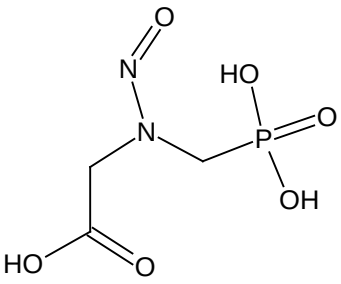
Validation of an analytical method for the determination of N-nitrosoglyphosate in technical grade glyphosate and glyphosate formulations by high performance liquid chromatography with tandem mass spectrometry detection

Fred A. Claussen, EPL Bio-Analytical Services

Abstract: N-nitrosoglyphosate (NNG) is an impurity of toxicological concern sometimes found in technical grade glyphosate and various formulations containing glyphosate or its salts. The United States Environmental Protection Agency and other global regulatory bodies have established maximum allowable concentrations of NNG in glyphosate formulations and technical materials. In most instances, the maximum allowable concentration is 1 part per million (ppm). Analytical methods for the determination of NNG are sparse in the scientific literature. The polar nature of NNG does not lend itself to most conventional reverse phase or normal phase liquid chromatographic techniques. Ion exchange high performance liquid chromatography (HPLC) is the most commonly employed method. However, it suffers from insensitive detection techniques. This research was conducted to evaluate a unique reverse phase HPLC stationary phase coupled with tandem mass spectrometry detection. The developed method was successfully validated for accuracy, precision and linearity. The method limit of quantitation (LOQ) was established at 0.5 ppm in glyphosate salt formulations and technical grade glyphosate acid.

Chemicals and Reagents: The chemical structure of NNG appears below.



The NNG analytical standard was obtained from Dr. Ehrenstorfer GmbH, Augsburg, Germany. A stock solution was prepared at a concentration of 100 µg/mL in deionized (DI) water. Dilutions of the stock solution were prepared in DI water at various concentrations. All glyphosate standard solutions were found to be stable for at least 90 days. DI water was obtained from a Barnstead NANOPure water system. Additional reagents used included:

Formic acid, 88%, ACS, J.T. Baker
Sodium hydroxide pellets, ACS, J.T. Baker
Hydrogen peroxide, 30%, ACS, Fisher Scientific

Equipment:

Analytical balances, capable of weighing to the nearest 0.1 mg
Class A volumetric glassware
HPLC autosampler vials, 1.5 mL with screw caps, Waters Corporation
HPLC column, ODS-AQ, 250 mm x 4.6 mm x 5 µm, YMC
HPLC, 2695 Separations Module, Waters Corporation
Mass spectrometer detector, Quattro micro™ API, Micromass

Procedures: Sub-samples (0.05 g) of technical grade glyphosate acid or formulated glyphosate end-use product were weighed into a 10 mL volumetric flask. An aliquot (0.1 mL) of 2.5N NaOH/0.3% hydrogen peroxide was added and the flask was brought to volume with DI water. The sample solution was then diluted 1:5 in DI water and analyzed by high performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS). An isocratic mobile phase was used consisting of 0.5% formic acid in DI water at a flow rate of 0.4 mL/minute. The run time was 10 minutes per injection. The expected retention time for NNG was 8.1-8.2 minutes. A solvent delay was used to divert flow away from the detector source from 0.0-7.5 minutes. This was necessary to keep the source clean due to the high level of active ingredient present in the sample solutions. The column was held at 50°C. The injection volume for sample and calibration standard solutions was 50 µL. Multiple reaction monitoring (MRM) was used with positive ion polarity to detect NNG. The MRM program appears in Table 1. The mass spectrometer was tuned periodically to maintain optimal sensitivity and mass accuracy by infusing NNG standard solutions and adjusting detector settings accordingly. The daughter ion spectrum for NNG displaying the molecular ion $[M+H]^+$ at a mass to charge ratio (m/z) of 199 and the daughter ion used for quantitation at m/z 168 appears in Figure 1. The daughter ion at m/z 168 represents collision cell fragmentation of the nitroso group (N=O). Representative chromatograms for a 0.5 ng/mL NNG standard, reagent blank, unfortified control, LOQ fortification and 10x LOQ fortification appear in Figures 2a-e.

Table 1. MRM Program for the Detection of NNG

Ion (m/z)		Dwell Time (s)	Cone Voltage	Collision Energy
Parent	Daughter			
199	168	3.0	16	9

The HPLC-MS/MS system was calibrated by analysis of external calibration standard solutions ranging from 0.25 to10 ng/mL NNG. Typically, at least four calibration standards were injected with each set of sample solutions. A linear regression plot of the peak area (y-axis) and the standard solution concentration in units of ng/mL (x-axis) was constructed. An inverse (1/x) weighting function was used. Sample solution concentrations were calculated using the linear regression equation $y = mx + b$; where y is the sample solution peak area, m is the slope of the linear regression plot, x is the sample solution concentration, and b is the y-intercept. Masslynx version 4.1 was used to collect the chromatographic data, construct calibration curves and calculate sample solution concentrations.

The method was validated using technical grade glyphosate acid and two end-use products containing glyphosate (Table 2). Sub-samples were fortified at the target LOQ of 0.5 ppm and at 10x the target LOQ (5 ppm). The target LOQ was equivalent to one-half the regulatory limit of 1 ppm.

Table 2. Technical Grade and End-Use Products used for Validation

Product	Product Type	Composition
Glyphosate Dimethylamine Salt (DMA)	End-Use	500-600 g/L Glyphosate DMA
Glyphosate Isopropylamine Salt (IPA)	End-Use	480-485 g/L Glyphosate IPA
Glyphosate Acid	Technical	>97% Glyphosate Acid

Results/Discussion: For each product evaluated, a total six replicate fortifications at the target LOQ (0.5 ppm) and 10x LOQ (5 ppm) were analyzed. Mean recovery, recovery range and relative standard deviation (RSD) for each spiking level for each product are presented in Table 3. The overall mean, range and RSD were also calculated. Mean recoveries at each spike level met acceptance criteria (mean recovery >70%) for all three products at each spiking level. Precision was also acceptable (RSD < 20%).

Table 3. Summary of Laboratory Fortification Recoveries

Product	**Fortification Level (ppm)**	**Replicates**	**Mean Recovery (%)**	**Recovery Range (%)**	**Relative Standard Deviation (%)**
Glyphosate DMA	0.5	6	95	82-104	9
	5	6	85	73-96	12
Overall	-	12	90	73-104	11
Glyphosate IPA	0.5	6	74	60-85	11
	5	6	78	73-88	7
Overall	-	12	76	60-88	9
Glyphosate Acid	0.5	6	94	85-109	14
	5	6	93	92-94	1
Overall	-	12	94	85-109	10

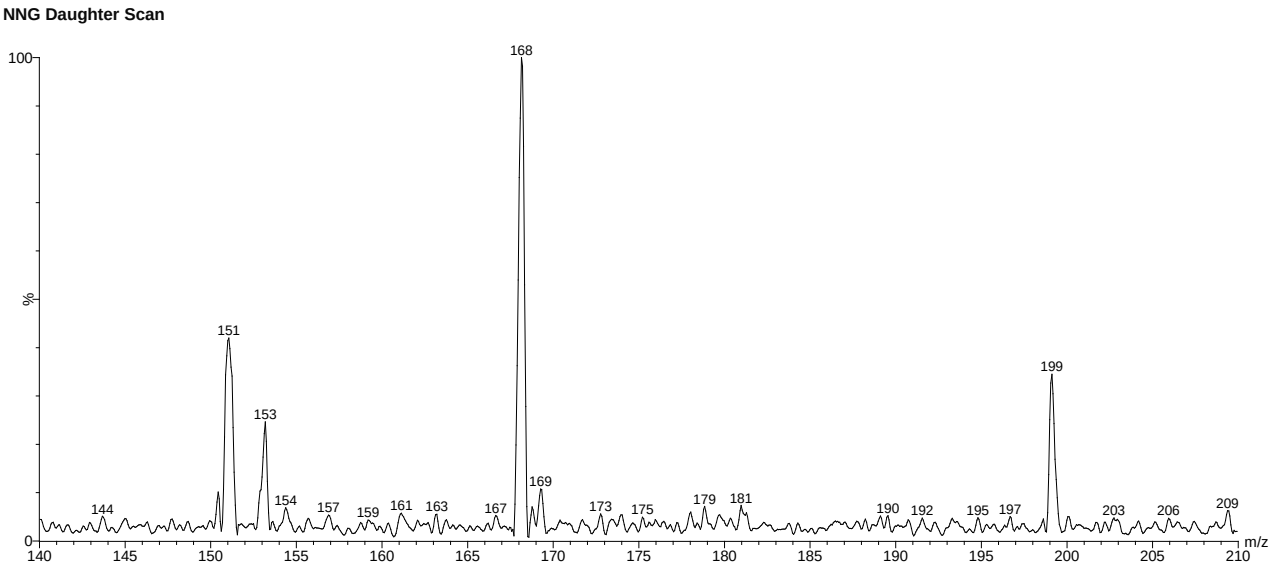
The linear working range for NNG was established by the analysis of external standard solutions. An optimum linear range of 0.25 to 10 ng/mL was established (Figure 3). Linear regression calibration plots of the peak area (y-axis) and the standard concentration in units of ng/mL (x-axis) consistently yielded a coefficient of determination (r^2) value of 0.995 or greater.

During the development phase of the research, several HPLC columns were evaluated. The YMC ODS-AQ was chosen because it provided the best retention and peak symmetry. The ODS-AQ can be used with 100% aqueous mobile phases and has a wide functional pH range.

The limit of detection (LOD) was determined by signal to noise measurements of calibration standards analyzed over the course of the research. A signal to noise ratio of three was used as a benchmark for the LOD. The method LOD was estimated at 0.1 ppm.

Conclusions: An analytical method for the determination of NNG in glyphosate technical and end-use products was successfully validated. The method demonstrated acceptable accuracy and precision for three products at fortification levels of 0.5 ppm and 5 ppm. The validated LOQ of 0.5 ppm is adequately sensitive to provide for accurate determination of NNG below the regulatory limit of 1 ppm. A method LOD of 0.1 ppm was estimated based on instrument signal to noise measurements. A 25x linear range was established and found to be reproducible from run to run.

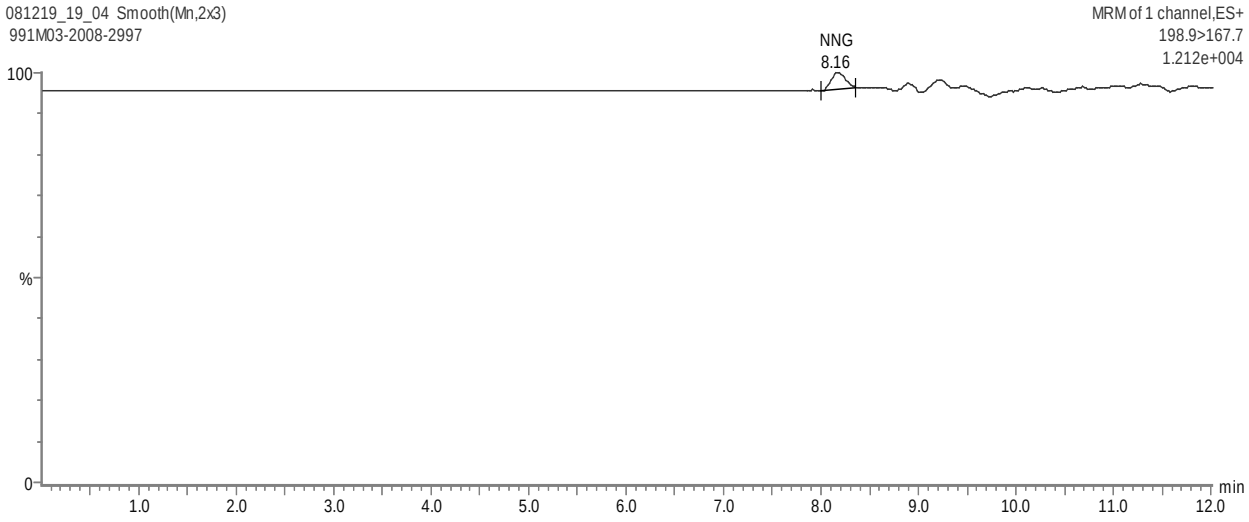
Figure 1. Daughter Ion Spectrum of NNG



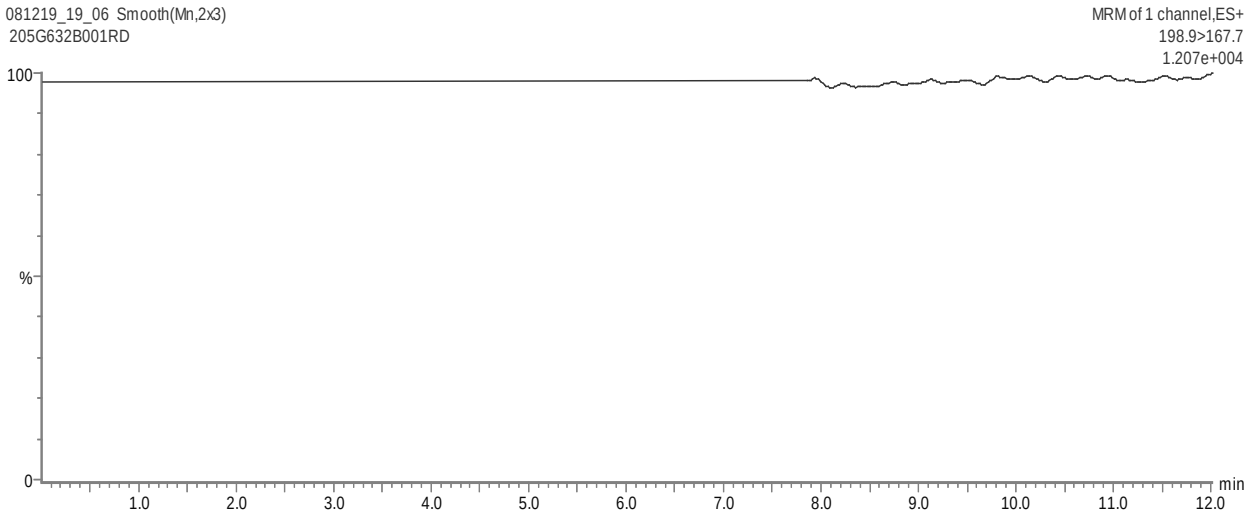
Molecular Ion at m/z 199. Quantitation ion at m/z 168.

Figure 2. HPLC-MS/MS Chromatograms

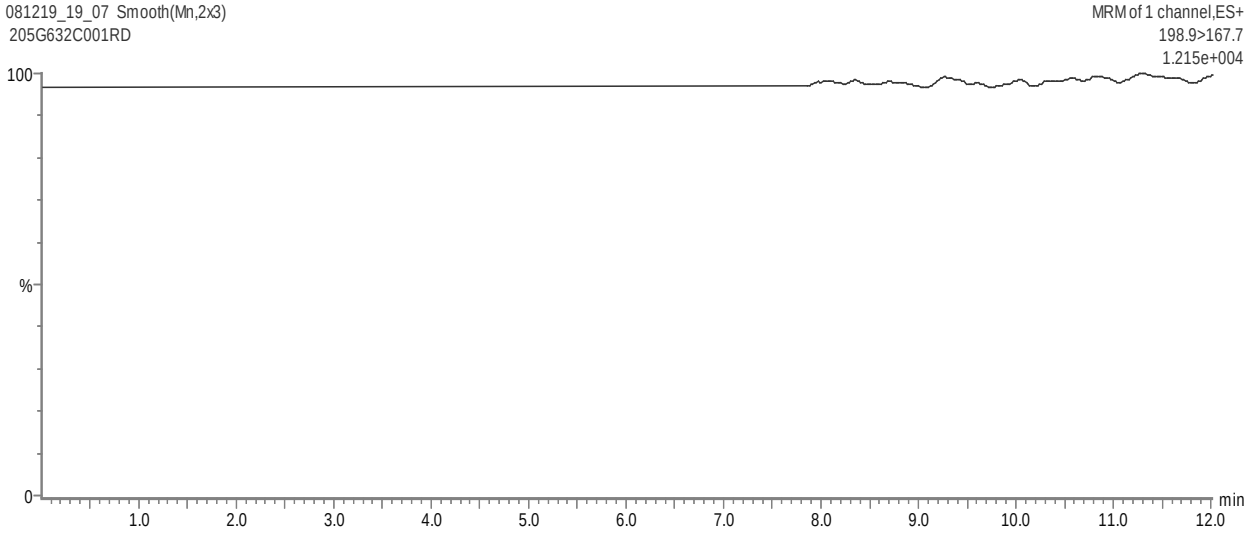
2a. NNG Standard, 0.5 ng/mL



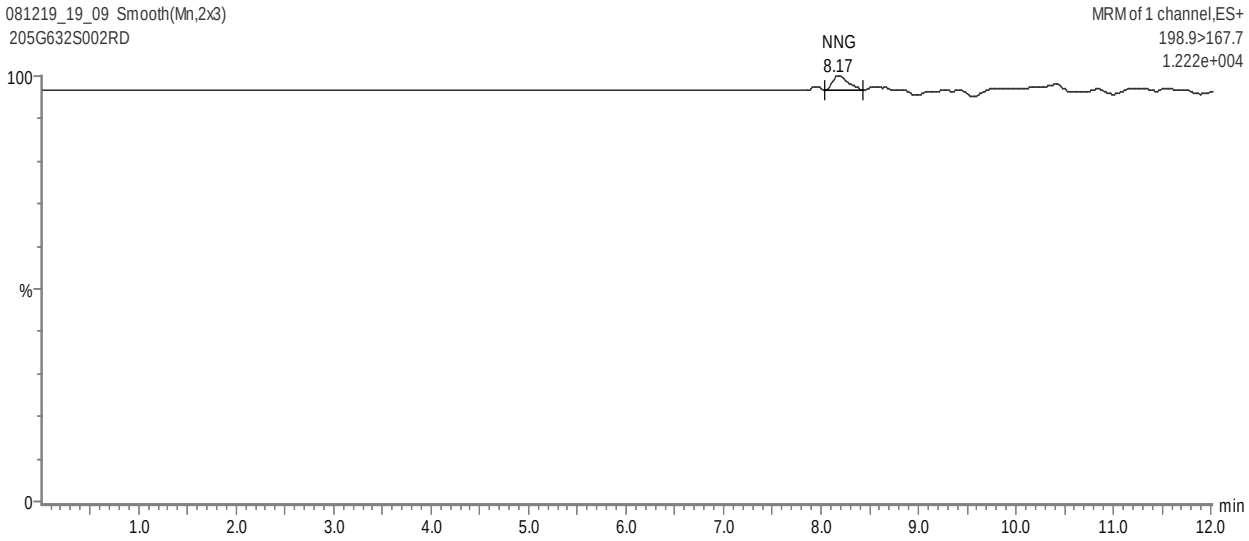
2b. Reagent Blank



2c. Control Glyphosate DMA



2d. 0.5 ppm NNG Fortification, Glyphosate DMA



2e. 5 ppm NNG Fortification, Glyphosate IPA

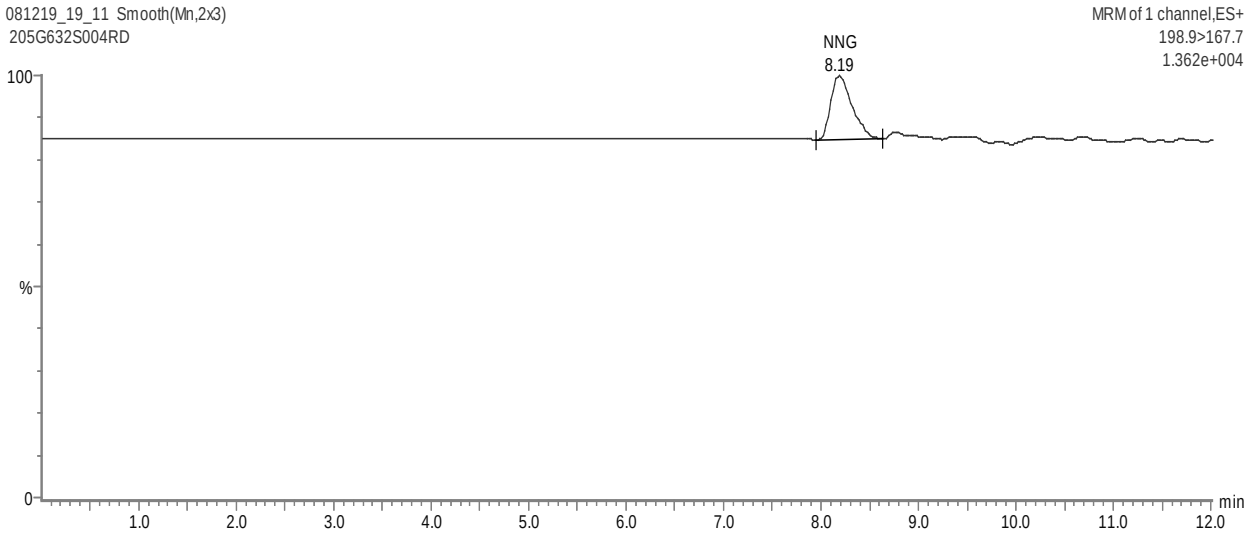


Figure 3. NNG Linear Range

Compound name: NNG
Correlation coefficient: r = 0.998782, r^2 = 0.997566
Calibration curve: 174.005 * x + -11.9208
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None