.

Monsanto's technique uses HBr instead of HCl. As a matter of fact, a slightly higher absorbance was obtained with HBr solutions that can be attributed to a stronger intrinsic acidity of HBr ($pK_a = -9$) with respect to HCl ($pK_a = -8$) [15–17] in highly concentrated acid media. However, even loosing sensitivity, HCl was preferred instead of HBr due to easiness of handling and security.

3.3. Influence of the temperature on optimization of desnitrosation reaction

In order to study the effect of temperature on desnitrosation, this variable was changed between room temperature and 98 °C. Optimized conditions obtained in previous items were employed. Standard solutions of nitrite were not assayed as temperature is not a critical variable for the obtention of the azo-dye, the reaction is fast even at room temperature [18]. Experiments ($n = 4$) show that sensitivity always increases with temperature (see Fig. 3). A value of 95 °C was selected. Higher values were not assayed since the appearance of bubbles is not compatible with the employment of a spectrophotometric flow through cell.

3.4. Optimization of the Griess reagents concentrations

The best yield for the Griess reaction was obtained by keeping a 1:10 relationship NED/SD. All the other operational variables were the same chosen before. The optimal signal was obtained for NED 0.03% (m/v), SD 0.3% (m/v), HCl 4.5 M. Results ($n = 4$) are shown in Fig. 4.

3.5. Working curve under batch conditions

In order to test the efficiency of desnitrosation and colour development, the molar absortivity of the azo-dye was calculated through the slope of the working curve of NNG. Found values were in good agreement (95%) with tabulated ones [19] (see Table 3).

Table 3
Comparison of molar absorptivities of the diazo dye

Calculated value	Experimental data	Bibliographic data
Molar absorptivity (L mol^{-1} cm^{-1})	$2.89 \times 10^4 \pm 1 \times 10^1$	2.70×10^4 [10]
		2.85×10^4 [17]

3.6. Coupling of the HPLC to the post-derivatization system

The optimized conditions found in the previous batch experiments were employed for the HPLC post-derivatization system displayed in Fig. 1. The mobile phase was changed with respect to Monsanto's proposal. In this way Na_2SO_4 0.0075 mol L^{-1} (pH 11.5) was chosen instead of $(NH_4)_2HPO_4$ mol L^{-1} (pH 2.1) as the latter produces a split of the analytical signal and a lost of sensitivity which can be attributed to the presence of two conformers of NNG in slow equilibrium in acidic media [20]. The volumetric flow of CR was set in order to obtain the minimal dilution of the sample ($q = 1.05$ mL min^{-1}) (see Fig. 5). The holding times inside the heating bath and the cooling bath were chosen as a compromise between sensitivity and sample throughput (heating holding time = 30 s, cooling time = 15 s).

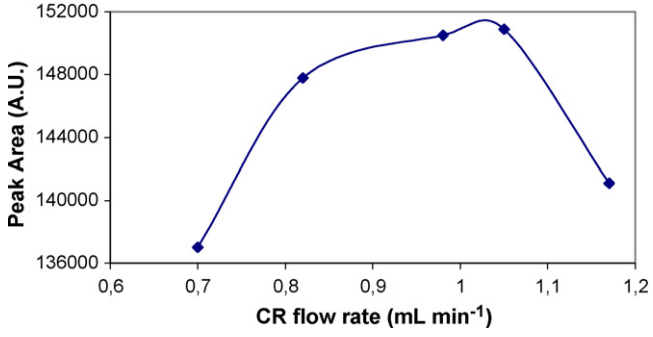

Fig. 5. Optimization of the CR flow rate. Measurement conditions: [NNG]: 1 mg L^{-1}, residence time in thermostatic bath at 95 °C: 30 s, carrier (DIW) flow rate: 1.0 mL min^{-1}, CR: [SD]: 0.3% (w/v), [NED]: 0.03% (w/v). [HCl]: 4.5 mol L^{-1}. Cooling time on ice bath: 15 s.

3.7. Figures of merit of the proposed methodology

The figures of merit of more relevance for the optimized coupled system are: sensitivity = $(5.89 \pm 0.01)\,L\,mg^{-1}$; dynamic linear range = 0.1–1.0 $mg\,L^{-1}$ and LOD (in the final solution) = 0.04 $mg\,L^{-1}$.

3.8. Analysis of the unknown samples

Several solid samples of glyphosate were provided by different customers of the Third Parties Analytical Service of our laboratory. The solid samples were treated as described earlier. A comparison between the regression parameters of the calibration curve (obtained with NNG standards) and the standard addition curve (obtained by spiking with NNG the dissolved samples) was performed in order to study matrix effects on analytical signal. Obtained values for the calibration curve and the standard addition curve are, respectively: $y = 5.89 \pm 0.01\,(L\,mg^{-1})x + 0.0865$ ($r^2 = 0.9969$), and $y = 5.50 \pm 0.01\,(L\,mg^{-1})x - 0.075$ ($r^2 = 0.9958$). Results show that matrix interferences are not relevant and that the calibration curve can be used for NNG determination in solid samples of technical glyphosate. The levels of NNG obtained with the described methodology were lower than the maximum tolerable values established by FAO and so, the samples were suitable for commercialization.

4. Conclusions

In the present work, several differences in analytical methodology were found in comparison to that of Monsanto. Experiments show that:

(1) SD, NED and the mineral acid used together are the best strategy to make easier the handling of reagents. Moreover, as the method described is a continuous flow method, the "all reagents together" approach reduces the number of confluences of the post-derivatization system and the number of mixing coils. As a matter of fact, the reduction of confluences and mixing coils decreases dispersion and/or dilution of the sample bolus and thus, sensitivity is improved.
(2) The complexity of the post-derivatization manifold employed by Monsanto – with a high number of confluences and mixing coils – obliges to the use of an air segmented flow system for minimizing dispersion. The absence of segmentation employed in our case, makes easier and faster the manifold operation.
(3) Proton concentrations of either HCl 4.5 $mol\,L^{-1}$ or HBr 1.2 $mol\,L^{-1}$ are efficient for NNG desnitrosation. HCl was preferred due to the easiness of handling and security.
(4) The use of a spectrophotometric flow cell of 8 μL reduces sample dispersion in the detector device and thus, the LOD.
(5) The employment of Na_2SO_4 as mobile phase avoids the splitting of the analyte signal found with $(NH_4)_2HPO_4$. Consequently, the sensitivity is enhanced with the first eluant.
(6) The consumption of reagents and the wastes generated during the analytical operations are reduced about 25%.

From the results obtained and summarized here, it can be observed that the coupled system designed for NNG colorimetric quantification in commercial glyphosate samples is quite satisfactory, since it reaches the LOD imposed by the regulations in a simpler and faster way than that described up to now.

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