CHAPTER III

METHODOLOGY

3.1 Site Selection Methodology

3.1.1 Site selection criteria

The criteria used for site selection for this study are as follows:

- A primarily agricultural area
- A high potential for glyphosate contamination in the natural water resources
- High usages of glyphosate herbicide and chemical fertilizers
- Soil with characteristics that encourage the adsorption of glyphosate; clayey soil was viewed as the most important characteristic in this study.

Nakorn Pa Tom province was selected as the soil sampling site because it met all of the criteria requirements as shown in Figure 3.1 and 3.2.

Figure 3.1. Soil sampling site at Nakorn Pa Tom province.

[a] Picture provided by Department of land development

Figure 3.2. The soil in Nakorn Pha Tom series.

3.1.2 Properties of soil in Nakorn Pa Tom province

The land use of soil in Nakorn Pa Tom series is suitable for planting the rice, cereal and sesame. The water drainage is badly and the permeability is slowly. The chemical properties of the soil samples are shown in Table 3.1

Table 3.1. Chemical properties of soil in Nakorn Pha Tom.

Depth (cm)	Organic Matter	Cation Exchange Capacity	Base Saturation	Available Phosphorus	Available Potassium	Enrich
0-25	Medium	Medium	High	High	High	High
25-50	Medium	Medium	High	High	High	High
50-100	Medium	Medium	High	High	High	High

[a] Information provided by Department of land development

3.2 Soil Sampling Methodology

3.2.1 Materials

- A high density polyethylene (HDPE) column, 35 cm in length with a 5.8 inner diameters.
- Auger.
- Hoe and Spade.

3.2.2 Sampling methodology

The soil sampling methodology have done in this study as shown in Figure 3.3 and explained by following:

- Remove the top 2-5 cm soil layers in order to eliminate the grass cover before sampling the soil.
- Start to put the soil columns into the soil in 15 deep by using auger
- Most of 60 soil columns were putted into the soil very close to each other to get the same soil properties.
- After that, hoe and spade were used for excavation the soil surroundings in order to bring the soil columns up from the soil.
- The 60 sampled soils were taken back to the laboratory to perform the leaching experiments.

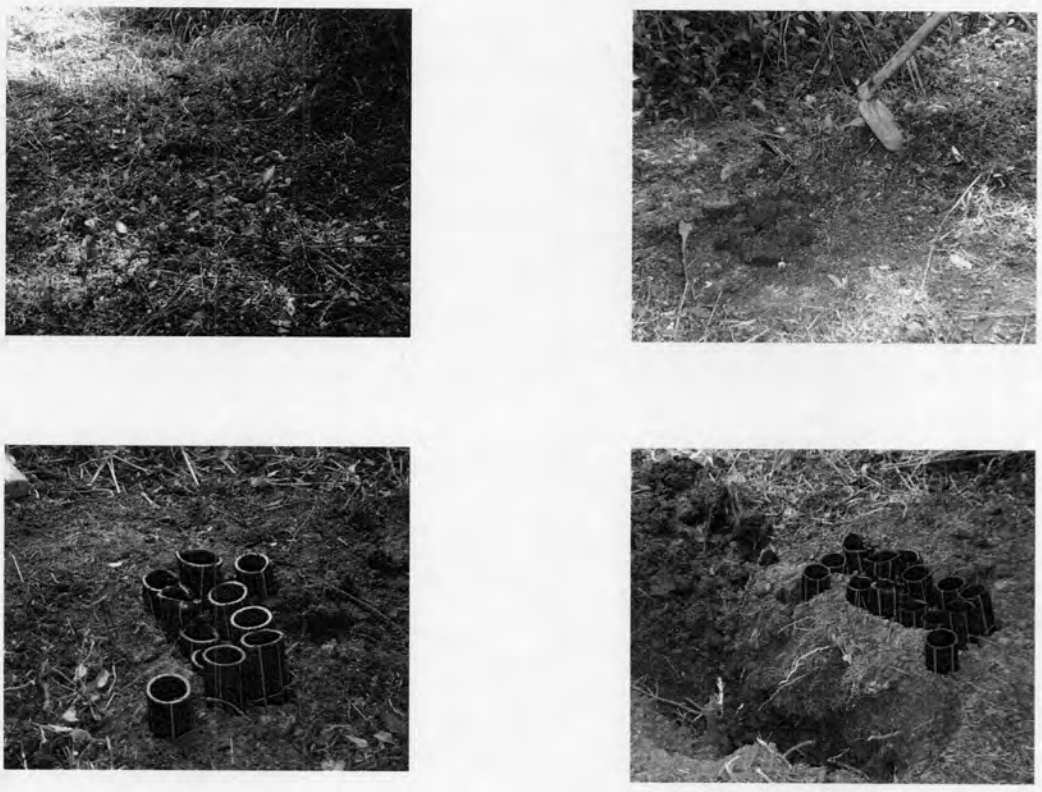

Figure 3.3. Soil sampling methodology.

3.2.3 Characteristics of sampled soil

Physical and chemical properties of soil sampled as shown in Table 3.2 and Table 3.3 were analyzed in order to know the properties of the soil. The analytical results were used to estimate the reactions among the soil chemicals, chemical fertilizer, and glyphosate herbicide that could occur during the monitoring period.

Table 3.2. Physical properties of the soil samples.

Soil texture		Texture classification
% Sand	23	Clayey soil
% Silt	32	
% Clay	45	

[b] Data provided by Kasetsart University

Table 3.3. Chemical properties of the soil samples.

Parameter	Value	Rate
pH	4.3	-
Organic Mater (OM)	2.2 %	Medium
Phosphorus (P)	19 mg/kg	Medium
Potassium (K)	344 mg/kg	Very high
Calcium (Ca)	2663	High
Magnesium (Mg)	18	Low
Extractable Fe	265.5 mg/kg	-
Extractable Al	56 mg/kg	-
Cation Exchange Capacity (CEC)	31 Cmol/kg	-

[b] Data provided by Kasetsart University

3.3 Experimental Design

The summarization of experimental detail for studying glyphosate in leachate and soil are shown in Table 3.4 and 3.5. And other details as follow;

3.3.1 Groups definition

In order to study the leaching behavior of glyphosate herbicide under and not under the influent of chemical fertilizer and two different rainfall conditions, the experimental works in this study were divided as follows:

3.3.1.1 Blank groups

The blank groups were setup for confirm that the soil sampled from the site have not been contaminated with glyphosate herbicide. The leachate from blank groups were collected, measured, and analyzed as well as the other groups. The results of analyzation of glyphosate in blank groups were used for comparison the different between amount of glyphosate in soil experiment and soil blank.

3.3.1.2 Control groups

The control groups were the groups set up for studying the behavior of glyphosate leaching without any influent of chemical fertilizer. The control groups were divided into 2 sub-groups as follows:

a) Summer Control Group (SC)

The group was setup for studying the behavior of glyphosate leaching without the influent of chemical fertilizer under summer condition.

b) Rainy Control Group (RC)

The group was setup for studying the behavior of glyphosate leaching without the influent of chemical fertilizer under rainy condition.

3.3.1.3 Experiment Groups

The groups were setup for studying the behavior of glyphosate leaching influenced by chemical fertilizer. The control groups were divided into 2 sub-groups as follows:

a) Summer Experiment Group (SE)

The group was setup for studying the behavior of glyphosate leaching influenced by chemical fertilizer under summer condition.

b) Rainy Experiment Group (RE)

The group was setup for studying the behavior of glyphosate leaching influenced by chemical fertilizer under rainy condition.

3.3.2 Chemical and application rate

The inner diameter of soil column used in this study was 5.8 cm. The equation used for calculation the surface area of soil column is ¶(r^2). Hence, the area of soil surface is 26.41 cm^2.

3.3.2.1 Glyphosate herbicide

a) Form and formula

Glyphosate applied in this study was in the liquid form of the "Roundup" trade name. The Formula of Roundup is comprised of 36% of glyphosate in [phosphonomethy] glycine form.

b) Application rate

The recommendation of the application rate of glyphosate is 6,815 g per 4,000,000 cm^2. Hence, the amount of glyphosate which should have been applied into soil column in this study equaled to 45 mg.

However, application rates of the glyphosate in real situations generally exceed the recommended rate; the glyphosate applied to this experiment was 135 mg or 3 times higher than the recommended application rate to ensure the necessary adsorption of glyphosate for the leaching experiment.

3.3.2.2 Chemical fertilizer

a) Form and formula

The chemical fertilizer applied in this study is a commonly used formula; in the solid form and made up of 16% total N_2, 16% P_2O_5, 16% K_2O, 0.6 % MgO, and 4.8 % CaO.

b) Application rate

The fertilizer was applied in 10 g per 26.41 cm^2 area.

3.3.3 Water application

To simulate natural conditions as closely as possible, the amount of water applied in the experiment was comprised of the amount of water applied for the purpose of agriculture and the amount of water precipitated by nature as rainfall.

3.3.3.1 The water applied for agricultural purposes

a) Source of data

Amount of water applied for this purpose was estimated from the amount of water required in a real situation.

b) Application rate

An area of 100 x 100 cm uses approximately 7.5 L of water per time per day. Thus the area of 26.41 cm^2 requires the approximately 10 ml of water per time per day. Hence, amount of water applied in the experiment was approximately 20 ml in order to generate the water applied for agricultural purposes 2 times per day.

3.3.3.2 Water applied as rainfall

a) Source of rainfall data

Rainfall data used in this study were collected from all the stations in the central part of Thailand over a 10-year period (1996-2005) provided by Thai Meteorological Department.

b) The rainfall data summarization and selection

Summarization procedures:

- Summarize the rainfall data to get the total amount of rainfall of each station in each month during 1996-2005.
- Then, summarize the total amount of rainfall data of each station in each month to get the representative total amount of rainfall precipitated in the central region for each month.

Selection procedure:

- The maximum amount rainfall in 2 months was selected to simulate the rainy season precipitation. Hence, the rainfall data of September and October were used to simulate the amount of rainfall during the rainy season.
- The minimum amount of rainfall in 2 months was selected to simulate the summer precipitation. Hence, the rainfall data of December and January were used to simulate the amount of rainfall during the summer.

c) Application rate

Amount of water applied for rainy season:

The total amount of rainfall in September and October was 441.98 mm over a period of 60 days. It was divided by 60 days to generate the daily water application along the monitoring period and calculated to determine the accurate amount of water for the 26.41 cm^2 area of the soil column.

Hence, the daily application rate of rainfall for the rainy season was approximately 20 ml per day.

Amount of water applied for summer:

The total amount of rainfall in December and January was 15.75 mm over a period of 60 days. It was divided by 60 days to generate the daily water application along the monitoring period and calculated to determine the accurate amount of water for the 26.41 cm^2 area of the soil column.

Hence, the daily application rate of rainfall for the rainy season was approximately 1 ml per day.

3.3.3.3 Total amount of water applied for rainy and summer seasons

a) The total amount of water applied for rainy

- Amount of water applied for agricultural purpose was approximately 20 ml per day.
- Amount of water applied as rainfall was approximately 20 ml per day
- Total amount of water applied daily for rainy season was 40 ml per day.

b) The total amount of water applied for summer

- Amount of water applied for agricultural purpose was approximately 20 ml per day.
- Amount of water applied as rainfall was approximately 1 ml per day.
- Total amount of water applied daily for rainy season was 21 ml per day.

Table 3.4 Experimental details for monitoring glyphosate in leachate.

Group	Group name	Chemical applied		Amount of water applied per day (ml)	Number of replicate
		Glyphosate Herbicide (135 mg)	Chemical Fertilizers (10 g)		
1	Blank	×	×	40	3
2	Summer Control (SC)	✓	×	21	3
	Rainy Control (RC)	✓	×	40	3
3	Summer Experiment (SE)	✓	✓	21	3
	Rainy Experiment (RE)	✓	✓	40	3

× refers to not applied. ✓ refers to applied.

Table 3.5 Experimental details for determination residual glyphosate in soil

Group	Group name	Soil column divided		Remark
		Number of replicate for Upper soil	Number of replicate for Lower Soil	
1	Blank	3	3	Use the same column
2	Summer Control (SC)	3	3	Use the same column for both in leachate and soil
	Rainy Control (RC)	3	3	
3	Summer Experiment (SE)	3	3	
	Rainy Experiment (RE)	3	3	

3.4 Experimental Setup

3.4.1 Experimental simulation

The experiments in this study simulated natural conditions to determine natural glyphosate leaching behavior. The soil columns were held with the supporting and stand in outdoor conditions throughout the 60-day monitoring periods to expose the soil to natural light, temperature, and humidity in order to mimic the seasonal effects on water flow and glyphosate leaching as shown in Figure 3.4.

The amounts of glyphosate, chemical fertilizers and water applied in this experiment were determined from the amounts applied in real situations based on both the application methodologies and the actual application rates.

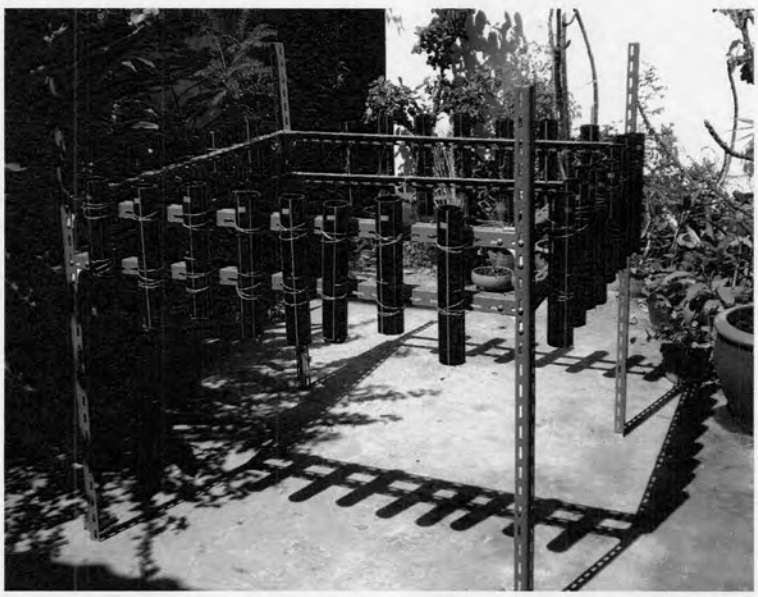

Figure 3.4. Experimental setup.

3.4.2 Leaching schematic diagram

Figure 3.5 shows the leaching schematic diagram for leachability test in this experiment.

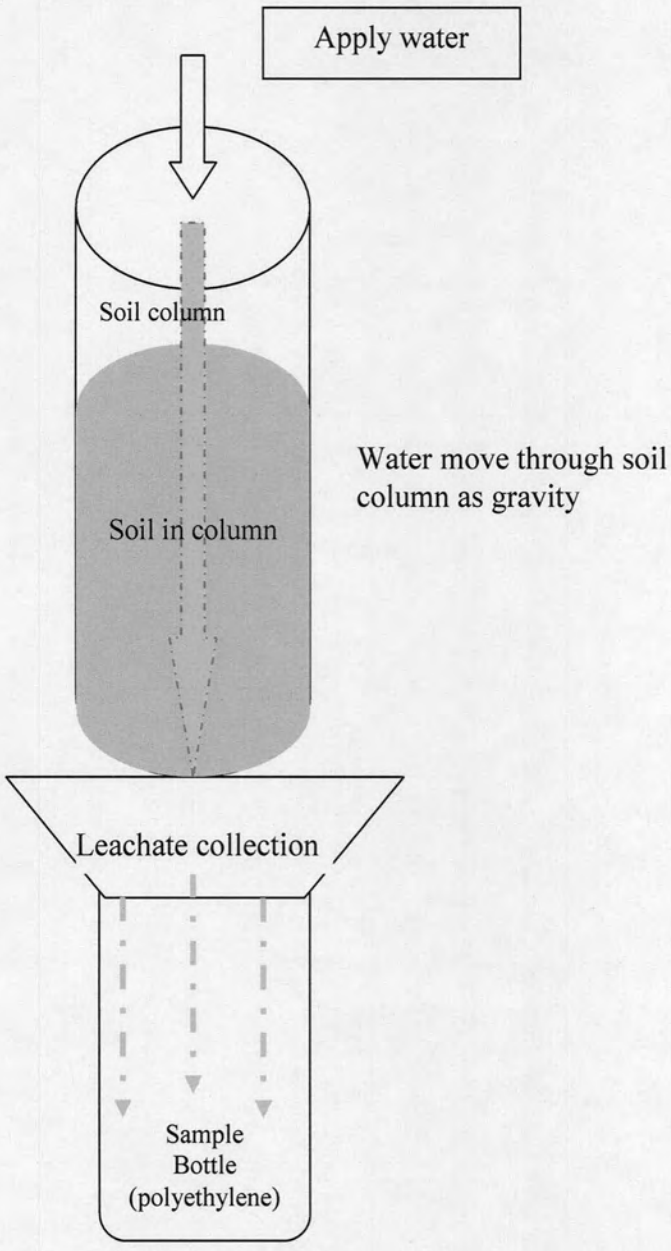

Figure 3.5. Leaching schematic diagram.

3.5 Experimental procedure

To clearly explain the experiment procedure, an experiment procedure flow chart was created as shown in Figure 3.6.

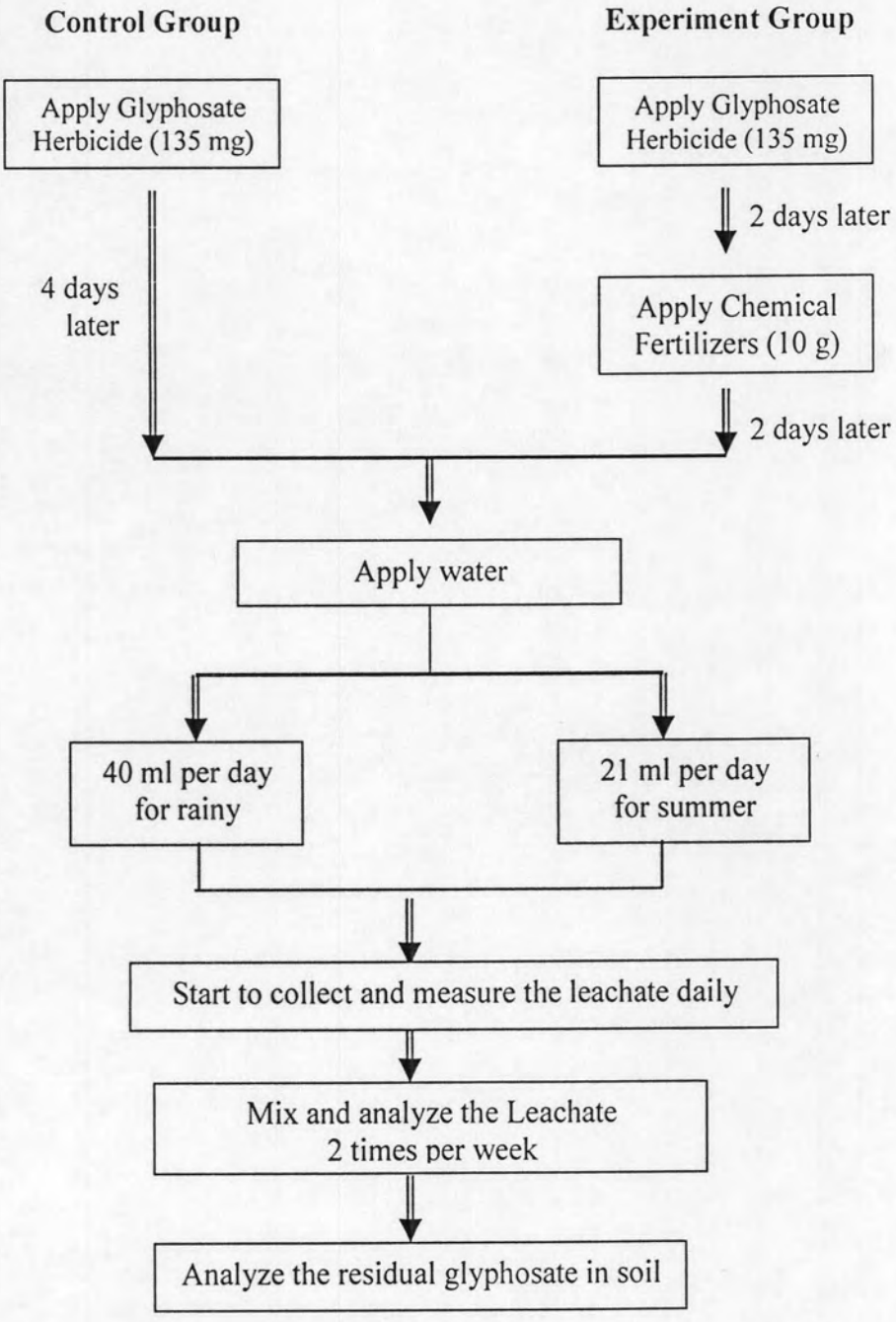

Figure 3.6. Experiment procedure flow chart.

3.5.1 Leachate sampling and preservation

The leachate concentrations from all groups (blanks, controls and experiments) were sampled and measured daily in the polyethylene bottles. Then, the leachates were stored at 4°C away from light and analyzed within a period of 2 weeks.

3.5.2 Extraction residual glyphosate and AMPA in the soil

After the monitoring period, the soil in the column was divided equally into 2 layers, the upper and lower soils, in order to extract the residue glyphosate. The divided soil from each column was mixed to obtain a homogenous mixture before extraction with 0.1 M of 25 ml potassium dihydrogen phosphate (KH_2PO_4) and centrifuged at 2500 rounds per minute by a centrifuge. The extracted solution was then separated. The extraction was replicated 3 times to get the final amount of the extracted solution of 75 ml. Then the extracted solution was analyzed for residue glyphosate by using the same method used to analyze the leachate. The recovery percentage is 80%.

3.6 Instruments and Analytical Method

3.6.1 Instruments

3.6.1.1 High Performance Liquid Chromatography (HPLC)

A module 1100 consists of automatic degasser, binary pump, autosampler, column thermostat, diode-array detector, and fluorescence detector (Agilent Technologies, Palo Alto, CA, USA).

3.6.1.2 Post-column derivatization instrument

A module PCX5200 consists of two post-column pumps, column thermostat, heated reactor, and ambient reactor (Pickering Laboratories, Inc., Mountain View, CA, USA).

3.6.2 Analytical method

Glyphosate and AMPA were analyzed according to EPA Standard Method 547, the determination of glyphosate in water by direct-aqueous-injection HPLC, post-column derivatization [21]. The chromatographic condition for analyzation of glyphosate and AMPA is shown in Table 3.6.

3.6.2.1 HPLC mobile phases

The mobile phases of HPLC used for glyphosate and AMPA analyses are as follows:

<u>Mobile phase A</u>: potassium dihydrogen phosphate (KH_2PO_4) buffer, pH 2 (used to re-equilibrate the glyphosate analytical column). The HPLC mobile phase A was prepared as follows:

- Weighed 1.00 g of potassium dihydrogen phosphate and dissolved with Milli-Q water 1,000.0 mL and then stored in a 1,000-mL solvent bottle.
- Added 4.70 mL of *o*-phosphoric acid (80%) into the same bottle using a 10.00 mL graduated pipette.
- Mixed the solvent completely.

Mobile phase B: potassium hydroxide (KOH) 0.3% (used to regenerate the glyphosate analytical column). The HPLC mobile phase B was prepared as follows:

- Weighed 1.50 g of potassium hydroxide in a 500-mL solvent bottle.
- Added 500 mL of Mill-Q water.
- Mixed the solvent completely.

3.6.2.2 Post-column derivatization reagents

The post column derivatization system had 2 reagents:

- Reagent 1: sodium hypochorite as the oxidizing reagent.
- Reagent 2: thioflour and *o*-phthaladehyde as OPA reagent.

a) The preparation of oxidizing reagent

- Weighed 0.950 g of potassium dihydrogen phosphate, 9.50 g of sodium chloride and 0.950 g of sodium hydroxide.
- Dissolved all of weighed chemical in 950.0 mL of Milli-Q water and stored in a 1,000-mL solvent bottle.
- Finally, added with 100.0 µL of 5% sodium hypochlorite solution.
- Mixed solution completely.

b) The preparation of OPA reagent

- Weighed 67.10 g of potassium tetraborate tetrahydrate, dissolved in 950.0 mL of Milli-Q water and stored in a 1,000-mL solvent bottle. This solution is called OPA diluent.
- Stored 5.0 mL of the OPA diluent in a 10.0-mL graduated cylinder for the following step.
- Weighed 0.1000 g of OPA, dissolved in 10.0 mL of methanol and stored in a 20-mL glass bottle.
- Added the OPA solution to the deoxygenated OPA diluent.
- Weighed 2.00 g of Thiofluor and dissolved in 5.0 mL of the OPA diluent in a 20-mL glass bottle and transferred into the OPA diluent.
- Mixed solution completely.

Table 3.7. The chromatographic conditions.

Parameter	Condition
Analytical column	Glyphosate column, K^+ form, 4.0 mm. ID × 150 mm
Mobile phase	A: KH_2PO_4 buffer, pH 2
	B: 0.3 % wt/v KOH
Gradient program	Time (min) % B
	0.0 0.0
	15.0 0.0
	17.0 100.0
	25.0 0.0
Flow rate	0.40 ml/min
Injection volume	5 µl
Column temperature	55 °C
Detector	Fluorescence:
	λ_{ex} = 330 nm, λ_{em} = 460 nm
Post-column	Reagent 1: sodium hypochorite (NaOCl)
	Pump1: 0.30 ml/min
	Reactor 1: operate at 36 °C
	Reagent 2: thioflour and OPA
	Pump 2: 0.30 ml/min
	Reactor2: operate at ambient temperature

3.6.3 The determinations of glyphosate and AMPA

After the sample was injected into the HPLC, glyphosate and AMPA were separated in the glyphosate cation exchange column. After glyphosate and AMPA were separated, it was sent into the post column to check for its fluorescence signal. In the post column, glyphosate was oxidized by hypochorite into glycine and then the glycine reacted with thioflour and OPA at pH 9-10 to produce a fluorescence signal. AMPA did not need to be oxidized because oxidation would reduce its fluorescence yield. Therefore, AMPA only reacted with thioflour and OPA. After that, both glyphosate and AMPA was passing through to the HPLC to detect their fluorescence activity with a fluorescence detector. The chromatogram of glyphosate and AMPA was shown in Figure 3.7

Limit of detection (LOD) of glyphosate determination by using HPLC with Post Column in this experiment is 0.2 ppm.

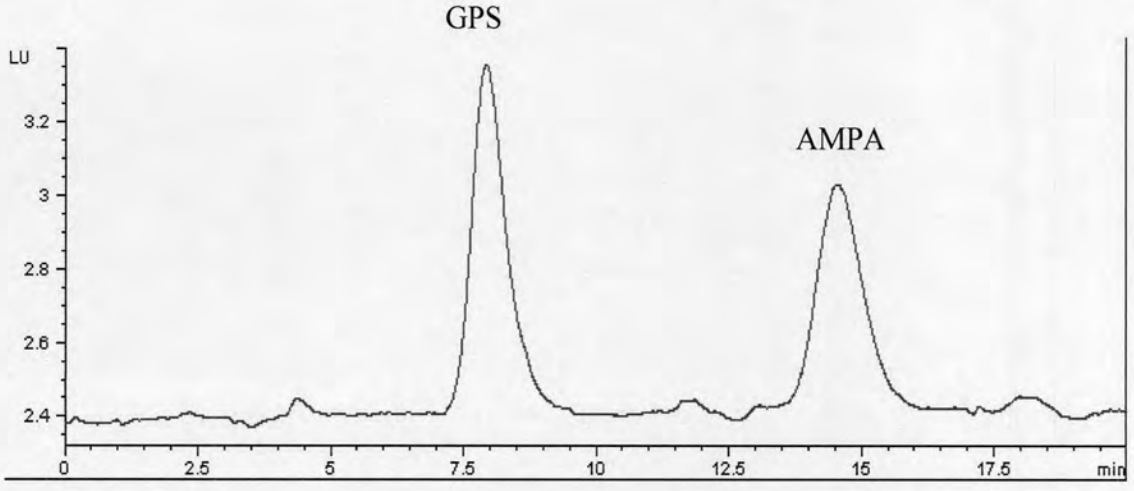

Figure 3.7. Chromatogram of glyphosate and AMPA.