OPTIMISATION OF HPLC-BASED METHODS FOR THE SEPARATION AND DETECTION OF HERBICIDE GLYPHOSATE AND ITS MAJOR METABOLITE IN WATER

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PREFACE

The following report describes the work that was carried at the Durban University of Technology, M L Sultan Campus, Department of Chemistry to investigate a suitable method for the separation and determination of levels for selected herbicides: glyphosate and AMPA, in dams and rivers around the Province of KwaZulu-Natal, South Africa.

DECLARATION

This dissertation is submitted in partial compliance with the requirements for the Masters Degree in Technology: Chemistry, in the Faculty of Applied Sciences at the Durban University of Technology

I, Lawrence Mzukisi Madikizela, do declare that this dissertation is representative of my own work. It has not been submitted before for any degree or examination in any other institution.

..... **……**

Signature of the student **Date of Signature**

APPROVED FOR FINAL SUBMISSION

..... **……**

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……

HOD: Prof. G. G. Redhi

DEDICATION

This dissertation is dedicated to the following people:

- To my deceased father, Mongezi Morris Madikizela who did everything he could to enable me to get a good education.
- My mother, Landiwe Nkwentsha, who brought me up under very difficult circumstances and supported me all the way through out this research.
- My aunt, Ntombentsha Nkwentsha for her support, encouragement throughout this work
- My late grandmother, Nomsa Madikizela who wanted me to get a very good education.

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Everyone involved in Eskom group at DUT for their input, helping hands, kindness and understanding.

Finally, my family and friends, for their patience, understanding, encouraging words and support throughout this work.

Without all of you I think I might have given up a long time ago, thank you.

ABSTRACT

Water storage dams play an important part in the collection and purification of water destined for human consumption. However, the nutrient rich silt in these dams promotes rapid growth of aquatic plants which tend to block out light and air. Glyphosate is universally used as the effective non-selective herbicide for the control of aquatic plants in rivers and dams. Invariably there is residual glyphosate present in water after spraying of dams and rivers with glyphosate herbicide. The amount of residual glyphosate is difficult to determine on account of high solubility of glyphosate in water. Thus a method of sample preparation and a sensitive HPLC method for the detection of trace amounts of glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) in water is required. A crucial step in sample preparation is pre-column derivitization of glyphosate with 9-fluorenylmethyl chloroformate (FMOC-Cl).

For sample pretreatment, water samples were derivatized with FMOC-Cl at pH 9, extracted with ethyl acetate and sample clean-up was carried out by passing a sample through the SPE cartridge. For SPE, recovery studies were done to choose a suitable cartridge for glyphosate and AMPA analysis. The following cartridges were compared, namely, C_{18}, Oasis HLB and Oasis MAX SPE cartridges. Best recoveries (101% for glyphosate and 90% for AMPA) were obtained using 500 mg of C_{18} solid-phase extraction cartridge. The eluent from SPE cartridge was injected into HPLC column. Three types of separation columns (namely; C_{18} column, silica based amino column and polymeric amino column) were compared for the separation of glyphosate and AMPA. The best separation of glyphosate and AMPA in water samples was achieved using a polymeric amino column and a mobile phase at pH 10 which contained a mixture of acetonitrile and 0.05 M phosphate buffer (pH 10) 55:45, (v/v) respectively.

The method was validated by spiking tap water , deionized water and river water at a level of 100 µg/l. Recoveries were in the range of 77% -111% for both analytes. The method was also used in determining the levels of glyphosate and AMPA in environmental samples. This method gave detection limits of 3.2 µg/l and 0.23 µg/l for glyphosate and AMPA respectively. The limits of quantification obtained for this method were 10.5 µg/l and 3.2 µg/l for glyphosate and AMPA respectively.

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CHAPTER 1

INTRODUCTION

1.1 GENERAL DISCUSSION OF HERBICIDES

Herbicides are chemicals that are used to kill unwanted plants. They are classified into two categories, namely, selective and non-selective herbicides [1].

- Selective herbicides are those herbicides that kill specific plants while leaving the desired crop relatively unharmed. Some of these herbicides act by interfering with the growth of the weed and are often based on plant hormones [1].
- Non-selective herbicides are herbicides that are used to kill all plants with which they come into contact and these are used mostly to clear waste grounds [1].

Long-term use of herbicides (as well as pesticides and fertilizers) leads to the pollution of soil and ground water.

1.1.1 Compounds selected for this study

The following compounds were considered for this study:

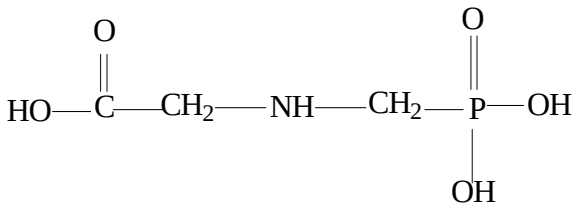
Common Name	**IUPAC Name**
(i) Glufosinate	Ammonium DL-homoalanin-4-(methyl) phosphinate
(ii) Glyphosate	*N*-(phosphonomethyl) glycine and
(iii) Aminomethylphosphinic acid	AMPA

Glyphosate and glufosinate are phosphorus containing amino acid-type herbicides. They are broad spectrum, non-selective, post-emergence herbicides. They are extensively used in various applications for weed control in aquatic systems and for vegetation control in non-crop areas [2, 3].

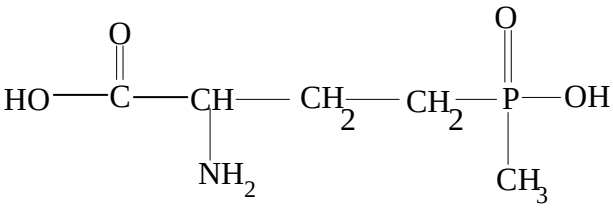
Aminomethylphosphonic acid (AMPA) is the major degradation product of glyphosate found in plants, water and soil; however AMPA can also originate from the degradation of phosphonic acids in detergents [3].

1.1.2 Structures of selected compounds

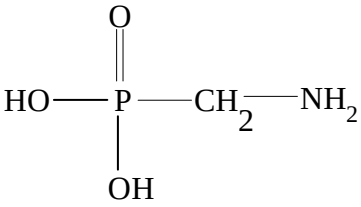
Glyphosate



Glufosinate



AMPA



1.2 Chemical properties of selected herbicides

Glyphosate and glufosinate are used in most countries. The regulatory bodies have set the maximum contaminant levels for these herbicides in water. The levels of herbicides in water have to be determined to ensure that water of appropriate quality is supplied for human consumption. These herbicides are the most difficult herbicides to analyse in various matrices such as water and soil compared to all the herbicides used in agriculture. It has been difficult

to obtain a simple method for the determination of these compounds at residue levels because of their chemical properties listed below.

- They lack chemical groups that could facilitate their detection [2]
- They are very polar compounds [2]
- They have low mass [3]
- They have low Volatility [3]
- They have an ionic character [3]
- The compounds have a high solubility in water (12 g/l and 13.7 g/l being the solubility of glyphosate and glufosinate, respectively at 25^0C) [4]
- They are insoluble in organic solvents [4]
- They show complexing behaviour [4] and
- They are amphoteric compounds [5]

Therefore a suitable method is needed for the analysis of these herbicides in water (dam and river water) that can accommodate the above properties. In this project the simplest method for the analysis of these herbicides is presented.

1.3 Historical account of the production of selected herbicides

1.3.1 Glyphosate

Glyphosate was first reported as a herbicide in 1971 and is now claimed to be the world's biggest selling herbicide by its manufacturer, Monsanto Company, situated in United States of America (USA) [6]. Glyphosate is known to be highly effective in killing weeds, not harmful to users and friendly to the members of the public and also to the environment. It is commonly known by the trade name of Roundup in South Africa. Some other trade names are used in different countries such as Spark in Sri Lanka and Thailand, Sunup in Indonesia, and Glycine in Korea [7].

1.3.2 Glufosinate

It is produced by AgrEvo, a joint venture established by the German chemical corporations Hoechst and Schering. The herbicide was first introduced into Japan in 1984. In the UK, glufosinate was first considered as an herbicide in 1984. It was not approved then (for

toxicological reasons) but was given provisional approval in 1991. US registration was achieved in 1993. The herbicide is now registered in more than forty countries and is marketed under a number of trade names including Basta, Rely, Finale, Ignite and Challenge [8]. The information about the usage of glufosinate in South Africa was not obtained.

1.4 Uses of Herbicides

Herbicides are used to control the growth of grasses, sedges, broadleaf weeds and woody plants. Herbicides are also used on various crops, orchards, plantations, pastures, lawns, gardens, forests, road verges, and water bodies. Other uses include pre-harvest desiccation of cotton, cereals, peas, and beans [7].

1.5 Sources of Herbicides in Water

These herbicides can be directly introduced into surface water during the spraying of water bodies such as dams and rivers to control aquatic weeds. Other sources are shown in the following diagram (Fig. 1). The diagram shows the areas that are suspected to be the major source of surface water contamination. The herbicides are manufactured in industries, residues of these herbicides can be carried to the surface water during the rainy season or residues can be carried to the waste water treatment plants, then water after treatment is sent to the surface water possible with trace amounts of herbicides. The herbicides used in agriculture and in road-sides can be carried to rivers due to water run-off.

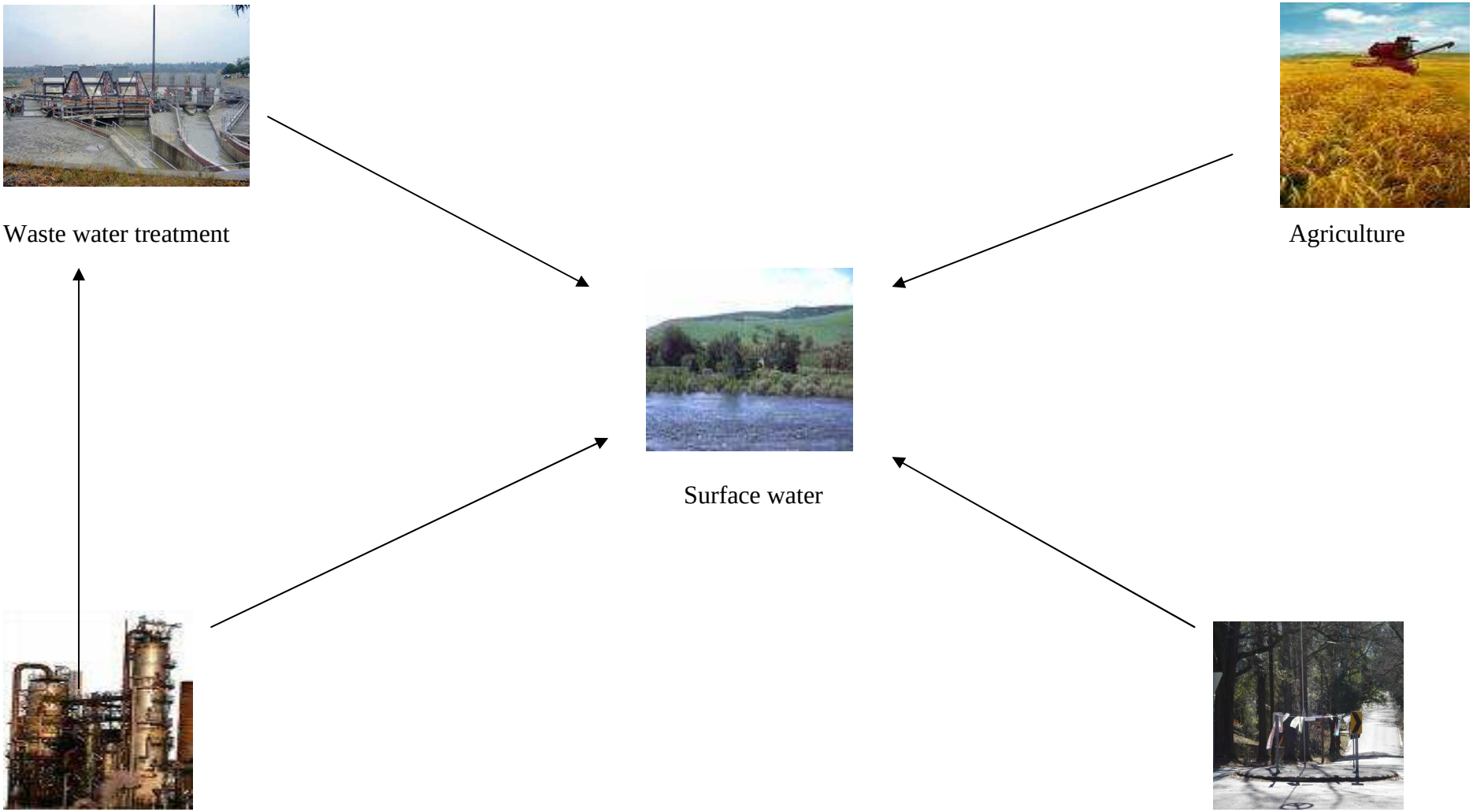


Figure 1: Sources of herbicides in water.

1.6 Glyphosate in Action

1.6.1 Action of Glyphosate in Plants

Glyphosate is effective in killing all plant types including grasses, perennials and woody plants. It works by being absorbed into the plant mainly through its leaves but also through stalk tissues. It is then transported to all other parts of the plant where it acts on various enzyme systems. It acts in plants by interfering with the enzyme called 5-enol-pyruvyl-shikimate-3-phosphate synthase that catalyses the sixth step in the shikimic acid pathway [6, 9, 10]. This shikimic acid pathway exists in higher plants and micro organisms but not in animals. Plants treated with glyphosate slowly die over a period of days or weeks (generally 1-3 weeks), and because the chemical is distributed throughout the plant, no part survives [4, 6]. The action of glyphosate in plants affects both photosynthesis and respiration process [4].

1.6.2 Glyphosate in the Environment

Glyphosate is inactivated when it comes into contact with soil since it is adsorbed onto soil particles. The mechanism of adsorption is not fully understood, but it is believed that glyphosate binds to soil in the same way as inorganic phosphates. Un-bound glyphosate is rapidly degraded (see Fig. 2) by microbial activity to carbon dioxide, phosphates and amino acids whereas bound glyphosate is degraded more slowly. In some instances glyphosate is not degraded but remains inactive in soil for several years [6]. Because of its adsorption to soil, glyphosate is not easily leached and is unlikely to contaminate ground water. However, it is used in water for the control of aquatic weeds, and it can be carried with eroded soil into surface waters where natural breakdown processes are much slower. Glyphosate has been detected in water but it is not continuously analysed due to the fact that it is difficult to isolate it and is not considered to be a major concern as a water contaminant [6]. Glyphosate has been found to inhibit anaerobic nitrogen fixation in soil [6].

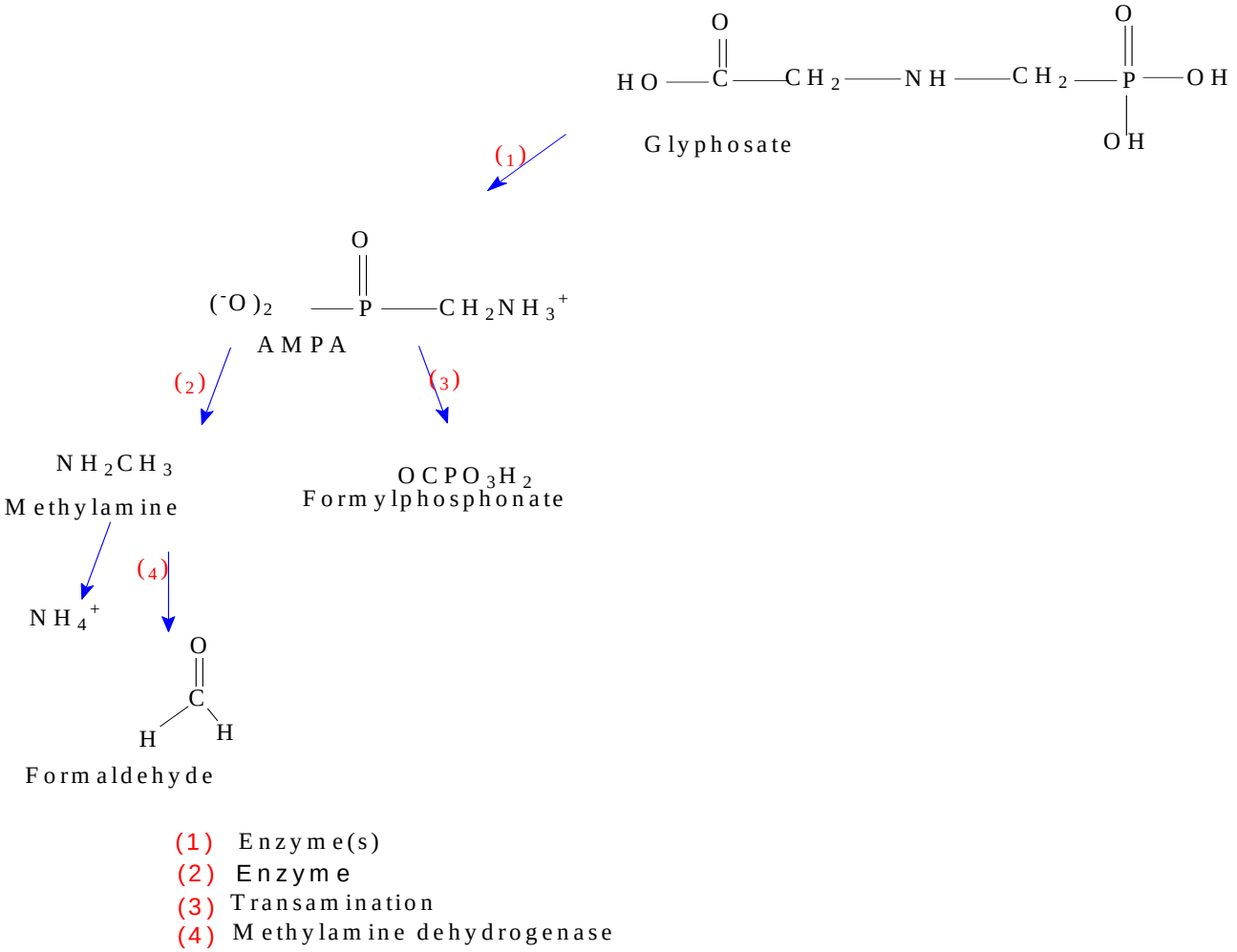


Figure 2: Degradation Pathway of glyphosate in soil [Schuette, 1998]

Glyphosate degrades more rapidly in soil than in water because of the large number of microbial organisms in soil compared to water. The primary metabolite of glyphosate is AMPA. The latter does also degrade to methyl amine and formylphosphonate (see Fig. 2), but the degradation is generally slower than that of glyphosate. This observation may be explained by postulating that AMPA is more strongly adsorbed by soil particles than glyphosate and that AMPA has a lower tendency to permeate the cell walls or membranes of micro organisms present in soil particles [11].

The rate for the degradation of glyphosate in water is slower compared to the rate of degradation in soil [6]. Glyphosate can be reduced in the environmental water by the use of chlorination reaction [12]. It is well known that water treatment plants are using disinfectants to kill or inactivate pathogenic microorganisms that occur in drinking water supplies. According to the study carried by Mehrsheikh et al [12], the chlorination of glyphosate converts glyphosate into other compounds such as carbon dioxide and methanediol. It was found that during the chlorination, the carboxylic acid carbon (C_1) of glyphosate is quantitavely converted into carbon dioxide. The methylene carbon (C_2) is converted into carbon dioxide and methanediol. In pH range of pH 6-9, carbon dioxide is the predominant product; and when pH is less than 6, the predominant product is methanediol [12]. Phosphonomethylene carbon (C_3) is converted into methanediol in all pH values, whereas, the nitrogen atom is converted into nitrogen gas and nitrate; and phosphorus produces phosphoric acid (the formation of these compounds is shown in Fig. 3) [12]. Chlorination process can be used by manufacturers of glyphosate or waste water treatment plants to stop glyphosate from polluting the environment, but glyphosate is widely used in rivers and dams to kill weeds.

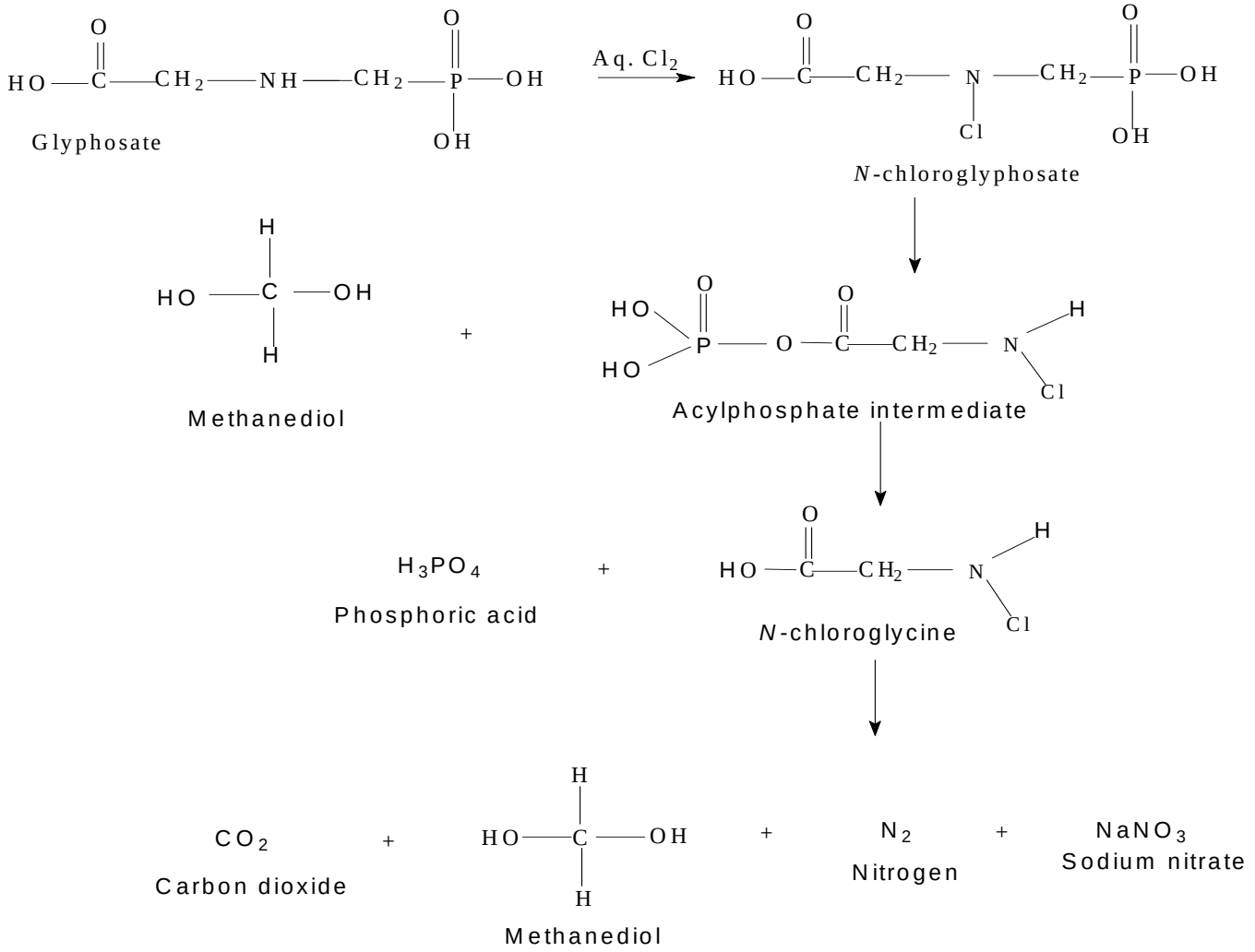


Figure 3: Degradation of glyphosate in the presence of chlorine [Mehrsheikh et al, 2006]

1.6.3 Toxicity of Glyphosate

The toxicity of glyphosate to mammals and birds is known to be relatively low. However, its broad spectrum of herbicidal activity has led to the destruction of habitats and food sources [6] for some birds and amphibians leading to population reductions. Fish and aquatic invertebrates are more sensitive to glyphosate and its formulations. Glyphosate's toxicity increases with higher water temperatures and higher pH values. Soil invertebrates as springtails, mites and isopods are also affected by glyphosate. This herbicide is also toxic to a range of bacteria, fungi, actinomycetes and yeasts. Although the toxicity of glyphosate is relatively low, a mixture of glyphosate with the surfactant polyoxyethylene amine (POEA), which is widely used, is more toxic [6].

1.7 Glufosinate in Action

1.7.1 Action of Glufosinate in Plants

Glufosinate is a naturally occurring compound which may be isolated from two species of Streptomyces fungi. It inhibits the activity of an enzyme known as glutamine synthetase in plants, which is necessary for the production of glutamine and for detoxification of ammonia [8, 9]. The presence of glufosinate in plants leads to reduced glutamine and increased ammonia levels in plant tissues. This causes photosynthesis to stop and the plant dies within few days (7 to 21 days). Respiration is also affected by the use of this herbicide [4]. Glufosinate also inhibits the same enzyme in animals [8].

1.7.2 Glufosinate in the Environment

The US Environmental Protection Agency (US EPA) classifies glufosinate as being persistent and mobile. Degradation is largely caused by microbial activity. The half life of glufosinate has been determined in laboratory studies and varies from 3 to 42 days in some studies [8] and up to 70 days in other studies [8]. The shortest half life tends to be in soils with a high clay and organic matter content. In sandy soils which overlie many aquifers, glufosinate has been found to be highly persistent due to the lack of biodegradation [8].

1.7.3 Toxicity of Glufosinate

Glufosinate is toxic to a number of aquatic animals including the larvae of clams and oysters, daphnia and some freshwater fish species. The commercial formulations are more toxic that the technical grade glufosinate [8].

There are other problems associated with the presence of organic chemicals in environmental water, regardless of the toxicity of these organic chemicals. There is a possibility that, low toxic compounds can contribute to the problems caused by organic compounds present in water in some organisations such as Eskom. Eskom power stations use raw water from dams to generate electricity. Eskom have ascertained that their raw water supplies contain high concentration of organics (approximately 5 mg/l) [13]. Although Eskom purifies their raw water, they do not have the guarantee that all the organics are removed during water purification. A call for the methods of analysis of organics in water has been made. It is necessary to be able to characterise qualitatively the total organic carbon and quantitatively its constituent compounds present in raw water in order to understand the behaviour of organic matter in the water treatment process. Therefore it was recognised that the presence of organic matter contributes to the impurity of the power plant cycle chemistry [13].

1.8 Levels of herbicides set by regulatory bodies

The highest level of a contaminant that is allowed in drinking water (the maximum contaminant level) of glyphosate for safe drinking water issued by the United States Environmental Protection Agency (US-EPA) is 0.7 µg/ml, and currently glyphosate is on the list of the US national primary drinking water contaminants [5, 14]. In Europe, the drinking water standard for any pesticide has been set at a level of 0.1 µg/l [15].

The maximum contaminant levels for these herbicides have also been set for food stuffs. These herbicides are also likely to be detected in food stuffs because genetically modified plants such as soybeans have been recently introduced into the market [16]. These herbicides are widely used to control weeds in areas where the genetically modified plants are planted. The following maximum residue limits has been set by Dutch regulation for glyphosate in food. For wheat and rye, barley and oats, mushrooms, linseed, rape seed and soybeans the limits are 5, 20, 50, 10, 10 and 20 mg/kg respectively [16]. This is due to the fact that these

herbicides are non-selective and they have low toxicity to mammals compared to other herbicides. In the European Union the maximum residue limits for the herbicide in food is set at the level of 0.1 mg/l (ppm) [17].

South Africa has regulations governing the maximum limits for pesticide residues that may be present in food stuffs. The maximum contaminant level for glyphosate in maize is set at 2.0 mg/kg. The maximum limit for both glyphosate and AMPA in sugar cane is 0.5 mg/kg [18].

To the best of my knowledge, the maximum contaminant levels of glufosinate have not been set, since this herbicide has not been used in South Africa.

CHAPTER 2

LITERATURE REVIEW

Glyphosate is widely used on a global scale. The low limits set by many countries for glyphosate, AMPA and glufosinate in matrices such as water; soil and food require very sensitive methods for the determination of these compounds residues in such matrices [3].

Several methods using a variety of analytical techniques for the separation and quantitation of glyphosate, AMPA and glufosinate in complex matrices have been reported. Among these methods employed by several researchers for the analysis of glyphosate, glufosinate and AMPA, include high performance liquid chromatography [3, 15, 16, 17, 19, 20, 21, 22, 23, 24, 25], gas chromatography [9, 26, 27], ion chromatography [5, 14, 28, 29] and capillary electrophoresis [4, 30, 31, 32, 33]. Some of these methods are reviewed in this chapter.

2.1 High Performance Liquid Chromatography (HPLC) Methods

HPLC is the method of choice for the analysis of glyphosate, AMPA and glufosinate because of the chemical properties mentioned in chapter 1. However the detection of these compounds with LC analysis is not very simple because they lack an inherent chromophore or fluorophore for the detection using the common detectors such as UV and fluorescence [2, 3]. For these reasons both pre-column and post-column derivatization procedures have been employed. The following procedures are generally used for the determination of glyphosate with HPLC:

(i) Pre-column derivatization with 9-fluorenylmethyl chloroformate and detection using fluorescence detector [16, 22, 24, 34, 35].

(ii) Post-column derivatization with o-phthalaldehyde and detection using fluorescence detector [19, 20, 36].

(iii) Pre-column derivatization with *p*-toluenesulphonyl chloride and detection using UV detector [17].

(iv) Pre-column derivatization with 9-fluorenylmethyl chloroformate using HPLC with UV detection [15].

(v) Pre-column derivatization with 9-fluorenylmethyl chloroformate and detection using mass spectrometry detector [3, 23, 25].

A brief review of the above HPLC procedures is presented in details below. These procedures differ in sample preparation methods; they also differ in a way that they use different amounts and different concentrations of derivatizing reagents. And as seen above three HPLC detection methods have been used for glyphosate analysis. These detection methods are fluorescence detection, UV detection and mass spectrometry detection.

2.1.1 Pre-column derivatization methods

For pre-column derivatization, 9-fluorenylmethylchloroformate (FMOC-Cl) is employed for various reasons, namely,

(i) To form fluorescent derivatives
(ii) To improve detection of the analytes and
(iii) To reduce the polar character of the analytes thus improving the analytes chromatographic retention [3].

The disadvantage of pre-column derivatization reaction is that FMOC-Cl reacts with the analyte to form FMOC-analyte and FMOC-OH in the presence of water. FMOC-OH is a highly fluorescing product, represented by a large peak in the chromatogram. The FMOC-OH peak often masks with the analyte peak and creates difficulties in analyte detection [24]. Several methods have been reported to remove the FMOC-OH; namely, column coupling technique and the use of organic solvents (solvent extraction) to extract FMOC-OH. The details of these two techniques are given in the following section.

2.1.1.1 Fluorescence Detection Methods

Miles et al [35] have developed an analytical method for the determination of glyphosate and AMPA in natural waters, namely, river water, rain water and lake water. The preparation of samples was carried out by adding potassium hydrogen phosphate to the samples. The samples were spiked and pre-concentrated by the use of rotary evaporation. Samples were then derivatized with borate buffer (pH 9), acetone and FMOC-Cl for 20 minutes at room temperature. Excess FMOC reagent and FMOC-OH were extracted using ethyl ether. Samples were then injected into an HPLC system; the analytes were then separated in an amino column using a mobile phase containing 75% (v/v) mixture of potassium hydrogen

phosphate (pH 6) in acetonitrile delivered at 1.0 ml/min. Recovery studies were performed with samples spiked with 0.01 mg/l and 0.05 mg/l of both glyphosate and AMPA. Percent recoveries were found to be in the range of 80 to 111%. The detection limits obtained when using this method were 10 µg/l for glyphosate and 5 µg/l for AMPA.

An approach that uses solvent extraction for the removal of FMOC-OH was reported by Nedelkoska et al [24]. They utilised ethyl acetate to remove FMOC-OH and used HPLC to determine the amount of glyphosate in water and plant material after pre-column derivatization with FMOC-Cl using single and coupled polymeric amino columns, respectively. Derivatization of samples was done by adding FMOC-Cl solution and borate buffer (pH 9) into the water sample. The derivatization reaction was maintained at room temperature for 30 minutes. After derivatization of water samples the excess amount of FMOC-Cl and FMOC-OH were removed by extracting the reaction mixture three times with ethyl acetate before the injection of the aqueous layer into the HPLC column. The separation of glyphosate and AMPA in water samples was achieved using a single polymeric amino column and mobile phase (at pH 10) which contained 55% (v/v) acetonitrile and 50 mM phosphate buffer. Rainwater samples spiked with glyphosate or AMPA at levels of 1 and 0.5 µg/l, respectively were used for validation of the method. The average recovery was 94% with relative standard deviation of 5.4% and the method detection limit of 0.16 µg/l.

The HPLC analysis of glyphosate was also done in plant material by Nedelkoska et al [24]. Two polymeric amino columns were coupled via a switching valve to separate interfering products from the analyte. The switching valve procedure with the coupled columns eliminated the large interfering species from the vegetation sample extracts thus enabling the detection of the glyphosate peak. This method was validated using grass samples spiked at the level of 1 mg/kg and gave a detection limit of 0.3 mg/kg for glyphosate and a recovery of 82.4% with relative standard deviation of 10.3%.

Hogendoorn et al [16] reported the use of a column coupling technique for the determination of glyphosate in cereal samples by means of pre-column derivatization with FMOC-Cl and detection with a fluorescence detector. Their study involved the overnight extraction of homogenized cereal sample with water, centrifugation of the sample, separation of the liquid from the cereal powder, passage of the clear layer through a C_{18} solid-phase extraction cartridge used for clean-up and collection of the eluent into a calibrated tube. Before sample

loading the solid phase extraction (SPE) cartridge was conditioned with acetonitrile, HPLC-grade water and standard or sample. Then a volume of sample or standard was passed through the SPE cartridge and collected. The collected eluent was derivatized with borate buffer (pH 9) and FMOC-Cl solution. This reaction was carried at room temperature for 15 minutes. The solution was then diluted 16 times with borate buffer (pH 9) prior to HPLC analysis. The latter involved a coupled column liquid chromatography with a fluorescence detector. A mixture of acetonitrile-0.05 M phosphate buffer (35:65, v/v), pH 5.5, was used as the mobile phase on a 30 x 4.6 mm I.D. column packed with 5 µm Hypersil ODS. This was coupled with a 5 µm Adsorbosphere NH_2 column. This method gave a limit of quantification of 0.5 mg/kg. The method was validated by analysing spiked cereal samples. The overall recovery of spiked samples was 86% (n = 10) with a repeatability of 6.5% and a reproducibility of 9.5%. The method was found to be effective for grain flour samples with aged residues stored for a period of 150 days. A throughput of at least 25 samples per day was obtained.

Sancho et al [34] investigated the use of coupled-column liquid chromatography for rapid determination of glufosinate in environmental water samples using 9-fluorenyl-methoxycarbonyl pre-column derivatization and large volume injection of the samples. Their method involved a five-fold pre-concentration of the sample where the water sample was evaporated to dryness with a rotary evaporator using a water bath temperature of 40^0C. Derivatization reaction was carried out by adding together an environmental water sample, borate buffer (pH 9) and FMOC-Cl in acetonitrile. The mixture was swirled and the reaction was allowed to take place at room temperature for 30 minutes. Thereafter, borate buffer (pH 9) was added with swirling to dilute the reaction mixture. A large volume injection (2 ml) was performed into a Nucleosil C_{18} column. LC analysis was performed using a mobile phase containing a mixture of acetonitrile-0.05 M phosphate buffer (pH 5.5) (35:65, v/v). The flow rate was 1 ml/min. This mobile phase was used to rinse the Nucleosil C_{18} column. The columns coupled were 30 x 4.6 mm I.D. separating column packed with 5 µm Nucleosil C_{18} and 250 x 4.6 mm I.D. column packed with 5 µm Adsorbosphere NH_2. Adsorbosphere NH_2 column was kept at 30^0C. The fraction of sample that has the analyte from Nucleosil C_{18} column was transferred to Adsorbosphere NH_2 column by mobile phase. The mobile phase containing a mixture of acetonitrile-0.1 M phosphate buffer (pH 5.5) (35:65, v/v) was used in Adsorbosphere NH_2 column. The flow rate was 1 ml/min. The method can be used for the determination of glufosinate in environmental water samples down to a level of 0.25 µg/l. If

necessary the detection limit can be lowered to 0.1 µg/l by using a rotary evaporator to pre-concentrate samples. The method has a sample throughput of about 50 samples per day. The method was tested by spiking drinking, ground and surface water samples at levels between 0.5 and 5.0 µg/l. The average recoveries were reported to be between 90 and 105% (n = 5 for each sample and spiked level) with relative standard deviations between 1 and 5%.

Sancho et al [22] have also investigated the use of coupled-column liquid chromatography for the determination of glufosinate, glyphosate and AMPA in environmental water samples using pre-column fluorogenic labelling. In their investigation the methodology had two approaches: a multi-residue method allowing the simultaneous determination of the three analytes in environmental water samples to a level of 1 µg/l and a single residue method that focused on the analysis of a single analyte to the sub-µg/l level. The two approaches involved a pre-column derivatization step with FMOC-Cl yielding highly fluorescent derivatives of the analytes which were then determined by coupled-column LC with fluorescent detection.

- Sample preparation in multi-residue analysis method: A water sample was derivatized with borate buffer (pH 9) and FMOC-Cl solution prepared in acetonitrile. The mixture was allowed to stand for 30 minutes at room temperature. Thereafter it was diluted with borate buffer (pH 9) and a large volume injection of the derivatized sample into a C_{18} column was performed.
- Sample preparation for a single-residue analysis method was the same: The only difference was the concentration of FMOC-Cl used (0.1 mg/ml of FMOC-Cl in acetonitrile was used instead of 1 mg/ml FMOC-Cl in acetonitrile).

In LC analysis, a mixture of acetonitrile and 0.05 M phosphate buffer (pH 5.5) (35:65, v/v) was used as the mobile phase. Reversed-phase C_{18} column (C-1) was coupled to a weak ion-exchange column (Adsorbosphere NH_2) (C-2). The separation in the first column was used to achieve sensitivity. By injecting large volume of samples, and using automated sample clean-up, the less polar interferences were removed, including the excess of FMOC-OH. The analytes were recovered from water samples at 0.50-10 µg/l fortification levels, with a sample throughput of at least 40 samples per day. However at pH 5.5 the LC lacks the stability of retention times for the analytes. Surface water samples spiked with 0.4 µg/l of each analyte were used to test the single residue method. The recoveries and relative standard deviations obtained for glyphosate, AMPA and glufosinate were 107% (RSD of 5%), 95% (RSD of 11%) and 92% (RSD of 10%), respectively.

The use of coupled-column liquid chromatographic method with pre-concentration procedure for the determination of glyphosate and AMPA residues in environmental waters was investigated by Hidalgo et al [37]. Pre-concentration was done on an Amberlite IRA-900 resin. When water samples were passed through the resin, analytes were trapped in the resin, which was then washed with HPLC-grade water. Analytes were then eluted with sodium chloride. The eluted analytes were derivatized using the borate buffer (pH 9) and FMOC-Cl reagent at room temperature for 30 minutes. The derivatized sample was then diluted with borate buffer (pH 9) prior to large volume (2 ml) injection into LC-LC-FD. For column coupling, a short (3 cm) C_{18} and Hypersil APS (amino column) columns were used. The mobile phase in both columns was a mixture of acetonitrile and 0.05 M phosphate buffer (pH 5.5) (35:65, v/v). The LOQ for the method was 0.1 µg/l. This LOQ was improved by the application of pre-concentration procedure on an Amberlite resin to 0.02 µg/l. The method was validated by spiking drinking, surface and ground water samples with glyphosate and AMPA, these samples were analysed without the pre-concentration step. Recoveries were 87-106% with relative standard deviations being lower than 8%. Drinking and ground water samples were spiked with glyphosate and analysed after pre-concentration on the resin. Recoveries obtained were ranging between 94% and 105%.

2.1.1.2 UV Detection Method

This method of detection is not widely used in glyphosate analysis because of its poor sensitivity compared to fluorescence detection method; hence little work has been done using this detection method. To achieve a high sensitivity (when using a UV detection method), a sample pre-concentration procedure is often required.

Khrolenko et al [17] investigated the use of HPLC with UV detector for the determination of glyphosate and AMPA in fruit juices using supported-liquid membrane pre-concentration method after derivatization with *p*-toluenesulphonyl chloride. In their work, supported-liquid membrane technique was used for the extraction of glyphosate and AMPA from fruit juices such as orange, grapefruit, apple and blackcurrant. As part of method development, water samples were spiked with both glyphosate and AMPA. The pH was adjusted to pH 11 with sodium hydroxide. A sample solution was pumped through the donor channel of the supported-liquid membrane. The acceptor phase consisting of sodium chloride or

hydrochloric acid was circulated in the acceptor channel with the same flow-rate as the donor phase during the extraction. After the extraction, the acceptor phase was neutralised to pH 7 with concentrated potassium hydroxide solution and then derivatized. Juice samples were spiked with 1 mg/ml of glyphosate and AMPA and centrifuged to remove solid particles. The samples were then filtered and treated using the same procedure as for water samples. Derivatization reaction was carried out by adding together the acceptor phase, phosphate buffer (pH 11) and *p*-toluenesulphonyl chloride solution (prepared in acetonitrile). This solution was then heated in a water bath at a temperature of 50^0C. The resulting solution was injected into an HPLC column. A C_{18} column was used for the separation of compounds. The mobile phase was a mixture of 0.06 M KH_2PO_4 buffer (adjusted to pH 2.3 with H_3PO_4) and acetonitrile (85:15, v/v). Elution was monitored at a wavelength of 240 nm. This method allowed the detection of both analytes in fruit juices at concentrations as low as 0.025 mg/l. The recoveries for glyphosate were 71.1, 72.1, 93.6 and 102.7% and for AMPA were 64.1, 64.6, 81.7, and 89.2% for orange, grapefruit, apple and blackcurrant juices, respectively.

Peruzzo et al [15] have investigated the use of UV detection with HPLC for the determination of glyphosate in surface waters, sediments and soil after pre-column derivatization with FMOC-Cl. For sample preparation, the extraction in soil and sediment samples was carried out by adding potassium dihydrogen phosphate solution into the samples followed by centrifugation. The extraction was repeated twice on solid residues, and then the extracts were filtered. The derivatization procedure for all the samples (water, soil and sediments) was the same. Derivatization was carried out by adding borate buffer (pH 9) and FMOC-Cl solution prepared in chloroform into the sample. The reaction was carried out for 24 hours at 40^0C in the dark. After 24 hours the reaction was stopped by the addition of phosphoric acid into reaction vessel and the derivatized glyphosate was injected into the HPLC. C_{18} column was used for the separation, with a mobile phase containing a mixture of 0.05 M phosphate buffer (pH 5.5): acetonitrile (65:35, v/v). The flow rate was set at 0.8 ml/min and the detection was done at 206 nm. The levels of glyphosate in surface waters ranged from 0.1 to 0.7 mg/l, while in sediments and soil the levels were between 0.5 and 5.0 mg/kg. The method gave a limit of quantitation (LOQ) of 0.1 mg/l and limit of detection (LOD) of 0.04 mg/l for water samples, and LOQ of 0.25 mg/kg and LOD of 0.1 mg/kg for sediment and soil samples. Recoveries of 86% were obtained for glyphosate in water.

2.1.1.3 MS Detection Methods

Ibanez et al [3] studied the use of liquid chromatography coupled to electrospray tandem mass spectrometry (LC-ESI-MS/MS) for the selective determination of glyphosate, glufosinate and AMPA residues in water and soil samples.

- Water samples were collected in plastic bottles and stored in a freezer before the analysis. Derivatization was carried out by adding to a water sample, an isotope labelled glyphosate (internal standard), borate buffer (pH 9) and FMOC-Cl reagent. The reaction was carried out overnight at room temperature. The samples were acidified with hydrochloric acid to pH 1.5. The acidified derivatized samples were injected directly into an on-line solid phase extraction (SPE)-LC-ESI-MS/MS system using an Oasis HLB cartridge column for SPE and a Discovery chromatographic column (C_{18}), for separation of analytes.
- Soil samples were extracted with potassium hydroxide. The extracts were 10-fold diluted with water and derivatized as water samples. The derivatized samples were injected into the LC-MS/MS system without pre-concentration via an a SPE cartridge.

The Oasis HLB SPE cartridges were conditioned with LC-grade water at pH 2.5. The method was validated through spiking and recovery studies. Water samples were spiked at 50 and 500 ng/l, and the soil samples were spiked at 0.05 and 0.5 mg/kg. In water samples, the mean recoveries were ranging from 89 to 106% for glyphosate (RSD < 9%), from 97 to 116% for AMPA (RSD < 10%), and from 72 to 88% for glufosinate (RSD < 12%). In soil samples, the mean recoveries were ranging from 90 to 92% for glyphosate (RSD < 7%), from 88 to 89% for AMPA (RSD < 5%), and from 83 to 86% for glufosinate (RSD < 6%). The detection limits for this method were as low as 5 ng/l in water and 5 µg/kg in soil.

Vreeken et al [25] developed a fully automated on-line solid-phase extraction-high performance liquid chromatography-electrospray ionization mass spectrometry (SPE-HPLC-ESI-MS-MS) method for the determination of glyphosate and AMPA in water. In their investigation, water samples were collected in polypropylene bottles and stored in a freezer at -20^0C before the analysis. Derivatization was carried out by adding FMOC-Cl solution to the sample together with borate buffer and the reaction was allowed to take place overnight at 37^0C. The reaction was stopped by adding phosphoric acid. The derivatized sample was passed over PLRP-s pre-concentration column then to the HPLC column and finally to an MS

for analysis. For HPLC, aqueous ammonium acetate (5 mM)-acetonitrile mobile phase and a 250 x 4.6 mm Inertsil column were used. Gradient elution was used in this investigation. The detection limits for both analytes were as low as 0.03 µg/l. Recoveries of 96% and relative standard deviations for repeatability of less than 8.4% were obtained. The method was developed for the drinking and surface waters but it can also be used for the determination of glyphosate and AMPA in waste water.

Ghanem et al [23] have developed an LC-MS method for the determination of glyphosate and AMPA in sewage sludge. For sample preparation, analytes were extracted with sodium hydroxide from a sludge sample and purified on a strong anion-exchange resin (AG-1 x 8 SAX). AG-1 x 8 SAX resin was packed in a 300 mm x 10 mm column using ultra pure water (pH 9). Sewage sludge sample was percolated through the resin and water was used for rinsing the resin. For derivatization, the resin was conditioned with a mixture containing 25 mM borate buffer (pH 9.2) / acetonitrile (30:70, v/v). Then the same mixture plus 20 mg of FMOC-Cl was added to the resin. Trapped analytes and FMOC-Cl were allowed to react for 10 minutes. The resin was reconditioned with a mixture containing borate buffer (pH 9.2 / acetonitrile (50:50, v/v). Then another FMOC-Cl in mixture was loaded to complete derivatization. The analytes were then eluted with a mixture containing sodium chloride / acetonitrile (70:30, v/v) at pH 5 and recovered into water at pH 3. The pH of the sample was then adjusted to pH 3 using hydrochloric acid. The sample was then pre-concentrated using an Oasis HLB SPE cartridge. Prior to the pre-concentration step, the Oasis HLB cartridge was conditioned with methanol followed by water and phosphate buffer (pH 3). The sample was then loaded, followed by rinsing of the cartridge with water which was dried under nitrogen. The analytes were eluted from the cartridge with methanol and concentrated under nitrogen prior LC analysis. For LC-MS/MS determination, the compounds were separated on a Discovery HS C_{18} column with a mobile phase consisting of 5 mM ammonium acetate buffer and acetonitrile at a flow rate of 0.15 ml/min at 24^0C. A gradient from 20% to 100% of acetonitrile was used within 12 minutes, and the final composition was maintained for 4 minutes. This method [23] was applied in the monitoring of sewage sludge contamination for a period of 1 year in France. The method gave the LODs of 20 and 30 µg/l, for glyphosate and AMPA respectively.

2.1.2 Post-column derivatization methods

In post-column derivatization, the analyte is normally derivatized with *o-phthalaldehyde* (OPA) and mercaptoethanol [3, 24] or with OPA and *N,N*-dimethyl-2-mercaptoethylamine [3]. Literature indicates that post-column derivatization procedure has been used for the determination of glyphosate in water. It has been approved by the US EPA [24]. This method has also been recommended for the determination of glyphosate in foodstuffs and the method is currently used in USA as an EPA method for the determination of glyphosate in drinking water [24]. A disadvantage of using this derivatization procedure is that OPA reagent can only react with a primary amine. Glyphosate being a secondary amine requires a hydrolysis step prior to the post-column OPA reaction [24]. As a consequence of this, more instrumentation is required to effect the determination of glyphosate and AMPA in water. Three studies using OPA as a post-column derivatization reagent are summarised below.

Abdullah et al [19] has determined glyphosate and AMPA in water using HPLC with post-column derivatization and a fluorescence detector. Water samples (after filtration) were extracted with dichloromethane to remove organic compounds. The aqueous phase which contained analytes was concentrated by rotary evaporation and the pH was adjusted to 10 with sodium hydroxide. The sample was passed through a Supelclean cartridge with an additional of Bio-Rad SAX resin in the hydroxide form packed on top of the Supelclean packing. The cartridge was then washed with deionised water to remove interferences and the analytes were eluted with sodium citrate buffer at pH 5.0. The analytes were determined by HPLC using a cation-exchange column. During the HPLC analysis, glyphosate was oxidized with calcium hypochlorite in the post-column reactor coil at 48^0C to form glycine. Glycine was reacted with OPA in the presence of mercaptoethanol (MERC) in the second coil to form a fluorophore at ambient temperature and was detected with a fluorescence detector. A switching valve was used to cause AMPA to by-pass the calcium hypochlorite and undergo reaction with the OPA-MERC reagent. When a switching valve was not used, it was observed that the calcium hypochlorite solution resulted in a decrease of the AMPA signal. The method detection limits were less than 2 µg/l and the average recoveries were >85% for both compounds.

An investigation based on analysis and degradation study of glyphosate and AMPA in natural waters by means of polymeric and anion-exchange solid-phase extraction columns followed

by ion-chromatography-post-column derivatization with fluorescence detection was conducted by Mallat et al [20]. In their study, the water sample was pre-concentrated by a two step procedure: In the first step, sample was percolated through a polymeric cartridge then through an anion-exchange column mechanism. For the first step, a Lichrolut EN SPE cartridge was used; it was conditioned with methanol followed by HPLC-grade water. The sample was percolated through the cartridge and collected. For the second step, a strong anion-exchange column was packed with Amberlite IRA 410, in the hydroxide form. Anion exchange column was conditioned with water followed by the percolation of water sample through the cartridge and elution with sodium citrate solution. The analytes were analysed by ion-exchange chromatography using a cation-exchange column and a mobile phase of potassium dihydrogen phosphate followed by post-column reaction with OPA followed by fluorimetric detection. This method gave them detection limits of 2 µg/l for glyphosate and 4 µg/l for AMPA. In a degradation study, Mallat et al [20] found that the degradation is affected by the microbial activity, temperature and photolysis.

The investigation carried out by Wigfield et al [36] was based on liquid chromatographic determination of glyphosate and AMPA residues in environmental water using post-column fluorogenic labelling. Environmental water samples were filtered and washed with methylene chloride. The organic layer was discarded and the aqueous layer was transferred to the anion-exchange column. The analytes were eluted from the column by gravity with potassium citrate solution (pH 5) and injected into the LC column. Ion-exchange column chromatography was coupled with post-column ninhydrin derivatization and absorbance detection at 570 nm. The validation of the method was carried out by spiking environmental water samples with glyphosate and AMPA at the levels of 6-60 µg/l (glyphosate) and 1-10 µg/l (AMPA). The average recoveries were ranging from 84% to 89%. The detection limits were 1.04 µg/l and 0.39 µg/l for glyphosate and AMPA, respectively.

2.2 Gas Chromatography (GC) Methods

Because of the properties of glyphosate, glufosinate and AMPA, GC is not widely used for the analysis of these compounds. When GC is used, it is performed after an extensive derivatization procedure that converts the analytes into sufficiently volatile and thermally stable derivatives [17]. The polar nature and high water solubility of both glyphosate and glufosinate limit the possibility of using the standard derivatization techniques generally

employed in GC analysis [26]. Therefore the derivatization involves the use of trifluoroacetic anhydride (TFAA) and trifluoroethanol, TFAA and diazomethane or heptafluorobutyric anhydride (HFBA) and 2-chloroethanol. The use of mixtures of fluorinated anhydrides and perfluorinated alcohols, such as TFAA and heptafluorobutanol, give derivatives of glyphosate that can be detected by GC-MS with high sensitivity and selectivity [26].

Kataoka et al [9] have developed a method that is based on the determination of glyphosate, glufosinate and AMPA in river water, soil and carrot samples by gas chromatography with flame photometric detection. 4-aminobutylphosphonic acid (ABP) was used as an internal standard, during the gas chromatographic analysis. Samples were prepared as follows:

- Water Samples: The samples were filtered and derivatized for GC analysis.
- Soil Samples: The internal standard was added to the soil sample and the analytes were extracted three times with sodium hydroxide followed by centrifugation. The supernatants were combined and made up to volume with distilled water before the derivatization prior to GC analysis.
- Carrots Samples: The internal standard was added to the carrot sample. The sample was homogenized in water, centrifuged and the precipitate was re-extracted two times with distilled water. The supernatants were combined and made up to volume with distilled water before the derivatization prior to GC analysis.

After the analytes have been extracted from water, soil, and carrot samples, they were derivatized into their *N*-isopropoxycarbonyl methyl esters using isopropyl chloroformate and diazomethane at room temperature. The derivatives were sufficiently volatile and stable within 20 minutes. The solvents were evaporated to dryness and the residue was dissolved in ethyl acetate and injected into the GC column. The column used in GC was a fused silica capillary column. The detection limits were 8, 12, 20 μg/l for AMPA, glyphosate and glufosinate respectively. The recoveries of the compounds in all samples were in the range of 91-106%.

Borjesson et al [27] have developed a method for the determination of glyphosate and AMPA in ground water and soil using a GC-MS.

- Treatment of ground water samples: pH of Ground water samples was adjusted to pH 2. The acidified ground water sample was applied on the Chelex column. The column was washed with water followed by diluted hydrochloric acid. The analytes were eluted from

the Chelex column with concentrated hydrochloric acid (HCl). The analytes were then passed through an AG 1-X8 column. AG 1-X8 column was prepared by adding an AG 1-X8 gel, chlorine form, into a polypropylene tube. After the gel has settled inside the tube, portions of both dilute and concentrated HCl were added and passed through the column by gravity. The analytes were eluted from the AG 1-X8 column with HCl. The sample was then evaporated to dryness; the residue was dissolved in a mixture of water-methanol-HCl and derivatized.

- Treatment of soil samples: Soil samples were extracted with sodium hydroxide and centrifuged before filtration. The pH of the sample was adjusted to 2.0 and treated identically to the water samples.

Samples were derivatized with trifluoroacetic anhydride and trifluoroethanol at 100^0C for one hour. Samples were acclimatised to room temperature, evaporated and re-dissolved in ethyl acetate prior to GC-MS analysis. The analytes were separated on a fused silica capillary column and MS was used for the detection. The limit of detection was 0.05 µg/l in ground water and 0.003 µg/g in soil for both glyphosate and AMPA. The limit of quantification was 0.1 µg/l for ground water and 0.006 µg/g for soil.

2.3 Ion Chromatography (IC) Methods

This is a technique introduced in the mid-1970's and it has been a useful tool for detecting ionic substances, and the hydrophilic substances (e.g. glyphosate) [28]. Very few detection methods for glyphosate and AMPA without derivatization have been reported.

You et al [5] have reported the use of ion chromatography with condensation nucleation light scattering detection (IC-CNLSD) method for the direct analysis of glyphosate and AMPA in water samples without the need for sample pre-treatment and derivatization. The environmental water samples were collected, filtered through 0.45 µm filters and injected directly into the cation-exchange column. Glyphosate and AMPA were separated using two 100 x 4.6 mm I.D. cation-exchange columns packed with polybutadiene-maleic acid-coated silica (µm) connected in series. The separation was performed at room temperature with nitric acid as the eluent at the flow rate of 0.5 ml/min. This method (IC-CNLSD) gave a limit of detection of 41 µg/l for glyphosate and 53 µg/l for AMPA. An application of the method was conducted on tap water and lake water, but both analytes were not detected.

Zhu et al [28] have developed a suppressed conductivity ion chromatography method for the analysis of glyphosate in water. The water samples were filtered and extracted with dichloromethane to remove organic interferences. The aqueous phase which contained the analyte was concentrated to a small volume by rotary evaporation and injected to the IC. The ion chromatography system was equipped with AS4SC separation column and a conductivity detector. The eluent contained a mixture of 9 mmol/l sodium carbonate (Na_2CO_3) and 4 mmol/l sodium hydroxide (NaOH) was used at a flow rate of 1.5 ml/min. The relative standard deviation for 9 injections was 0.49% and the recovery was in the range of 96.36 to 103.18% when the sample was spiked at the level of 1-50 $\mu g/ml^{-1}$. The method was linear in the range of 0.042 – 100 µg/ml. The limit of detection was 0.042 µg/ml when using the signal to noise ratio of 3. The method was used for the determination of glyphosate in lake water with no glyphosate detected.

In terms of sensitivity, the above IC methods are not good enough for the quantitative analysis of these herbicides at low levels.

Guo et al [14] have investigated the use of ion chromatography coupled with inductively coupled plasma - mass spectrometry detection for the determination of glyphosate and phosphate in water. In their study, water samples were filtered and injected into the LC module without any further treatment. A polymer anion-exchange column (Dionex IonPac AS16, 4.0 mm x 250 mm) was used for the separation of the analytes and the eluent used was citric acid. After the analytes were eluted from the column, they were directly detected by ICP-MS at m/z = 31. The detection limits were 0.7 µg/l for both analytes. The recoveries were in the range of 97.1 to 107.0% and relative standard deviations of ≤7.4% (n=30) were obtained. The sensitivity of glyphosate obtained in Guo et al [14] study was much higher than the sensitivity obtained when using ion chromatography with suppressed conductivity detector, study done by Zhu et al [28].

Bauer et al [29] have studied the method of analysis for glyphosate, AMPA and two other polar organic micro-pollutants namely ethylenediamine tetraacetate (EDTA) and diacetonketogulonic acid (DAG) in water using ion chromatography with electrospray mass spectrometry detection. In their investigation, the water sample was directly injected into an anion exchange column without any further sample preparation step except filtration. Two eluents were prepared; eluent A was a mixture of 1.3 mmol/l of disodium carbonate and 2.0

mmol/l of sodium hydrogen carbonate (pH 10.30), and eluent B was a mixture of 13 mmol/l of disodium carbonate and 20 mmol/l of sodium dihydrogen carbonate (pH 10.08). The gradient program was started with 95% of eluent A and 5% of eluent B, held for 10 minutes. For the following 20 minutes, eluent A was reduced to 50% and after 5 minutes the eluent A was adapted again to the starting conditions and the stationary phase was equilibrated for 5 minutes before the next injection. The analysis of these polar organic micro pollutants in ground and surface water has been achieved in this investigation without additional sample preparation at a concentration level of 1 µg/l.

2.4 Capillary Electrophoresis (CE) Methods

Some researchers have published some work that is based on the determination of glyphosate and AMPA in water using capillary electrophoresis [4, 30, 31, 33].

Cikalo et al [30] have developed a method for the analysis of glyphosate and AMPA in water using CE with indirect UV detection because the analytes had no significant UV absorbance. The method was based on the separation of glyphosate and AMPA. Glyphosate and AMPA were separated within 4 minutes using pH 7.5 with phthalate as supporting electrolyte and containing tetradecyltrimethyammonium bromide (TTAB) as an electro-osmotic flow modifier. The limit of detection for glyphosate was found to be 0.8 µg/ml and a linear response was found in the calibration range of 1.7 – 170 µg/ml for glyphosate and 1.1 – 110 µg/ml for AMPA.

Chang et al [4] have investigated a CE method for the analysis of glyphosate, AMPA and glufosinate using indirect fluorescence detection. Since there are no fluorophores in the analytes, fluorescein was employed as the buffer fluorophore and an argon-ion laser was used to induce the fluorescence background. The analytes were separated within 5 minutes with a fluorescein solution at pH 9.5. Detection limits for all three analytes were reported to be in the µM range. After the method has been developed it was applied in the commercial herbicide analysis. It has been reported [4] that the developed method can be applied to the direct analysis of glufosinate and AMPA in ground water but the application for the direct analysis of glyphosate in ground water was found to be problematic. Chang et al [4] have also reported that their method can be applied for the quality control of herbicide products.

Corbera et al [31] have reported a sample pre-concentration method for CE analysis of glyphosate and AMPA in natural water. The method was investigated to improve the sensitivity in the analysis of both glyphosate and AMPA using a slightly modified method published by Cikalo et al [30]. The analytes were pre-concentrated using a strong anion-exchange resin (Amberlite IRA-900). After the resin was rinsed with water, a water sample spiked with both glyphosate and AMPA was loaded onto the resin. Interfering matrix components were removed from the resin with ultrapure water and analytes were eluted with sodium chloride. Eluted analytes were analysed using CE method developed by Cikalo et al [30]. Recoveries ranging from 84% to 87% for glyphosate and from 85% to 98% for AMPA were obtained with RSD of <6% for glyphosate and RSD <5% for AMPA. The method allowed the determination of 85 ng/ml of glyphosate and 60 ng/ml of AMPA. The effect of salt content in samples on extraction efficiency was investigated and a linear relationship was established for AMPA with R^2 = 0.996. A comparison of the investigated method and an HPLC method with UV-vis detection and pre-column derivatization with *p*-toluensulphonyl chloride was carried out. No differences in results were found (using t- and f- statistical tests).

Jiang et al [33] have investigated the use of solid phase extraction (SPE) technique for the pre-concentration of the herbicide glyphosate for analysis by CE. The work involved the clean-up of the sample using an SPE cartridge containing 300 mg of a mixture of strong base anion exchange resin (chloride form) and strong acid cation exchange resin (hydrogen form) at a mass ratio of 60:40. The spiked river water sample was then pre-concentrated using a 200 µl micro-pipette tip packed with 50 mg of Bio-Rad AG-X8 anion exchanger beads. Glyphosate was eluted with hydrochloric acid at a flow rate of 0.1 ml/min. The effluent was collected in a borosilicate glass vial for derivatization. Glyphosate was converted into glycine with sodium borate buffer (pH 10.4) and calcium hypochlorite solution. The mixture was placed in a 60^0C water bath for 5 minutes, then sodium cyanide and naphthalene-2,3-dicarboxaldehyde were added. The mixture was allowed to react in the dark at room temperature for 3 minutes, before the CE analysis. The separation was performed at 12 kV across a 37 cm long fused capillary using 50 mM SDS and 20 mM sodium borate pH 9.3 running buffer. The limit of detection for glyphosate was 0.04 nM.

A method for the analysis of glyphosate and AMPA in soya beans has been reported by Chiu et al [32]. The method is based in a capillary electrophoresis coupled with electrochemiluminescence (ECL) detection. Soya bean samples were milled to powder form

and extracted with water. Acetonitrile was added to precipitate the proteins followed by centrifugation. The supernatant obtained after centrifugation was filtered and injected into CE-ECL. The method was tested by analyzing glyphosate in both transgenic and non transgenic soybeans. Glyphosate was not detected in both transgenic and non transgenic soybeans. The samples were then spiked with 12 µg/g of glyphosate. The recovery was 92.7 ± 3.5% (n =3) and the limit of detection for glyphosate in soybean was 0.6 µg/g.

2.5 Aims and Objectives of this project

In the light of the very brief survey above, this research was undertaken to:-

- Develop a rapid method for sample preparation.
- To overcome the problems encountered in the analysis of glyphosate and AMPA using HPLC and fluorescence detection.
- Determine a sensitive and rapid method for the analysis of both glyphosate and AMPA in water.
- Use the above method for the determination of concentrations of glyphosate and AMPA found in water samples from dams and rivers.

The work was also motivated by the need to have a method that is within the ambit of the expertise and affordability of laboratories charged with monitoring residual glyphosate in drinking water supplies. At early stages of this research glufosinate was considered as target analyte. However, the analysis of glufosinate was not pursued further due to the difficulty of separating it from the other two analytes.

CHAPTER 3

PRINCIPLES ON WHICH THE CHOSEN TECHNIQUES ARE BASED

This chapter will discuss all the techniques and instruments used when carrying out this investigation, theory of each technique and instrument. The details about the experimental procedures and the use of techniques will be explained in this section.

3.1 High Performance Liquid Chromatography

3.1.1 Introduction to HPLC

Liquid Chromatography (LC) is a separation technique based on a different distribution rate of sample components between a stationary phase and a liquid mobile phase. HPLC is the LC technique with the ability to separate sample compounds in a given time with a given resolution [38]. The selectivity of HPLC technique is based on the different types of stationary phases and on the mobile phase. The basic liquid chromatograph consists of six basic units, namely, the mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

3.1.2 Basic components of HPLC

3.1.2.1 The mobile phase supply system

The mobile phase is the liquid that plays an important part in the chromatographic system by carrying the sample solutes (glyphosate and AMPA derivatives) through the stationary phase. The liquid mobile phases are used to adjust the chromatographic separation and retention in liquid chromatography. The mobile phases are usually stored in solvent reservoirs depending on properties of the eluent to be used. Special solvent reservoirs are available such as, brown laboratory bottles for light-sensitive eluents and for inorganic ions, polyethylene containers are used because additional inorganic ions may be dissolved from the glass material [38].

3.1.2.2 The pump and programmer

The pump is responsible for the constant flow of the mobile phase, which is necessary for the quantification and reproducibility of an analysis. Pumps are used to pump the mobile phase from solvent reservoirs to the various parts of the instrument. Good pumps should be able to deliver a solvent at a flow rate of up to 10 ml/min. They should be equipped with high and low pressure limits. To avoid damage, the pump or column should be chemically inert. The pump heads should not warm up to avoid the evaporation of the solvents and bubbles [38].

3.1.2.3 The sample valve

This is the valve that operates in the loop filling mode. The sample is usually injected into the column while the system is at high pressure. The sample is injected into a sample loop (20 µl). While the sample is injected, the mobile phase at high pressure moves through the sample valve directly to the column. Then the valve is rotated (so that the mobile phase passes through sample loop), sweeping the sample into the column. 0.02 ml to 5 ml sample loops are available commercially [38, 39].

3.1.2.4 The column

The column is part of the instrument where the separation of the sample components injected into the HPLC takes place. Usually for complex matrices, a special separation technique called column switching or column coupling is used.

Column Switching Technique

This is the coupling of two columns usually with different selectivities by means of manual or automatically driven high pressure valve. Column switching is widely used in the analysis of very complex samples (example, drug analysis in blood). In column switching technique, analytes are selectively removed from the sample matrix. This technique is applied in on-line sample cleanup, backflush technique, and multidimensional chromatography [38]. In column coupling technique the switching valve is very important. It must work reliable under high pressures and must be chemically inert to the sample and the mobile phase.

This technique is very suitable for sample cleanup. For example, for this research it is used for the determination of glyphosate and AMPA in water after precolumn derivatization with FMOC-Cl. During the derivatization process, it was observed that the derivatizing reagent (FMOC-Cl) is very reactive, in aqueous solution it rapidly gets converted into FMOC-OH and the derivatization procedure always provides a large excess of flourogenic FMOC interferences and FMOC-glyphosate (analyte) [16]. These FMOC interferences are easily removed when using the column coupling technique, coupling of C_{18} column with amino column was previously reported [16]. The analyte, glyphosate derivative, has a little retention on C_{18} column and adequate retention on amino column. The clean-up is obtained by transferring unretained analyte to amino column. All interferences with more C_{18} retention (example, FMOC-OH) are retained on C_{18} column and sent to waste by the rinsing mobile phase during the separation of the analyte on the amino column [16].

3.1.2.5 The detector

The detector works by detecting every component of the sample eluted from the separation column. A good detector in the HPLC should have the following characteristics:

- High sensitivity
- Good selectivity
- Fast response
- Wide range of linearity
- No contribution to column band broadening

Depending on the properties of the analytes, the following detectors are used in HPLC [38, 39]:

- Refractive index
- UV-Vis absorption
- Diode-array
- Mass spectrophotometer (MS)
- Fluorescence
- Evaporative light scattering detection (ELSD)

Glyphosate, AMPA and glufosinate are the examples of the compounds in which UV-Vis absorption, mass spectrophotometer and fluorescence detector can be used for their analysis after derivatization of analytes. These compounds have to be derivatized before being detected by UV, MS and fluorescence detectors. In this research the main detector used is fluorescence detector, therefore the principle of operation of fluorescence detector is explained below.

3.1.3 Fluorescence detector

3.1.3.1 Introduction to fluorescence detection

When light is emmited by molecules that are excited by electromagnetic radiation, the phenomenon is called photoluminescence. If the release of electro-magnetic energy is immediate, or stops on the removal of the excitation radiation, the substance is said to be fluorescent. Fluorescence detector is used to monitor the fluorescing substances and is widely used in trace analysis because it provides high selectivity and sensitivity. Due to the fact that only few compounds are able to fluoresce, resulting in a high selectivity [38].

3.1.3.2 Principles of operation of fluorescence detectors

Fluorescence involves the emission of radiation from a molecule which has attained an excited electronic state after the absorption of radiation. Fluorescence process involves excitation and emission step, and this process in explained below.

In excitation step, absorption of a photon causes the solute molecule to move to an excited electronic state and this absorption results in the transition of the solute molecule from its ground singlet to its first excited singlet state. In most cases the absorbed energy will become dissipated through a series of vibrational transitions which will result in the production of heat. This process occurs when the energy levels for the excited and ground states overlap. When there is no overlap between energy levels the most convenient method by which the excited molecule can lose energy is through the emission of a photon [40].

In the fluorescence detector, the sample that passes the detector cell is irradiated by a beam of light with a certain intensity I_0 and excitation wavelength. Flourescing solutes are able to emit

radiation with a characteristic wavelength which converted into an electric signal and recorded. The emitted radiation is not monochromatic, therefore a monochromator needs to be inserted into the beam in order to enhance selectivity [38]. The fluorescence radiation is emitted in all directions and the detectors are usually operated in a right angle configuration. A lamp (xenon or deuterium source) passed radiation through a monochromator (excitation monochromator) into the flow cell through which the column effluent passes. Fluorescence radiation is collected at right angles to the excitation beam, where it is passed through a second monochromator (emission monochromator), and then to a photomultiplier [40].

To produce more fluorescing analytes, pre- or post column derivatization is needed [38].

3.1.4 Derivatization

3.1.4.1 Introduction to derivatization process

Although there is a wide range of separation and detection systems available in HPLC, there is still arise the cases where either the selectivity or sensitivity of a particular analysis may be considered in adequate and in such cases derivatization is necessary [40]. The aims for the derivatization of analytes are stated below:

- Improvement in the detectability of the analyte(s)
- Improvement of the resolution of the analyte(s)
- Establishment of solute identity
- Improvement of the selectivity of the analyte in a complex matrix
- Improvement of the chromatographic behaviour such as peak shape of the analyte
- Improvement of the stability of the analyte during chromatography
- Achievement of more desirable physical properties of the analyte.

The derivatization of the analyte can be performed in two ways; before (pre-column derivatization) the chromatographic separation column or after the chromatographic separation column (post-column derivatization) [40].

3.1.4.2 General Introduction to Pre-column Derivatization

There are no restrictions on reaction kinetics provided that a complete reaction can be achieved in a reasonable time. There is a choice of changing reaction conditions to optimize reaction time and yield. The solvent that is incompatible with the chromatographic mobile phase may be used provided that this solvent is removed before the injection. This reaction also forms excess reagents and byproducts; these can be separated from the analyte either on the chromatographic system or by using a pre-chromatographic clean-up step. Each sample is handled individually. An internal standard should be used in cases where the derivatization reaction is incomplete [40].

3.1.4.3 General Introduction to Post-column Derivatization

The derivatization reaction should be reproducible without the necessity of being quantitative. Sample preparation is limited. Accuracy and precision of the derivatization can be enhanced through automation of the derivatization procedure. The choice of reaction conditions is limited because the derivatization must be carried out in the chromatographic mobile phase. Band broadening usually occurs, leading to loss of chromatographic resolution. Additional equipment is required to perform this reaction such as post-column reactor [40].

3.2 Ion Chromatography (IC)

3.2.1 Introduction to IC

Separation of ionic solutes is an important and rapidly growing field in analytical chemistry nowadays. Ion chromatography is a chromatographic technique that is used to determine all ions which carry one or two charges. The technique can be used for the analysis of anions, cations, organic acids and amines plus analytes such as carbohydrates. This technique has been used in many important fields such as the following:

- The routine investigation of aqueous systems such as drinking water, rivers, effluents and rain water.
- For the analysis of ions in chemical products, foods, cosmetics, pharmaceuticals etc.
- Ultra-trace analysis such as in the semi-conductor and power industry [41].

The ion-exchange is divided into two main groups namely non-suppressed ion chromatography and suppressed ion chromatography.

- Non-suppressed IC comprises those methods in which an ion-exchange column is used to separate a mixture of ions, with the separated solutes being passed directly to the detector.
- Suppressed ion chromatography contains an additional device called suppressor. This device is inserted between the ion-exchange separator column and the detector. The function of the suppressor is to modify both the eluent and the solute in order to improve the detectability of the solutes with a conductivity detector. The suppressor requires a regenerate (or scavenger) solution to enable it to operate for extended periods [40].

3.2.2 Separation in IC

The separation of the solutes is performed as ions using an ion exchange resin or bonded silica, or it is possible to convert the ions to polar molecules and separate them on a reverse phase system [41].

3.2.3 Mechanism of suppression in IC

In the case of anion-exchange separations, cations from the eluent are replaced by hydrogen ions from the suppressor which, in turn, react with the eluent anion to form an undissociated weak acid. In the case of cation-exchange separations, anions from the eluent are replaced by hydroxide ions from the suppressor, which in turn react with the eluent cation to form an undissociated weak base. Solute ions which are the conjugate of strong acids or bases do not react under these conditions, but the exchanged hydrogen ion or hydroxide ion is eluted with the sample band and thereby increases its conductance. The suppressor reduces the background conductance of the eluent and simultaneously enhances the detectability of the solute ions [40].

3.2.4 Ion Chromatographic Detection

The detection is commonly performed by using conductivity, UV absorption, refractive index or electrochemical detection [41]. Conductivity is a good general purpose method; the others

usually exhibit high sensitivity for a reduced number of ions and so tend to be used as selective detection systems.

In conductivity detection, the detector gives the conductance reading that is due to various components in the sample. There is a baseline conductance reading that is due to the various ions in the mobile phase buffer. When a band of solute ions elutes from analytical column (the conductance increases) the solute ions displace the mobile phase ions in this region [40].

In UV absorption detection, there is a direct and indirect UV detection. In direct UV detection there is a UV transparent mobile phase and the solute ions absorb UV. Indirect UV detection uses a UV absorbing mobile phase with solute ions that do not absorb, thus producing a decrease in absorbance as the solute band passes through the detector.

Electrochemical detection is used for low level or selective analysis of the three important anions I^-, SO_3^{2-} and CN^-.

Refractive index detection is used mainly for borate and polyphosphonates [40].

3.3 Sample Preparation Techniques

Solvent and solid-phase extraction are the two techniques used for separating mixtures of substances, either by selective transfer between two immiscible liquid phases or between a liquid and a solid phase.

3.3.1 Solvent Extraction

Solvent extraction is based on the extraction of nonpolar, uncharged species from an aqueous solution into an immiscible organic solvent, or the extraction of polar or ionized species into an aqueous solution from an organic solvent. The purpose of extraction is to separate components of a mixture by exploiting differences in their solubilities in two immiscible liquids. Substances reach an equilibrium distribution through intimate contact between the two phases, which are then physically separated to enable the species in either phase to be recovered for completion of the analysis. This is actually done by dissolving the sample in a

suitable solvent then shaking the solution with a second immiscible solvent. When the equilibrium distribution of the two solutes differs, a separation takes place [37].

Solvent extraction is widely used for sample pre-treatment or clean-up to separate analytes from matrix components that would interfere with their detection or quantitation. It is also used to pre-concentrate analytes present in samples at very low levels and which might otherwise be difficult or imposible to detect or quantify [37]. When this research was carried out, ethyl acetate was used to perform a separation between glyphosate derivative and FMOC-OH. Glyphosate derivative is insoluble in organic solvents; therefore the extraction was easy to perform.

3.3.2 Solid-phase Extraction (SPE)

3.3.2.1 Introduction to SPE

This is a relatively new technique and it has been widely used for the sample pre-treatment or the clean-up of dirty samples [37]. This is non-equilibrium, exhaustive technique used for the removal of chemical constituents from a flowing liquid sample via retention on a contained solid-phase sorbent and subsequent recovery of selected constituents by elution with a suitable solvent from the sorbent [42]. The technique has been applied for the analysis of environmental, clinical, biological and pharmaceutical samples [37].

3.3.2.2 Advantages of SPE

The advantages of SPE include the following [21]:

- Reduced analysis time compared to liquid-liquid extraction.
- Reduced labor (because SPE is faster and requires less manipulation).
- Reduced organic solvent consumption and disposal, which results in reduced analyst exposure to organic solvents.
- Reduced potential for formation of emulsions.

3.3.2.3 Solid-phase sorbents

Solid-phase sorbents are generally either silica or chemically-modified silica similar to the bonded phases used in HPLC but of larger particle size (40-60 µm in diameter) [40]. Solutes interact with the surface of the sorbent through van der Waals forces, dipolar interactions, H-bonding, ion-exchange and exclusion. Hydrocarbon-modified silicas (like C_{18}) are non polar, and therefore hydrophobic, but are capable of extracting a very wide range of organic compounds from aqueous solutions. However, they do not extract very polar compounds well and these are best extracted by unmodified silica, alumina or florisil, all of which have a polar surface. Ionic and ionizable solutes are readily retained by an ion-exchange mechanism using cationic or anionic sorbents. Weak acids can be extracted from aqueous solutions of high pH when they are ionized and weak bases from aqueous solutions of low pH when they are protonated [37].

Sorbents are either packed into disposable cartridges, fabricated into disks or incorporated into plastic pipette tips or well plates.
Most cartridges are made from a polypropylene syringe barrel and are usually packed with 25-500 mg of sorbent. The sorbent generally occupies only the lower half of the cartridge and the upper half is for the sample solution or solvents [37].

SPE sorbents should be porous with large surface areas, be free of leachable impurities, exhibit stability toward the sample matrix and the elution solvents, and have good surface contact with the sample solution [42].

3.3.2.4 Requirements for successful SPE

A high, reproducible percentage of the analytical solutes must be taken up by the solid extractant.
The solutes must be completely eluted from the solid particles. The sorption process must be reversible [42].

3.3.3 General methodology of SPE

The methodology when the sorbent is packed in a cartridge generally consists of four steps namely sorbent conditioning, sample loading or retention, rinsing and elution [37, 42]. These four steps are explained below.

Step 1: Sorbent conditioning:

In this step, the cartridge is flushed through with the sample solvent to wet the surface of the sorbent and to create the same pH and solvent composition as those of the sample, thus avoiding undesirable chemical changes when the sample is applied.

Step 2: Sample loading or retention:

The sample solution (liquid sample) is passed through the cartridge. Two things may happen when the sample is passed through the cartridge, the anlytes of interest can be retained in the cartridge whilst the matrix components pass through or the matrix components can be retained while the analytes of interest pass through.

Step 3: Rinsing:

This step is for the removal of all those components that are not retained by sorbent during the retention step or is for rinsing of cartridge to remove undesired components.

Step 4: Elution:

This is the final step and is used for the recovery of the analytes retained in cartridges; otherwise the matrix free solution and rinsings from the second and third steps are combined for quantitative recovery of the analytes before completion of the analysis [37].

CHAPTER 4

EXPERIMENTAL

This chapter will present the list of all chemicals used, it will also describe the preparation of these chemicals and mention the techniques used for the analysis of glyphosate and AMPA in water. All the samples collected and storage of these samples prior to HPLC analysis will be discussed. The chapter will also discuss the analysis methods for the determination of concentrations of glyphosate and AMPA in water.

4.1 Materials

The following materials were purchased and used in this research. Standards of Glyphosate (99.9%), glufosinate (94.7%) and AMPA (≥ 99%) were purchased from Sigma (Missouri, USA). HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). Analytical reagent (AR) grade of sodium dihydrogen phosphate, disodium hydrogen phosphate, disodium tetraborate decahydrate, hydrochloric acid (HCl) (32%), potassium hydroxide (KOH), succinic acid, formic acid, acetic acid, ammonium acetate and FMOC-Cl were purchased from Merck (Darmstadt, Germany). HPLC grade ethyl acetate was purchased from Sigma (Steinheim, Germany). Buffer solutions (phosphate) of pH 4 and pH 7 were purchase from BDH Laboratory (Poole, UK). Double de-ionised water was obtained from a Milli-RO15 purification water system from Waters (Massachusetts, USA) and all the preparations of solutions in water were prepared using this double de-ionised water.

4.2 Equipment

4.2.1 High performance liquid chromatography instrument

HPLC is the main instrument used in this research. The HPLC instrument consisted of a TSP model AS 100 auotosampler (San Jose, USA), TSP model P100 LC pump (pump 1) (San Jose, USA) and a model RF-10AXL fluorescence detector (Shimadzu, Tokyo, Japan). EZChrom chromatography data system (version 6.6) was obtained from Scientific software (San Ramon, California, USA) and used for recording of chromatograms. Samples and standards were injected using a Rheodyne 7010 injector equipped with a 20 micro litre (µl)

sample loop (California, USA). Columns used were Polymeric amino column of 250 x 4.6 mm I.D purchased from Advanced Separation Technologies (New Jersey, USA), pH 2-13; Spherisorb amino column of 250 x 4.6 mm I.D purchased from Waters (Massachusetts, USA) and 125 x 4 mm I.D Lichrospher C_{18} column purchased from Merck (Darmstadt, Germany). The temperature of columns during HPLC and IC analysis was maintained at 25^0C by using a column block heater purchased from Jones Chromatography LTD (Llanbradach, Wales).
For column switching technique, the second LC pump (pump 2) was used, this was a Waters 501 HPLC pump purchased from Waters (Massachusettes, USA).

4.2.2 Ion chromatography

Another instrument used was ion chromatography (IC), it consisted of Waters 510 HPLC pump (Milford, USA), Hamilton PRP- X100 of 100 x 4.1 mm I.D separation column (Nevada, USA). Waters 430 conductivity detector (Milford, USA) and the chromatograms were recorded using a Varian star chromatography workstation version 3 (California, USA). Standards were injected using a Rheodyne 7010 injector equipped with a 20 µl sample loop (California, USA).

4.2.3 Solid phase extraction

The SPE vacuum manifold used was purchased from Supelco (Bellefonte, PA, USA). The use of the following SPE cartridges was investigated for the sample clean-up before the injection of samples into the HPLC instrument. Sep-Pak Vac 3cc (500 mg) C_{18}, Oasis MAX 6cc (150 mg) and Oasis HLB 6cc (200 mg) cartridges all purchased from Waters (Massachusetts, USA). Strata C_{18}-U 100 mg, Strata C_{18}-E 100 mg cartridges were obtained from Separations (New Jersey, USA).

4.2.4 pH meter

A model 691 pH meter was used (Metrohm, Herisau, Switzerland).

4.3 Preparation of solutions

4.3.1 Preparation of solutions for HPLC Analysis

100 mg/l stock solutions of glyphosate, glufosinate and AMPA were prepared in double deionised water. A 100 mg/l mixture of all these three standards was prepared in double deionised water. 100 mg/l mixture was used to prepare other solutions in water ranging from 0.005 mg/l to 50 mg/l using the method of serial dilution.

0.125 M borate buffer (pH 9) was prepared in water and 0.05 M of FMOC-Cl was prepared in acetonitrile. 0.05 M phosphate buffers (pH 5.5 and pH 10) were prepared in water from solutions of disodium hydrogen phosphate and sodium dihydrogen phosphate in water and used in HPLC. 40 mM of succinic acid (pH 2.72) was prepared in double deionised water and used as the mobile phase in ion chromatographic analysis.

A mixture of acetonitrile-0.05 M phosphate buffer (pH 10) (55:45, v/v) was used as the mobile phase for the work that uses a polymer amino column. A mixture of acetonitrile-0.05 M phosphate buffer (pH 5.5) (35:65, v/v) was used as the mobile phase for the work that uses a C_{18}, silica based amino column and a column coupling technique.

A Metrohm 691 pH meter was used to measure the pH of borate buffer, phosphate buffer, succinic acid and the mobile phases used in HPLC. The pH values of all these solutions were adjusted with 4 M KOH solution and 32% (v/v) HCl solution. 4 M potassium hydroxide solution was prepared in water. The pH meter was optimized and calibrated using BDH Laboratory buffer solutions (phosphate) of pH 4 and pH 7.

4.3.2 Preparation of solutions for IC analysis

100 mg/l stock solutions of glyphosate, glufosinate and AMPA were prepared in double deionised water. A 100 mg/l mixture of all these three standards was prepared in double deionised water. 100 mg/l mixture was used to prepare 10 mg/l and 1 mg/l in water using the method of serial dilution. The solutions were filtered through 0.45 μm membrane filter paper, and injected into IC without further treatment.

4.4 Samples

A full description of water samples used in this research is presented in this section. Some of the samples were collected to test the effectiveness of the method of analysis and sample preparation method; and some were collected in areas that were suspected to be contaminated by glyphosate. All the samples were collected in areas of Kwa-Zulu Natal Province (South Africa). All water samples were collected in plastic containers to avoid the possible adsorption of analytes onto glass surfaces; and kept in a refrigerator at 4^0C until they were analyzed, to avoid the decomposition of the analytes and to prevent any possible chemical reactions that might take place.

4.4.1 Effluent water sample

An effluent water sample was collected from a chemical company situated in south of the port city of Durban. One of the products this chemical company is making is glyphosate. Therefore an effluent water sample was collected to test the effectiveness of the sample preparation method developed in this research, and to test the presence of both glyphosate and AMPA in this sample and their levels if they are present. Another objective of collecting the sample from this company was to check if this company was contaminating the environment with the herbicide glyphosate.

4.4.2 Umdloti River

This river is situated in north of the City of Durban. This river was once suspected of being contaminated by herbicides. The Mercury Newspaper published an article (Weed control could delay) on 15 February 2006; the article was about the fish that were found dead after the river was sprayed with herbicides to remove aquatic plants. After this article was published in the Newspaper there was a lot of debate about the testing of herbicides in water. The samples were collected in this river because at once the glyphosate herbicide was used in this river.

4.4.3 Amanzimtoti river

Amanzimtoti river is situated in south of Durban. There was no information obtained regarding the use of herbicides in this river. The sample was collected from the river mouth.

4.4.4 Hammersdale dam

The dam in situated on the Sterkspruit tributary in a small town called Hammersdale. Hammersdale dam is a major breeding ground for the water hyacinth weeds. This dam stores water and the water stored in this dam is purified for human consumption. The nutrient rich silt in this dam promotes rapid growth of aquatic plants which tend to block out light and air. The herbicide, glyphosate, is widely used in this dam to control hyacinth weeds. The growth of these weeds has a significant impact on water health and reduces the benefits that people could derive from a more healthy water (river) ecosystem.

4.4.5 Stream water sample

This water sample was collected from the first stream found south of Durban international airport.

4.5 Derivatization

Derivatization was done in both the standards and field samples.

4.5.1 Derivatization of standards

Derivatization reaction was carried out by adding 1.80 ml of 0.125 M borate buffer (pH 9) followed by the addition of 12.00 ml of 0.05 M FMOC-Cl into a 10.20 ml of the standard. Derivatization reaction was carried out at room temperature for 30 minutes. After 30 minutes reaction time, the solution obtained was extracted three times with 6.0 ml of ethyl acetate to remove the excess amount of FMOC-Cl and FMOC-OH. Then the standards were filtered through a 0.45 μm membrane filter paper prior to HPLC analysis.

4.5.2 Derivatization of field samples

Prior to the derivatization, field samples were homogenised and sub-samples were obtained. These sub-samples were filtered through a 0.45 µm membrane filter paper. Then the sub-samples were then derivatized in the same way as the standards. After derivatization, the solution obtained was extracted with ethyl acetate to remove FMOC-Cl, FMOC-OH and the interfering organic compounds that may be present in the field samples. Other interferences are then removed by using solid phase extraction technique. The solid phase extraction technique was only used in field samples for sample clean-up to remove non-polar interferences.

4.6 Sample Clean-up

For sample clean-up, a suitable SPE cartridge was investigated and used. The interferences were removed by passing the sample through a 500 mg C_{18} SPE cartridge. The cartridge was first conditioned by passing 3.0 ml of acetonitrile followed by passing 3.0 ml of deionised water and finally 2.0 ml of standard or sample obtained after derivatization with FMOC-Cl through the C_{18} SPE cartridge. All these fractions were sent to waste using a flow rate of 1 ml/min. Then the derivatized sample was transferred through the cartridge using a flow rate of 1 ml/min and collected in a vial. The collected sample was then filtered through a 0.45 µm nylon filter paper before the injection onto the HPLC column.

4.7 HPLC Analysis

The flow rate of the mobile phase was 1 ml/min and the column was maintained at a temperature of 25^0C. A volume of 20 µl of the solution obtained after sample clean-up (SPE) was injected onto polymeric amino column, which was connected directly to the fluorescence detector. The analytes were separated in the polymeric amino column and thereafter were etected with the fluorescence detector. The emission wavelength used in fluorescence detector was 315 nm and the excitation wavelength was set at 266 nm. Quantitation of both analytes was done by using external calibration method with standard solutions of both analytes in water obtained after derivatization.

CHAPTER 5

RESULTS AND DISCUSSION

High performance liquid chromatography comprises ion chromatography and partition chromatography. In view of the fact that the analytes to be determined were extremely polar or tending towards ionic species, it was decided to use IC as a technique and compare it with partition chromatography.

5.1 Ion chromatography for glyphosate and AMPA analysis

IC is a chromatographic technique that is used for the separation and detection of ionic substances. Glyphosate is an amino acid that has a strongly ionized phosphate group having the pk_a values of 0.78, 2.29, 5.96 and 10.98, and it is a hydrophilic substance [23 and 28]. According to Zhu et al [28], IC can determine this hydrophilic substance quickly and conveniently.

The details of IC equipment used in this research are described in the experimental section (chapter 4, section 4.2).

5.1.1 IC analysis

The standards or a mixture of standards were directly injected into the Hamilton PRP-X100 column without any further preparation except filtration. The mobile phase used in IC analysis was a 40 mM succinic acid (pH 2.72) and the flow rate was set at 1.7 ml/min. The column was directly connected into a non-suppressed conductivity detector.

5.1.2 Results and discussion for IC analysis

5.1.2.1 Detection of the compounds

In order for the glyphosate, glufosinate and AMPA to be detected in IC, they have to be ionized. These compounds are ionized by adjusting the pH in the mobile phase. In this study it was discovered that the pH of the mobile phase affects the migration of glyphosate,

glufosinate and AMPA. This is in general agreement with the results obtained by You et al [5]. Figures 4 and 5 show the different structures of glyphosate and AMPA ions that are formed at various pH values [5]. The structure of glufosinate is almost the same as the structure of glyphosate. Thus glyphosate and glufosinate behave in similar ways. Glyphosate, glufosinate and AMPA are zwitterionic compounds [5], and as shown in Figs. 9 and 10, the change in pH values changes the distributions of the analytes. The pH of the mobile phase used in this work is 2.7. At this pH, glyphosate and glufosinate form an ion of charge -1 and AMPA forms a species with zero net charge.

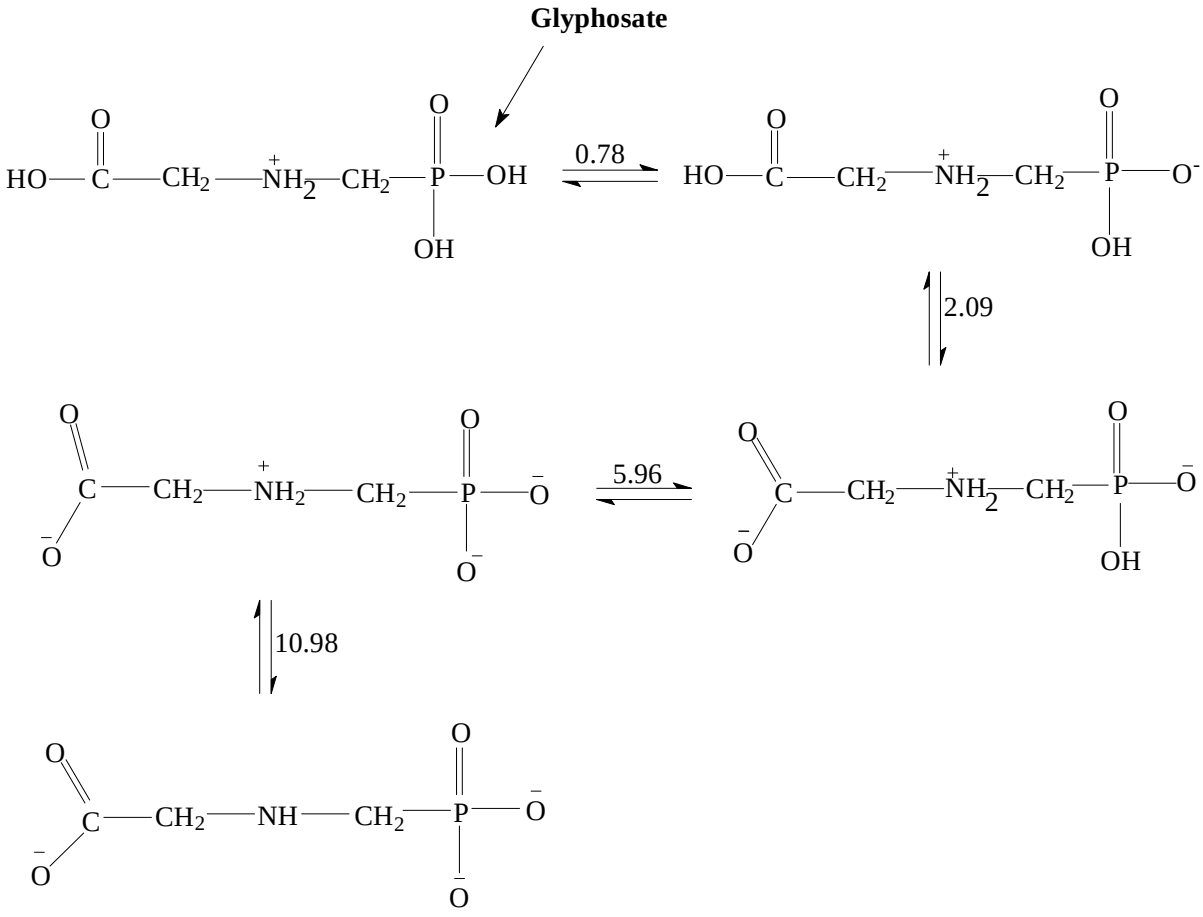


Figure 4: Ionization of glyphosate [5].

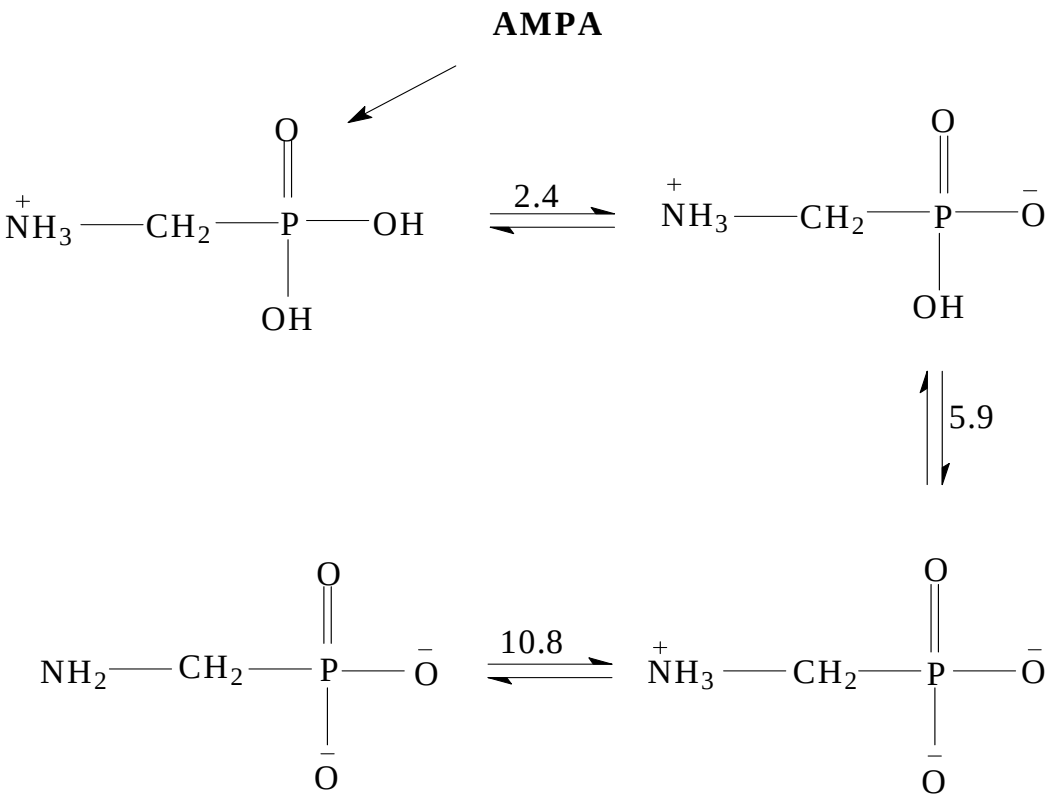


Figure 5: Ionization of AMPA [5].

Glyphosate and glufosinate were detected but AMPA was not detected. This means that the non-suppressed conductivity detector, used in this project, detects only the ions of charge -1. AMPA can be detected if the pH of the mobile phase is changed to greater than 5.9. However, at pH above 5.9, glyphosate does not form the ion of charge -1; instead it forms the ion of charge -2. Therefore the simultaneous analysis of these three compounds is impossible at the selected pH. AMPA cannot be determined simultaneously with glyphosate using this method.

When using the experimental conditions described in chapter four for IC analysis, glyphosate and glufosinate are well separated. The retention times obtained for the separation of glyphosate and glufosinate are 8 and 4 minutes respectively.

5.2 HPLC-FD for glyphosate and AMPA analysis

The use of partition chromatography, to overcome the problems encountered when using IC, was investigated. In partition chromatography, prior to the sample/standard injection to the analytical column, the sample or standard has to be derivatized to form fluorescence detectable compounds. Factors affecting the derivatization process were investigated and are described in sections 5.2.2 and 5.2.3.

5.2.1 Derivatization of glyphosate and AMPA

Due to the lack of fluorophores in both glyphosate and AMPA, these compounds were derivatized with FMOC-Cl, prior to their separation on an analytical column, to form the fluorescing compounds that can be detected using a fluorescence detector. The measured amount of water sample was added prior to the addition of FMOC-Cl reagent. When the volume of water sample or standard was added to the reaction mixture after the addition of FMOC-Cl reagent, a precipitation reaction took place. The derivatization reactions of both glyphosate and AMPA with FMOC-Cl are shown in Fig. 6 below. During this research, non-fluorescing compounds namely; glyphosate and AMPA were analysed as the fluorescing compounds, FMOC-glyphosate (same as glyphosate-FMOC) and AMPA-FMOC (same as FMOC-AMPA), respectively after derivatization.

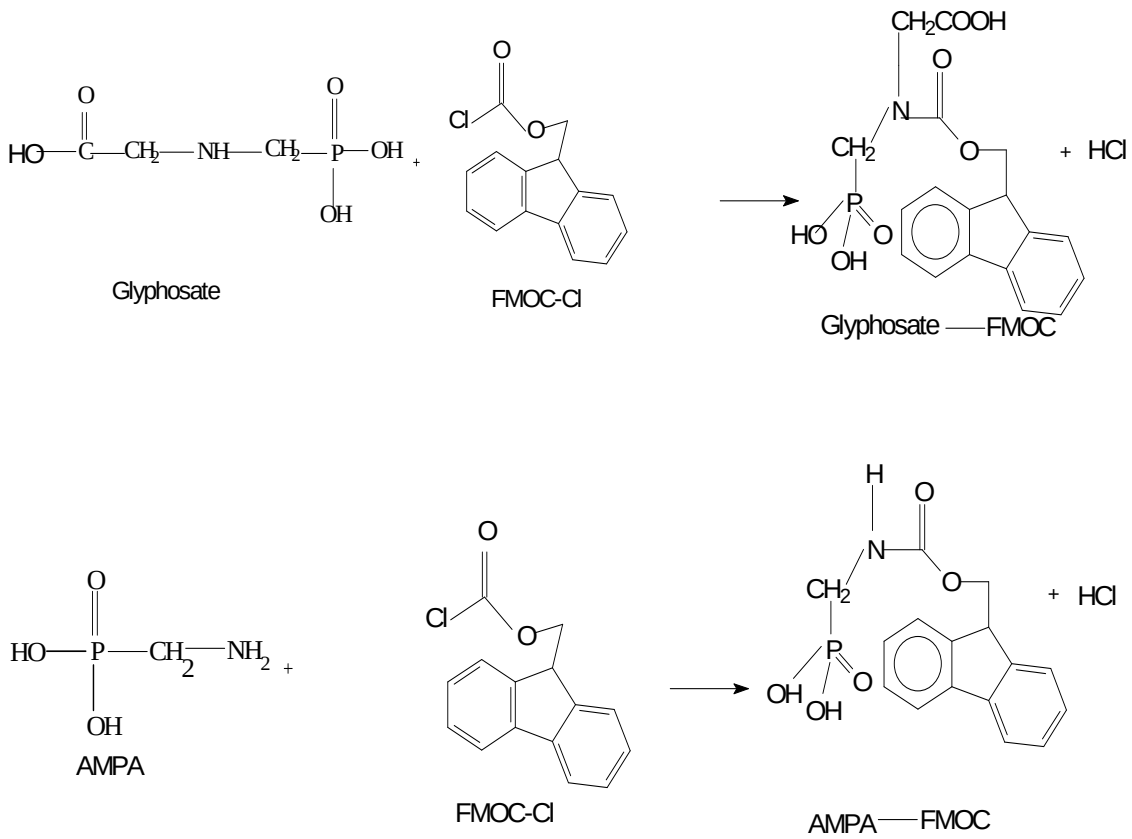


Figure 6: Derivatization reaction of both glyphosate and AMPA

5.2.2 Optimization of derivatization

Two factors can affect the quantity of the derivative, namely duration of the derivatization process (reaction time) and the concentration of the derivatizing reagent (FMOC-Cl). Both of these factors were optimised in this work.

5.2.2.1 The effect of reaction time on derivatization reaction

The effect of time on the derivatization process was investigated in order to determine the optimal time for the derivatization of both glyphosate and AMPA to form fluorescing products. This was necessary to use the minimum reaction time to ensure that the analytes were converted completely into detectable forms or into fluorescing compounds. Initially, the derivatization was started by derivatizing only 100 μg/L of glyphosate with 4 mM of FMOC-Cl at pH 9 for 15 minutes. The pH of the reaction mixture was controlled by the addition of

0.125 M borate buffer (pH 9). Borate buffer also enhances the ionisation of both analytes. The structures of the derivatization products are shown in Fig. 6. This derivatization method gave a low yield of glyphosate-FMOC product. To improve the quantity of glyphosate-FMOC product, the following reaction times were investigated; 15 minutes, 30 minutes, 45 minutes and 60 minutes. A high amount of the glyphosate-FMOC was obtained in a 30 minutes reaction time. Both 45 minutes and 60 minutes reaction gave the same amount of glyphosate-FMOC as for the 30 minutes reaction. Therefore, 30 minutes reaction time was chosen and this reaction time was used throughout this project. While investigating the effect of reaction time on derivatization reaction, the concentration of derivatizing reagent and the pH were kept constant at 4 mM FMOC-Cl and at pH 9, respectively. The data for this is summarised in Table 1 below and presented graphically (Fig. 7).

After the time for derivatization reaction was optimised, a series of standards in the range between 5 ppb and 100 ppb of glyphosate were prepared, derivatized and injected into the HPLC-FD system. The peak areas for these standards are shown in Table 2. A calibration line was drawn (shown in Fig. 8) and the results are depicted in Fig. 7.

Table 1: Reaction times vs peak areas of FMOC-glyphosate

Reaction time	Peak area
15	1475939
30	2027176
45	2025600
60	2026342

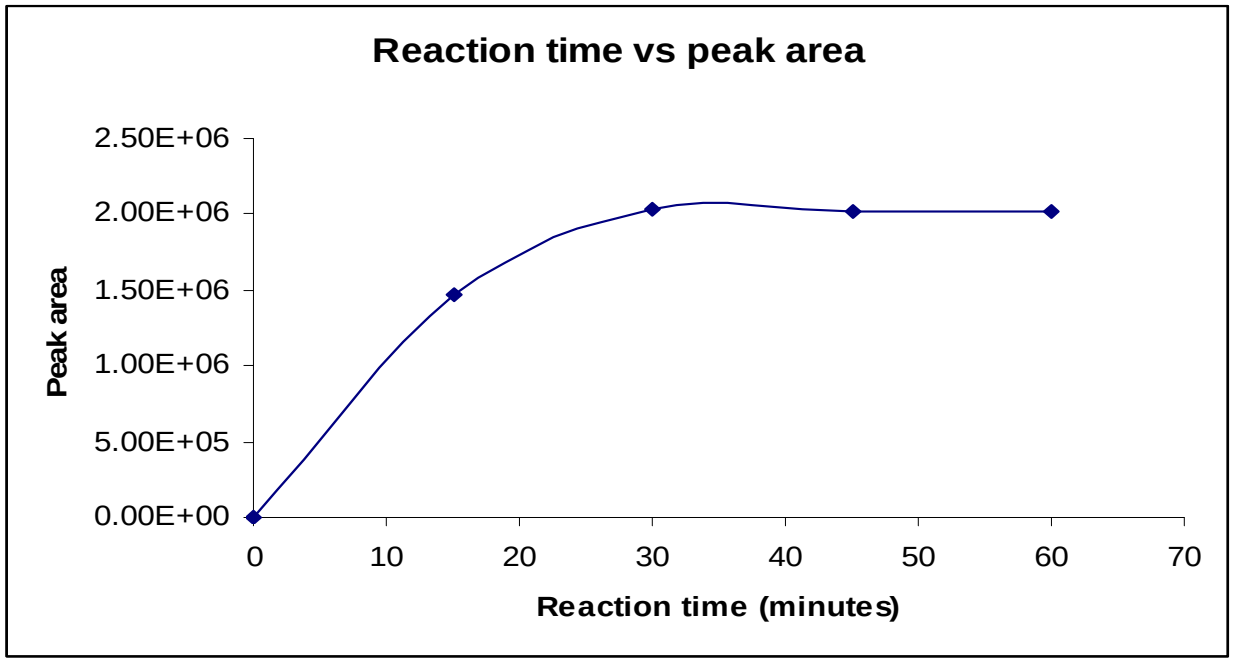


Figure 7: A profile showing the effect of reaction time in the formation of glyphosate-FMOC. This profile demonstrates the change of retention of glyphosate-FMOC with respect to the increase of derivatization reaction time.

Table 2: Peak areas of glyphosate-FMOC standards

Concentration (ppb)	Peak area
5	100116
10	118078
50	1132104
100	2132726

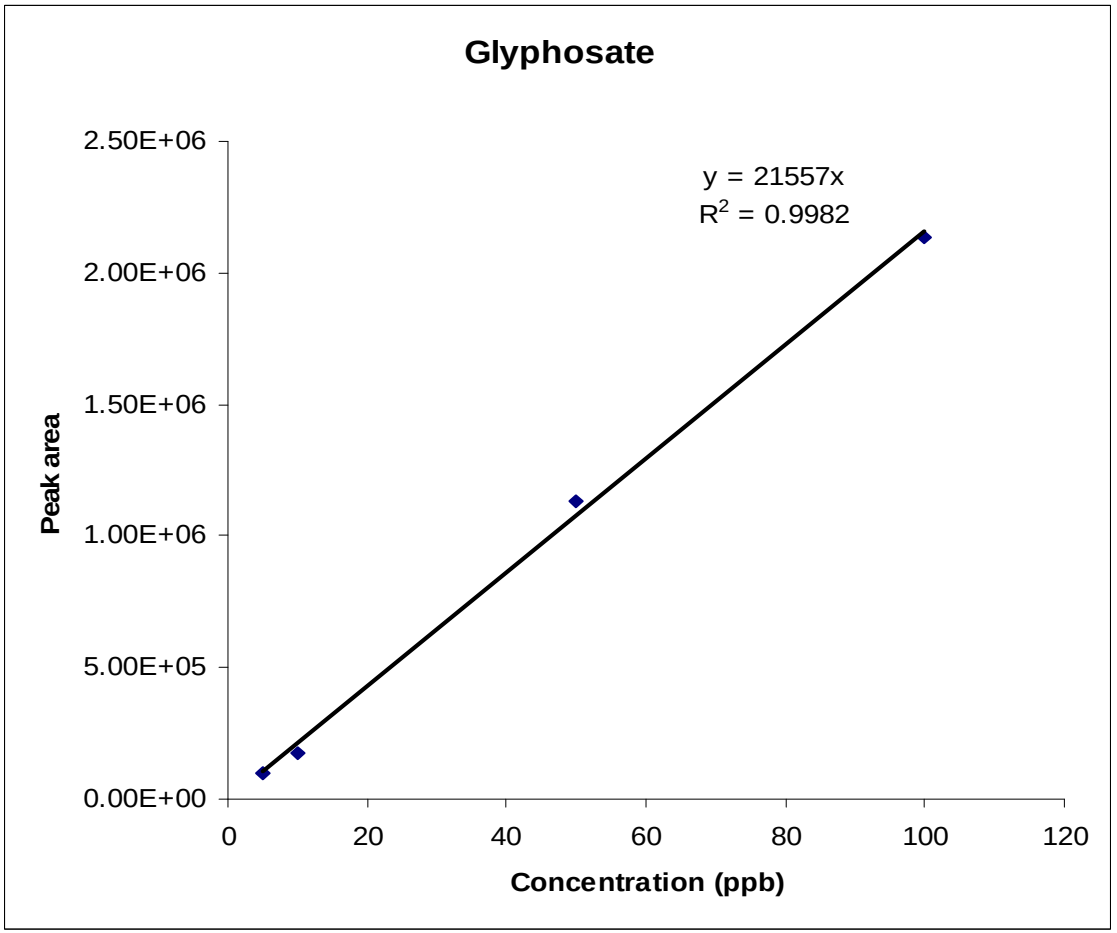


Figure 8: Calibration line of glyphosate-FMOC after optimisation of derivatization reaction.

5.2.2.2 The effect of the concentration of FMOC-Cl on derivatization reaction

After optimising the reaction time, the effect of concentration for FMOC-Cl reagent was investigated. The concentrations investigated were in the range between 0.1 mM and 3.0 mM. The following concentrations were compared, based on peak areas obtained in HPLC for glyphosate-FMOC formed: 0.10 mM, 0.25 mM, 0.50 mM, 1.0 and 3.0 mM. The highest peak area was obtained when using 0.5 mM of FMOC-Cl reagent. Since FMOC-Cl is relatively insoluble in water and it has a low stability in water, FMOC-Cl was prepared in acetonitrile. The results are summarised in Table 3 and shown graphically in Fig. 9.

Table 3: The effect of FMOC-Cl concentration on derivatization reaction

FMOC-Cl Concentration /mM	Peak Areas
0.10	2254192
0.25	13180432
0.50	24583963
1.0	25397821
3.0	25236740

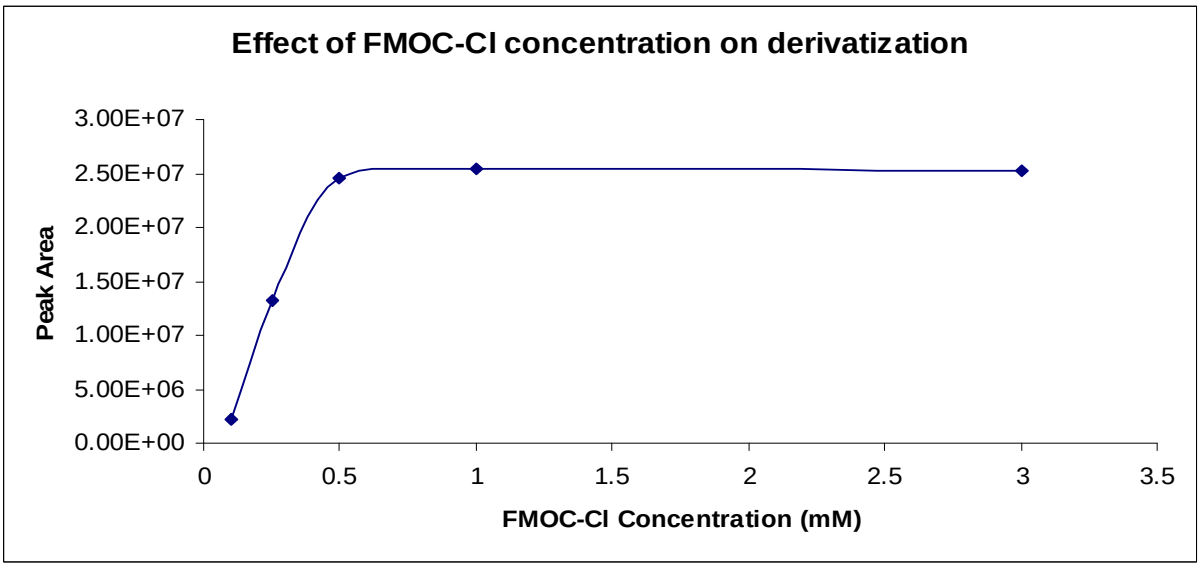


Figure 9: A profile showing the effect of FMOC-Cl concentration in the formation of glyphosate-FMOC.

This profile demonstrates the change in peak areas with respect to the change in the concentration of FMOC-Cl reagent.

Overall, a high amount of glyphosate-FMOC was formed by the reaction of glyphosate and 0.5 mM of FMOC-Cl after 30 minutes. This reaction was carried out using a reaction ratio of 0.7: 1 of glyphosate to FMOC-Cl. FMOC-Cl is added in excess to ensure the complete derivatization of glyphosate and the excess amount of FMOC-Cl, left after derivatization, is removed with an organic solvent (ethyl acetate).

5.3 Calibration of HPLC with fluorescence detector

A multipoint calibration was performed in the optimization and the calibration of the high performance liquid chromatography. A blank and five standard solutions of glyphosate were prepared in the range from 5 to 500 ppb and were used to construct the calibration line as shown in Fig. 10. For AMPA, a blank and five standard solutions of AMPA were prepared from 0.1 to 100 ppb and the calibration curve was constructed and is shown in Fig. 11.

The principle of calibration is based on the equation of the straight line which is as follows:-

$$y = mx + c$$

In the above equation, y represents the peak area of the analyte, m is the slope or gradient of the straight line, x is the concentration of the analyte and c is 0.

Table 4: Concentrations and areas of analytes for calibration lines

Glyphosate		**AMPA**	
Concentration (ppb)	**Peak area**	**Concentration (ppb)**	**Peak area**
0.00	0.00	0.00	0.00
5.0	188658	0.1	163100
10	284989	1.0	332476
50	1966402	10	1785022
100	4218533	50	7446917
		100	15843901

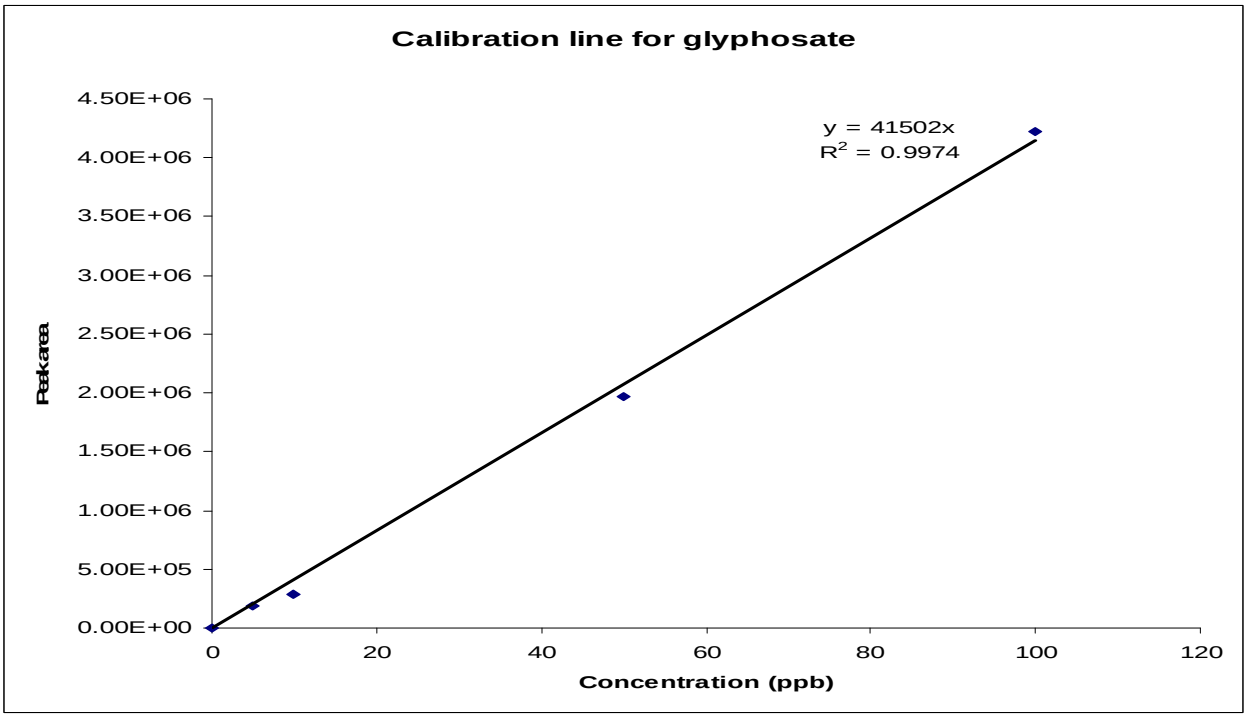


Figure 10: Calibration line for glyphosate by HPLC-FD.

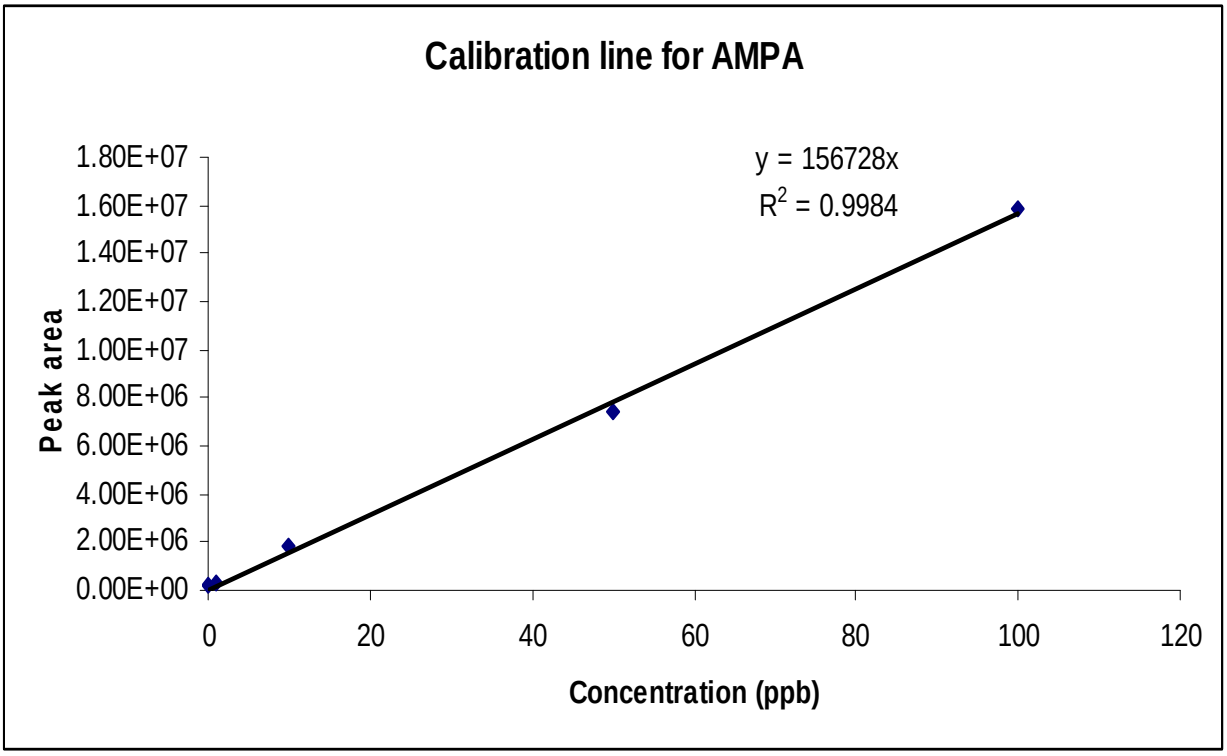


Figure 11: Calibration line for AMPA by HPLC-FD.

5.4 HPLC-FD reproducibility

The reproducibility of the HPLC-FD system was determined as follows: Deionised water was spiked to give 100 ppb of both glyphosate and AMPA. Recovery studies were undertaken. The calculations of percent recoveries were done using the calibration lines shown in Figs. 10 and 11 for both analytes. The results obtained are shown in Table 5.

Table 5: Percent recoveries

Compound	**% recovery**			**Average**	**SD**	**% RSD**
Glyphosate	89	90	90	89.5	0.473	0.528
AMPA	101	99	100	99.9	0.473	0.473

5.5 Analytical column selection

Although FMOC-Cl has been used widely as a pre-column derivatising reagent, it has a disadvantage. A drawback of using FMOC-Cl as a pre-column derivatizing reagent is that, it tends to react with any water present in the reaction vessel, to form a highly fluorescing product called FMOC-OH [14] according to the following reaction:

$$\text{FMOC-Cl} + H_2O \longrightarrow \text{FMOC-OH} + \text{HCl}$$

FMOC-Cl is a very reactive reagent and in aqueous solutions the FMOC-Cl reagent rapidly converts into FMOC-OH (according to the above reaction). Therefore, the derivatization reaction always produces a large excess of fluorogenic FMOC-OH interferent [16]. Water is always present in the reaction vessel because glyphosate standards are prepared in water. Glyphosate is soluble in water but insoluble in organic solvents, therefore the standards cannot be prepared in organic solvents. FMOC-Cl is soluble in acetonitrile but is not soluble in water and it is highly reactive with water to form the FMOC-OH product. Nedelkoska et al [24] reported that in the chromatogram for glyphosate determination, FMOC-OH is represented by the large peak in front of the FMOC-AMPA peak. FMOC-OH peak obscures the analytes peaks in the analysis and this creates difficulties in the analysis of glyphosate and AMPA. This difficulty was also experienced in this study (Fig.12). Several efforts were made

to overcome the problem. One attempt involved the elimination of FMOC-OH by using a column switching technique and extracting FMOC-OH with an organic solvent. In the light of this, a suitable analytical column was found by the trial and error method for the separation of the interfering FMOC-OH product and FMOC-glyphosate (analyte). The analytical columns tried are discussed in detail in sections 5.5.1, 5.5.2 and 5.5.3. For the selection of analytical columns only the standards were used to investigate the behaviour of FMOC-glyphosate, FMOC-AMPA and FMOC-OH in the columns.

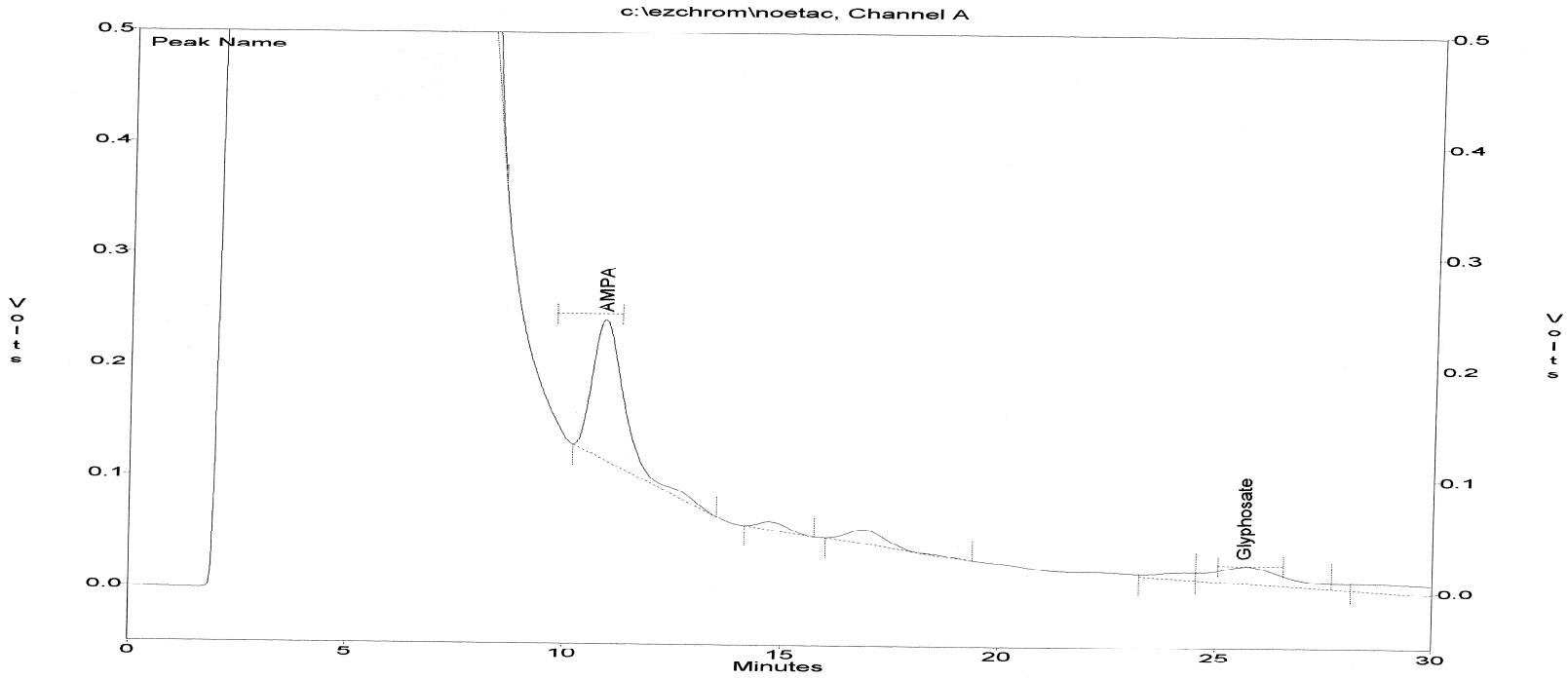


Figure 12: A chromatogram for 100 µg/l mixture of glyphosate and AMPA after derivatizing with FMOC-Cl.

This chromatogram shows FMOC-OH peak (large front peak) overlapping the FMOC-AMPA peak. HPLC mobile phase (pH 10) contained 55 % (v/v) acetonitrile in water, and 50 mM phosphate buffer.

5.5.1 C_{18} Column

Lichrospher C_{18} column was the first column investigated in this study for the separation of FMOC-glyphosate, and the interfering FMOC-OH. This column showed that the derivatization reaction between glyphosate and FMOC-Cl not only produced FMOC-glyphosate but also produced FMOC-OH and some unknown fluorescing by-products. The chromatogram (Fig.13) clearly revealed the presence of these fluorescing by-products. The chromatogram in Fig.13 shows FMOC-OH peak interferes with the analysis of glyphosate. Hence when this C_{18} column was employed, the limit of detection obtained for glyphosate was 0.1 mg/l. This detection limit is not good enough for glyphosate determination in water.

The separation shown in the chromatogram, Fig.13, was achieved by using the Lichrospher C_{18} column with a mobile phase containing a mixture of acetonitrile and 50 mM phosphate buffer (pH 5.5) with a ratio of 35: 65 (v/v) acetonitrile to phosphate buffer, and a flow rate of 0.5 ml/min. When using 0.5 ml/min as the flow rate of the mobile phase, the retention time for glyphosate was found to be 3.3 minutes and when using 1 ml/min the retention time for glyphosate was 1.8 minutes.

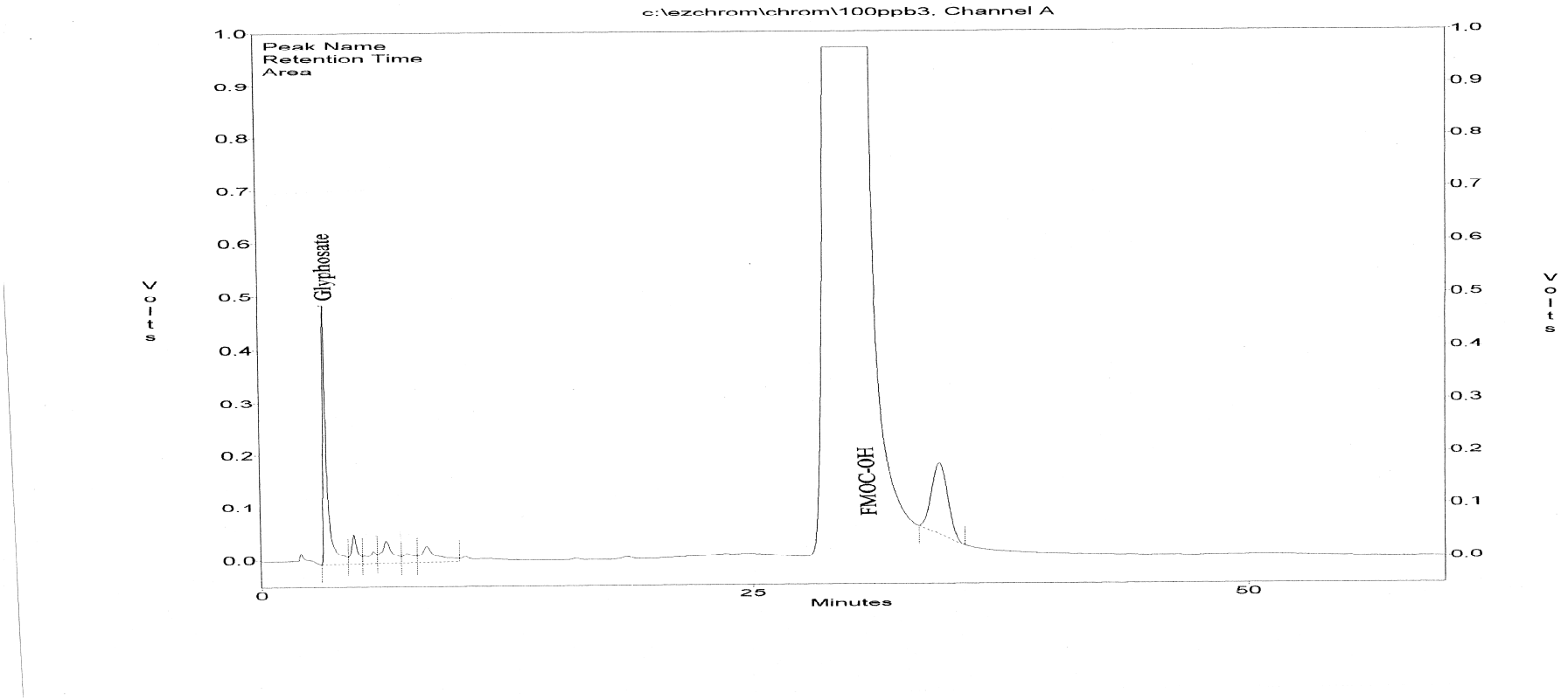


Figure 13: Chromatogram showing separation of glyphosate on C_{18} column.

A chromatogram demonstrating the separation of FMOC-glyphosate (0.1mg/l) from FMOC-OH using a C_{18} column. The front peak represents FMOC-glyphosate and the large peak represents FMOC-OH.

In order to achieve the best separation the mobile phase composition was varied. This was done by changing the amount of acetonitrile in the mobile phase in order to achieve the best separation. When the amount of acetonitrile in the mobile phase was increased, the retention time for glyphosate was found to result in the non-separation of the analyte from the interferents. This behaviour is illustrated in Fig.14.

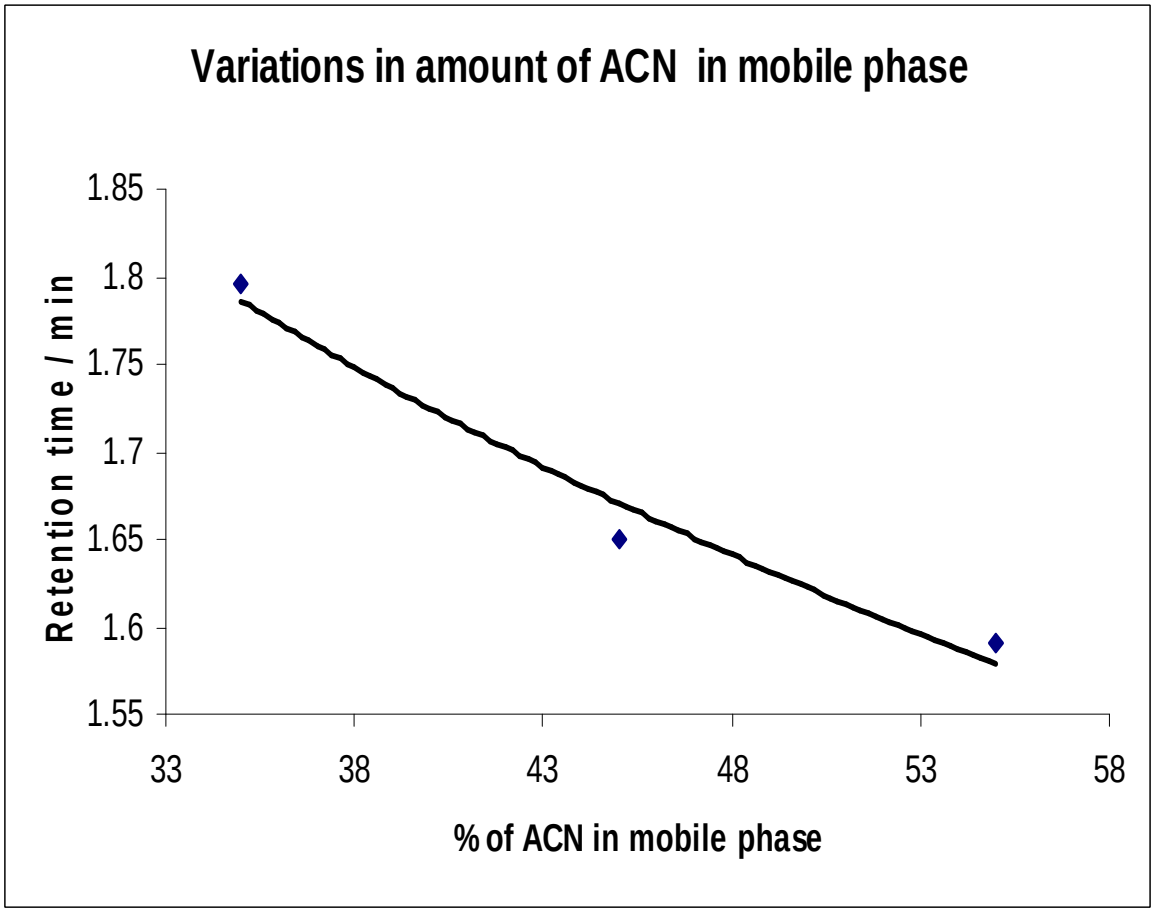


Figure 14: Changes in retention time of glyphosate due to acetontrile in mobile phase.

In view of the above set-back it was decided to vary the pH of the mobile phase. However changing the pH of the mobile phase did not improve the separation between the interferences and the analyte. Increasing the pH of the mobile phase was actually reducing the retention time of glyphosate slightly. The results obtained for the investigation of the effect of pH are shown in Table 6. These results show the change in retention time of the analyte while changing the pH of the mobile phase. The pH can only be changed over a small range because low and high pH levels reduce the lifetime of the column. Silica based columns usually degrade under high alkaline or under high acidity conditions.

As a further test of the suitability of the selected C_{18} column a calibration graph [see Fig.15] was plotted for glyphosate standard solutions in the range of 10 to 1000 µg/l. From Fig.15 it is clear that the relationship between peak area and concentration of glyphosate was not linear in chosen concentration range. This is probably due to the fact that this column is unable to separate glyphosate from the impurities that results from the derivatization reaction although FMOC-OH is separated.

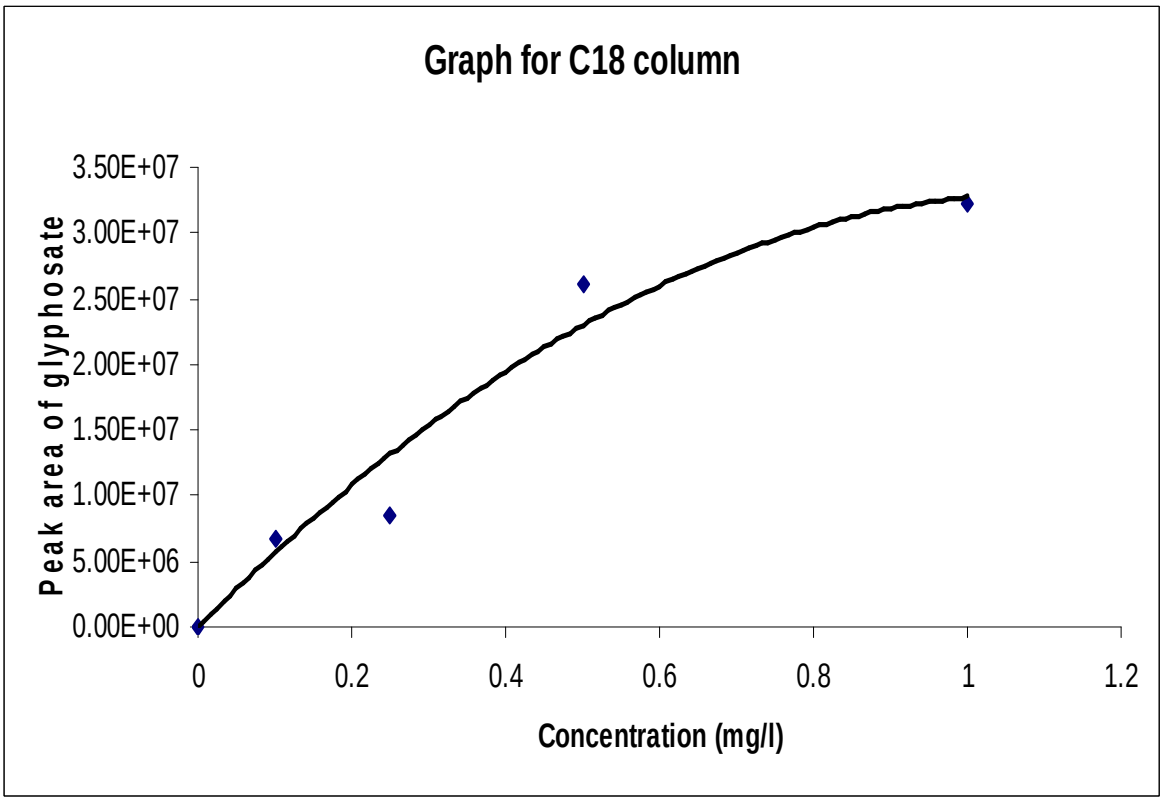


Figure 15: The response of glyphosate when using C_{18} column.

According to the results obtained when using a C_{18} column, it is clear that this column is not suitable for the determination of glyphosate in water using HPLC with a fluorescence detector. Two other analytes, namely glufosinate and AMPA were injected into the same column. From their retention times and chromatograms it can be seen that poor separation was obtained for the three analytes. The retention times are shown in Table 7.

Table 6: Effect of pH of the mobile phase on retention times

pH of the mobile phase	Retention time of the analyte /min
4	1.8
5.5	1.8
7	1.7

Table 7: Retention times of analytes using C_{18} column

Analyte	Retention time (minutes)
Glyphosate	1.8
Glufosinate	2.0
AMPA	2.2

5.5.2 Silica-based amino column

The second column investigated in this research was a silica-based spherisorb amino column. In this column, a mobile phase of pH 5.5 was used. In this mobile phase the analytes get ionised yielding anionic compounds. According to the literature [16, 22 & 34], the separation of FMOC-glyphosate and interferences arising from the derivatization reaction is preferably performed on an amino-bonded silica based column in combination with aqueous phosphate solution.

In this method the two peaks were completely resolved using the same mobile phase as used in C_{18} column [a mixture of acetonitrile and 50 mM phosphate buffer (pH 5.5) with a ratio of 35: 65 (v/v) acetonitrile to phosphate buffer] and the mobile phase flow rate was set at 1 ml/min. This method suffers from the non-reproducibility of the retention times. The

retention times were changing from run to run for identical conditions. This was expected since the phosphate solution at the selected pH (pH 5.5) does not have any buffer capacity [19]. According to Sancho et al [34], at pH 5.5 the phosphate buffer does not have any buffer capacity and therefore fluctuations in retention times should be expected. Sancho et al [34] also suggested that 0.05 M citrate buffer (pH 5.5) would be more suitable for glyphosate determination when using silica based amino column in comparison with 0.05 M phosphate buffer (pH 5.5). The use of citrate solution with amino column was previously reported by Sancho et al [19]. When using the combination of citrate solution with silica based amino column they observed a considerable band broadening of the FMOC-glyphosate peak. Sancho et al [34] also tried a mixture of phosphate buffer and citrate buffer but this did not improve the problem of band broadening of the analyte situation.

When using the silica-based amino column a plot of glyphosate standard solutions against peak areas was linear for standard solutions in water, for concentrations between 0.1 and 10 mg/l (R^2 = 0.9953 when n = 6). The chromatogram and the calibration graph are shown in Fig.16 and Fig.17 respectively. The limit of detection was determined for glyphosate for this method and was found to be 0.1 mg/l, using signal/noise ratio of 3. Based on this detection limit, it was concluded that this method suffered from poor sensitivity. By comparison, the HPLC methods reported in the literature, have been shown to have detection limits of less than 0.005 mg/l for glyphosate [3, 16 and 25].

To improve the sensitivity in silica-based amino column method, a method of removing FMOC-OH and all interfering derivatization products was investigated. This was done by coupling both C_{18} column and amino column discussed above.

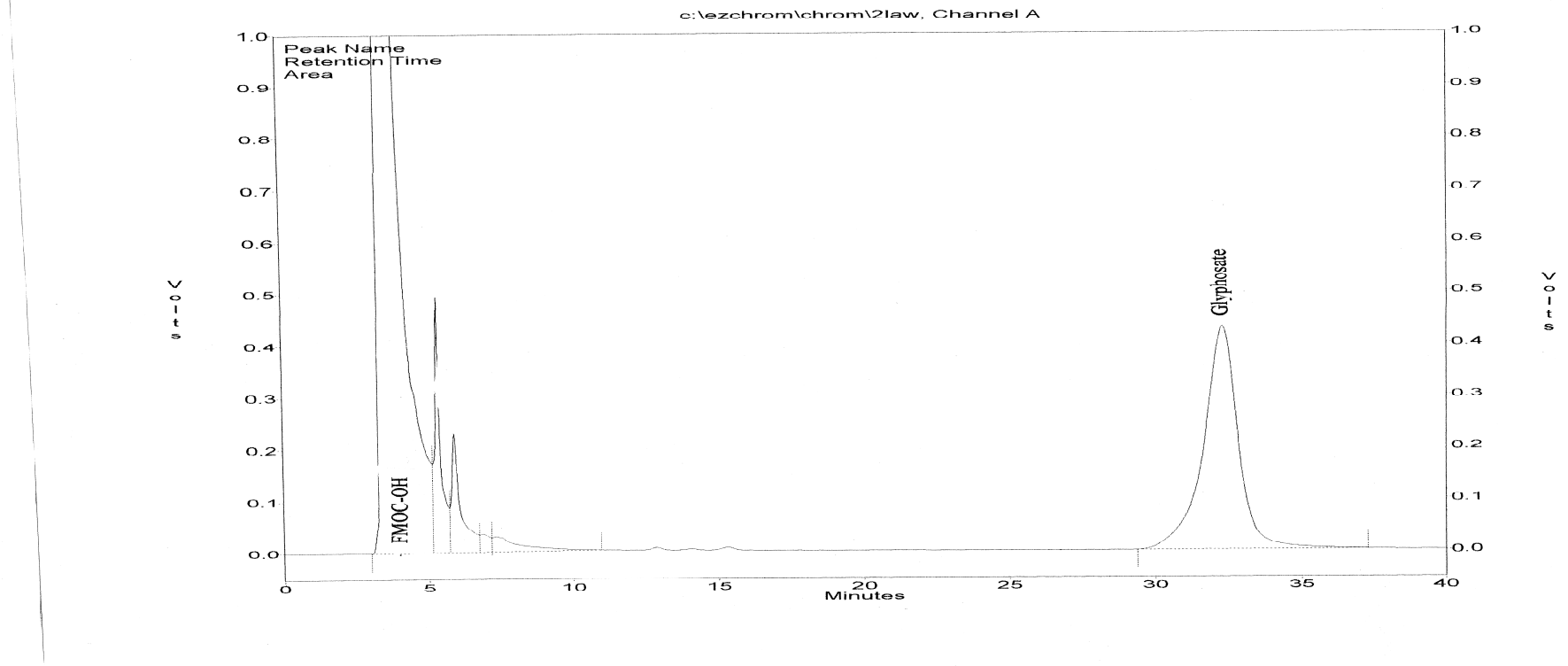


Figure 16: Chromatogram showing separation of glyphosate on spherisorb amino column.

This chromatogram demonstrates the separation of FMOC-glyphosate (2 mg/l) from FMOC-OH.

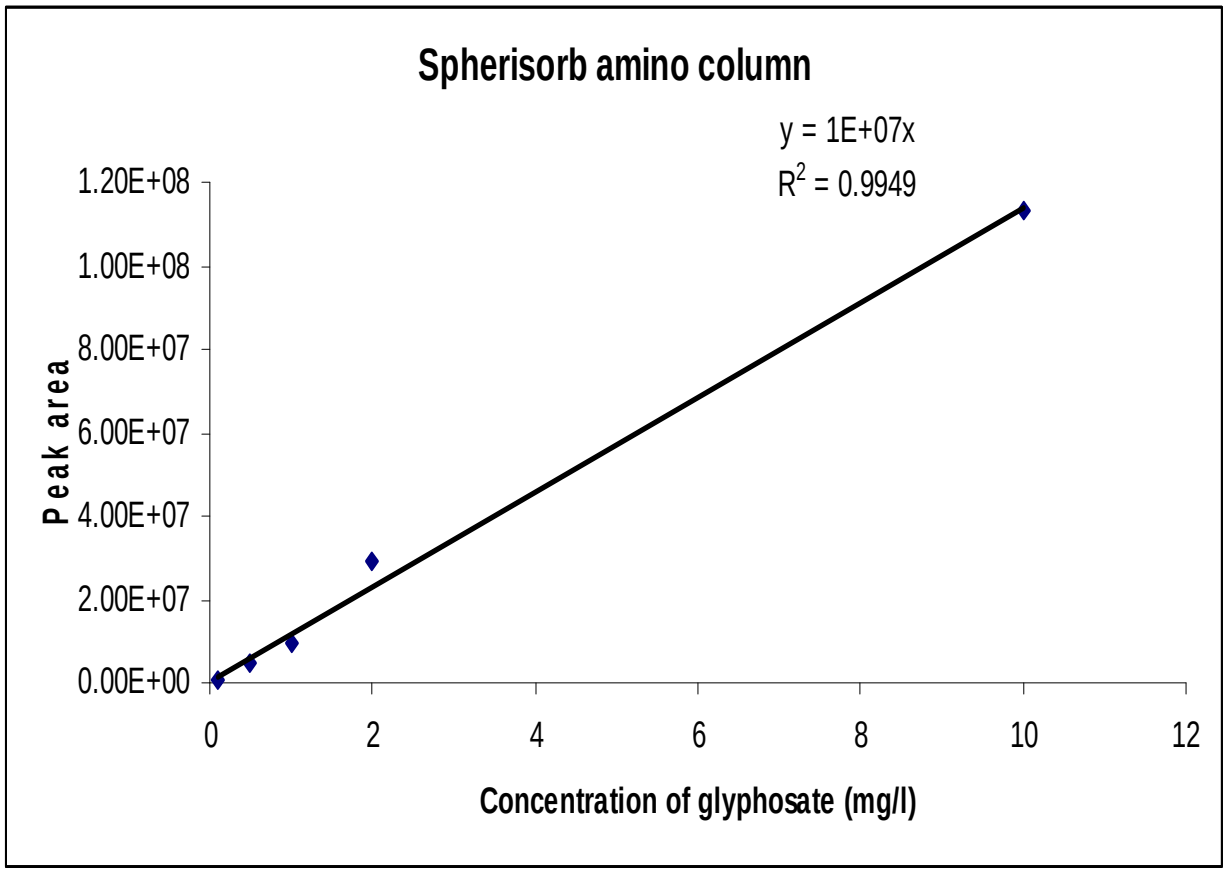


Figure 17: The response of glyphosate when using spherisorb (silica-based) amino column.

5.5.3 Coupling of C_{18} and amino column

A silica-based amino column and a C_{18} column were coupled to attain the following two objectives:

- To improve the detection limit obtained when using the silica-based amino columns,
- To remove FMOC-OH product and other interfering products produced from a pre-column derivatization of glyphosate.

Fig. 18 shows two schematic diagrams of the chromatographic system set-up for the column switching technique. The column coupling technique used two columns and two pumps.

Diagram A: Loading position

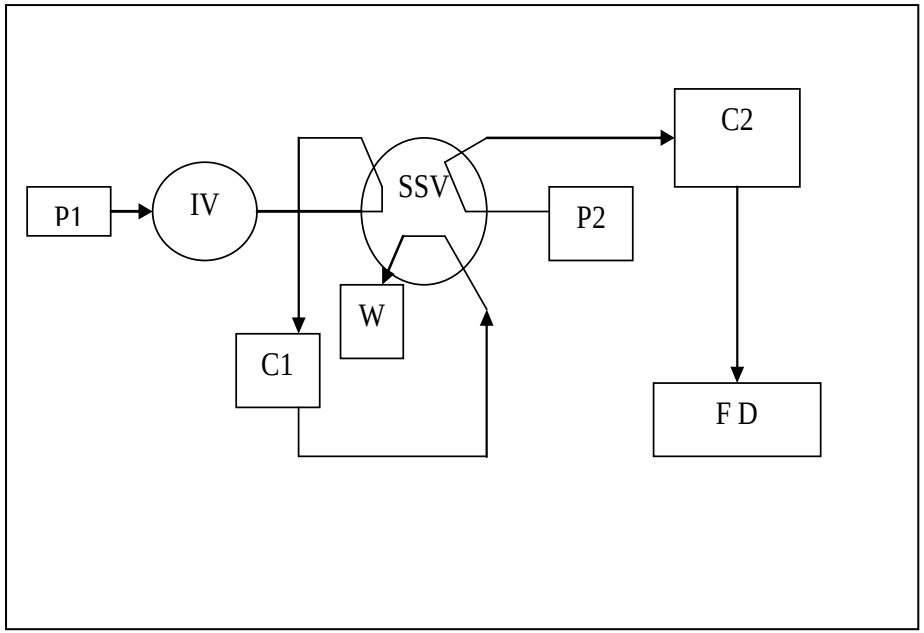


Diagram B: Injecting position

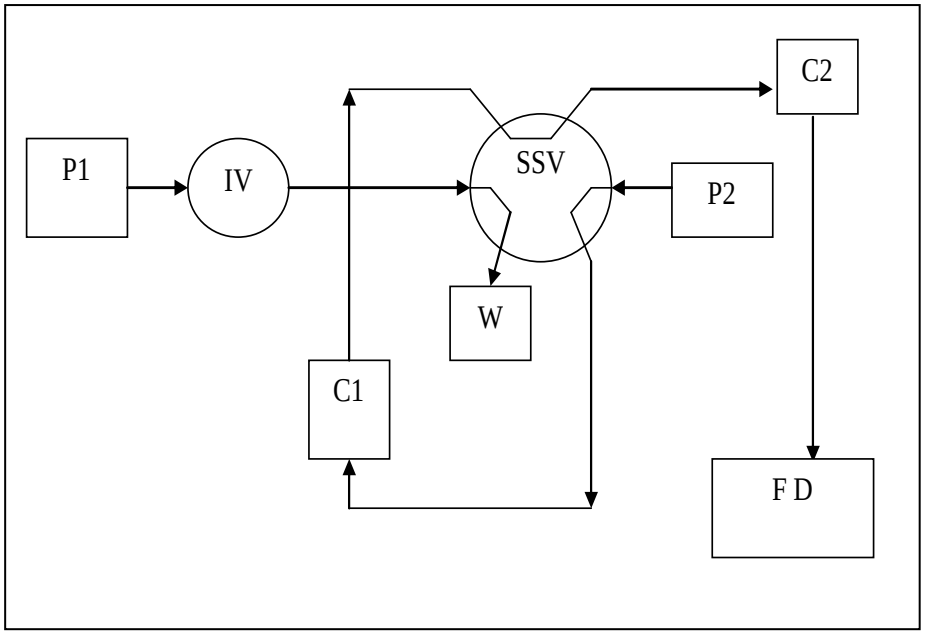


Figure 18: Schematic diagrams of column switching HPLC system.

Symbols in diagrams: P1: first LC pump; P2: P100, second LC pump; SSV: six-port switching valve; IV: Rheodyne injection volume; C1: pre-separation column (C_{18} column); C2: analytical column (amino column); W: waste bottle; F D: fluorescence detector.

5.5.3.1 Chromatographic system

As illustrated in both diagrams (Fig. 18, A and B) a pre-separation column which is a C_{18} column was coupled to the analytical column (silica-based amino column) through a six-port switching valve. Analytical column was housed in a column block heater, and the column was kept at a temperature of 25^0C and this column (C2) was the main separation column for this work. In this column-coupling technique, a volume of 20 µl of glyphosate standard obtained after derivatization with FMOC-Cl at pH 9 was injected into the pre-separation column when the switching valve was in loading position (Fig. 18, diagram A). The unretained analyte (FMOC-glyphosate) was passed through the pre-separation C_{18} column with a mobile phase containing a mixture of acetonitrile and 0.05 M phosphate buffer (pH 5.5) at a ratio of 35: 65, v/v acetonitrile to phosphate buffer solution by pump 1 at a flow rate of 0.5 ml/min, and simultaneously the derivatization interferents including FMOC-OH product were retained in C_{18} column by a reverse phase mechanism. Five minutes after the standard was injected, the switching valve was switched to the injecting position (Fig. 18, diagram B) and the analyte was transferred to the analytical column in a back-flush mode and applying a strong mobile phase consisting of 35% (v/v) acetonitrile in 0.1 M phosphate buffer (pH 5.5) delivered by pump 2 at a flow rate of 1.0 ml/min. While the analyte was being separated in analytical column (C2) by an ion exchange mechanism, the derivatization interferences and FMOC-OH were sent from C_{18} column to waste without passing the second analytical column.

5.5.3.2 Separation of interferences in C_{18} column

In this procedure the first column (C_{18} column) was used for the purpose of pre-separating the un-wanted (interfering) derivatization products. This was done by injecting the standard or sample directly into C_{18} column. In this LC integrated standard pre-separation set-up, the standard was first fractioned into derivatization interferences and analyte by the use of the Lichrospher C_{18} pre-separation column. When the glyphosate standard was injected into the C_{18} pre-separation column, the highly polar FMOC-glyphosate was transferred to the second column without adsorption, while the FMOC-OH and less polar interferences are adsorbed and retained in the C_{18} column. In this way, the packing material in first column provides a direct extraction of less polar interferences, and the separation of less polar interferences from a highly polar analyte. In order to create enough selectivity in the system and to get high

recovery of the analyte in the pre-separation step, it was necessary to optimise the pre-separation, the composition of the mobile phase as well as the switching time of the six-port switching valve.

After optimization, a mobile phase of acetonitrile-phosphate buffer, pH 5.5 (35:65, v/v) was used. In this approach, FMOC-glyphosate was ionised and resulted in little C_{18} retention on C1 and adequate retention on the amino column (C2). The efficient removal of derivatization interferences was obtained by transferring an almost unretained small analyte-containing fraction to the amino column on C2. All interferences with more C_{18} retention such as FMOC-OH were retained and sent to waste by the rinsing mobile phase during the separation of the analyte on C2.

5.5.3.3 Separation on second column (C2)

During the derivatization reaction (Fig. 6), glyphosate reacts with FMOC-Cl (an acid chloride) via its amine group, yielding an anionic compound, FMOC-glyphosate. According to the literature [22, 34], the separation of FMOC-glyphosate is preferably performed on an amino-bonded silica column in combination with aqueous phosphate solution. C2 was directly connected to the fluorescence detector. Therefore, the analyte, after elution from the second column (C2), is monitored by a fluorescence detector set at an emission wavelength of 315 nm and an excitation wavelength of 266 nm.

5.5.3.4 Results and discussion based on column switching technique

The chromatogram obtained in column switching technique for 1 mg/l (1 ppm) of glyphosate standard is shown in Fig. 19. This chromatogram shows that less polar interferences including FMOC-OH arising from derivatization reaction can be separated and effectively removed from the glyphosate chromatogram. A series of glyphosate standards were prepared and injected. The calibration graph of this method using the mentioned parameters was constructed. The response for glyphosate standards was found to be linear with concentrations ranging between 0.1 and 10 mg/l with $R^2 = 0.9979$ (n = 5). The calibration graph is shown in Fig. 20. The detection limit of glyphosate when using the column coupling technique was found to be 0.05 mg/l. This was found by using the signal to noise ratio of 3.

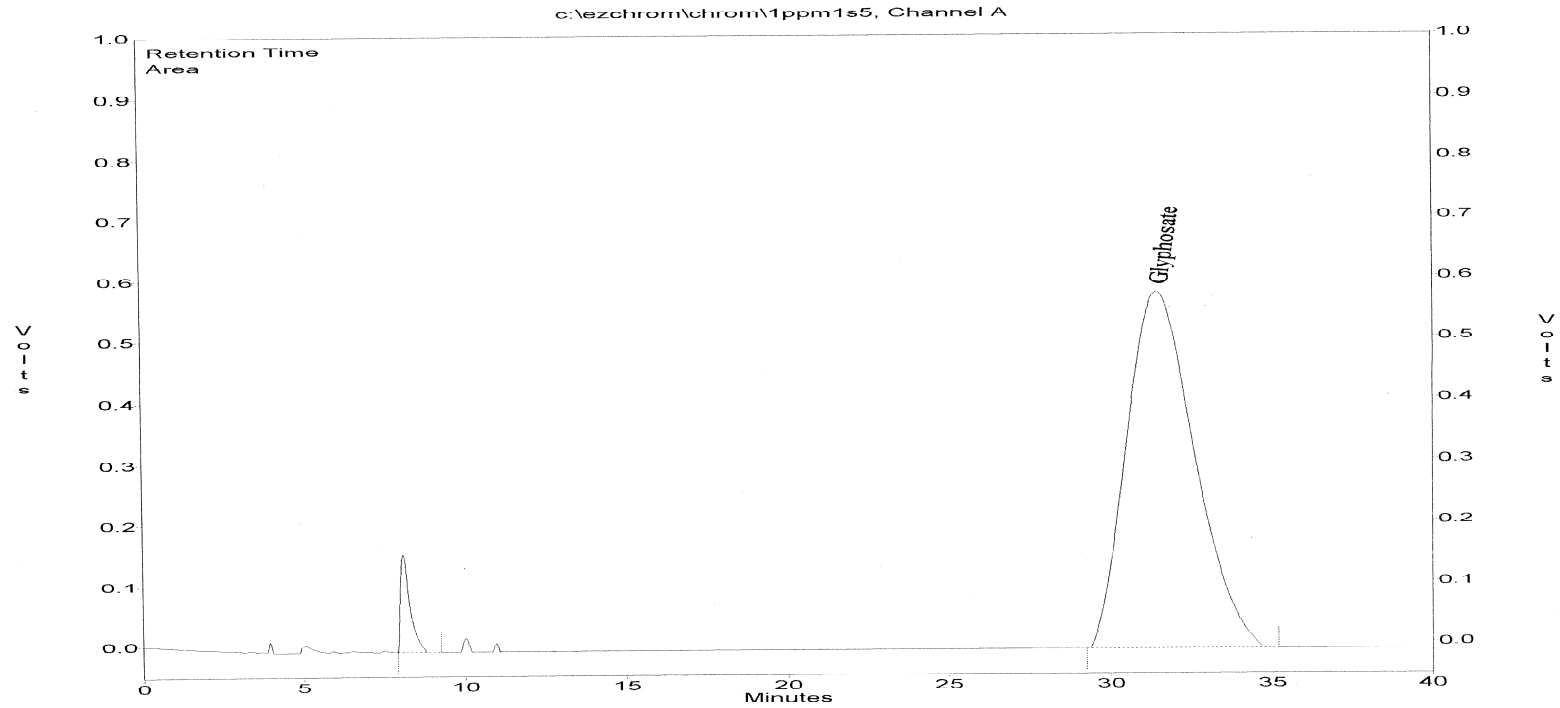


Figure 19: Chromatogram showing glyphosate in column switching technique.

A chromatogram of 1 mg/l (ppm) glyphosate standard obtained in column coupling technique.

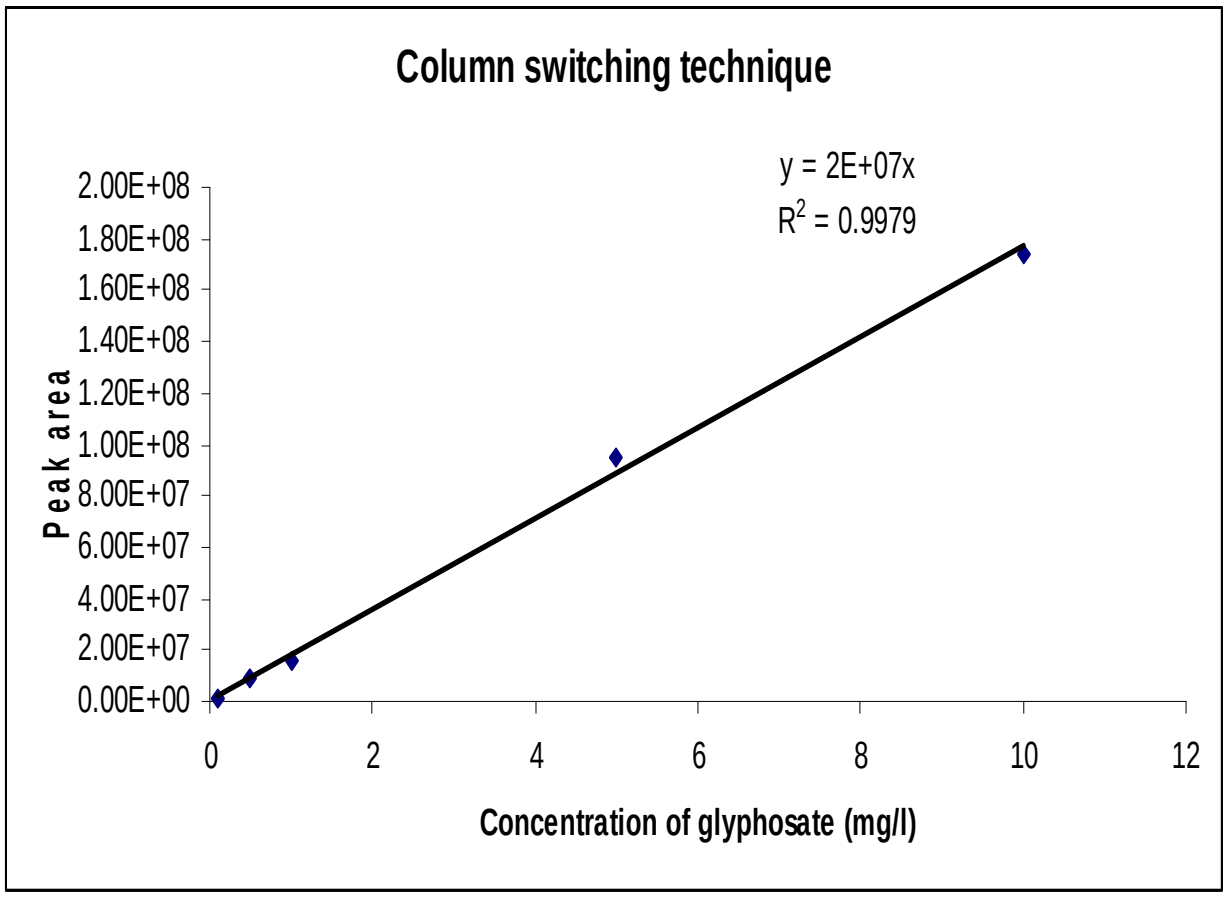


Figure 20: The response of glyphosate when using column switching technique.

Aiming for a detection limit of 0.7 mg/l which is the maximum contaminant level of glyphosate in drinking water (set by US-EPA), a good sensitivity was obtained in the column-coupling technique. In literature, the coupling of short C_{18} column and amino column is reported by Hogendoorn et al [16] and Tadeo et al [26] with much better detection limits. Hogendoorn et al [16] published a paper that is based in glyphosate determination in grain samples down to a level of 0.5 mg/kg using the coupling of short (30 x 4.6 mm I.D.) C_{18} column and amino column. When the two columns were coupled in this work, it was observed that the retention times for the glyphosate peak were not stable; therefore AMPA was not investigated although the objectives for the use of this technique were achieved. The retention times for glyphosate peak were fluctuating.

5.6 Polymer amino column for separation of glyphosate and AMPA

The polymer amino column was investigated to overcome the problems encountered when using silica based columns (C_{18} and amino column). As stated previously, for a silica-based amino column at pH 5.5 the retention times are not reproducible from run to run. To overcome the fluctuation of retention times, the use of a polymer amino column at high pH (pH 10) was investigated. Silica based amino columns cannot be used at high pH values because they usually degrade at high alkaline conditions [24]. The degradation of a silica based amino column occurred after few weeks when this column was used at pH 10 in this research. Sancho et al [34] reported that there is a gradual decrease in efficiency of the silica-based amino column after continuous usage for about two months at high alkaline conditions. In view of this problem, it was decided to find a more robust amino separation column for glyphosate analysis which can tolerate a greater pH range and can be easily regenerated.

A polymer amino column was chosen on the basis of its properties, namely;

- It has a good mechanical strength,
- It has a good chemical stability and
- It is capable of working over a wide pH range (pH 2-13).

5.6.1 Preparation of solutions before injection into polymer amino column

In this method the standards were prepared as for silica based columns. The method for the derivatization of standards in C_{18} column was adapted and used on the polymer column. The standards were derivatized with FMOC-Cl. The latter was added in excess to ensure complete derivatization of the analyte.

Once formed FMOC-OH and other interferences from derivatization reaction were removed by extracting the reaction mixture with organic solvent. Ethyl acetate is the only organic solvent investigated for the removal of interferences from derivatization reaction in this research. The use of ethyl acetate has been reported [24]. Solvent extraction was performed with ethyl acetate after 30 minutes of pre-column derivatization of glyphosate with FMOC-Cl. The reaction mixture was extracted three times with 6 ml of ethyl acetate and by doing

this the interferences arising from the derivatizing reaction are removed (for more information see experimental section).

5.6.2 Separation of glyphosate and AMPA

The two compounds, glyphosate and AMPA were well separated using a polymeric amino column with a mobile phase containing a mixture of acetonitrile-0.05 M phosphate buffer (pH 10) (55:45, v/v). The flow rate of the mobile phase was 1 ml/min and the temperature of the column was kept at 25^0C. The chromatogram obtained when using these conditions is shown in Fig. 21.

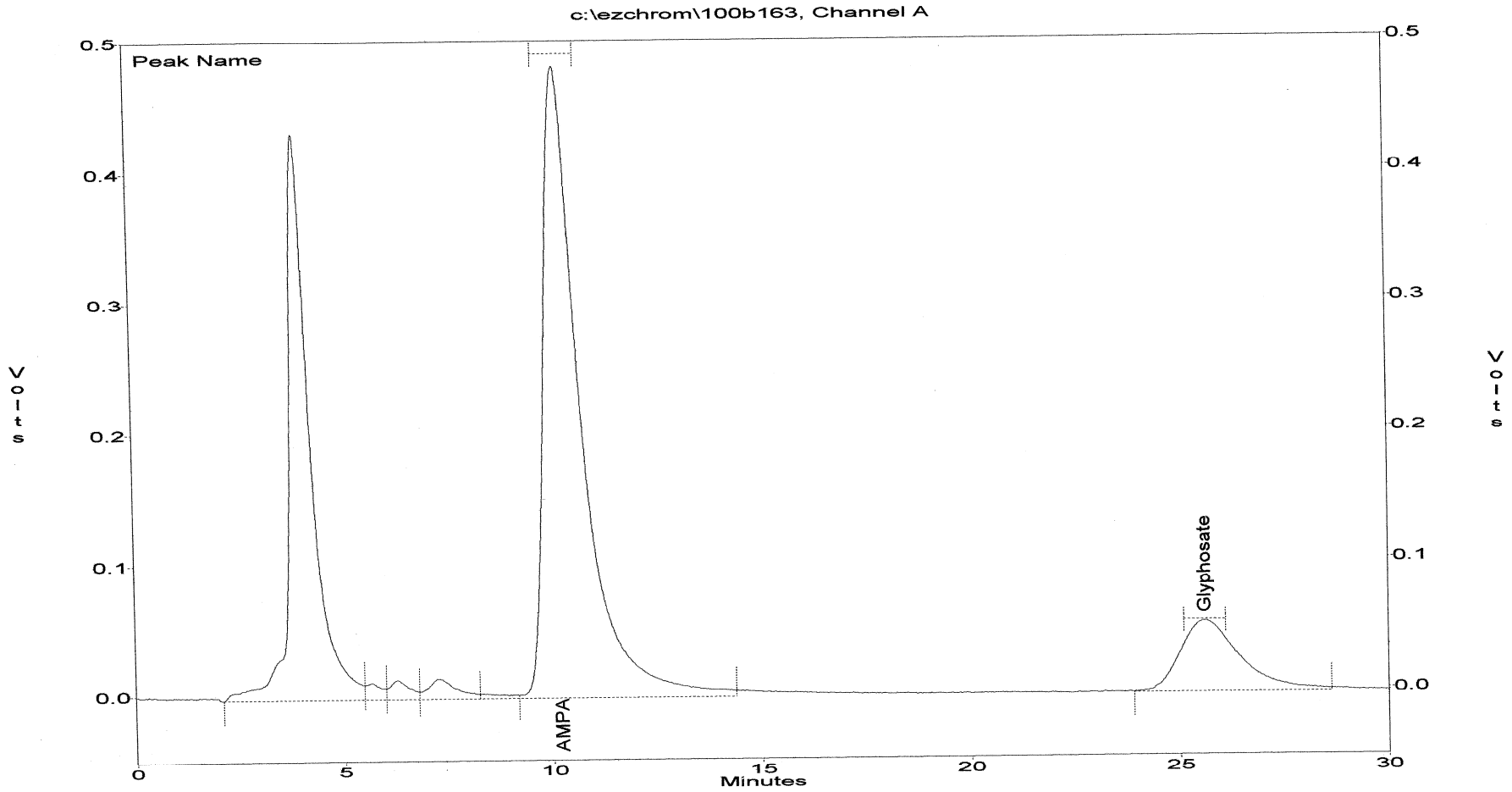


Figure 21: The separation of glyphosate and AMPA derivatives on a polymeric amino column after the removal of FMOC-OH with ethyl acetate. HPLC mobile phase (pH 10) contained 55% (v/v) acetonitrile in water, and 50 mM phosphate buffer.

5.6.3 Results and discussion for polymer amino column

5.6.3.1 Behaviour of glyphosate and AMPA on polymeric amino column

Secondary amides are formed from the reactions between the FMOC-Cl and both glyphosate and AMPA in a mobile phase of acetonitrile-phosphate buffer at pH 10. The resulting amides are negatively charged species; the structures of which are shown in Fig. 22. The amino groups of the column at pH 10 are positively charged. The interactions of these positively charged groups with the derivatives of glyphosate and AMPA may be ascribed to an ion-exchange mechanism, possibly complemented by hydrophobic interaction with the polymeric backbone of the column [24].

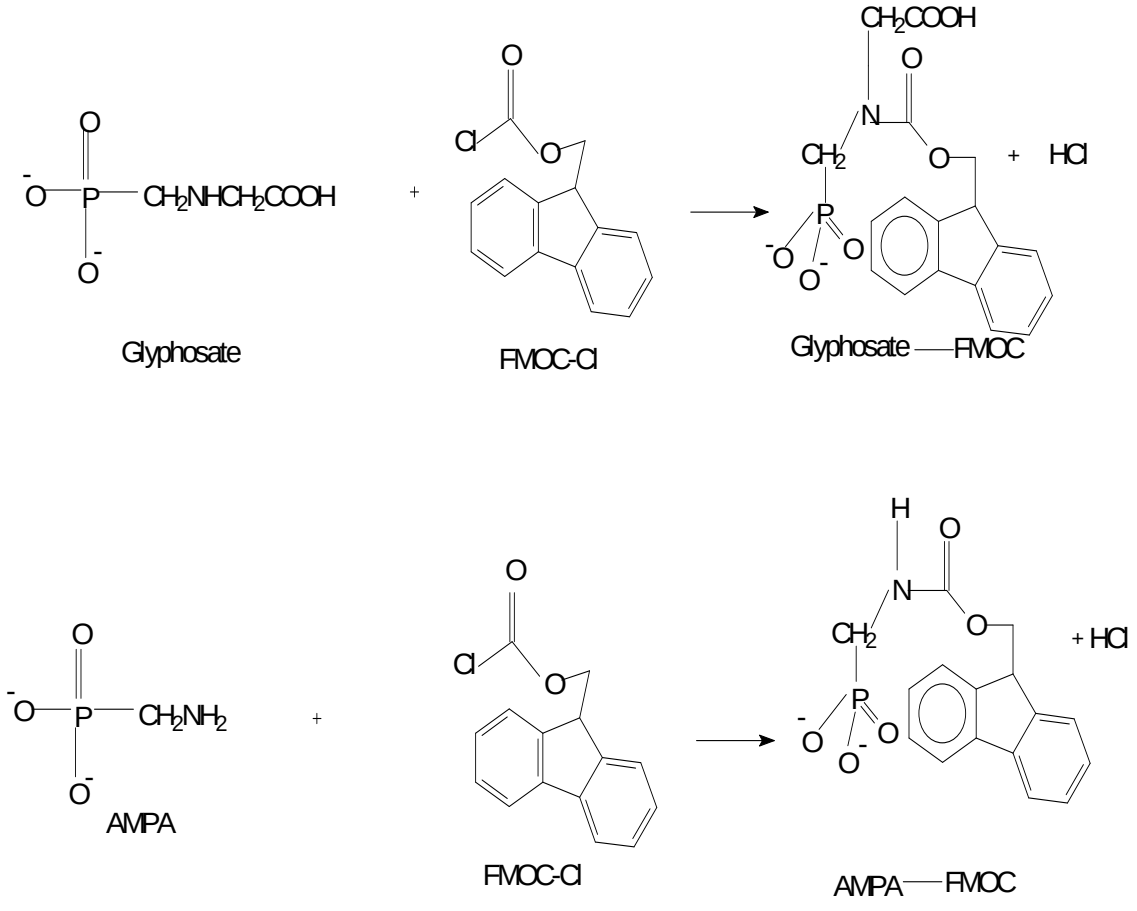


Figure 22: The structures of amides formed at pH 10.

With regard to separation, AMPA elutes prior to the elution of glyphosate. This order of elution is attributed to the presence of an extra CH_2COOH group in glyphosate structure which provides more ionic interaction with the column. The mobile phase of acetonitrile-phosphate buffer at pH 10 gives rise to complete ionisation of the carboxylic group of the glyphosate and AMPA molecule. The anionic species reacts more strongly with the positively-charged amino groups of the stationary phase of the column. Therefore there is greater retention for glyphosate on a polymer amino column.

5.6.3.2 Amount of acetonitrile in the mobile phase

The effect of acetonitrile concentration on the elution of glyphosate and AMPA was investigated. This was one by changing the percent of acetonitrile in the mobile phase; the range investigated was between 35 – 60% (v/v) of acetonitrile. It was found that the elution of both glyphosate and AMPA from the polymeric amino column decreased with increasing percentages of organic modifier. Optimal separation occurred when using 55% (v/v) of buffered acetonitrile in the mobile phase. When the mobile phase contained 35% (v/v) of acetonitrile, longer retention times for both analytes were observed. This implies that the mobile phase was too weak for eluting both glyphosate and AMPA from polymer amino column. It was observed that as the concentration of acetonitrile increases the retention times for both analytes was decreasing, these results are also depicted on Figs. 23 and 24.

Table 8: Variation of retention times with changes of % acetonitrile in mobile phase

Glyphosate		**AMPA**	
% acetonitrile	**Retention time (min)**	**% acetonitrile**	**Retention time (min)**
35	38	35	19
40	33	40	15
50	23	50	11
55	23	55	10
60	21	60	9

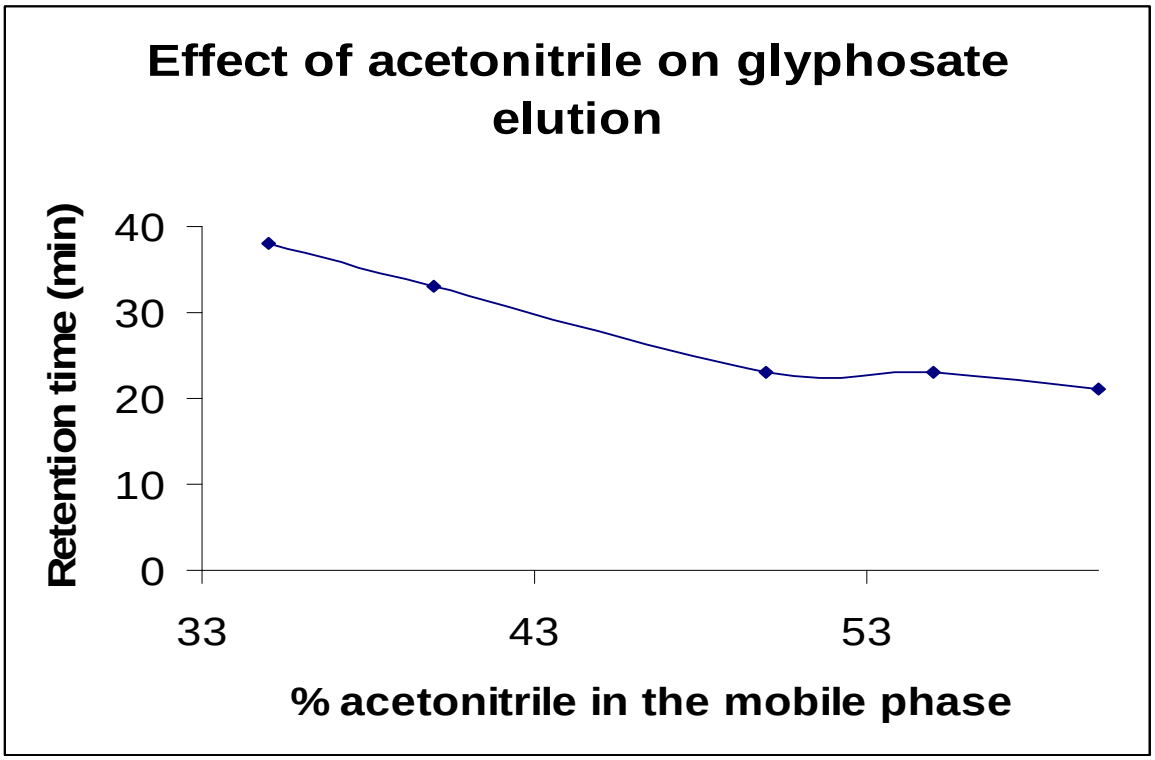


Figure 23: Effect of acetonitrile in glyphosate elution.

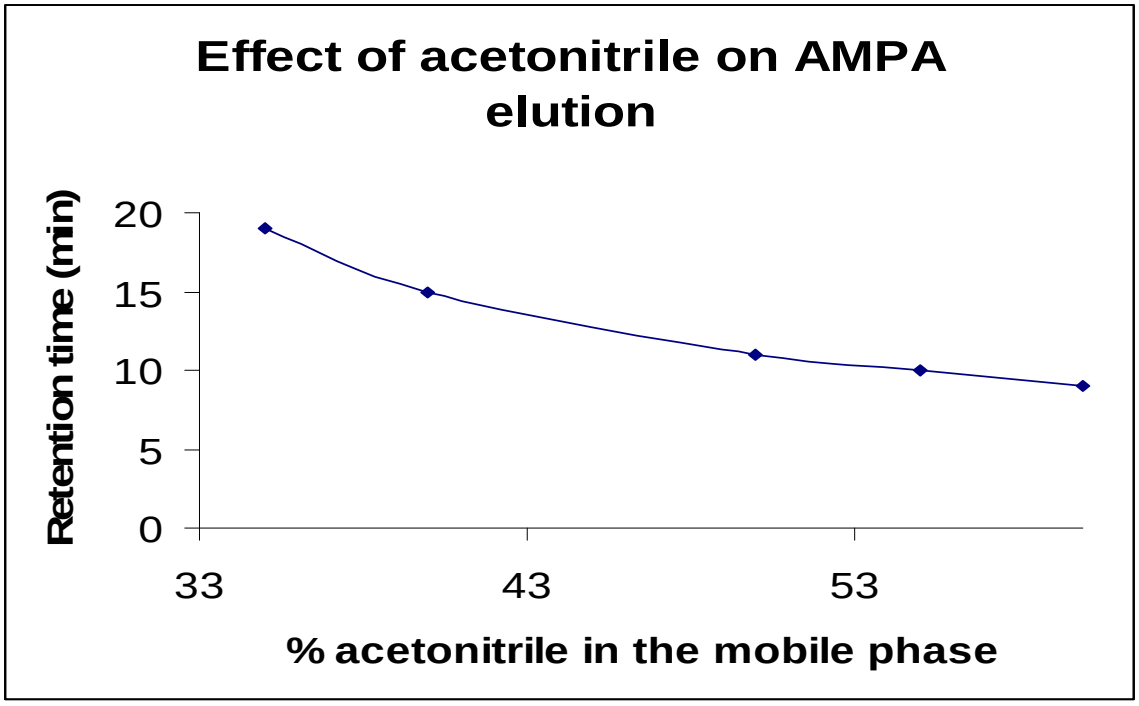


Figure 24: Effect of acetonitrile in AMPA elution.

5.6.3.3 Calibration

After a good separation of both glyphosate and AMPA was achieved by the use of a polymeric amino column, a multipoint calibration was performed for both compounds. This was done to construct a calibration graph. As shown in Figs. 25 and 26 the curves for both analytes were linear.

Table 9: Results for calibration lines of glyphosate and AMPA

Glyphosate		**AMPA**	
Concentration (ppb)	**Peak area**	**Concentration (ppb)**	**Peak area**
0.0	0.00	0.0	0.00
5.0	100116	1.0	539928
10	198078	5.0	2712844
50	1132104	10	4428310
100	2132726	50	22206930
		100	50870276

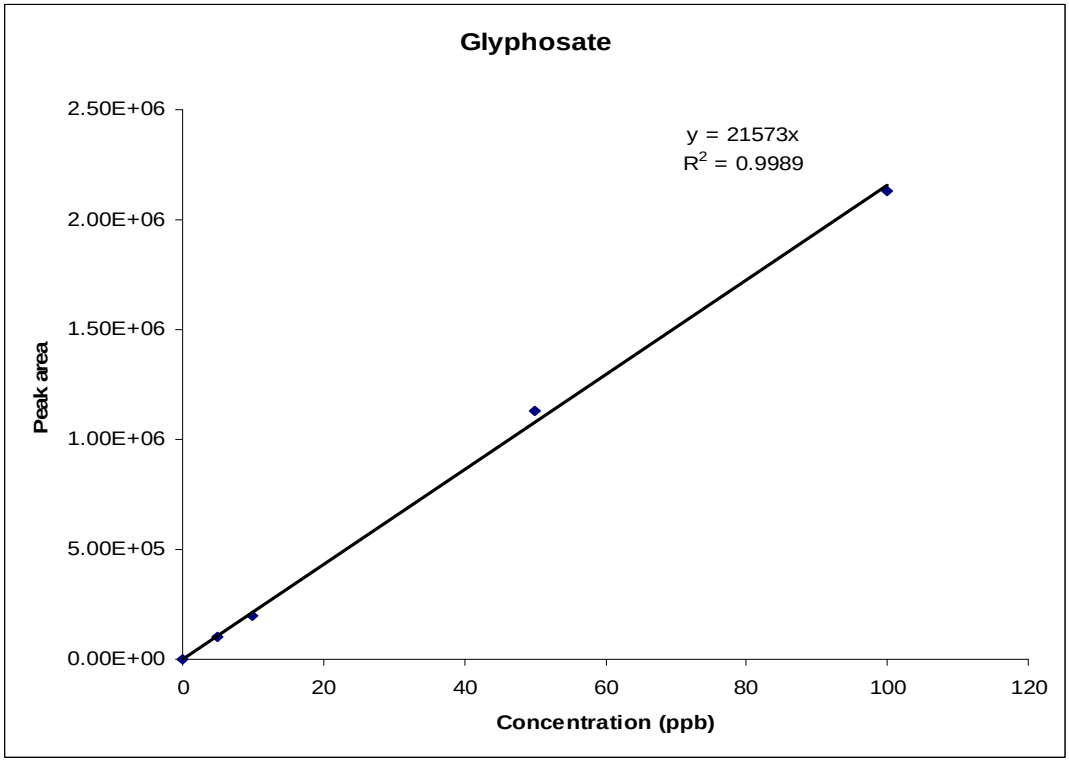


Figure 25: Polymer column; calibration line for glyphosate.

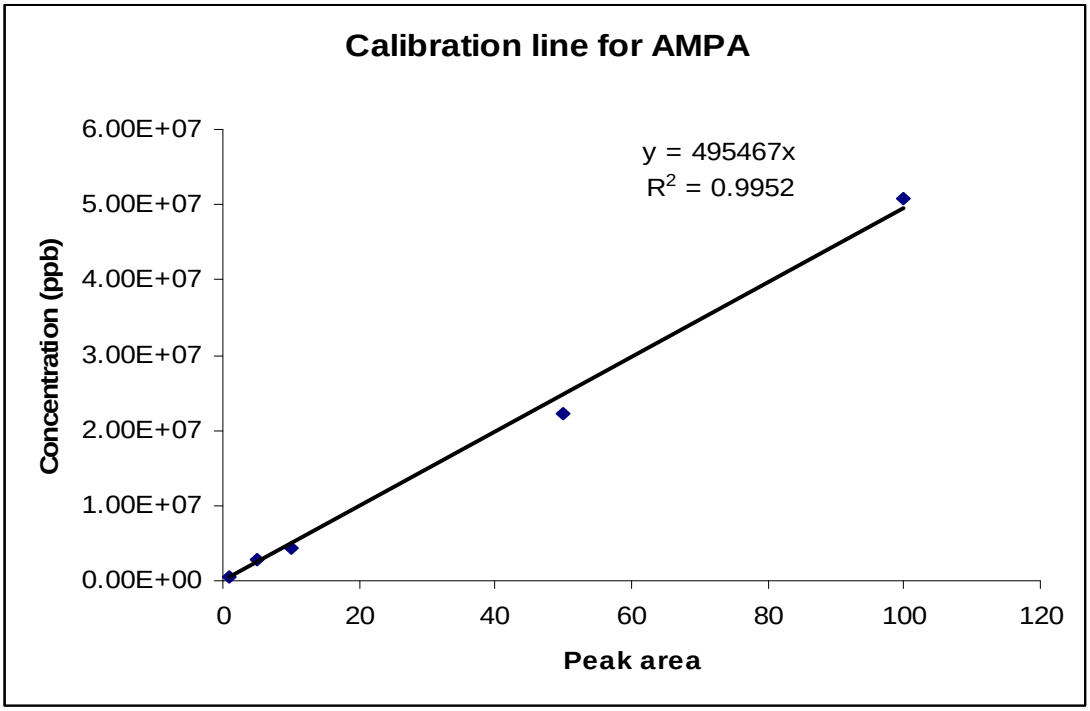


Figure 26: Polymer column; calibration line for AMPA.

5.7 Investigation of the efficacy of SPE in sample clean-up procedure

For the determination of glyphosate and AMPA in environmental water samples, an additional step was required in sample preparation procedure. This additional step was used for the separation of non-polar interferences from the highly polar analytes, glyphosate and AMPA. A sample clean-up step was investigated; this step being necessary for samples with high amount of interferences and in this work a suitable SPE cartridge was required for the removal of interferences. Thus SPE cartridges packed with different sorbents were investigated. This was done by doing the recovery studies of 100 μg/l (ppb) mixture of both glyphosate and AMPA.

5.7.1 Strata C_{18}-E and Strata C_{18}-U cartridges

The packing material in both of these cartridges is 100 mg of C_{18} sorbent. This is a non-polar sorbent with a tendency of retaining the non-polar compounds. Hogendoorn et al [16] have reported the use of C_{18} SPE cartridges for the extraction of glyphosate from cereal samples with an overall recovery of 86%. At early stages of the investigation the method used by Hogendoorn et al [16] for SPE was adapted and the method was optimised to get better recoveries. For calculation of recoveries, the calibration graphs of both glyphosate and AMPA were plotted. These graphs are shown in Figs. 27 and 28.

Table 10: Concentrations and peak areas of analytes for calibration lines

Glyphosate		**AMPA**	
Concentration (ppb)	**Peak area**	**Concentration (ppb)**	**Peak area**
10	229836	5.0	698155
50	1546402	10	1318966
100	4218533	50	5728962
500	24177676	100	13153118

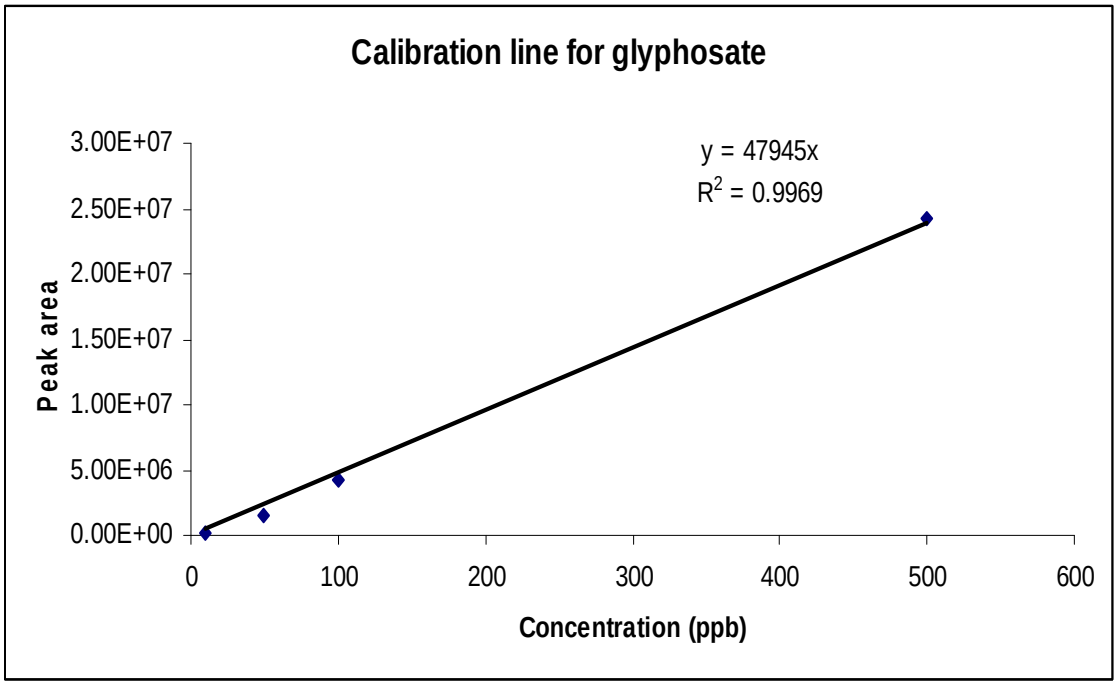


Figure 27: Calibration line for glyphosate.

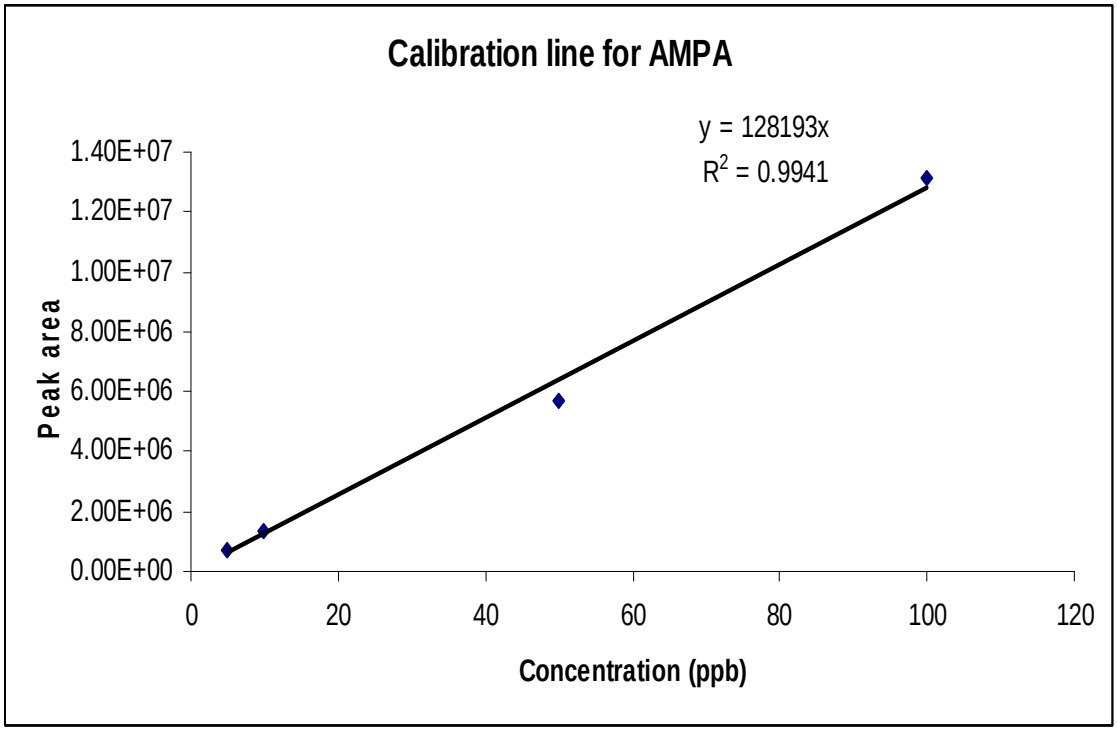


Figure 28: Calibration line for AMPA.

5.7.1.1 SPE methodology for Strata cartridges

The conditioning of the cartridges was carried out, sequentially, with 3 ml of acetonitrile, 3 ml of water and 1 ml of standard, at a flow rate of 1 ml/min; these quantities were sent to waste. Then 1.5 ml of the standard was transferred through the cartridge at a flow rate of 1 ml/min and collected into a tube. The collected standard was then derivatized, extracted with ethyl acetate before the injection into the HPLC column.

5.7.1.2 Calculation of % recoveries

Strata C_{18}-E

Glyphosate

$y = 47945\,x = 1250539$

x = 1250539/47945 = 26.1 ppb

% recovery = 26.1 ppb / 100 ppb x 100%
= 26.1%

The 100 ppb standard gave a peak of area = 1250539, which gives a recovery of 26.1%.

Table 11: % recoveries for Strata C_{18} cartridges

Cartridge type	% recovery	
	Glyphosate	**AMPA**
Strata C_{18}-E	26.1	96.0
Strata C_{18}-U	13.2	90.7

5.7.1.3 Optimization of SPE methodology

To improve the percent recovery of glyphosate, the SPE method was optimized. The following two changes in SPE methodology were made.

- The amount of standard passed through the cartridge was increased to 2.5 ml.
- The standard obtained after derivatization and solvent extraction was transferred through the cartridge.

These two procedures did not improve the percent recovery of glyphosate; therefore another cartridge packed with 500 mg of C_{18} was investigated.

5.7.2. Sep-Pak Vac 3cc (500 mg) C_{18} cartridges

5.7.2.1 Methodology for Sep-Pac cartridges

The conditioning of the cartridges was carried, sequentially, with 3 ml of acetonitrile, 3 ml of water and 1 ml of standard at a flow rate of 1 ml/min; these quantities were sent to waste. Then 1.5 ml of the standard was transferred through the 500 mg SPE cartridge at a flow rate of 1 ml/min and collected into the tube. The collected standard was then derivatized, extracted with ethyl acetate before the injection into the HPLC column.

- This method is adapted from that reported by Hogendoorn et al [16] and percent recoveries are reported in Table 12 A.
- The results, indicated in Table 12 B and 12 C, were obtained by passing the standard obtained after derivatization through the cartridge and increasing the volume of derivatized standard passed through the cartridge to 3 ml, respectively. Acceptable recoveries were obtained when 3 ml of the derivatised standard was passed through the cartridge. Therefore the same conditions were applied in the case of environmental water samples.
- The recoveries obtained when spiking tap water with 100 ppb mixture of glyphosate and AMPA are shown in Table 13.

Table 12 A: % recoveries for 500 mg C_{18} cartridges

Cartridge type	% recovery	
	Glyphosate	**AMPA**
500 mg C_{18} SPE cartridge	21.7	96.0

Table 12 B: % recoveries obtained when passing derivatized standard through the 500 mg C_{18} cartridge

Cartridge type	% recovery	
	Glyphosate	**AMPA**
500 mg C_{18} SPE cartridge	57.6	107

Table 12 C: % recoveries obtained when passing 3 ml of derivatized standard through the 500 mg C_{18} cartridge

Cartridge type	% recovery	
	Glyphosate	**AMPA**
500 mg C_{18} SPE cartridge	105	95.2

Table 13: % recoveries obtained when spiking tap water

Cartridge type	% recovery	
	Glyphosate	**AMPA**
500 mg C_{18} SPE cartridge	101	90.2

5.7.3. Oasis HLB 6cc (200 mg) SPE cartridges

Good recoveries were obtained using 500 mg of C_{18} SPE cartridges but due to the limitations regarding the use of C_{18} SPE cartridges the use of Oasis cartridges was investigated for sample clean-up in the analysis of glyphosate and AMPA in water using HPLC with fluorescence detector.

C_{18} SPE cartridges have limitations because they are not hydrophilic or water wettable therefore during the extraction, care was taken to ensure that the sorbent stays wet before loading the sample to avoid low analyte recoveries. To overcome the reverse phase limitations, HLB cartridges are normally used. HLB cartridges are packed with a copolymer known as poly (divinylbenzene-co-N-vinylpyrrolidone) that exhibits both hydrophilic and lipophilic retention characteristics [43]. HLB has a tendency of retaining both polar and non-polar compounds [43].

5.7.3.1 Oasis HLB cartridge methodology

The following solvents were prepared and used for SPE clean-up.

Eluting solution	Composition of eluting solution
(1)	LC-grade water at pH 2.5 was adjusted with formic acid.
(2)	Ammonium acetate (5 mM) pH 4.8 was adjusted with acetic acid.
(3)	A mixture of 90% acetonitrile and 10% of 5 mM ammonium acetate pH 4.8.
(4)	A mixture of 50% acetonitrile and 50% of 5 mM ammonium acetate pH 4.8.
(5)	A mixture of 10% acetonitrile and 90% of 5 mM ammonium acetate pH 4.8.

In SPE, the cartridge was conditioned with 7 ml of LC-grade water (pH 2.5) at a flow rate of 1 ml/min. An aliquot of 4.3 ml of 100 ppb standard obtained after derivatization was loaded into the Oasis HLB cartridge at a flow rate of 1 ml/min followed by washing with acidified water (pH 2.5). After washing, the analytes were eluted with solvent no: (3).

5.7.3.2 Results for Oasis HLB extraction

Poor recovery of < 10% was obtained for AMPA with no recovery for glyphosate. Solvent no (4) and (5) were used for elution of analytes but the recoveries were not improved. It was concluded that a suitable solvent is required for the elution of analytes to improve recoveries

because all the fractions of solvents collected from the cartridge contained no analytes, therefore the analytes were not recovered from the SPE cartridge.

5.7.4. Oasis MAX 6cc (150 mg) SPE cartridges

Oasis MAX cartridges contain a mixed-mode polymeric sorbent with a reversed-phase and an anion-exchange functionalities [43]. In Oasis Max, strong anion-exchange quaternary amine groups are on the surface of a poly (divinylbenzene-co-N-vinylpyrrolidone) copolymer. During this work different methods were investigated.

5.7.4.1 MAX cartridge methodology

100 ppb standard containing a mixture of glyphosate and AMPA was prepared in water; the pH of the standard was 6.3. The cartridge was conditioned by passing 2 ml of methanol, 4 ml of 0.5 M sodium hydroxide and 2 ml of HPLC-grade water, respectively at a flow rate of 1 ml/min. After conditioning, the cartridge was rinsed with 4 ml of 0.5 M sodium hydroxide and 2 ml of water respectively. Therefore, 20 ml of the standard was loaded into the cartridge and washed with 2 ml of HPLC-grade water. The retained analytes were eluted with 4 ml of 0.5 M hydrochloric acid in acetonitrile. The eluted sample was dried under nitrogen gas and reconstituted in water; the pH was adjusted back from 1.95 to 6.3 prior to derivatization.

5.7.4.2 Results and method optimization

Glyphosate was not recovered and 44% recovery of AMPA was achieved. The method was optimized by decreasing the amount of sample loaded to 15 ml, this improved the AMPA recovery to 48% but still glyphosate was not recovered. Further method optimization was carried by increasing the volume of hydrochloric acid from 4 to 8 ml for the elution step, this yielded 50% of AMPA and 22% of glyphosate recovery. The volume of hydrochloric acid was increased further to 12 ml, but this did not improve the recoveries.

5.8 Analysis of environmental water samples

After the SPE method had been developed, it was applied in determination of residual glyphosate and AMPA in environmental water samples. The environmental water samples were collected from the following areas namely; industrial effluent water, Umdloti river, Amanzimtoti river mouth and Hammersdale dam. The preparation of these samples was discussed in section 4.4. Fig. 29 is the chromatogram for Hammersdale dam water sample prepared without the use of SPE cartridge. Fig. 30 is the chromatogram for the same sample after the matrix interferences have been removed by the use of C_{18} SPE cartridge. The samples were prepared and injected into the polymer amino column.

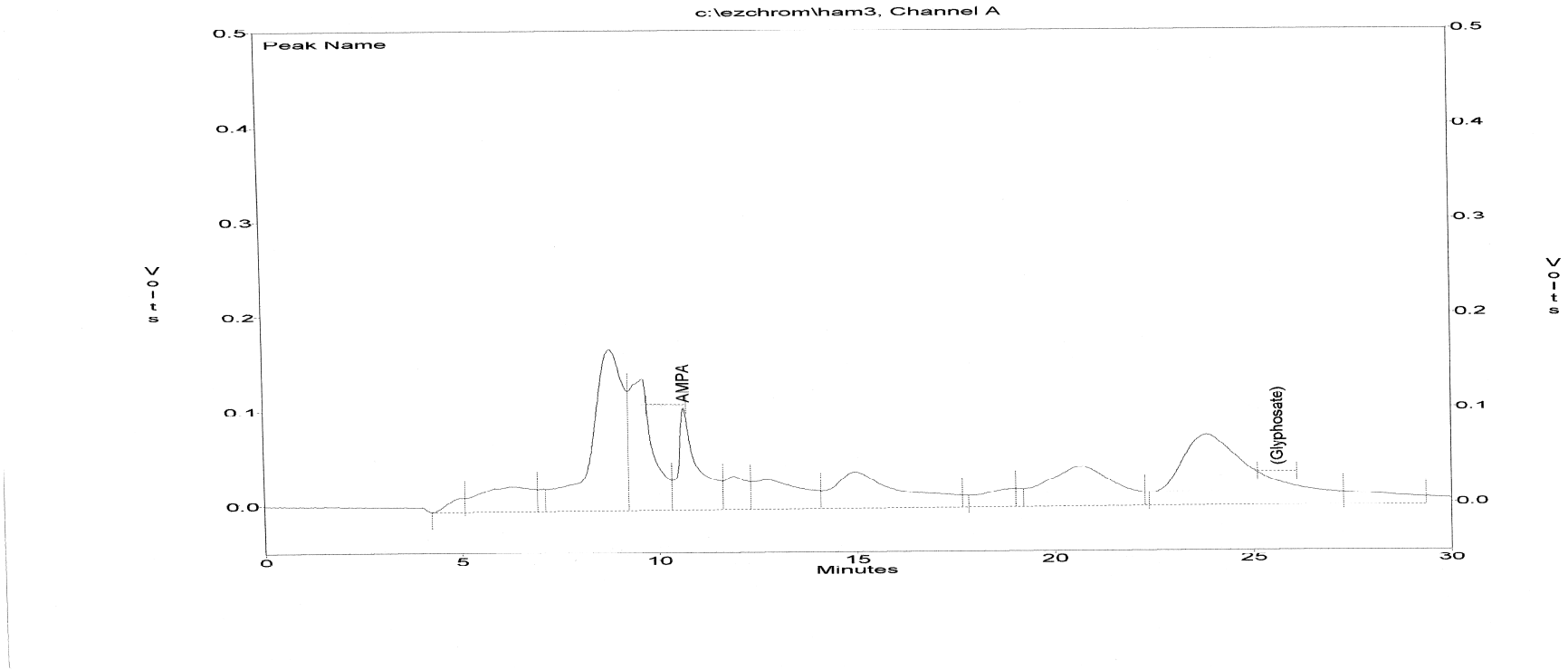


Figure 29: Interferences in field sample.

Chromatogram demonstrates the matrix interferences in the sample obtained from Hammersdale dam. The sample was injected into a polymeric amino column after derivatization with FMOC reagent. HPLC mobile phase (pH 10) contained 55% (v/v) acetonitrile in water, and 50 mM phosphate buffer.

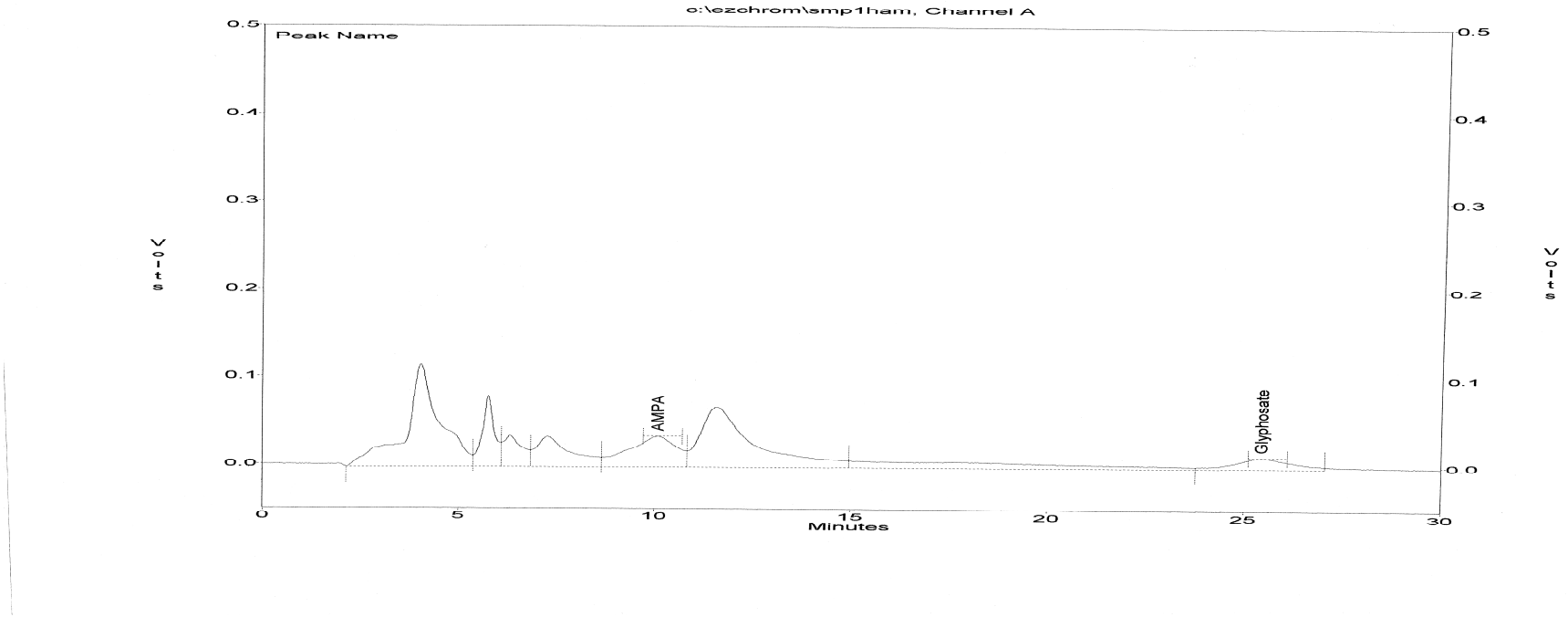


Figure 30: Removal of interferences using SPE.

Chromatogram of Hammersdale dam water sample after the removal of matrix interferences using SPE. The sample was cleaned-up on SPE procedure described in the text (sample preparation), then injected in polymer amino column using the HPLC mobile phase (pH 10) contained 55% (v/v) acetonitrile in water, and 50 mM phosphate buffer.

5.8.1 Levels of glyphosate and AMPA in field samples

For field water samples, the presence and the levels of glyphosate and AMPA were determined. The calibration graphs for both analytes were plotted and used for determining the amount of analytes present in environmental samples (graphs in Figs. 31 and 32).

Table 14: Concentration and peak areas of analytes used for quantitative analysis

Glyphosate		**AMPA**	
Concentration (ppb)	**Peak area**	**Concentration (ppb)**	**Peak area**
0.0	0.00	0.0	0.00
5.0	213165	0.5	142159
10	432104	1.0	354353
50	2693494	5.0	698176
100	5689459	10	1266045
		50	6716395
		100	13048668

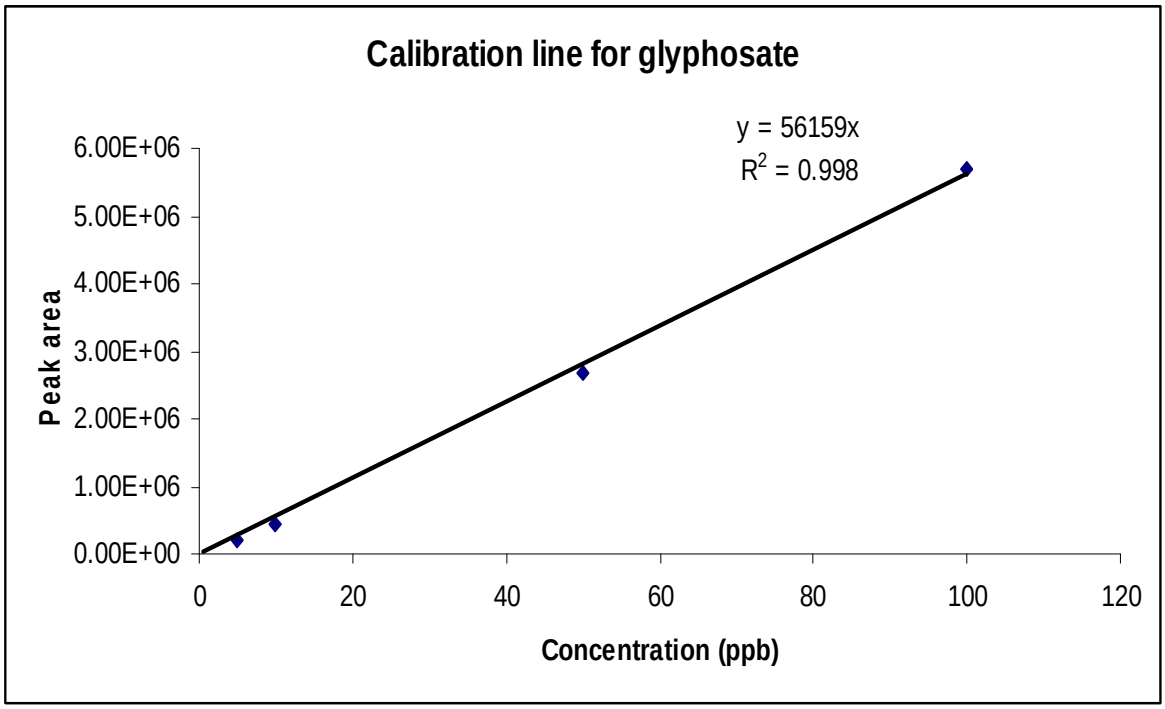


Figure 31: Field samples; calibration line for glyphosate.

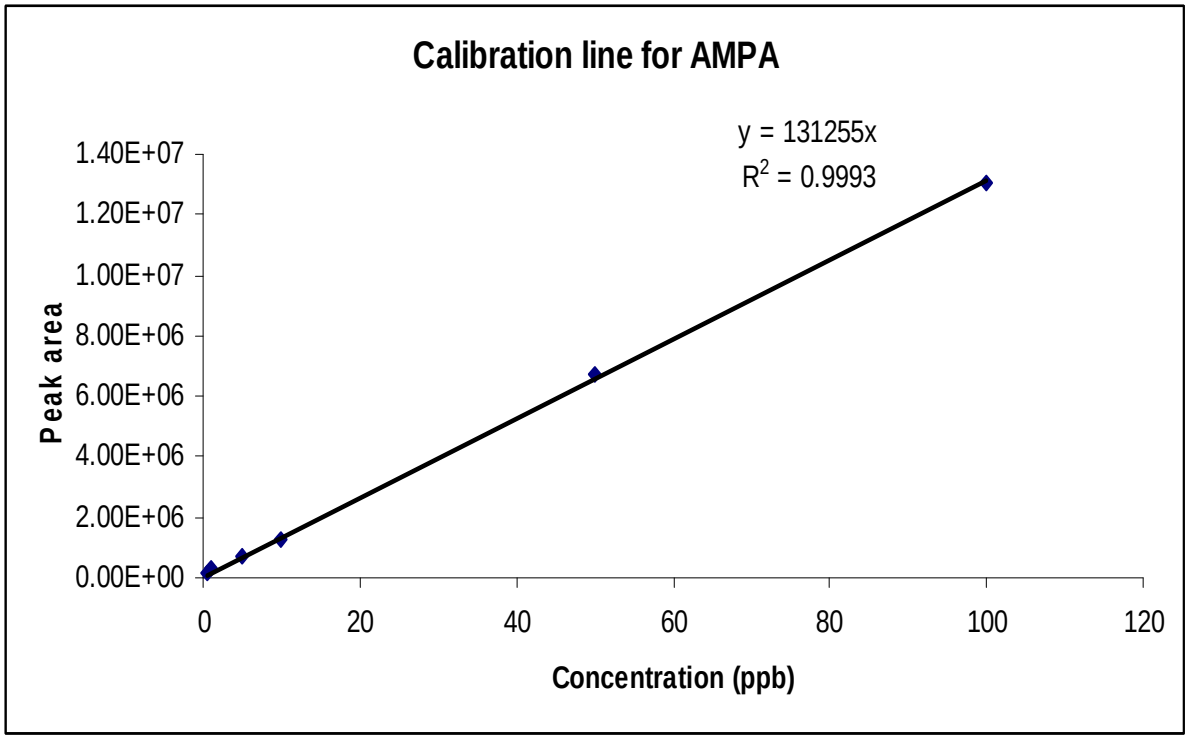


Figure 32: Field samples; calibration line for AMPA.

The samples were collected in Hammersdale dam a day after the dam was sprayed with glyphosate, and hence traces of both glyphosate and AMPA were detected in dam samples. None of the analytes were detected in Umdloti River and only AMPA was detected in industrial effluent, and Amanzimtoti river mouth.

Table 15: Performance of HPLC-FD for standard solutions

Method	**Analyte**	**Response**
Retention time (minutes)	Glyphosate	25.4
	AMPA	10.8
LOD (µg/l)	Glyphosate	3.2
	AMPA	0.23
LOQ (µg/l)	Glyphosate	10.5
	AMPA	3.2
Linearity range (µg/l)	Glyphosate	5-100
	AMPA	0.5-100
Calibration curve	Glyphosate	y = 56159x
	AMPA	y = 134107x
R^2	Glyphosate	0.9983
	AMPA	0.9996

Table 16: Concentrations of analytes detected in water

Sample	**Glyphosate (ppb)**	**AMPA (ppb)**	**N**
Hammersdale	Below LOQ	5.00	3
Umdloti river	Not detected	Below LOQ	3
Effluent	Not detected	3.24	3
Amanzimtoti river mouth	Not detected	Below LOQ	3
Stream sample	Not detected	3.15	3

Table 17: Concentrations of AMPA in samples not cleaned-up with SPE

Sample	Concentration of AMPA (ppb)		Concentration of glyphosate (ppb)	
	No SPE	SPE	No SPE	SPE
Stream	3.15	3.35	Not detected	Not detected
Umdloti	0.68	0.71	Not detected	Not detected
Amanzimtoti river mouth	0.30	0.86	Not detected	Not detected

5.9 Method validation

The method was validated by spiking deionised water, tap water and umdloti river water with 100 $\mu g l^{-1}$ of each analyte.

Table 18: Results for spiked deionised water

Analyte	% recoveries			Average	SD	% RSD
Glyphosate	110	111	111	111	0.577	0.520
AMPA	78.5	79.4	78.8	79	0.458	0.580

Table 19: Results for spiked tap water

Analyte	% recoveries			Average	SD	% RSD
Glyphosate	84.4	84.5	83.5	84	0.325	0.386
AMPA	109	111	109	109	1.154	1.059

Table 20: Results for spiked umdloti river water

Analyte	% recoveries			Average	SD	% RSD
Glyphosate	93.5	92.5	94	93	0.601	0.646
AMPA	77	76.8	77.5	77	0.361	0.469

5.10 Presence of AMPA in water

The presence of AMPA in dam and river water can be due to the following several possible sources, namely

1. Glyphosate degradation to form AMPA.
2. AMPA can also originate from several phosphonates. These phosphonates can be regarded as precursors for the environmental occurrence of AMPA. These phosphonates are used in corrosion inhibitors, soaps, shampoos and detergents. This can cause AMPA to be more widely spread in the environment even without the use of glyphosate.

5.11 Analytical methods employed for glyphosate and AMPA analysis

The method discussed in this project was compared with other analytical methods developed for the analysis of glyphosate and AMPA in various matrices. It was found that the LOD's found in this work for glyphosate and AMPA, generally agrees with the results reported in literature. Table 21 gives a summary of various sample preparation procedures, analytical methods and LOD's obtained for those methods.

A manuscript (Appendix H) on this work is being finalised for submission to Water South Africa.

Table 21: Summary of analytical methods used for glyphosate and AMPA analysis

Sample Preparation	Derivatization	Matrix	Analysis	Detection	LOD	Reference
Online-SPE	Pre-column	Water, soil	HPLC	MS	5 ng/L for water, 5 μg/kg for soil, (Both)	[3]
SPE	Non	Water	CE	UV	85 μg/L (GLY), 60 μg/L (AMPA)	[4]
Non	Non	Water	IC	CNLSD	53 μg/L (GLY)	[5]
Non	Pre-column	Water	HPLC	UV	40 μg/L (GLY)	[15]
SPE	Pre-column	Cereal	HPLC	FD	0.5 mg/kg (GLY)	[16]
SLM	Pre-column	Fruit Juices	HPLC	UV	25 μg/L (Both)	[17]
LLE, SPE	Post-column	Water	HPLC	FD	< 2 μg/L (GLY)	[19]
SPE	Post-column	Water	HPLC	FD	2 μg/L (GLY), 4 μg/L (AMPA)	[20]
Non	Pre-column	Water	CC-LC	FD	1 μg/L (Both)	[22]
SPE	Pre-column	Water, plant	HPLC	FD	0.16 μg/L (GLY in water), 0.3 mg/kg (GLY in plant)	[24]
Online-SPE	Pre-column	Water	HPLC	MS	0.03 μg/L (Both)	[25]
LE, AE	Pre-column	Water, soil	GC	MS	0.05 μg/L (GLY in water), 0.003 μg/g (GLY in soil)	[27]
LLE	Non	Water	IC	SCD	42 μg/L (GLY)	[28]

SE, Centrifuge	Non	Soybeans	CE	ECL	0.06 μg/mL (GLY), 4.04 μg/mL (AMPA)	[32]
Non	Pre-column	Natural water	HPLC	FD	10 μg/L (GLY), 5 μg/L (AMPA)	[35]
Non	Post-column	Water	HPLC	FD	1 μg/L (GLY) 0.4 μg/L (AMPA)	[36]
SPE	Pre-column	Water	HPLC	FD	3.2 μg/L (GLY) 0.23 μg/L (AMPA)	This Work

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Removal of derivatization interferences

- **Solvent extraction versus column coupling technique**

In this study the two methods of removing derivatization interferences were solvent extraction and column coupling technique. All derivatization interferences were effectively removed by using the column coupling technique. Nevertheless, this technique suffers from the instability of retention times at low pH values (pH 5.5). The column coupling mode could not be used at high pH values. Furthermore, the columns degrade at pH 10. Therefore solvent extraction is a preferred technique since it can be used at low and high pH values.

6.2 Conclusion for HPLC columns

6.2.1 Silica-based columns

The sensitivity for the HPLC methods discussed in this research is good for the determination of glyphosate in water since the detection limits of 0.1 mg/l and less were obtained. Although a good sensitivity was achieved in this research there are other problems that limit the use of the HPLC methods for glyphosate analysis when using silica-based columns for separation. A calibration line is not linear for the method that uses a C_{18} column. As for the other two methods that use silica based amino column and column coupling technique, a good linear response was obtained but the methods suffered from the instability of retention times for the analyte. The problem with these methods arises from the formation of fluorescing FMOC-OH during the derivatization reaction. As mentioned by other researchers (literature review in section 2.1.1), the FMOC-OH creates problems in glyphosate analysis.
The major problem of silica-based columns is their degradation at high and low pH values. Because of its mechanical strength and resistance to degradation, a polymer amino column was used for the separation of glyphosate and AMPA at pH 10. Ethyl acetate was used to remove the derivatization by-products.

6.2.2 Polymer amino column

The interferences arising from the derivatization process were removed with organic solvent (ethyl acetate). For the determination of glyphosate and AMPA in different matrices, a polymeric amino column was found to be most suitable. From this study it appeared that the hydrophobic interaction of analytes with the polymeric backbone of the column complements the ion exchange mechanism. The chromatographic method that uses a polymeric amino column for the separation of glyphosate and AMPA after pre-column derivatization of analytes with FMOC-Cl and removal of derivatization interferences with an organic solvent has been demonstrated and is simple. This method can be used for the direct determination of glyphosate and its major metabolite, AMPA, in water. For the application of the method for field samples, a suitable sample clean-up procedure (solid-phase extraction) was required.

6.3 Conclusion for IC analysis

In view of the ionic character of the target analytes, IC should be the method of choice. IC method used in this study was found to be simpler than HPLC methods in that it does not require the derivatization of analytes (therefore there are no FMOC-OH interferences). IC method is fast and inexpensive but it has the following drawbacks.

1. The major degradation product of glyphosate, known as AMPA, could not be determined simultaneously with glyphosate using this method.
2. In the absence of modification, IC lacks the level of sensitivity required for the trace analysis of glyphosate and AMPA (since 1 mg/l is the limit of detection for unmodified IC).

- **Recommendations**:

To attain the µg/l levels (permissible in drinking water nationally and internationally) using IC, the use of modifications such as column-suppression, pre-concentration and more sensitive detectors is recommended.

6.4 Conclusion on Solid phase extraction

Acceptable percent recoveries were obtained using SPE cartridges packed with 500 mg of C_{18} sorbent. Therefore these cartridges were used for clean up in the determination of glyphosate and AMPA in environmental water samples. The recoveries obtained in this work when using 500 mg C_{18} SPE cartridges are much better compared to the overall recovery of 86% obtained by Hogendoorn et al [16] when using the same packing material (C_{18} sorbent).

Low percent recoveries (≤ 44%) were obtained when using the Oasis cartridges although the use of HLB cartridges has been previously reported [3] with acceptable recoveries. During this investigation, it was found that the analytes were not well eluted from the cartridge.

- **Recommendation**

A suitable solvent combination has to be investigated for elution of analytes from Oasis HLB and Oasis MAX cartridges.

6.5 Overall conclusion

- To produce high amount of fluorescing analytes, derivatization reaction was carried out with FMOC-Cl reagent at pH 9. The effect of derivatization time and the effect of FMOC-Cl concentration were investigated. It was discovered that the optimal amounts of the fluorescing products are produced by using 0.5 mM of FMOC-Cl reagent and derivatization reaction was complete in 30 minutes at room temperature.
- A rapid method for sample preparation has been described in this report. Derivatization and organic interferences were effectively removed in a solvent extraction step. The existing SPE method developed by Hogendoorn et al [16] for the clean-up of cereal samples was optimized and used for the clean-up of environmental water samples. This simple and fast SPE method was used to eliminate the relatively non-polar interferences present in river, dam and effluent water samples.
- A sensitive and rapid HPLC method was determined for the simultaneous determination of glyphosate and AMPA in water. The separation of glyphosate and AMPA in this method was achieved by using a polymeric amino column and a mobile phase at pH 10.

- The methods developed for sample preparation and HPLC analysis were applied for the determination of concentrations of glyphosate and AMPA in water samples collected from dam, river and industrial effluent. Whereas trace amounts of analytes were detected in dam water, neither of them were detected in river water. Only AMPA was detected in industrial effluent water.
- The current method can be applied in routine analysis of environmental water samples with small amount of samples available.

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APPENDICES

Appendix A: Abbreviations

POEA	Polyoxyethylene amine
US EPA	United States Environmental Protection agency
M	Molar or moles per litre
µg/ml	Micrograms per millilitre
µg/l	Micrograms per litre
ng/l	Nanograms per litre
mg/l	Milligrams per litre
mg/kg	Milligrams per kilogram
mg	Milligram
ml	Millilitre
L	Litre
µl	Micro litre
ppm	Parts per million
ppb	Parts per billion
GLY	Glyphosate
AMPA	Aminomethylphosphinic acid
HPLC	High performance liquid chromatography
HPLC-FD	High performance liquid chromatography – fluorescence detector
LC	Liquid Chromatography
GC	Gas Chromatography
IC	Ion Chromatography
ICP-MS	Inductively Coupled Plasma-Mass spectrometry
FMOC-Cl	9-fluorenylmethylchloroformate
OPA	o-phthalaldehyde
MERC	Mercaptoethanol
SPE	Solid-phase extraction
LC-ESI-MS/MS	Liquid chromatography coupled to electrospray tandem mass spectrometry
MS	Mass spectrometry
SD	Standard deviation

% RSD	Percent relative standard deviation
^{0}C	Degrees Celsius
%	Percent
ACN	Acetonitrile
LOD	Limit of detection
LOQ	Limit of quantification
CNLSD	Condensation nucleation light scattering detection
SLM	Supported liquid membrane
SCD	Suppressed conductivity detection
FD	Fluorescence detection
SE	Solvent extraction
ECL	Electro-chemiluminescence
CC-LC	Coupled-column liquid chromatography
FPD	Flame photometric detector
LE	Ligand-exchange
AE	Anion-exchange

Appendix B: Explanation of equations used in text

Limit of detection (LOD) = S/N = 3

Where S/N is signal to noise ratio

Limit of quantification (LOQ) = S/N = 10

Standard deviation (SD) = $\sqrt{\sum (x_i - \bar{x})^2 / (n-1)}$

x_i is the measured value, $\bar{x}$ is the mean or average and n is the number of measured values.

Mean (Average), $\bar{x} = (x_1 + x_2 + x_3 + ... + x_n) / n$

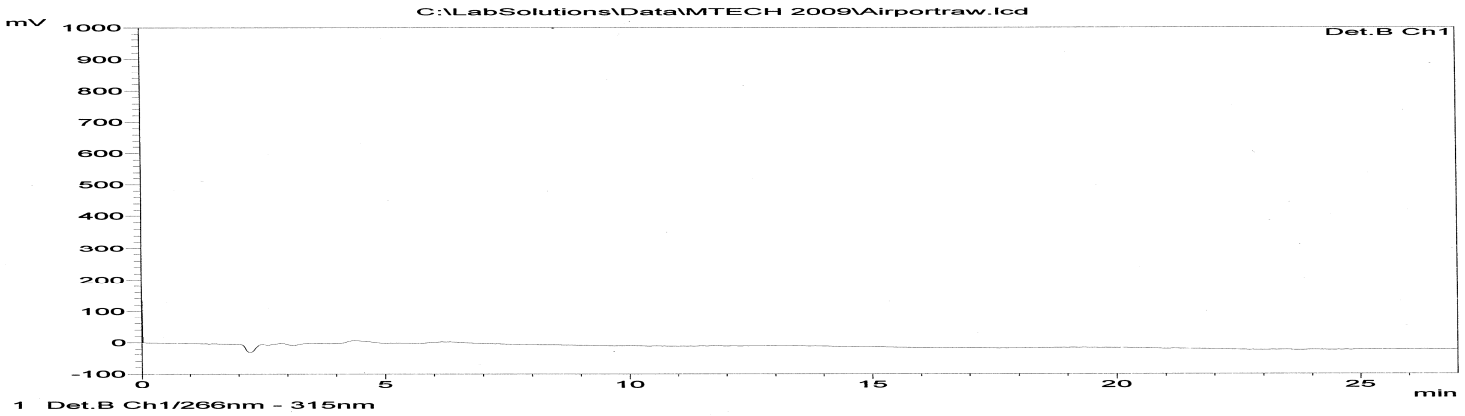
Relative standard deviation (% RSD) = (SD / $\bar{x}$) x 100

Appendix C: Chromatograms for samples injected without being derivatized

1. Stream sample

Raw samples

2009/05/19 11:24:30 1 / 1



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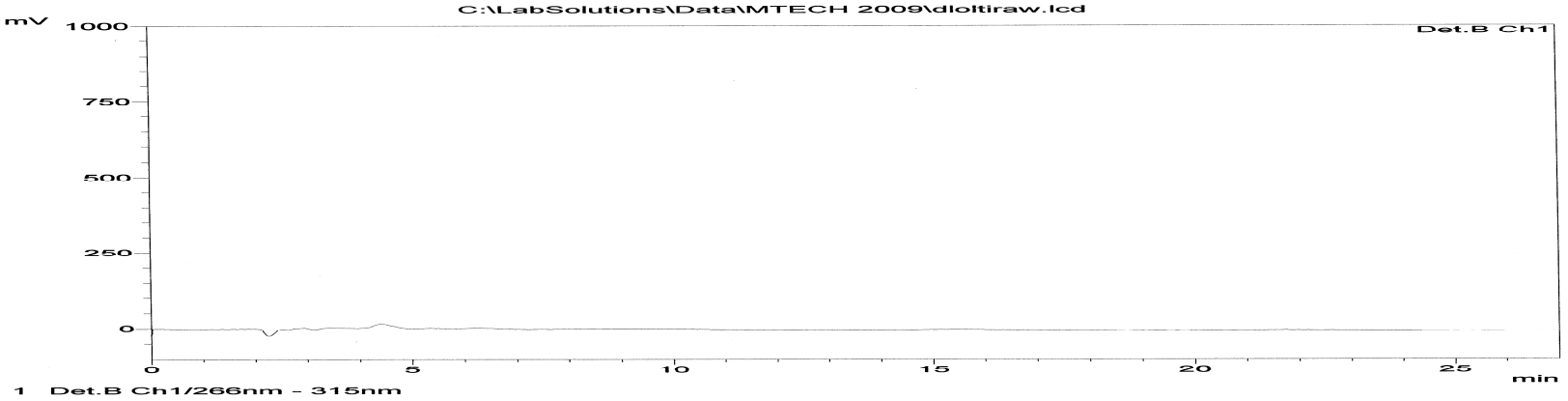
2. Umdloti sample

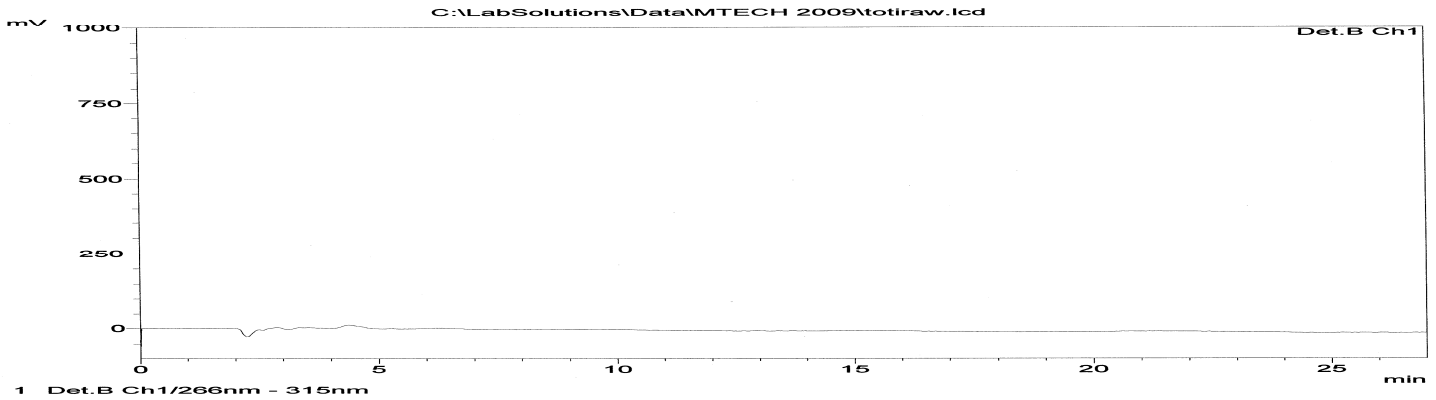
2009/05/19 11:20:49 1 / 1



C:\LabSolutions\Data\MTECH 2009\dloltiraw.lcd

3. Amanzimtoti sample

2009/05/19 11:23:12 1 / 1



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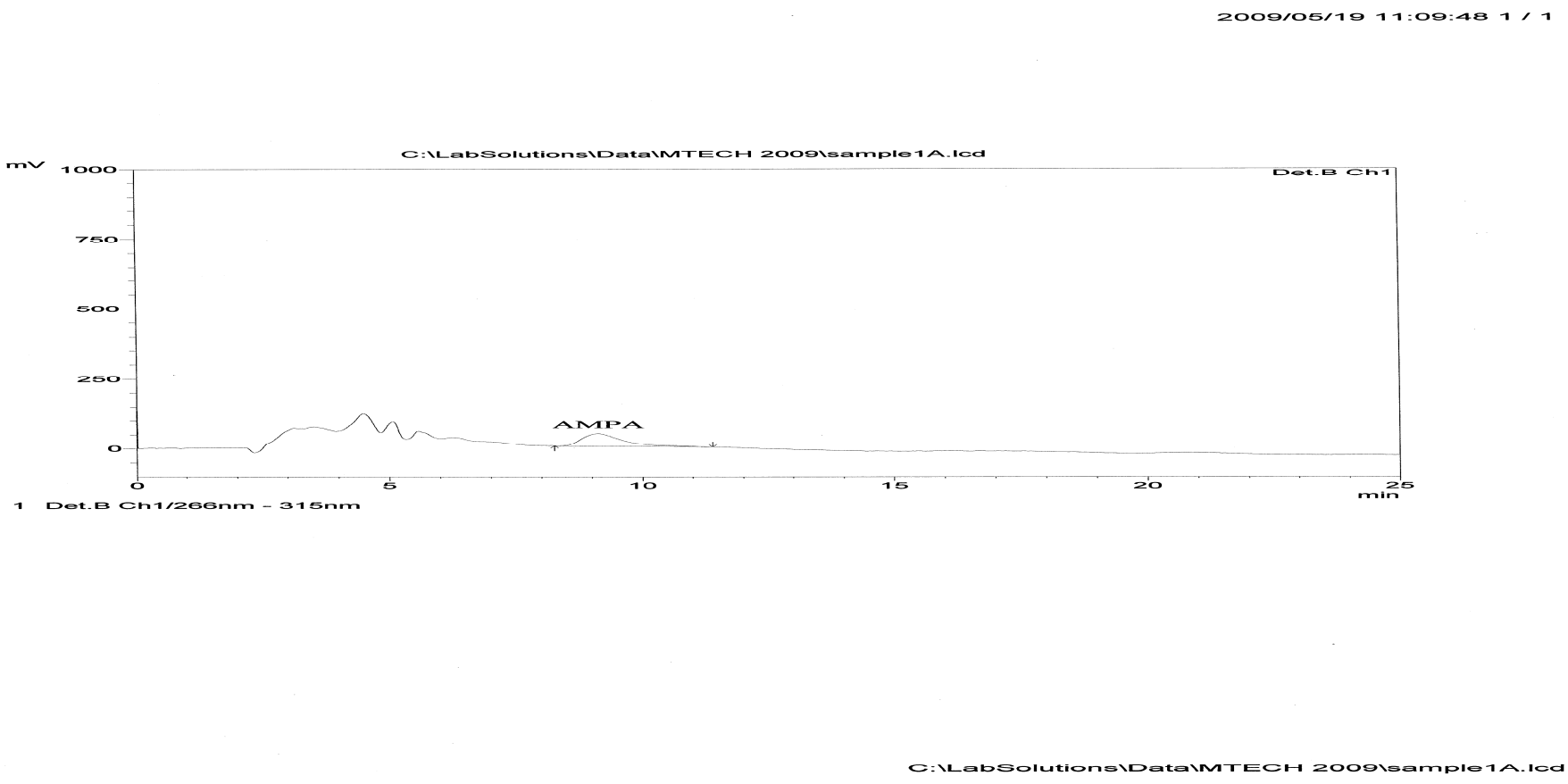
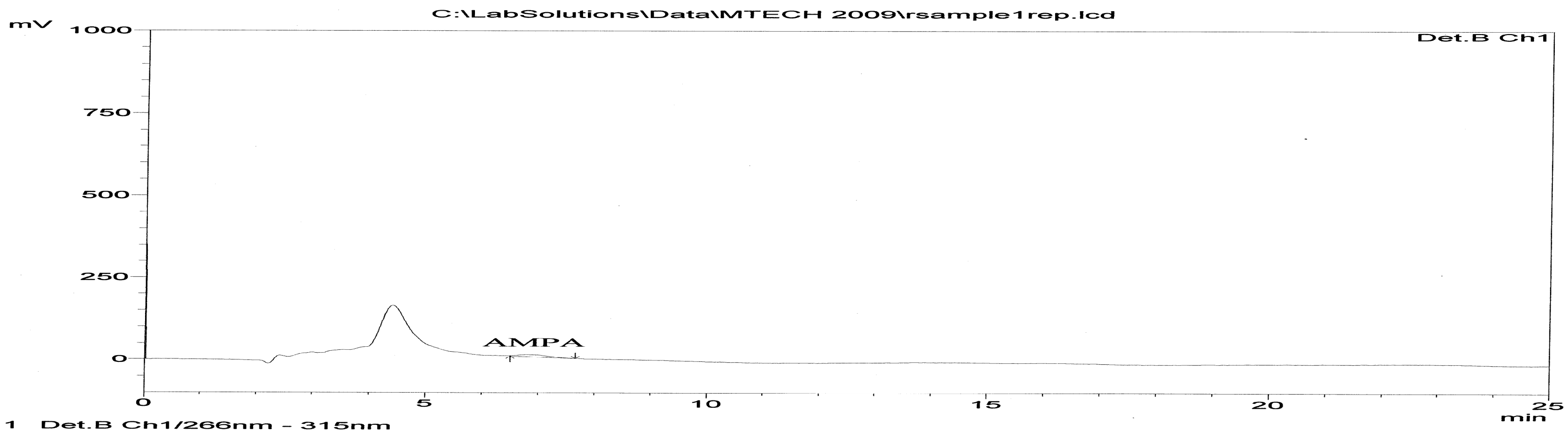
Appendix D: Chromatograms for derivatized samples

1. Stream sample



2. Umdloti sample

2009/05/19 10:51:07 1 / 1

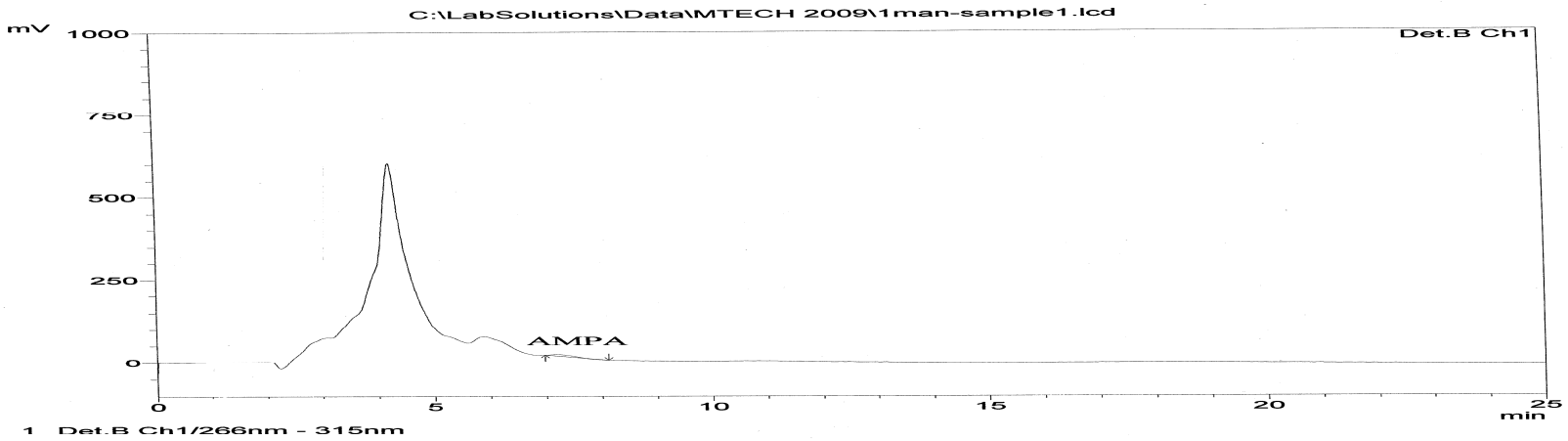


C:\LabSolutions\Data\MTECH 2009\rsample1rep.lcd

3. Amanzimtoti sample

Amanzimtoti

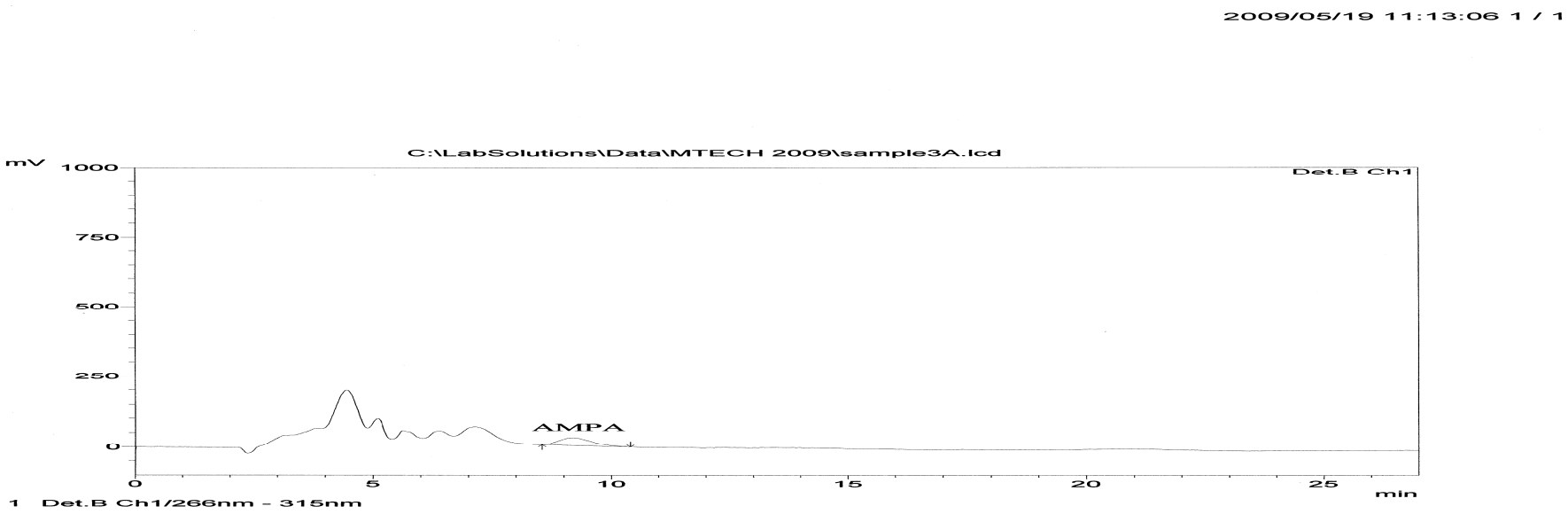
2009/05/19 11:07:41 1 / 1



C:\LabSolutions\Data\MTECH 2009\1man-sample1.lcd

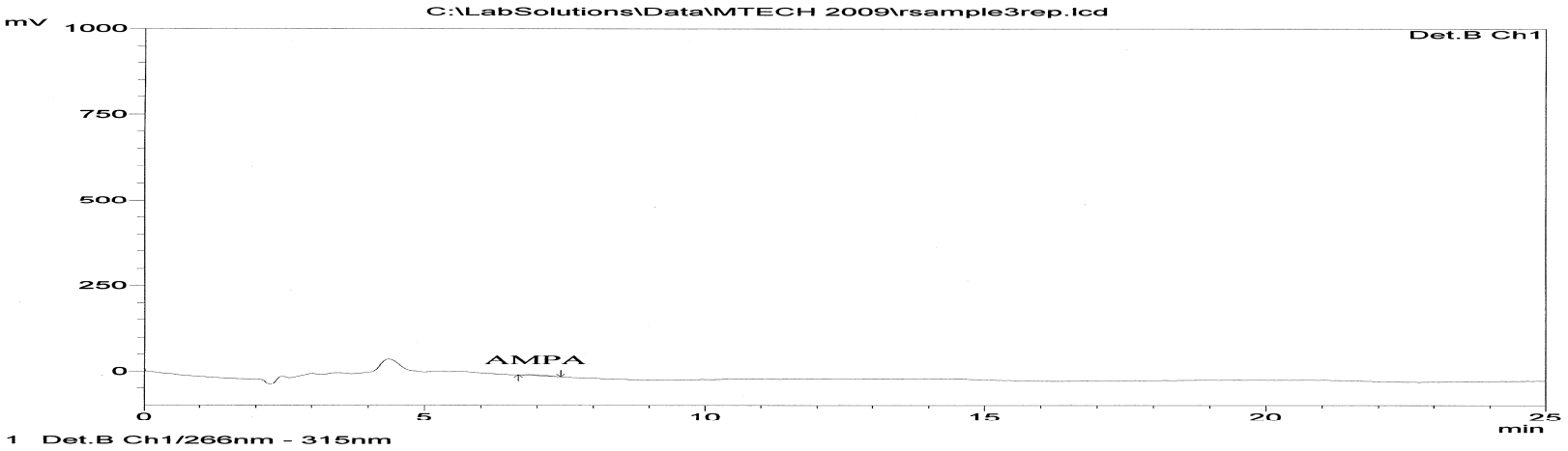
Appendix E: Chromatograms for samples derivatized followed by SPE clean-up

1. **Stream sample**



2. Umdloti sample

2009/05/19 10:52:11 1 / 1



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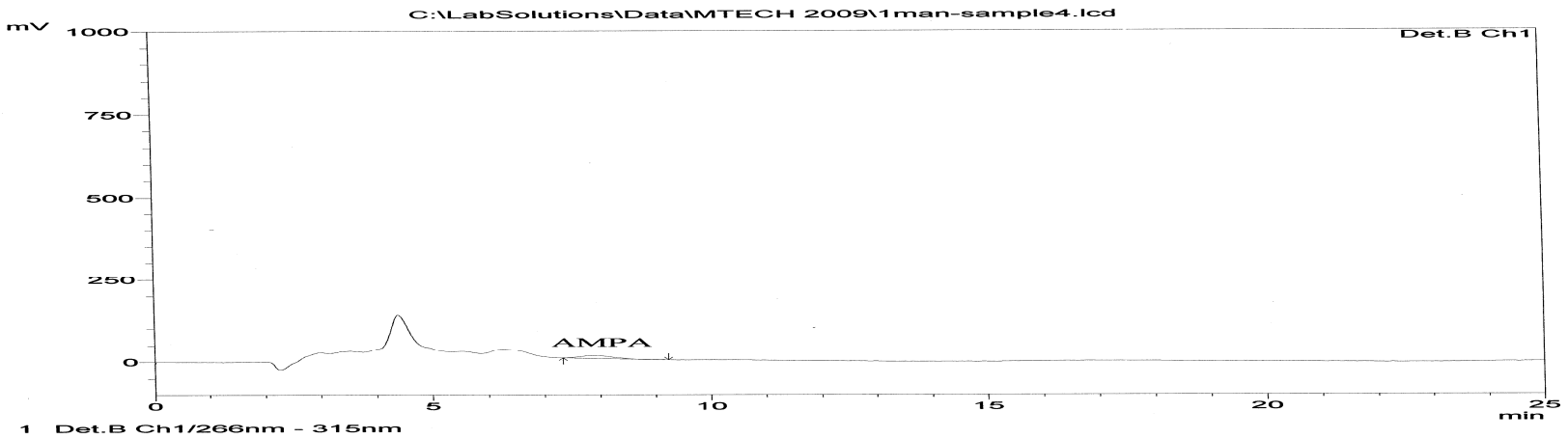
3. Amanzimtoti sample

2009/05/19 11:04:54 1 / 1



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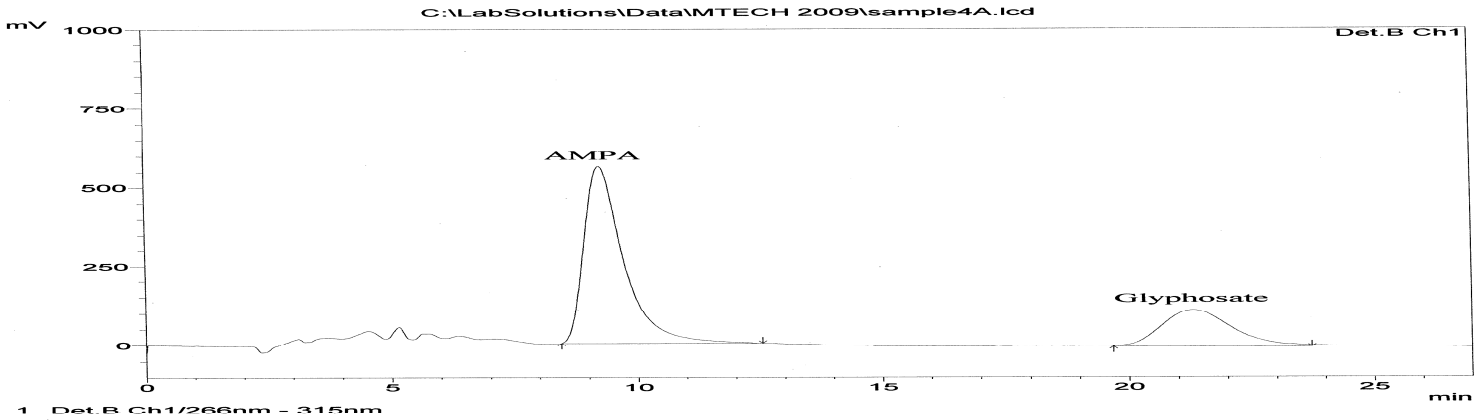
Appendix F: Chromatograms for spiked samples.

These samples were spiked with 100 mg/l of both glyphosate and AMPA, then derivatized with FMOC-Cl followed by SPE.

1. **Stream sample**

Airport Sample

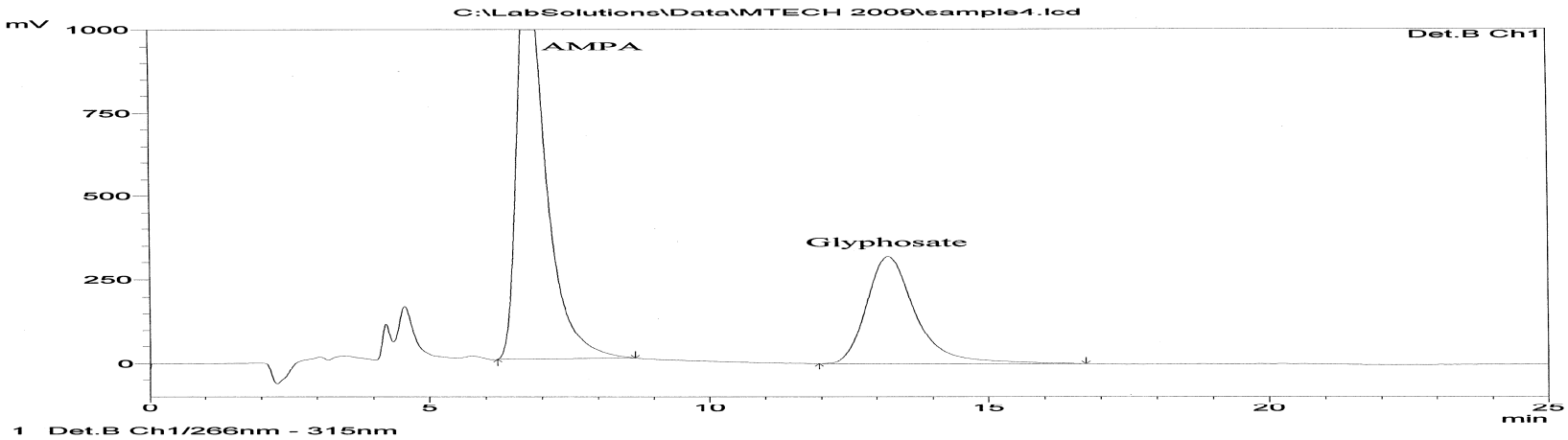
2009/05/19 11:14:21 1 / 1



C:\LabSolutions\Data\MTECH 2009\sample4A.lcd

2. Umdloti sample

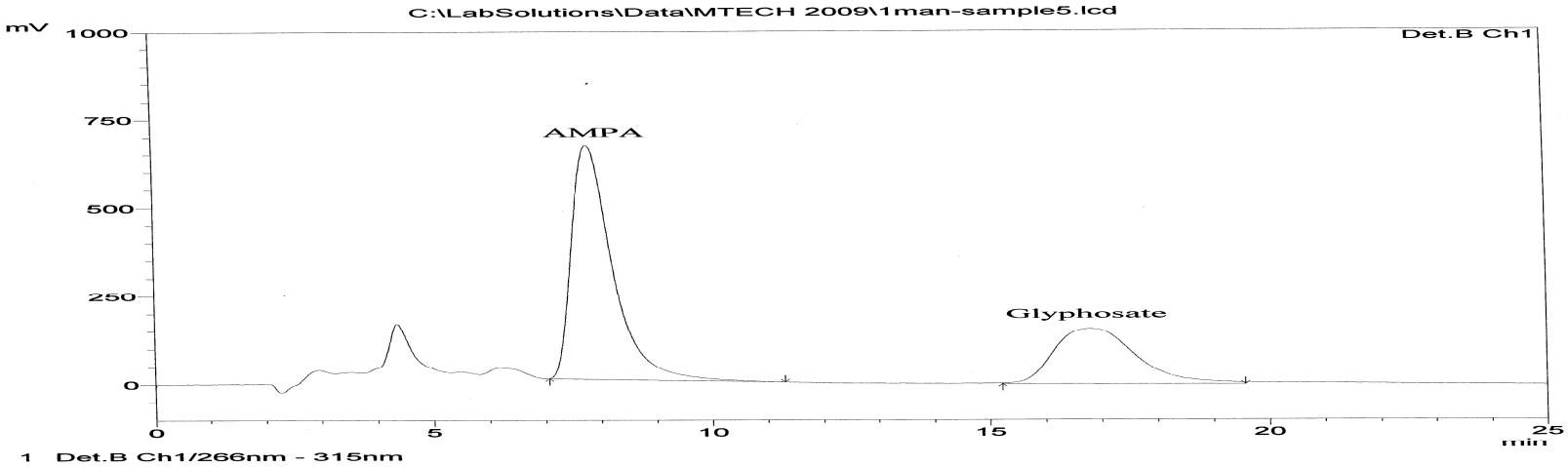
2009/05/19 10:55:33 1 / 1



C:\LabSolutions\Data\MTECH 2009\sample4.lcd

3. Amanzimtoti sample

2009/05/19 11:06:06 1 / 1



C:\LabSolutions\Data\MTECH 2009\1man-sample5.lcd

Appendix G: SPE apparatus.