

METHOD 547

**DETERMINATION OF GLYPHOSATE IN DRINKING WATER BY DIRECT-AQUEOUS-
INJECTION HPLC, POST-COLUMN DERIVATIZATION, AND
FLUORESCENCE DETECTION**

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1.0 SCOPE AND APPLICATION

- 1.1 This method describes a procedure for the identification and measurement of Glyphosate (N-phosphonomethyl glycine) in drinking water matrices. Single laboratory accuracy and precision data have been determined for this method.
- 1.2 Glyphosate was found to rapidly decompose in chlorinated waters¹. It is therefore unlikely that the analyte will be evidenced in tap water except as separate glycine and N-phosphonomethyl moieties, neither of which is applicable to this method.

Analyte	Chemical Abstract Services Registry Number
Glyphosate	1071-83-6

- 1.3 The method detection limits (MDL, defined in Section 13.0) for glyphosate are listed in Table 1². The MDLs for a specific sample may differ from those listed.

2.0 SUMMARY OF METHOD

- 2.1 A water sample is filtered and a 200 μ L aliquot injected into a cation exchange HPLC column. Separation is achieved by using an isocratic elution. After elution from the analytical column at 65°C, the analyte is oxidized with calcium hypochlorite. The product (glycine) is then coupled with o-phthalaldehyde-2-mercaptoethanol complex at 38°C to give a fluorophor, which is detected by a fluorometer with excitation at 340 nm and detection of emission measured at >455 nm^{1,3}.

3.0 DEFINITIONS

- 3.1 Laboratory Duplicates (LD1 and LD2) -- Two sample aliquots taken in the analytical laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 give a measure of the precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

- 3.2 Field Duplicates (FD1 and FD2) -- Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.
- 3.3 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.
- 3.4 Field Reagent Blank (FRB) -- Reagent water placed in a sample container in the laboratory and treated as a sample in all respects, including exposure to sampling site conditions, storage, preservation and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.
- 3.5 Laboratory Performance Check Solution (LPC) -- A solution of method analytes, surrogate compounds, and internal standards used to evaluate the performance of the instrument system with respect to a defined set of method criteria.
- 3.6 Laboratory Fortified Blank (LFB) -- An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the method is in control, and whether the laboratory is capable of making accurate and precise measurements at the required method detection limit.
- 3.7 Laboratory Fortified Sample Matrix (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.
- 3.8 Stock Standard Solution -- A concentrated solution containing a single certified standard that is a method analyte, or a concentrated solution of a single analyte prepared in the laboratory with an assayed reference compound. Stock standard solutions are used to prepare primary dilution standards.
- 3.9 Calibration Standard (CAL) -- A solution prepared from the primary dilution standard solution and stock standard solutions of the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.
- 3.10 Quality Control Sample (QCS) -- A sample matrix containing method analytes or a solution of method analytes in a water miscible solvent which is used to fortify reagent water or environmental samples. The QCS is obtained from a

source external to the laboratory, and is used to check laboratory performance with externally prepared test materials.

4.0 INTERFERENCES

- 4.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in the chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by analyzing laboratory reagent blanks as required by Section 10.2.
 - 4.1.1 Glassware must be scrupulously cleaned⁴. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. Glassware should then be drained dry, and heated in a laboratory oven at 400°C for several hours before use. After drying and cooling, glassware should be stored in a clean environment to prevent any accumulation of dust or other contaminants.
 - 4.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required to achieve necessary purity.
- 4.2 Samples may become contaminated during shipment or storage. Field blanks must be analyzed to determine that sampling and storage procedures have prevented contamination.
- 4.3 The extent of matrix interferences may vary considerably from source to source, depending upon the nature and diversity of the matrix being sampled. No interferences have been observed in the matrices studied.
- 4.4 The extent of interferences that may be encountered using liquid chromatographic techniques has not been fully assessed. Although the HPLC conditions described allow for a unique resolution of the compound covered in this method, other matrix components may interfere.

5.0 SAFETY

- 5.1 The toxicity or carcinogenicity of chemicals used in this method has not been precisely defined. Each chemical should be treated as a potential health hazard, and exposure to these chemicals should be minimized. Each laboratory is responsible for maintaining awareness of OSHA regulations regarding safe handling of chemicals used in this method⁵. A reference file of material data handling sheets should be made available to all personnel involved in the chemical analysis.

6.0 APPARATUS AND EQUIPMENT

- 6.1 Sampling Equipment -- For discrete or composite sampling.
 - 6.1.1 Grab sample bottle -- 60 mL screw cap bottles (Pierce No. 13075 or equivalent) and caps equipped with a teflon-faced silicone septa (Pierce No. 12722 or equivalent). Prior to use, wash vials and septa as described in Section 4.1.1.
- 6.2 Glassware
 - 6.2.1 Autosampler vials -- Glass, 3.7 mL, with teflon-lined septa and screw caps. (Supelco, #2-3219, or equivalent)
 - 6.2.2 Volumetric flask -- 1000 mL and 100 mL
- 6.3 Balance -- Analytical, capable of accurately weighing 0.0001 g.
- 6.4 pH Meter -- Capable of measuring pH to 0.01 units.
- 6.5 Filtration Apparatus
 - 6.5.1 Macrofiltration -- To filter mobile phase and derivatization solutions used in HPLC system. Membrane filter, 0.2 μ mesh, 47 mm diameter, Nylon 66 (Alltech, #2034 or equivalent)
 - 6.5.2 Microfiltration -- To filter samples prior to HPLC analysis. Use 0.45 μ filters (Gelman Acrodisc - CR or equivalent)
 - 6.5.3 Helium -- For degassing solutions and solvents.
- 6.6 Syringes
 - 6.6.1 One 250 μ L glass syringe, with blunt tip needle for manual injection.
 - 6.6.2 Three to five mL disposable hypodermic syringes with Luer-Lok tip.
 - 6.6.3 Micro syringes -- Various sizes.
- 6.7 Instrumentation -- A schematic diagram of the analytical system is shown in Figure 1.
 - 6.7.1 A high performance liquid chromatograph (HPLC) capable of injecting 200 μ L aliquots and utilizing an isocratic pumping system with constant flow rate of 0.5 mL/min.
 - 6.7.2 Column -- 250 x 4 mm, Bio-Rad, Aminex A-9. Column specifications: K⁺ form, packed at 65°C, pH = 1.9. This column was used to generate the method performance statements in Section 13.0. Different HPLC

columns may be used if requirements described in Section 10.3 are met. Use of guard columns is recommended.

- 6.7.3 Guard column -- C₁₈ packing - (Dupont, Zorbax Guard Column or equivalent). An alternative guard column similar in composition to the analytical column may also be used provided the requirements of Section 10.3 are met.
- 6.7.4 Column oven -- Fiatron, Model CH-30 and controller, Model TC-50, or equivalent.
- 6.7.5 Post Column Reactor (PCR) -- Capable of mixing reagents into the mobile phase. Reactor to be equipped with pumps to deliver 0.5 mL/min of each reagent; mixing tees; two 1.0 mL delay coils, both thermostatted at 38°C; and constructed using teflon tubing (Kratos Model URS 051 and URA 200 or equivalent).
- 6.7.6 Fluorescence detector -- Capable of excitation at 340 nm and detecting of emission >455 nm. A Schoeffel Model 970 fluorescence detector was used to generate the validation data presented in this method.
- 6.7.7 Data system -- A strip chart recording of the detector response must be provided as a minimum requirement. The use of a data system to calculate retention times and peak areas is recommended but not required.

7.0 REAGENTS AND CONSUMABLE MATERIALS

7.1 HPLC Mobile Phase

- 7.1.1 Reagent water -- Reagent water is defined as water of very high purity, equivalent to distilled-in-glass solvents.
- 7.1.2 Mobile phase -- 0.005 M KH₂PO₄ (0.68 gm) in 960 mL reagent water, add 40 mL HPLC grade methanol, adjust pH of solution to 1.9 with concentrated phosphoric acid then filter with 0.22 μ filter and degas with helium before use.

7.2 Post-column Derivatization Solutions

- 7.2.1 Calcium hypochlorite solution -- Dissolve 1.36 g KH₂PO₄, 11.6 g NaCl and 0.4 g NaOH in 500 mL deionized water. Add 15 mg Ca (C10)₂ dissolved in 50 mL deionized water and dilute solution to 1000 mL with deionized water. Filter solution through 0.22 μ membrane filter and degas with helium before use. It is recommended that this solution be made fresh daily.
- 7.2.2 O-phthalaldehyde (OPA) reaction solution

7.2.2.1 2-Mercaptoethanol (1+1) -- Mix 10.0 mL of 2-mercaptoethanol and 10.0 mL of acetonitrile. Cap and store in hood.

CAUTION: Stench.

7.2.2.2 Sodium borate (0.05 N) -- Dissolve 19.1 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) in 1.0 L of reagent water. The sodium borate will completely dissolve at room temperature if prepared a day before use.

7.2.2.3 OPA reaction solution -- Dissolve 100 ± 10 mg of o-phthalaldehyde (mp 55-58°C) in 10 mL of methanol. Add to 1.0 L of 0.05 N sodium borate. Mix, filter through 0.45 μ membrane filter, and degas. Add 100 μ L of 2-mercaptoethanol (1+1) and mix. Make up fresh solution daily unless the reagent solution is protected from atmospheric oxygen. The solution can be stored in glass bottles under atmospheric conditions at 4°C for up to two weeks without appreciable increases in background fluorescence or stored under nitrogen for indefinite periods.

NOTE: Fluoraldehyde (Pierce Chemical), a commercially formulated OPA reaction solution, may be substituted for Steps 7.2.2.1 through 7.2.2.3.

7.3 Sample Preservation Reagents

7.3.1 Sodium thiosulfate -- Granular, ACS grade or better (Fisher, S-446).

7.4 Stock Standard Solution (1.00 μ g/mL)

7.4.1 Accurately weigh and dissolve 0.1000 g of pure glyphosate in 1000 mL of deionized water. Larger or smaller volumes may be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight may be used without correction to calculate the concentration of the stock standard.

8.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 Collect samples in glass containers (Section 6.1.1). Conventional sampling practices⁶ are to be followed.

8.2 Sample Preservation -- Treatment of samples to remove residual chlorine will eliminate the possibility of glyphosate losses due to chlorine during storage. Chlorine is destroyed by adding 100 mg/L of sodium thiosulfate to the sample.

8.3 Sample Storage -- Samples should be stored at 4°C away from light and analyzed within two weeks. A preservation study⁷ has demonstrated the stability of glyphosate in frozen samples for up to 18 months. The analyst

should verify appropriate sample holding times applicable to the sample under study.

9.0 **CALIBRATION**

- 9.1 Establish liquid chromatographic operating conditions indicated in Table 1.
- 9.2 Prepare a minimum of three calibration standards of glyphosate by serial dilution of the stock standard solution in deionized water. One of the calibration standards should correspond to a glyphosate concentration near to, but above the MDL. The other concentrations should comprise the range of concentrations expected for the samples, or, otherwise, define the working range of the detector.
- 9.3 Analyze each calibration standard and tabulate peak area against concentration (in $\mu\text{g/L}$) injected. The results may be used to prepare a calibration curve for glyphosate.

Alternatively, if the ratio of response to concentration (response factor) is constant over the working range (<10% relative standard deviation), linearity through the origin can be assumed and the average ratio or response factor can be used in place of a calibration curve.

- 9.4 The working calibration curve must be verified on each working day by the measurement of a minimum of two calibration check standards, one at the beginning and one at the end of the analysis day. These check standards should be at two different concentration levels to verify the calibration curve. For extended periods of analysis (greater than eight hours), it is strongly recommended that check standards be interspersed with samples at regular intervals during the course of the analyses. If the response for the analyte varies from the predicted response by more than $\pm 20\%$, the test must be repeated using a fresh calibration standard. If the results still do not agree, generate a new calibration curve.

10.0 **QUALITY CONTROL**

- 10.1 Minimum quality control (QC) requirements are initial demonstration of laboratory capability, analysis of laboratory reagent blanks, laboratory fortified matrix samples, laboratory fortified blanks and QC samples.
- 10.2 Laboratory Reagent Blanks (LRB) -- Before processing any samples, the analyst must demonstrate that all glassware and reagent interferences are under control. Each time a set of samples is extracted or reagents are changed, a LRB must be analyzed. If within the retention time window of the analyte of interest the LRB produces a peak that would prevent the determination of that analyte, determine the source of contamination and eliminate the interference before processing samples.

10.3 Initial Demonstration of Capability

10.3.1 Prepare laboratory fortified blanks (LFBs) at an analyte concentration of 250 µg/L. With a syringe, add .250 mL of the stock standard (Section 7.4) to at least four 100 mL aliquots of reagent water and analyze each aliquot according to procedures beginning in Section 11.0.

10.3.2 The glyphosate recovery (R) values determined in Section 10.3.1 should be within ±30% of the R values listed in Table 2 for at least three of four consecutive samples. The relative standard deviation (Sr) of the mean recovery (R) should be less than 30%. If the analyte of interest meets the acceptance criterion, performance is judged acceptable and sample analysis may begin. For analytes that fail this criterion, initial demonstration procedures should be repeated.

10.3.3 The initial demonstration of capability is used primarily to preclude a laboratory from analyzing unknown samples via a new, unfamiliar method prior to obtaining some experience with it. It is expected that as laboratory personnel gain experience with this method the quality of the data will improve beyond the requirements stated in Section 10.3.2.

10.4 The analyst is permitted to modify HPLC column, HPLC conditions, or detectors to improve separations or lower analytical costs. Each time such method modifications are made, the analyst must repeat the procedures in Section 10.3.

10.5 Laboratory Fortified Blanks (LFB)

10.5.1 The laboratory must analyze at least one LFB sample per sample set (all samples analyzed within a 24-hour period). The fortified concentration of glyphosate in the LFB should be 10 times the MDL. Calculate accuracy as percent recovery (R). If R falls outside the control limits (See Section 10.5.2.), the analysis is judged out of control, and the source of the problem must be identified and resolved before continuing analyses.

10.5.2 Until sufficient data become available from within their own laboratory, usually a minimum of results from 20-30 analyses, the laboratory should assess laboratory performance against the control limits in Section 10.3.2. When sufficient internal performance data become available, develop control limits from the mean percent recovery (R) and S_R of the percent recovery. These data are used to establish upper and lower control limits as follows:

$$\text{UPPER CONTROL LIMIT} = R + 3S_R$$

$$\text{LOWER CONTROL LIMIT} = R - 3S_R$$

After each 5-10 new recovery measurements, new control limits should be calculated using only the most recent 20-30 data points.

10.6 Laboratory Fortified Sample Matrix

10.6.1 The laboratory must add a known fortified concentration to a minimum of 10% of the routine samples or one fortified sample per set, whichever is greater. The fortified concentration should not be less than the background concentration of the original sample. Ideally, the fortified concentration should be the same as that used for the laboratory fortified blank (Section 10.5). Over time, samples from all routine samples sources should be fortified.

10.6.2 Calculate the accuracy as R for the analyte, corrected for background concentrations measured in the original sample, and compare these values to the control limits established in Section 10.5.2 from the analyses of LFBs.

10.6.3 If the recovery of any sample falls outside the designated range, and the laboratory performance for the analyte is shown to be in control (Section 10.5), the recovery problem encountered with the dosed sample is judged to be matrix related, not system related. The result for the analyte in the original sample is labeled suspect/matrix to inform the data user that the results are suspect due to matrix effects.

10.7 Quality Control Samples (QCS) -- Each quarter the laboratory should analyze at least one QCS (if available). If criteria provided with the QCS are not met, corrective action should be taken and documented.

10.8 The laboratory may adopt additional quality control practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. For example, field or laboratory duplicates may be analyzed to assess the precision of the environmental measurements or field reagent blanks may be used to assess contamination of samples under site conditions, transportation and storage.

11.0 PROCEDURE

11.1 Sample Cleanup -- The cleanup procedure for this direct aqueous injection HPLC method is limited to the filtration procedure described in Section 11.2.3. Applying only filtration, no interferences were evidenced in the analysis of tap water, ground water and municipal effluent. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must demonstrate that the recovery of the analyte is within limits specified by the method.

11.2 Analysis

11.2.1 Table 1 details the recommended HPLC-PCR operating conditions. An example of the chromatography achieved under these conditions is shown in Figure 2.

11.2.2 Calibrate the system daily as described in Section 9.0.

- 11.2.3 Filter samples using 0.45 μ Acrodisc filters (Section 6.5.2) and inject 200 μ L of sample into the HPLC-PCR system for analysis.
- 11.2.4 Record resulting peak sizes in area units.
- 11.2.5 If the response for a glyphosate peak in a sample chromatogram exceeds the working calibration range, dilute the sample with reagent water and reanalyze.
- 11.2.6 Some changes in analyte retention time may be observed following the analysis of matrices with moderate to high ionic strength. The equilibration of the analytical column with the mobile phase will minimize this problem.

NOTE: The use of alternative analytical columns is mentioned in Section 6.7.2.

11.3 Identification of Analytes

- 11.3.1 Identify a sample component by comparison of its retention time to the retention time of a reference chromatogram. If the retention time of an unknown compound corresponds, within limits (Section 11.3.2), to the retention time of the standard, then identification is considered positive.
- 11.3.2 The width of the retention time window used to make identification should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation in retention time can be used to calculate a suggested window size for a compound. However, the experience of the analyst should weigh heavily in the interpretation of chromatograms.
- 11.3.3 Identification requires expert judgement when sample components are not resolved chromatographically. When peaks obviously represent more than one sample component (i.e., broadened peak with shoulder(s) or valley between two or more maxima), or any time doubt exists over the identification of a peak in a chromatogram, appropriate confirmatory techniques such as use of an alternative detector which operates on a physical/chemical principle different from that originally used, e.g., mass spectrometry, or the use of an alternative separation technology, e.g., anion exchange chromatography, must be employed.

12.0 CALCULATIONS

- 12.1 Determine the concentration (C) of glyphosate in the sample by direct comparison with the calibration curve described in Section 9.0, or alternatively, by means of the equation below derived from the calibration data.

$$C (\mu\text{g/L}) = \frac{A}{RF}$$

where: A = Area of glyphosate peak in sample.

RF = Response factor derived from calibration data.

- 12.2 For samples processed as part of a set where laboratory fortified blank and/or laboratory fortified matrix recoveries fall outside control limits in Sections 10.5 and 10.6, data for the affected samples must be labeled as suspect.

13.0 METHOD PERFORMANCE

- 13.1 Method Detection Limits (MDL) -- The MDL is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above the background level². The concentrations listed in Table 1 were obtained using reagent water, ground water and dechlorinated tap water.
- 13.2 Single-laboratory precision and accuracy results at several concentrations in drinking water matrices are presented in Table 2.

14.0 REFERENCES

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3. Cowell, J.E. "Analytical Residue Method for N-phosphonomethyl Glycine and Aminomethyl phosphonic Acid in Environmental Water," Monsanto Company, Method Number 86-63-1, 1987.
4. ASTM Annual Book of Standards, Part 31, D3694, "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.
5. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. ASTM Annual Book of Standards, Part II, Volume 11.01, D3370-82, "Standard Practice for Sampling Water", American Society for Testing and Materials, Philadelphia, PA, 1986.

7. Cowell, J.E. "Storage Stability of Glyphosate in Environmental Water," Monsanto Company, 1988.

TABLE 1. ANALYTICAL CONDITIONS AND METHOD DETECTION LIMITS FOR GLYPHOSATE

Matrix¹	Retention Time (min)	MDL² (µg/L)
RW	13.5	6.00
GW	13.7	8.99
TW-T	11.8	5.99

Conditions:

Column:	250 x 4 mm, Bio-Rad, Aminex A-9 (Specifications as per Subsection 6.7) thermostatted at 65°C.
Mobile Phase:	0.005 M KH ₂ PO ₄ - water:methanol (24:1) buffered at pH = 1.9 (Section 7.0).
Elution Mode:	Isocratic
Flow Rate:	0.5 mL/min
Injection Volume:	200 µL
PCR:	Calcium Hypochlorite flow rate = 0.5 mL/min., OPA solution flow rate = 0.5 mL/min, reactor temperature = 38°C.
Detector:	Excitation wavelength at 340 nm and detection emission at 455 nm.

¹RW = reagent water,

GW = ground water,

TW-T = tap water spiked after dechlorination treatment.

²All MDL data were generated from spiked samples at 25 µg/L.

TABLE 2. RECOVERY OF GLYPHOSATE IN REPRESENTATIVE DRINKING WATER MATRICES

Fortified Concentration (µg/L)	Matrix¹	Number of Replicates	Mean Recovery %	Relative Standard Deviation %
2500	RW	8	102	1.96
	GW	8	103	1.25
	TW-T	8	99.2	1.74
700	RW	8	101	2.65
	GW	8	98.7	2.01
	TW-T	8	96.4	1.80
250	RW	8	95.6	3.91
	GW	8	101	1.77
	TW-T	8	98.0	1.75
25	RW	8	96.0	9.07
	GW	8	96.0	12.3
	TW-T	8	108	6.57

¹RW = Reagent water,
 GW = Ground water,
 TW-T = Tap water spiked after dechlorination treatment.

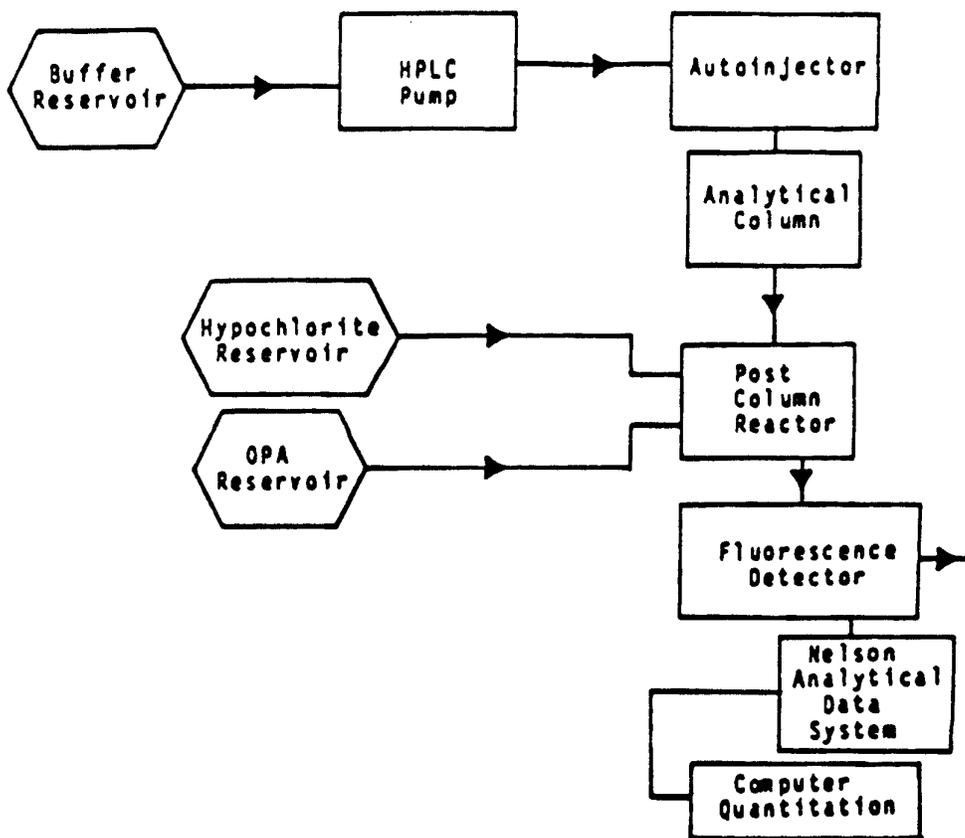


Figure 1. HPLC, Post-Column Reactor System

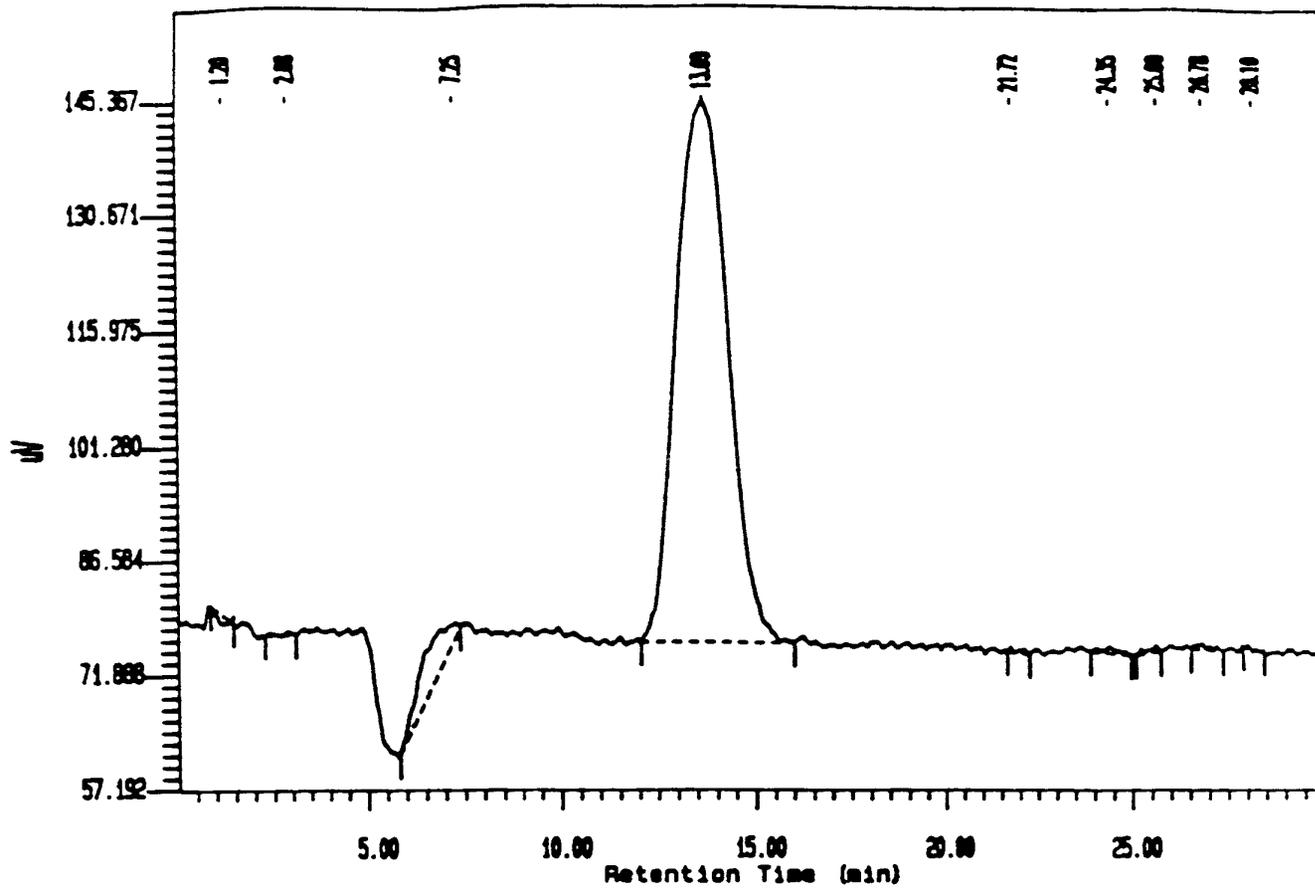


Figure 2. Liquid Chromatogram of glyphosate at 250 ug/L. Conditions are as stated in Table 1.