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A SIMPLE HPLC METHOD FOR THE DETERMINATION OF  
2,4 - DINITROPHENYL DERIVATIVES OF  
GLYPHOSATE AND AMINOMETHYLPHOSPHONIC ACID  
APPLICABLE TO PLANT STUDIES

by

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B. Sc. (Honours Biology/Geography),  
University of Windsor, 1985

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE  
in the Department of  
Biological Sciences

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SIMON FRASER UNIVERSITY

August 1990

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ISBN 0-315-78345-1

APPROVAL

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Degree: Master of Science

Title of Thesis:

A SIMPLE HPLC METHOD FOR THE DETERMINATION OF 2,4-DINITROPHENYL DERIVATIVES  
OF GLYPHOSATE AND AMINOMETHYLPHOSPHONIC ACID APPLICABLE TO PLANT STUDIES

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## ABSTRACT

A procedure was developed for the determination of glyphosate (GLYPH) and aminomethylphosphonic acid (AMPA) using high pressure liquid chromatography (HPLC) with UV detection at 370 nm. Leaves, stems and roots of bean plants (Phaseolus vulgaris cv. Topcrop) were fortified with GLYPH and AMPA. Plant samples were homogenized in water, and then filtered. The filtrate was adjusted to pH 8 and sorbed onto an anion exchange resin column. GLYPH and AMPA were eluted with 50 mL of 1 M sodium chloride. An aliquot of the eluate was derivatized with 2,4-dinitrofluorobenzene (2,4-DNFB) to convert these chemicals to their corresponding 2,4-dinitrophenyl derivatives. Further cleanup of the derivatized product involved organic extractions of the aqueous solution with dichloromethane and water-saturated isobutanol, with final extraction into 0.1 M sodium tetraborate. The aqueous solution was acidified for HPLC analysis. Recoveries of aqueous solutions containing GLYPH and AMPA (from 38 to 3,750 ug) were determined from the anion exchange column by comparison with standards analyzed without column chromatography. Most of the GLYPH and AMPA was eluted in 50 mL of 1 M sodium chloride, with recoveries of greater than 68% obtained for the aqueous solutions processed through the anion exchange column.

Detector response for amounts of GLYPH and AMPA standards separated by HPLC was linear over the range tested (25-250 ng injected) and passed through the origin ( $R^2 = 0.999$  for both GLYPH and AMPA). Coefficients of variation for replicate standards of GLYPH and AMPA were all less than 4%, with the exception of GLYPH at 0.5 ppm. The minimum level of quantitation in aqueous standards was 5 ng injected for GLYPH and 3 ng injected for AMPA. A substantially lower detector response was observed for GLYPH compared with AMPA for the same amount injected. Recoveries of GLYPH from plant homogenates ranged from 91 to 136% at fortifications of 38 to 938 ppm. Recoveries of AMPA from these homogenates ranged from 70 to 100%. Coefficients of variation were all less than 10% ( $n = 4$ ). This method is discussed in terms of its applicability to plant studies.

## ACKNOWLEDGEMENTS

I would like to express sincere thanks to Dr. J. Rahe, Dr. S. Szeto, Dr. H.R. MacCarthy, and Dr. G. Lister for their help and advice in the completion of this work. I am extremely grateful to Sunny Szeto for his guidance and patience, and the knowledge I have gained working with him at the Agriculture Canada station. The support of friends throughout the course of my graduate work is greatly appreciated; these friendships have made the experience truly worthwhile.



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## GLOSSARY

ai = active ingredient

AMPA = aminomethylphosphonic acid

CCREM = Canadian Council of Resource and Environment Ministers

C.V. = coefficient of variation

diam = diameter

2,4 - DNFB = 2,4 - dinitrofluorobenzene

DNP = dinitrophenyl

EC = electron capture

EPSP = 5-enolpyruvylshikimic acid-3-phosphate

FMOCCl = 9-fluorenylmethyl chloroformate

FP = flame photometric

GC = gas chromatography

GLYPH = glyphosate

ha = hectare

HFBA = heptafluorobutyric anhydride

HPLC = high pressure liquid chromatography

MAC = maximum acceptable concentration

mAU = milli absorbance units

MTBSTFA = N-methyl-N-(tert-butyldimethylsilyl)  
trifluoroacetamide

NP = nitrogen-phosphorus

OPA-MERC = o-phthalaldehyde-mercaptoethanol

PAM = Pesticide Analytical Manual

SD = standard deviation

TBDMS = tert-butyldimethylsilyl

TFAA = trifluoroacetic anhydride

TFE = trifluoroethanol

TLC = thin layer chromatography

UV = ultra-violet

Note: In general, abbreviations follow the CBE Style Manual,  
5th edition, 1983.

## CHAPTER 1

### INTRODUCTION

#### 1.0 General Discussion on Pesticides

Pesticides have become an indispensable yet menacing component of human existence. These chemicals have conferred tremendous benefits on mankind with respect to controlling the vectors of serious human diseases, and play an important role in maintaining silvicultural and agricultural productivity. However, they may also result in the contamination of our environment and our food. As such, they are the topic of controversial debate.

Knowledge about the immediate or delayed consequences of pesticide use is fundamental to evaluating their necessity. Acute and chronic toxicities of pesticides are a foremost consideration. People want to know if their food and water are safe for consumption. The fate of pesticides and their metabolites in treated crops and in the environment must, therefore, be addressed. Their potential as contaminants of groundwater and surface water systems, and the risk of long range transport of certain persistent pesticides underlies the



possibility of these chemicals being found far from areas of use. The effectiveness and affordability (i.e. cost/benefit ratio) of a pesticide also warrants evaluation, primarily by the applicator.

Our reliance on pesticides and other chemicals is unlikely to diminish in the near future. The world's population continues to grow, and consequently the need for food continues to place significant stress on the fraction of the work force that supplies it for the remainder. The mechanization of crop production and the extensive use of herbicides in North America have resulted in high efficiency of labour inputs (McEwen and Stephenson 1979). Very few food crops are now produced on an economically competitive basis without some type of pesticide input to control insects, weeds or pathogens, as well as to satisfy consumer expectations in terms of aesthetics (Coats 1987). The small proportion of the earth's arable land is continually threatened by the strength of other competing uses (such as residential and industrial expansion) and the lack of government foresight. The fact is, we must accept our present dependence on pesticides yet maintain a respect for these chemicals and our environment.

Confronting the scientific face of pesticides is the public perception. Increased awareness has raised fears, both real and perceived, of the potential consequences of pesticide

residues in our food, water and environment. Low-level residues of pesticides are virtually unavoidable components of our diet (Coats 1987). The media, often walking the edge of sensationalism, tend to ignite public emotions; but more importantly, motivate the desire for knowledge of and responsible action on what might otherwise remain as tragic indifference. Responsibility partially lies with the general public to maintain a keen awareness of local and national issues, and to pressure politicians accordingly. Only then can we feel a certain degree of assurance that our environment is being managed prudently.

Man is choosing a new direction towards a more environmentally compatible means of co-existence. Previous developments of pesticides and use patterns may provide insight for the future development of effective pest management practices that are agreeable with this new direction. It is understandable that we have come to depend on pesticides to maintain a certain level of productivity and profitability. However, consumers are often not aware that their expectations regarding the cosmetic appeal of the food they buy have dictated a degree of pesticide use. Cultural and technological changes have also freed families from having to produce and store most of their own food. These circumstances have inadvertently placed limitations on the food producers, wholesalers and retailers. Obviously, a change in personal attitudes to these issues will

facilitate the establishment of progressive attitudes towards pesticide use.

### 1.1 General Discussion on Pesticide Analysis

Concerns over pesticide use stem from the potentially adverse effects of pesticides on human and animal health, as well as on the general state of the environment. To understand fully the effects of pesticides, information is needed: on their inherent toxicities to and effects on all living species, including non-target organisms; on their chemical and physical properties; on their formulations and potential adjuvant metabolites; on user associated risks; and on field use characteristics. Other important considerations for a comprehensive understanding of the fate of these chemicals include their movement and biotransformation in both target and non-target organisms, their fate and persistence in the global environment, and their interactions with other chemical substances. The extent to which food commodities are shipped around the world today necessitates the surveillance of potential pesticide residues in food imports and exports. This knowledge is essential to allay public health concern over residues in food and water. Therefore, the ability to measure these chemicals over time in various matrices is

essential to addressing this concern.

Pesticide analysis is as much a science of its own as it is an interdisciplinary field. A multitude of techniques for the separation, purification, identification, and measurement of chemicals have developed from the uniqueness of every analytical situation. The diversity of chemical and physical properties of pesticides as well as their use patterns have provided this stimulus. The continual evolution of technological capabilities has provided increased versatility for analytical methods, as well as increased specificity and sensitivity in detection systems.

Sensitive and efficient analytical technology provides the scientific basis for the development of common sense approaches to pesticide use. The establishment of sound policies is needed to regulate the use of pesticides, while protecting humans and their environment. In Canada, the Health Protection Branch of Health and Welfare Canada is responsible for assessing the potential risks to Canadians of dietary exposure to toxic substances. The Canadian Drinking Water Quality Guidelines (Health and Welfare Canada 1987) include maximum acceptable concentrations (MAC's) for about a dozen insecticides and herbicides. National guidelines for edible tissues (fish specifically) exist for only a few chemicals; DDT and Mirex are the only pesticides for which

guidelines exist (MacDonald and Smith 1990). The Canadian Council of Resource and Environment Ministers (CCREM; now the Canadian Council of Ministers of the Environment or CCME) has established water quality guidelines applicable to inland surface waters and groundwater in Canada (CCREM 1987). A number of pesticide guidelines have been recommended for the major water uses. The water uses addressed include raw water for drinking water supply, recreation and aesthetics, the protection of freshwater aquatic life, agricultural uses, and industrial water supplies.

The positive identification of pesticide residues in any given matrix (whether this be soil, air, water, plant or animal tissues), requires some sort of extraction and purification protocol. Sample cleanup procedures are needed to distinguish the compounds of interest from the background organic matrix, and these become more important and challenging as attempts are made to lower the detection levels (Touchstone and Dobbins 1983). Standard chemical methods such as organic extraction, filtration and/or centrifugation are used in combination with chromatographic techniques to isolate the desired compounds from potentially interfering substances. Many important physicochemical properties may affect the feasibility and efficiency of these procedures. The fewer the steps used in any given protocol, the simpler, more cost effective, and less time consuming it is. Thus, the most direct protocol for

obtaining the target substances in a fraction suitable for measurement contributes to increased accuracy, reliability, reproducibility and safety.

Sample cleanup is followed by the detection and quantification of the desired compounds. Chromatographic procedures are used to separate the compounds as well as potentially to identify and quantify them. This stage of analysis may require a derivatization step to enable chromatographic equipment to detect the derivatives formed. As well, all chromatographic methods require the use of a reference compound to ensure the positive identification of a compound and to enable instrument calibration curves to be determined (Siggia and Hanna 1979). Quality control procedures are important for ensuring a high degree of confidence in the data generated. Finally, statistical analyses of the data aid in the interpretation and significance of results.

## 1.2 Focus on the Herbicide Glyphosate

Glyphosate, the common name for N-phosphonomethylglycine, is patented by Monsanto Co., St. Louis, MO. It is the active ingredient in the water-soluble herbicides Roundup<sup>®</sup>, Vision<sup>®</sup>, Clear-It<sup>®</sup>, Sidekick<sup>®</sup>, and Rustler<sup>®</sup> (which also contains 2,4-D).

Glyphosate is a broad spectrum, non-selective, post-emergence herbicide that is applied to the foliage of target plants. Introduced in 1971, this chemical has been registered for use in Canada since 1976 (CCREM 1987). The commercial product Roundup<sup>®</sup> is registered in Canada for weed control in specific crops (barley, corn, oats, potatoes, soybeans, sugar beets, and wheat) and industrial and non-agricultural areas (rights-of-ways, industrial sites, roadsides, pasture renovation, and recreational land) (CCREM 1987). Recommended application rates are 1.08 - 1.68 kg active ingredient (ai) per ha for annual weeds and 1.20 - 5.76 kg ai per ha for perennial weeds. Vision<sup>®</sup> is registered in Canada for the control and suppression of herbaceous weeds, weedy brush, and trees in silviculture (CCREM 1987). Recommended application rates are 1.07 - 2.14 kg ai per ha. A summary of glyphosate's general characteristics is provided in Table 1.

Glyphosate has many useful attributes as a herbicide, most notably its water solubility (specifically that of the monoisopropylamine salt used in formulation). Glyphosate has systemic qualities that make it particularly effective against deep-rooted perennial species (Worthing 1983). Translocation from the treated foliage to areas of above and below ground meristematic activity takes place within a few days in some species (Caseley and Coupland 1985; Sandberg et al. 1980;

Table 1. Physicochemical and biological characteristics of glyphosate and Roundup\*.

Characteristics		Reference
<u>Glyphosate:</u>		
chemical formula	C <sub>3</sub> H <sub>8</sub> NO <sub>5</sub> P	The Merck Index 1989
molecular weight	169.07	The Merck Index 1989
form	white solid, crystalline	The Merck Index 1989
melting point	230°C (decomposes)	The Merck Index 1989
photodecomposition	negligible	Rueppel et al. 1977
vapour pressure	negligible	Bronstad and Friestad 1985
solubility, water	12 g/L at 25°C	The Merck Index 1989
solubility, organic	insoluble, most solvents	The Merck Index 1989
pKa's (25°C)	1 = 2.32 2 = 5.86 3 = 10.86	Wauchope 1976
shelf life	very stable in water	Bronstad and Friestad 1985



Table 1. Continued.

Characteristics		Reference
acute oral LD <sub>50</sub>	3.8 g/kg (rabbit) 4.3 g/kg (rats)	Atkinson 1985
96 h LC <sub>50</sub>	86 ppm (trout) 11 ppm (trout; Roundup <sup>R</sup> )	Atkinson 1985
48 h LC <sub>50</sub>	780 ppm ( <u>Daphnia</u> ) 5.3 ppm ( <u>Daphnia</u> ; Roundup <sup>R</sup> )	Atkinson 1985
<b>Roundup<sup>R</sup>:</b>		
chemical formula	C <sub>6</sub> H <sub>17</sub> N <sub>2</sub> O <sub>5</sub> P (as the mono- isopropylamine salt)	The Merck Index 1989
formulation	356 g/L glyphosate, 480 g/L isopropyl- amine salt	CCREM 1987
recommended rate	1.08 - 1.68 kg ai per ha (annual weeds)	CCREM 1987
	1.20 - 5.76 kg ai per ha (perennial weeds)	CCREM 1987
symptoms of treated plants	yellowing and wilting of shoots, progresses from new to older tissues, visible in 2-4 days for annuals and 7-10 days for perennials	Ross and Lembi 1985

Schultz and Burnside 1980). Glyphosate's primary site of action involves the inhibition of 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase, an enzyme of the shikimic acid pathway (Ray 1989). The absence of this pathway in animals is an important factor in its low mammalian toxicity. Toxicity testing on a wide range of species has shown that glyphosate is a relatively non-toxic pesticide (Atkinson 1985), however toxicity may be increased by the surfactants used in its commercial formulation (Folmar et al. 1979; CCREM 1987; Tooby 1985).

The realized and anticipated use of glyphosate has prompted a variety of studies addressing its environmental fate. The major metabolite of glyphosate is aminomethylphosphonic acid (AMPA) (Putnam 1976; Rueppel et al. 1977; Sandberg et al. 1980); a compound with low biological activity (Cole 1985). Studies indicate that AMPA is a nonpersistent metabolite (Newton et al. 1984; Roy et al. 1989; Rueppel et al. 1977). Glyphosate is strongly adsorbed onto soil particles where it may be decomposed mainly by microbial action (Torstensson 1985). Rates of glyphosate degradation vary considerably depending on the type of soil, with half-lives ranging from a few days to several months or years. Damage to crops grown in the following year is not a major concern due to glyphosate's general lack of residual activity (Ross and Lembi 1985). Chances of aquatic contamination are limited when glyphosate

is applied according to label instructions and recommended buffer zones along water bodies are respected (Bronstad and Friestad 1985; Feng et al. 1990; Roy et al. 1989). However, glyphosate does have the potential to enter surface and subsurface waters when used near aquatic environments. This has been demonstrated by reports of glyphosate residues in water from direct overspray in forestry operations (Newton et al. 1984), from runoff (Edwards et al. 1980), and from irrigation canal discharges (Comes et al. 1976; Bowmer 1982). Low exposures to, and rapid elimination of, glyphosate in herbivores and omnivores indicate that food supplies contaminated with glyphosate residues are unlikely to be a threat to mammals (Newton et al. 1984). Reviews of the fate and biological consequences of glyphosate in the aquatic environment are covered by Tooby (1985) and the CCREM report (1987). Bioaccumulation of glyphosate in fish tissues is considered unlikely.

Levels of glyphosate residues in our food and water are of primary concern for the protection of humans and the environment. The Canadian guideline (MAC) for glyphosate in drinking water is 280 ug/L (280 ppb; Health and Welfare Canada 1987). The U.S. Environmental Protection Agency recommends a limit of 500 ug/L (500 ppb) in drinking water. This agency has also established maximum permissible residue levels in a variety of crops and meat organs (Atkinson 1985). These

levels range from 0.1 ppm in grain crops to 15 ppm in cotton and soya beans (for hay and forage). Canadian water quality guidelines do exist for glyphosate in groundwater and inland surface waters (CCREM 1987). The concentration of glyphosate in water should not exceed 65 ug/L (65 ppb; interim guideline) for the protection of freshwater aquatic life, and should not exceed 280 ug/L (280 ppb) for livestock watering.

Glyphosate sales have increased over the past few years (CCREM 1987), and they are likely to increase in the future. Monsanto's patent for this chemical is due to expire in 1990, thus creating the opportunity for a competitive market to develop over a generic version of Roundup<sup>®</sup>. The development of glyphosate tolerant plants has already been accomplished at the scientific level, and is expected to be introduced to commercial agriculture within the next couple of years (Comai et al. 1985; Shah et al. 1986). This accomplishment in plant biotechnology will almost certainly have an impact on the sales of Roundup<sup>®</sup> once the new seeds are readily available.

Glyphosate, as a herbicide, warrants full evaluation as a safe and effective chemical. Optimization of glyphosate efficacy requires full knowledge of its behaviour in plants and how this is affected by environmental factors (Caseley and Coupland 1985). Glyphosate's use must be regulated and its levels monitored in our food. The fate of glyphosate in the

environment also needs to be well established. Compliance with appropriate guidelines, in place and to be established, will ensure adequate protection of humans and their environment. Therefore, a sensitive and widely applicable method of analyzing for glyphosate and its potential metabolites is essential to addressing all of these concerns.

Many analytical methods for glyphosate have been developed over the past 15 years since the first method was published. These methods are to be reviewed in the next chapter. However, the difficulties inherent in the analysis of glyphosate and its potential metabolites in various matrices continue to challenge the versatility of methods and their analytical capabilities. Instrumentation has become increasingly sophisticated and expensive, thus the availability of specific equipment may dictate the method used. Therefore, it is advantageous to the scientific community to have available in the literature a variety of analytical protocols to choose from. Major attempts at reducing the complexity and length of analytical protocols have also led to the development of diverse approaches to the analysis of glyphosate and AMPA.

Difficulties encountered in the detection and quantitation of glyphosate stem from two intrinsic characteristics. Firstly, glyphosate's polar nature makes tedious its separation from

potentially interfering compounds. The major challenge to any analytical method is the development of efficient cleanup protocols for the sample involved. In fact, this step may dictate the degrees of sensitivity and specificity possible at the detection and quantitation stages. Secondly, the need to increase the sensitivity for detection and quantitation of the chemical at low levels requires the formation of a derivative. A number of derivatizing reagents for glyphosate and AMPA have been employed, however no reagent will be specific to these chemicals only.

The objective of this research was to develop a simplified, alternative method for the analysis of glyphosate and AMPA, utilizing high pressure liquid chromatography (HPLC) and ultraviolet (UV) detection. Sources of inconsistencies in the method involving the derivatization reaction, sample cleanup, and high pressure liquid chromatographic analysis, were identified and overcome where possible in order to develop a reliable and reproducible method. The procedure involves aqueous extraction of fortified plant tissues and sample cleanup using an anion exchange resin column. Glyphosate and AMPA were derivatized with 2,4-dinitrofluorobenzene (2,4-DNFB) for detection and quantification. Overall, the method is evaluated in terms of sensitivity, specificity, efficiency, reproducibility, and applicability for glyphosate and AMPA analysis in plant tissues.

## CHAPTER 2

### REVIEW OF ANALYTICAL METHODS FOR GLYPHOSATE

#### 2.0 Overview

Important criteria used in choosing an analytical technique primarily include sensitivity, accuracy, precision, and selectivity (Morrison and Skogerboe 1965). Practical considerations such as overall purpose, availability of reference standards, sampling design, cost and availability of equipment, and predicted time expenditures are significant as well. Many labs do not have the convenience of both gas and high pressure liquid chromatographs and their various detectors, thus the choice of method may be dictated by this circumstance alone. The adoption of a suitable method from the available literature is certainly the ideal approach if method development is not a goal. However, this often does not preclude the need for refinements in the method adopted, as particular needs will vary for every analytical situation.

A variety of methods for the analysis of glyphosate (GLYPH) exist already, and quite often they include the co-analysis of AMPA. A review of analytical techniques for GLYPH residues

was published by Bardalaye et al. (1985). Since then, many new analytical methods have been added to the literature, demonstrating the continuing need to search for sensitive, reproducible, yet versatile methods for GLYPH analysis.

The techniques used include colorimetry, polarography, thin layer chromatography (TLC), gas chromatography (GC), and high pressure liquid chromatography (HPLC). Most of the earlier contributions to the literature on GLYPH analysis were based on GC, in which the major difficulty was to give volatility to GLYPH and AMPA. More recently, the methods have been dominated by the use of HPLC systems which entail their own problems with derivatives of these compounds. Each technique used for analysis is reviewed in the following sections.

## 2.1 Colorimetry

In colorimetry, it is first necessary to convert the GLYPH to a coloured derivative to enable its detection and measurement. The concentration of the sample is determined by the difference in intensity of the incident and transmitted radiation in the UV and visible regions of the electromagnetic spectrum (Cheng 1965). GLYPH has been measured colorimetrically as a phosphomolybdate heteropoly blue complex



at 830 nm (Glass 1981) and as its ninhydrin derivative at 570 nm (Ekstrom and Johansson 1975). Ekstrom and Johansson (1975) reported the quantitative determination of GLYPH in standard solutions using an amino acid analyzer; the limit of quantitation for GLYPH was 3.4 ug. A minimum detection limit of 50 ug GLYPH was realized in the method by Glass (1981). Recoveries of the herbicide in distilled water, river water and run-off water fortified from 1 to 20 ppm, ranged from 91% to 108%.

Colorimetric methods offer relatively simple and rapid procedures for the estimation of a substance with the use of common laboratory instrumentation. However, this approach would not be generally applicable to field samples because the methods lack specificity for GLYPH. These methods require that the samples be essentially free from interfering contaminants to ensure accuracy and sensitivity in the results obtained.

## 2.2 Polarography

Polarography is a chronoamperometric technique in which a dropping mercury electrode is used. Mass transport takes place by diffusion with the voltage held constant (Day and

Underwood 1967). A limiting or diffusion current is obtained which is proportional to the concentration of the electroactive species in the bulk of the solution. The main problem in polarographic analysis is to measure this current accurately. GLYPH itself is polarographically inactive, whereas its N-nitroso derivative (the nitroso group is reduced) has enabled its polarographic analysis (Bronstad and Friestad 1976; Friestad and Bronstad 1985). However, oxygen and nitrite must be removed completely to preclude interferences.

Differential pulse polarography was successfully used to determine GLYPH residues in natural waters with a limit of detection of 0.035 ppm (Bronstad and Friestad 1976). The reduction of GLYPH nitrosamine was found to be very sensitive to pH changes in acidic media; the peak current continues to increase with decreasing pH. Friestad and Bronstad (1985) modified their earlier method in order to accommodate crop and soil samples. Additional cleanup procedures included cation and anion exchange columns, and time-consuming evaporation steps were eliminated. The detection limit was reported to range between 0.5 and 1 ppm, depending on the sample type and size. Mean recoveries were between 63% and 68% for various cereals, and were greater than 70% for other crops and soil, at fortification levels up to 4 ppm. The major limitation to

polarographic analysis is that AMPA is not amenable to nitrosation and subsequent detection.

### 2.3 Thin Layer Chromatography

Chromatography consists of a diverse but related group of practical techniques that permit the separation, identification, and quantification of components in a sample (Fried and Sherma 1986). The techniques essentially involve the differential migration of the applied sample through a system containing a stationary phase and a mobile phase. The stationary phase may be either a solid or a liquid, and the moving phase may be either a liquid or a gas (Day and Underwood 1967).

Thin-layer chromatography (TLC) is a mode of liquid chromatography in which the sample is applied as a small spot to the origin of a thin, stationary, sorbent layer supported on a glass, plastic, or metal plate (Fried and Sherma 1986). The mobile phase, often consisting of a mixture of solvents, moves through the stationary phase by current or most often by capillary action. Compounds, such as GLYPH and AMPA, not naturally coloured or fluorescent, need a detection reagent for visualization and quantification. The positions of the

separated compounds (or zones) can be used for qualitative identification, and the compounds may be eluted for further characterization by other techniques.

A procedure using TLC for detection and quantification of GLYPH was published by Young et al. (1977). The method involved aqueous extraction of GLYPH from the roots of Canada thistle (Cirsium arvense) plants treated with 40 mg ai per plant. Extracts were reacted with sodium nitrite to form the N-nitroso derivative, then separated on silica gel plates. These were irradiated with UV light to induce photolytic cleavage of the derivatives to produce the primary amine (i.e. AMPA). Fluorescamine, a primary amine specific reagent, was used for the formation of a fluorophore which was quantitatively measured by external standard comparison or a fluorescence spectrophotometric scanner. The detection limit of GLYPH was 5 ppm in plant tissues with recoveries of greater than 75% at this level.

Separation of GLYPH from potential metabolites (including AMPA, glycine and sarcosine) was successfully demonstrated by Sprankle et al. (1978). This method used cellulose plates developed with ethanol:water:15 N ammonium hydroxide :trichloroacetic acid:17 N acetic acid (55:35: 2.5:3.5 g: 2, v/v/v/w/v; v=mL). Visualization was accomplished with a ninhydrin spray.

Ragab (1978) described a one-dimensional TLC procedure for the separation of GLYPH and AMPA. The solvent systems involved varying ratios of methanol:water:0.5 M sodium chloride, followed by ninhydrin visualization. Two types of cellulose plates were compared as well as two different ninhydrin sprays to improve visualization. The method was tested on fortified distilled water samples, with the limits of detection for GLYPH and AMPA being about 0.1 ug and 0.05 ug, respectively.

#### 2.4 Gas Chromatography

Gas-liquid chromatography (GLC or more often GC) is a very sensitive method for analyzing the components of a sample which is rapidly vaporized. The separation of components takes place as they travel through a long column (25-60 m with 0.25-0.5 mm i.d. for the widely used capillary columns) (Vaughan and Zakrevsky 1988). The rate of separation is a function of the components' affinities for the carrier gas (inert) and the liquid stationary phase which coats the column as a thin film. The retention time is the length of time that a specific compound spends in travelling through a specified column under a given set of conditions.

Common detector systems used with gas chromatographic equipment include: flame photometry (FP), nitrogen-phosphorus (NP), and electron capture (EC). Flame ionization and thermal conductivity detectors may also be used, but they are the least sensitive of all the detectors mentioned. Detector response is measured through the modification of an electrical signal which is displayed as a peak on a recorder (Vaughan and Zakrevsky 1988). The height of and area under the peak are proportional to the amount of the compound present and the compound-specific sensitivity (response factor) of the detector. Derivatization is often necessary to enhance volatility in the target compounds, or to add halogens to groups of target compounds which do not tend to generate sufficient detector response. The identification of components may require further characterization by other methods.

A number of analytical methods have been reported for GLYPH involving GC and requiring either a single or double step derivatization scheme. The selection of derivatizing reagents for GLYPH and AMPA is made difficult by the presence of multiple functional groups and the lack of GLYPH solubility in solvents other than water (Bardalaye et al. 1985). Derivatization results in the esterification, alkylation or silylation of the carboxylic and phosphonic acid groups, as well as the acylation of the amino group. The incorporation

of halogens during this process also offers the choice of either FP or EC for detection (Deyrup et al. 1985). The FP detector would be chosen if high sensitivity is not a requirement, since it is much more selective than EC detection.

The first GC method for GLYPH and AMPA was published by a group at the Agricultural Research Department of Monsanto (Rueppel et al. 1976). It involved a two-step derivatization reaction with trifluoroacetic acid and trifluoroacetic anhydride (TFAA), followed by esterification with ethereal diazo-n-butane. Structures were verified by mass spectrometry and proton magnetic resonance. However, the efficiency of derivatization was quite low.

Monsanto's modification of this method included replacement with diazomethane or O-methyl-N,N-dicyclohexylpseudourea in the second derivatization step and an extensive sample cleanup protocol applicable to a number of crops and soil (Pesticide Analytical Manual or PAM 1977). Methods subsequently published criticized the PAM method for being extremely time consuming and dangerous due to the distillation and use of diazomethane. GLYPH and AMPA, isolated in separate fractions during column chromatography, required individual derivatization schemes. Further cleanup of derivatized fractions was necessary to reduce GC interferences, and was

specific for each chemical. When adapted for use in other laboratories, large residue losses, many chromatographic interferences, poor reproducibility, and extreme cleanup problems in certain crops were reported (Guinivan et al. 1982a).

Guinivan et al. (1982a) based their method on 2-chloroethyl N-heptafluorobutyryl derivatives of GLYPH and AMPA, which are amenable to quantification by FP, NP, or EC detection. Derivatization consisted of a two-step process, in which the compounds were alkylated first (using BCl<sub>3</sub> and 2-chloroethanol) followed by acylation (using heptafluorobutyric anhydride). A great deal of sample cleanup was required, especially in blueberries, due to large amounts of extractable sugars and pigments. Even though they claim their procedure requires less sample handling time than the PAM procedure (1977), it is still quite lengthy and involves many sample cleanup and concentration steps. However, other crops may require fewer cleanup requirements than those necessary for blueberries. Guinivan et al. (1982a) demonstrated significantly lower limits of detection compared with those achieved by the PAM procedure (1977). The use of Monsanto's methods in their laboratory achieved minimum detectability for standards of both compounds at 16.7 ng injected (FP detection), whereas the methods used by Guinivan et al. (1982a) resulted in minimum levels of detection of 0.015 ng



GLYPH injected and 0.025 ng AMPA injected (EC detection). However, at these levels, some interferences were encountered with the AMPA peak. Recovery from fortified blueberry samples (15 g) at the 0.05 ppm level was about 66% for GLYPH and 102% for AMPA. Mass spectral studies verified the structures and fragmentations of these new derivatives (Guinivan et al. 1982b).

The first attempt at a single-step derivatization method for GC analysis involved the reagent N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), which introduced tert-butyldimethyl-silyl (TBDMS) groups at sites having active hydrogens (Moye and Deyrup 1984). The efficiency of derivatization was initially low, but better chromatographic response was obtained when the glass test tubes were pre-coated with a dilute solution of phosphoric acid in ethanol. The authors claimed that analyses of these compounds at the ppm level were obtainable, but no recovery data or limits of quantitation were given.

Deyrup et al. (1985), recognizing the limitations to this particular method, changed to a new process that successfully provided a reliable single-step derivatization. A mixture of trifluoroethanol (TFE) and trifluoroacetic anhydride (TFAA) (also used by Monsanto; PAM 1977) or heptafluorobutyric anhydride (HFBA) reagent (used by Guinivan et al. 1982a)

converted both GLYPH and AMPA into derivatives amenable to GC analysis with either FP or EC detection. Excellent reproducibility of replicate derivatizations was obtained. Average recoveries from fortified deionized water samples were greater than 90% at the ppb level. However, a reasonable explanation for AMPA values greater than 100% (ranging from 103% to 122%) could not be offered. The EC detector showed extreme sensitivity, as 0.01 ng amounts of GLYPH and AMPA were detectable. The expected structures of derivatives were confirmed by mass spectral studies. This method also demonstrated an ability to select from various mixtures of alcohols and anhydrides to produce derivatives with varying chromatographic retentions, thus enabling the manipulation of retention time of these derivatives if interference was observed.

Analyses of GLYPH residues in kiwi fruit and asparagus were accomplished to support U.S. registration (Seiber et al. 1984). These crops were not amenable to analysis by earlier methods due to the numerous interferences that prohibited quantitation at the required sub-ppm levels. The procedure described was adopted or modified from previous procedures reported in the literature. The successful determination of GLYPH was due to the incorporation of a silica gel HPLC cleanup step prior to GC analysis for which baseline adjustment was required. Preliminary investigations also

indicated that the method was applicable to AMPA. The method could be adopted for other crops, some of which may not require the HPLC step. The detection limit was found to be about 0.05 ppm for both crops. Mean recoveries for either kiwi or asparagus were reported to be greater than 80% at fortification levels of 0.05 ppm to 0.25 ppm.

Finally, another GC method developed for residues in soils used an NP detector (Roy and Konar 1989). This method was based on that of Deyrup et al. (1985). The addition of phosphoric acid to the soil samples was explored as a means of increasing GLYPH recovery. GLYPH recoveries from fortified sand, clay and organic soil samples averaged 48%, 52%, and 78% respectively at fortification levels ranging from 0.035 to 1 ppm. AMPA recoveries were 43%, 50%, and 65%, respectively.

## 2.5 High Pressure Liquid Chromatography

HPLC provides high resolution, speed, sensitivity, and automation in a range of analytical applications (Johnson and Stevenson 1978). The stationary phase is bonded to a porous polymer and contained within a narrow-bore, stainless steel column. The mobile phase is forced through the column under considerable pressure. The successful chromatographic

separation of sample components results from a compromise between chromatographic resolution, sample capacity, and analysis time.

Derivatives in HPLC are invariably prepared to improve the response of a substance towards a particular detector (Poole and Schuette 1984). Detectors used in liquid chromatography must function with high precision, sensitivity and stability, and must not significantly affect the separation achieved by the column (Gilbert 1987). Photometers used for various purposes include UV, fluorescence, infra-red, and refractive index detectors.

Liquid chromatography, compared with gas chromatography, is more applicable to GLYPH and AMPA analysis because of the water-soluble and ionic nature of these compounds (Bardalaye et al. 1985). Mobile phases for reverse-phase HPLC include water mixed with either methanol or acetonitrile. However, to enable their measurement by photometric detectors, pre-column or post-column derivatization is necessary to produce coloured, fluorescent or UV-absorbing compounds.

An exception to employing derivatization was presented in Monsanto's first published reports with respect to formulation and technical samples (Burns and Tomkins 1979; Burns 1983). GLYPH and its impurities were separated using an anion

exchange HPLC column. Peak fractions were collected for identification and quantitation by nuclear magnetic resonance and colorimetric assay, with a detection limit of 2.5 ppm (Burns and Tomkins 1979). Burns (1983) used a method in the laboratory for a number of years before it was subjected to a collaborative study in order to demonstrate the method's reproducibility. Highly concentrated samples were used along with UV detection at 195 nm and the previous knowledge of GLYPH retention time. However, it was apparent that the method was intended for formulation and technical samples only, specifically for quality control.

Two derivatizing reagents are predominant in the literature concerning GLYPH detection by fluorescence. These are 9-fluorenylmethyl chloroformate (FMOCCL) and o-phthalaldehyde-mercaptoethanol (OPA-MERC). FMOCCL is a pre-column derivatizing reagent specific for primary and secondary amines which results in high quantum yields in aqueous based solvent systems (Moye and Boning 1979). Reaction of FMOCCL with GLYPH takes place rapidly under alkaline conditions and excess reagent can be efficiently removed. The reagent, however, also derivatizes alcohols under some conditions, and may interfere with AMPA analysis (Moye and St. John 1980). OPA-MERC is a post-column derivatizing reagent specific for primary amines. The post-column procedure forms derivatives on-line but it requires equipment modification and expertise

(Miles et al. 1986). An important consideration in the decision to use this approach is the requirement to cleave GLYPH to yield a primary amine. This is usually performed by pumping calcium hypochlorite into the system prior to derivatization.

A report comparing these two methods of derivatization demonstrated that special HPLC columns may be required (Moye and St. John 1980). The OPA-MERC method required a quaternary ammonium plastic bead column which demanded long equilibration times with the slightest change in solvent programming. The FMOCCl method worked best with silica particle columns, but their performance deteriorated rapidly when they became loaded with crop co-extractives.

Moye and Boning (1979) presented the first pre-column derivatization procedure using FMOCCl for fluorometric detection of GLYPH and AMPA. A detection limit of 0.1 ng in aqueous standards was realized. Derivatization and subsequent chromatographic analysis of other amino acids indicated that manipulation of mobile phase parameters may be necessary for resolution of these compounds with the AMPA peak.

Glass (1983) investigated the applicability of Moye and Boning's method (1979) to GLYPH-fortified soil and natural water samples. Modifications to the method adopted included

sample cleanup using a strong anion exchange column, the use of an amine phase column for HPLC analysis, and slight adjustments in the percentage of mobile phase constituents to reduce elution times. Minimum levels of GLYPH quantitation were 100 ppb in river water, 25 ppm in silt and sandy loams, and 100 ppm in clay loam soil. Recoveries averaged greater than 80% in water samples and ranged from 18% to 55% in soil samples. The average percent derivatization of GLYPH using FMOCCl was found to be much lower than that reported by Moye and Boning (1979).

Another pre-column derivatization procedure was applicable to the determination of GLYPH and AMPA residues in natural waters (Miles, et al. 1986). Sample preparation consisted of filtration and rotary evaporation steps before derivatization with the FMOCCl reagent. A number of HPLC anion exchange columns were evaluated as well as two fluorescence detectors. Minimum detectable quantities reported were 0.01 ppm GLYPH and 0.005 ppm AMPA. Recoveries from natural waters were greater than 80%. Interferences precluded the quantitation of AMPA at lower fortification levels.

This method was also used to determine the extent of sorption/desorption of GLYPH to several soils and clay materials (Miles and Moye 1988). They used various solvents with differing pH to extract GLYPH from soils varying in

amounts of clay and organic matter. Quantitation limits included 1 ppm for high clay soils and 0.5 ppm for sandy soils. In general, recoveries from fortified soils were found to decrease as the percentage of clay or organic matter increased.

A new approach to the analysis of GLYPH and AMPA was presented by Lundgren (1986). This consisted of pre-column derivatization with 2,4-dinitrofluorobenzene (2,4-DNFB). This reagent is specific for primary and secondary amines in aqueous solutions. These derivatives were quantified with reverse-phase, ion-pair HPLC using tetraethylammonium bromide as a counterion reagent and UV detection at 405 nm. Fortified soil samples yielded minimum detectable quantities at 0.05 ppm GLYPH and 0.1 ppm AMPA, which is an improvement over the method by Glass (1983). Recoveries were also relatively higher, with greater than 90% obtained from clay loam and sandy soils. However, relatively lower recoveries (55%) were obtained in soils high in organic matter.

A critical comparison of HPLC pre-column (FMOC1) and post-column (OPA-MERC) fluorogenic labelling of GLYPH and AMPA was provided by Moye and St. John (1980). However, due to interferences encountered with AMPA in the pre-column derivatization method, crop recoveries were only determined by the OPA-MERC method. GLYPH and AMPA were eluted in separate



fractions from a preparatory column, necessitating separate HPLC analyses. At the 0.1 ppm level, crop recoveries were greater than 61% for AMPA (82% in cucumbers) and greater than 70% for GLYPH (96% in cucumbers). Overall, recoveries were generally higher for GLYPH than for AMPA. GLYPH was not completely converted to the primary amine, even though adequate sensitivity and reproducibility were achieved. The procedure used here was comparable to that of Monsanto's method (PAM 1977), except that Moye and St. John (1980) reduced sample cleanup to a single cation exchange column.

A simplified HPLC procedure was reported by Moye et al. (1983) and was based upon the post-column fluorogenic labelling approach just described (Moye and St. John 1980). Improvements in chromatographic efficiency, resolution and sensitivity were claimed. A number of crops were analyzed using a simplified extraction and cleanup procedure (a single cation exchange column). Separation of GLYPH and AMPA on the anion exchange HPLC column used previously could not be improved upon, thus two columns were employed for the separate analyses of these compounds. This apparently did not affect sample throughput. Crop recoveries were consistently greater than 70% at the 0.1 ppm level for both GLYPH and AMPA (with the exception of 61% recovery of AMPA in cranberries). Crops with high sugar contents (e.g. blueberries) occasionally

slowed the chromatographic flow and produced interference with the GLYPH peak.

Archer and Stokes (1984) also utilized CPA-MERC post-column derivatization for analysis of GLYPH residues in blackberries. An acid reflux step, used to hydrolyse possible conjugates of GLYPH and plant constituents, improved the efficiency of ion exchange column cleanups that followed. All other steps were similar to previously published schemes. This method was sensitive to 0.05 ppm in blackberries with recoveries greater than 80%.

The Monsanto group published and validated their updated methodology as an inter-laboratory study (Cowell et al. 1986). This method attempted to combine various aspects of previous methods along with advances made in their own lab to provide a simplified procedure applicable to a variety of different matrices. Cleanup involved aqueous-organic extractions, ligand exchange, anion exchange and HPLC post-column reaction with fluoraldehyde (the OPA reagent) and fluorescence detection. Analyses of blind fortified samples, analyst fortified samples, and control samples were made by five different analysts. The overall recovery for the study was 81% for GLYPH and 79% for AMPA, with standard deviations of 14% and coefficients of variation equal to 17%.

Thompson et al. (1989) provided an alternative HPLC method for the quantitation of GLYPH and AMPA in organic and mineral soils, sediments and foliage. Ion exchange column chromatography was followed by post-column derivatization with ninhydrin, and absorbance detection at 570 nm. Limits of detection were 0.01 ppm for sediments (dry mass) and 0.1 ppm for hardwood foliage (dry mass). Mean recovery efficiencies for GLYPH were as follows: bottom sediment 84%, suspended sediment 66%, organic soils 79%, mineral soils 73%, alder leaf litter 81%, and salmonberry leaf litter 84%. Coefficients of variation were less than 14% on mean recovery for all substrates.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.0 Materials and Reagents

Glyphosate (N-phosphonomethylglycine; purity = 99%) was a gift from the Monsanto Chemical Company (St. Louis, MO). Aminomethylphosphonic acid (AMPA; purity = 99%) was purchased from the Sigma Chemical Company (St. Louis, MO). All stock solutions were prepared with distilled, deionized water. Serial dilutions were made where necessary. These solutions, along with the pure chemicals, were kept at 4°C.

The derivatizing reagent was prepared by dissolving 1 g of 2,4-dinitrofluorobenzene (2,4-DNFB) (Sigma Chemical Company, St. Louis, MO) in 100 mL of analytical grade acetone (1% w/v). The 2,4-DNFB reagent is a potentially carcinogenic compound and must be handled accordingly. Dichloromethane was of HPLC grade and isobutanol was of analytical grade. A 0.1 M solution of sodium tetraborate was prepared for use during the derivatization scheme. Deionized, distilled water was used at all times. A 1 M sodium bicarbonate solution and a 1 M solution of sodium chloride was prepared for use in anion

exchange chromatography. The buffer used for liquid chromatography was a 0.05 M sodium phosphate solution which was adjusted to pH 2.1 with concentrated phosphoric acid. Methanol was of HPLC grade.

Bean plants (Phaseolus vulgaris cv. Topcrop) were germinated and grown in growth pouches (CanLab) using a commercial hydroponic nutrient solution (Plant-Prod Hydroponic Fertilizer, Bramalea, Ontario). Plants were 12 days old when sampled, with the first trifoliolate leaves just emerging. These were grown under a bank of alternating fluorescent tubes and Gro-lights at an approximate day/night temperature regime of 25/20°C and a 14 h photoperiod.

### 3.1 Apparatus

Plant tissue extraction and cleanup required a Polytron homogenizer, Model PCU-1 (Brinkmann Westbury, NY). The homogenate was suction filtered using a Buchner funnel lined with Whatman #4 filter paper (7 cm diam). Column chromatography was performed using AG 1-X8 strong base, anion exchange resin, 50-100 mesh, chloride form (Bio-Rad Laboratories, Richmond, CA), and a glass column (1 cm i.c. x 37 cm) equipped with a Teflon stopcock and an attachable

250 mL glass reservoir. An IEC vortex mixer and an IEC table top centrifuge were used during the steps following derivatization of GLYPH and AMPA.

### 3.2 Instrumentation and Operation

The HPLC system consisted of a Varian 5000 liquid chromatograph, a Hewlett Packard Model 1040A UV detection system controlled by a Hewlett Packard 85 B computer, and a Hewlett Packard Model 3392A integrator and Model 7470A plotter. For HPLC, the variable wavelength UV detector was programmed at 370 nm. GLYPH and AMPA were chromatographed on a reverse-phase ALTEX ULTRASPHERE ODS C<sub>18</sub>, 5 µm particle column (4.6 mm id X 25 cm) (Beckman).

Operating conditions consisted of a 0.05 M sodium phosphate buffer (pH 2.1; solvent A)/methanol (solvent B) mobile phase. The methanol was degassed for approximately 45 min before turning on the pump. The flow rate was consistently maintained at 1 mL/min. The injection loop was flushed with phosphate buffer and a blank injection was made before chromatographing samples. Injection volumes ranged from 20 to 200 µL. Step-wise gradient operation consisted of 30% B at 0 min, 35% B at 6.5 min, 50% B at 7.5 min, 50% B at 12.5 min,

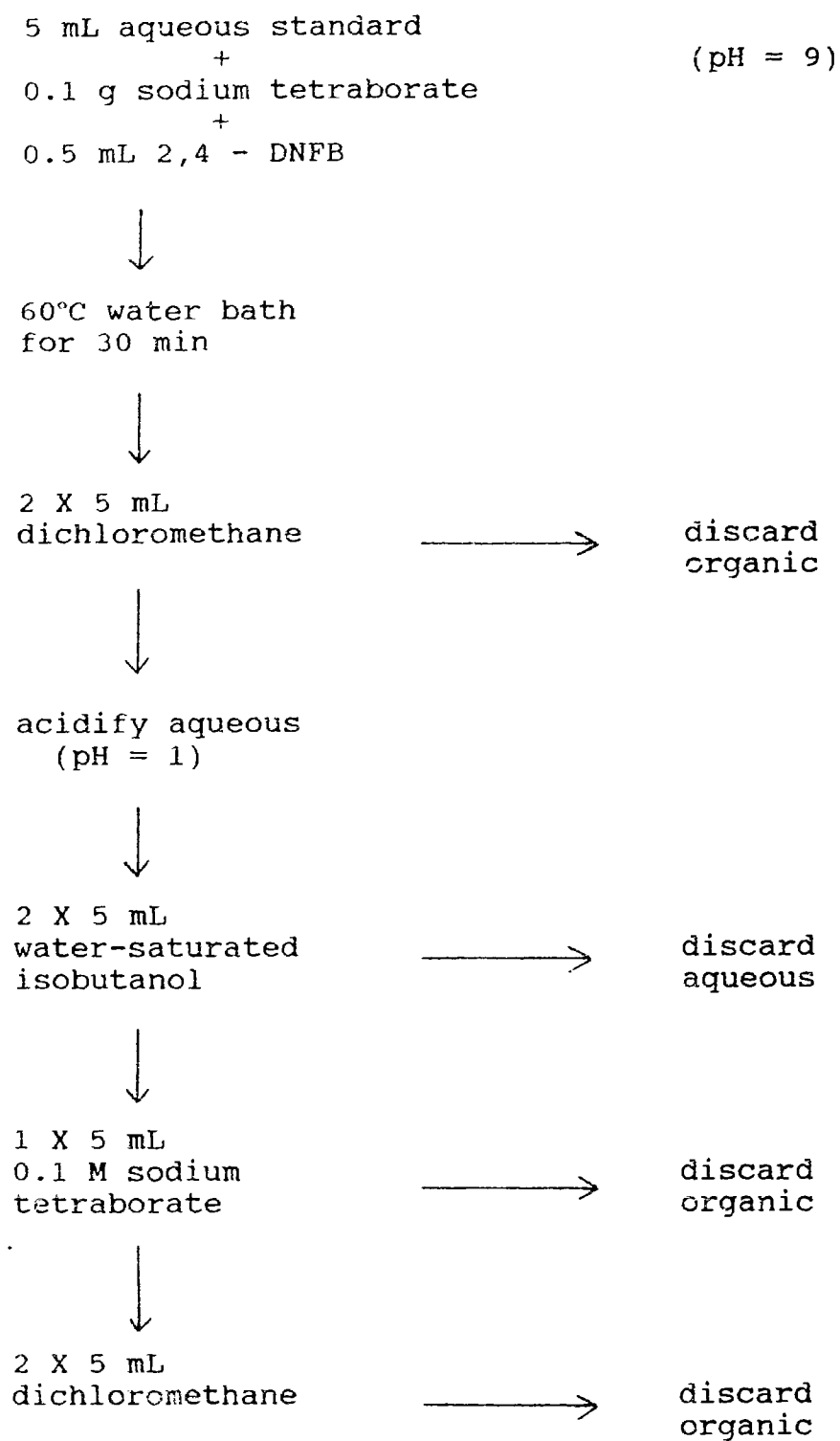
and 30% B at 13.5 min. Sample analysis time was programmed for 18 min. The mobile phase was allowed to equilibrate for at least 15 min longer when samples were derived from plant tissues. The integrator monitored the chromatographic signal, integrated peaks, and expressed results as unit areas. GLYPH and AMPA were quantified using the external standard technique.

The HPLC was maintained every day after chromatography by flushing the system with water for one h. This was followed by a 10 min flush with methanol. The injection loop was also flushed with methanol. This was necessary because of the low pH of the buffer used and to prevent microbial growth from occurring within the system when it was not in use.

### 3.3 Derivatization Scheme

A flow diagram of the derivatization scheme is shown in Figure 1. Five mL of a standard aqueous solution containing both GLYPH and AMPA at known concentrations were pipetted into a 30 mL Corex test tube. Control samples consisted of 5 mL of deionized, distilled water. The standard solution was adjusted to pH 9 by adding approximately 0.1 g of sodium tetraborate and vortexing until dissolved. This step was

Figure 1. Flow diagram of the derivatization scheme followed.





essential for the reaction to proceed. The test tube was kept protected from the light from this point onwards to prevent photodegradation of the product. One-half mL of the 2,4-DNFB reagent was added to the tube and mixed thoroughly. Derivatization was carried out for 30 min at 60°C in a water bath, after which time the test tube was removed and cooled to room temperature.

The aqueous solution was extracted twice, each time with 5 mL of dichloromethane. For each extraction, the solvents were mixed by vortexing for 1 min, and then centrifuged at 7000 rpm for about 5 min to speed phase separation. All subsequent extractions consisted of vortexing and centrifugation in this manner. Upon phase separation, the organic phase was removed with a Pasteur pipette and discarded. The aqueous phase was acidified to pH 1 with a few drops of 6 M hydrochloric acid and extracted twice with 5 mL of water-saturated isobutanol.

The isobutanol phase (upper layer), containing most of the DNP-GLYPH and DNP-AMPA, was quantitatively transferred to a clean test tube with a Pasteur pipette. The combined isobutanol phases were then extracted once with 5 mL of 0.1 M sodium tetraborate. The isobutanol layer was removed and discarded, and the remaining aqueous solution was extracted twice, each time with 5 mL of dichloromethane, which was discarded. The aqueous basic solution containing the

derivatized products was quantitatively transferred to an 8 mL screw cap vial (wrapped in tinfoil) and stored at 4°C until HPLC analysis.

The reproducibility of the method and linearity of detector response were evaluated. Dilutions were made of a stock solution containing 100 ug/mL each of GLYPH and AMPA, giving solutions of 0.5, 1, 5, and 50 ppm of each chemical. Four replicates of 5 mL aliquots of each solution were derivatized as described. The 0.5 and 1 ppm samples were acidified to pH 1 with 6 M hydrochloric acid, and the samples representing the 5 and 50 ppm concentrations were diluted with phosphate buffer mobile phase (pH 2.1) 1:10 and 1:100, respectively, prior to injection.

#### 3.4 Anion Exchange Calibration

Resin conversion and conditioning were performed on the same day for sample cleanup purposes. Approximately 7 g of the anion exchange resin (AG 1-X8) were hydrated in a small beaker of water for about one h. The slurry was poured into a glass column, and the resin was allowed to settle. Any resin adhering to the sides of the column was rinsed down with water. A glass wool plug was placed on the top of the resin

bed to prevent disturbance and trap particles. About 100 mL of water was rinsed through this anion exchange column. Conversion of the resin from the chloride form to the bicarbonate form required approximately 400 mL of 1 M sodium bicarbonate as a counterion solution. The column was finally rinsed with another 100 mL of water.

Recovery was determined for the GLYPH and AMPA standards put through the anion exchange column. Dilutions of a 100 ug/mL GLYPH and AMPA stock solution gave standards of 0.5, 1, 5, and 50 ppm concentrations of each chemical. Four replicate samples of each concentration were prepared. A 75 mL aliquot of the standard was adjusted to pH 8 with a few drops of 1 M sodium hydroxide and then loaded onto a conditioned resin column at a flow rate of 1 mL/min.

After loading the sample, about 25 mL of water were used to rinse the resin bed of non-adsorbed materials, which was subsequently discarded. GLYPH and AMPA were then eluted with exactly 50 mL of 1 M sodium chloride at a flow rate of 1 mL/min. Eluates were collected in glass-stoppered bottles, sealed with parafilm, and stored at 4°C. The next day, a 5 mL aliquot of each sample was taken and derivatized as described. Anion exchange efficiencies were determined by comparing peak areas of those standards put through the anion exchange column to those that were not. A flow diagram summarizing the steps

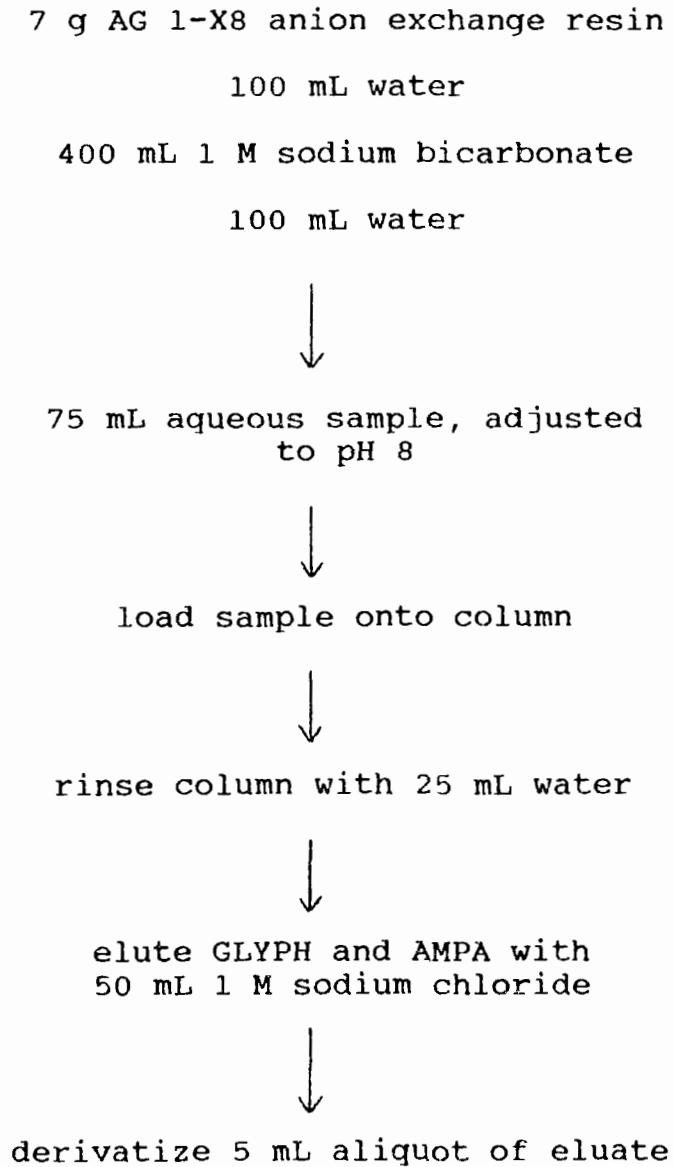
involved in the anion exchange cleanup protocol followed is shown in Figure 2.

### 3.5 Sample Preparation and Fortification of Plant Tissues

Bean plants were grown in growth pouches as described. The roots were rinsed with distilled water and blotted dry with a paper towel. The plants were divided into leaves, stems and roots, and 1 g or 4 g (fresh weight) of tissue was taken for sample fortification (with four replicates per fortification level). Samples were cut into relatively smaller pieces and put into 100 mL glass beakers. A leaf tissue sample weighing 4 g was spiked with 10 mL of an aqueous solution of GLYPH and AMPA (375 ug/mL). All other tissue samples weighed 1 g, and were spiked with 1 mL of an aqueous solution containing the appropriate amounts of GLYPH and AMPA (prepared from a stock solution of 375 ug/mL GLYPH and AMPA). Spiked samples were then allowed to equilibrate for about 2 h. Concentrations of GLYPH and AMPA with respect to fortified tissues ranged from 38 to 938 ug/g.

A Polytron homogenizer was used to grind the tissue in 50 mL of water. An additional 10 mL of water was used to rinse the Polytron blade after thorough homogenization. The homogenate

Figure 2. Flow diagram of the anion exchange cleanup protocol.



was suction filtered through a Buchner funnel lined with Whatman # 4 filter paper. The residue left on the filter paper was rinsed with approximately 20 mL of water. A plant sample size of 4 g required that the homogenate be split into approximately equal volumes and each filtered separately. The filtrate (about 80 mL) was adjusted to pH 8 and loaded onto a freshly conditioned anion exchange column. The sample retained on the column was processed as described in the anion exchange calibration section. Concentrations of GLYPH and AMPA in the 50 mL eluates ranged from 0.5 to 50 ppm. The 5 mL aliquots of plant sample eluates taken were neutralized prior to derivatization.

## CHAPTER 4

### RESULTS AND DISCUSSION

#### 4.0 Stability of Standards

Concentrated stock solutions of 100 ug/mL GLYPH and AMPA in deionized, distilled water were stored at 4°C, and were found to be stable for at least 6 mo. Standards prepared during this time period resulted in consistent chromatographic responses when analyzed by HPLC. This observation is supported by other published knowledge of the stability of GLYPH and AMPA in aqueous solutions. A solution containing 0.7 g of GLYPH in 100 mL of water acidified with sulphuric acid was found to be stable for at least 3 mo at room temperature (Bronstad and Friestad 1976). Friestad and Bronstad (1985) reported that analytical standards of 1 mg/mL GLYPH were stable for years at room temperature. In another report, concentrations of standards ranging from 0.005 to 10 ug/mL of GLYPH and AMPA were refrigerated for over 6 mo with no observed degradation (Miles et al. 1986).

#### 4.1 Derivatization Scheme

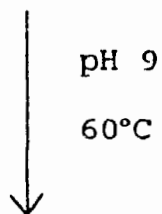
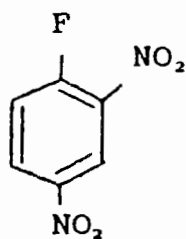
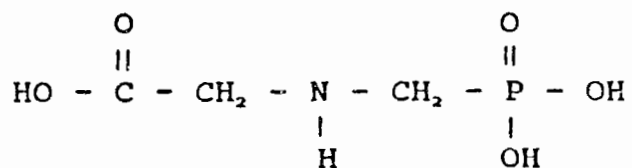
The 2,4-DNFB reagent is well-known for the derivatization of primary and secondary amines in aqueous solutions (McIntire et al. 1953; Day et al. 1966; Rosmus and Deyl 1972). The process of dinitrophenylation converts compounds such as GLYPH and AMPA to their corresponding 2,4-dinitrophenyl derivatives (i.e. DNP-GLYPH and DNP-AMPA). The degree of sample cleanup is very important to the sensitivity and specificity of GLYPH and AMPA detection because the 2,4-DNFB reagent will react with a number of other primary and secondary amines to form products that may interfere with subsequent GLYPH and AMPA chromatography. Figures 3 and 4 show a diagrammatic representation of these reactions.

The 2,4-dinitrophenyl chromophore has a molar extinction coefficient of  $> 10,000$  measured at 254 nm and provides detection limits for derivatized compounds at a low nanogram level (Poole and Schuette 1984). UV detection at 370 nm was chosen as the compromise between DNP-GLYPH and DNP-AMPA which were found to have absorption maxima at about 375 and 360 nm, respectively. Spectra plots showing absorption maxima are shown in Figure 5. The chromatographic results reported here pertain to DNP-GLYPH and DNP-AMPA and not to the actual compounds of GLYPH and AMPA.

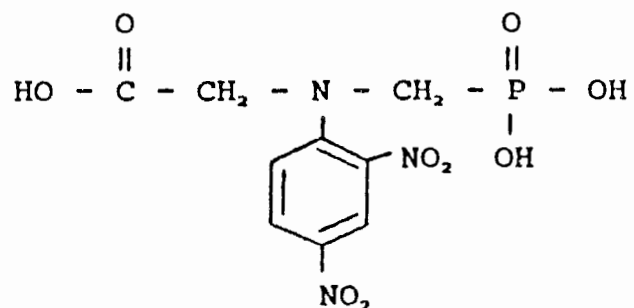


Figure 3. Diagrammatic representation of the derivatization of GLYPH with 2,4-DNFB.

GLYPH:



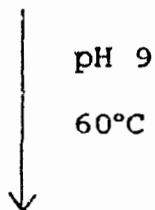
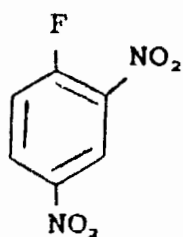
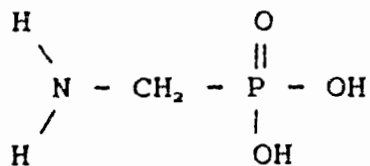
DNP-GLYPH:



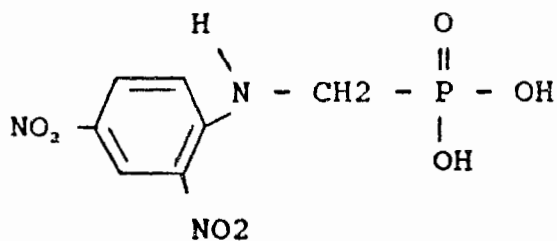
+ HF

Figure 4. Diagrammatic representation of the derivatization of AMPA with 2,4-DNFB.

AMPA:



DNP-AMPA:



OR:

+ HF

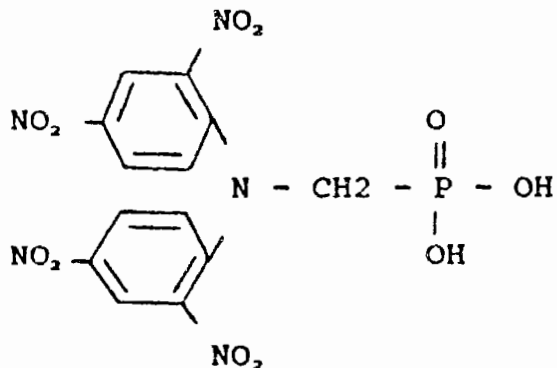
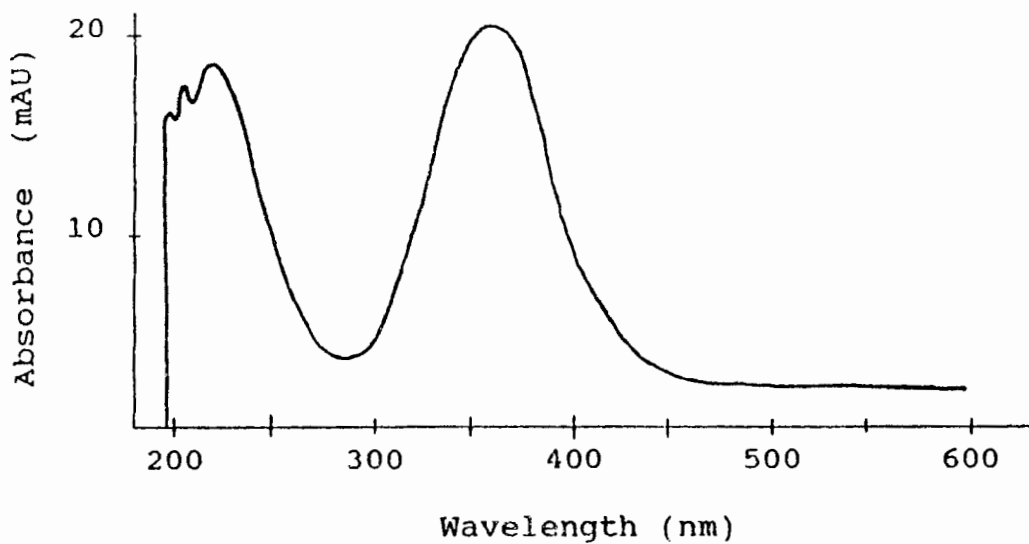
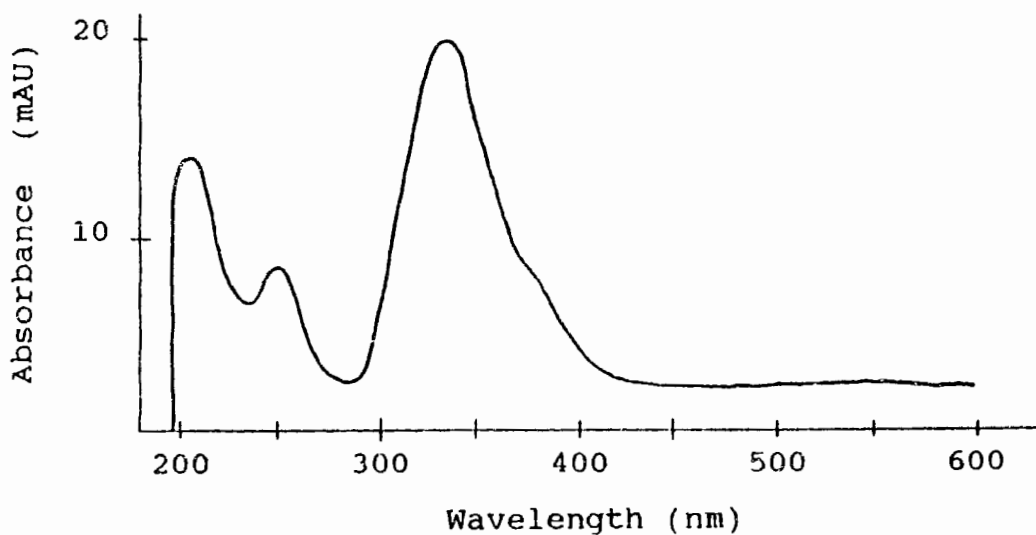


Figure 5. Spectra plots showing the absorption maxima of DNP-GLYPH and DNP-AMPA.

DNP-GLYPH:



DNP-AMPA:



The pH of the aqueous solution was very important for obtaining consistent chromatographic results. A pH of 9 is required for the reaction to take place between primary or secondary amines with 2,4-DNFB (Day et al. 1966; Rosmus and Deyl 1972). The reaction time of 30 minutes was chosen based on published procedures involving this reagent under similar conditions (McIntire et al. 1953; Day et al. 1966; Rosmus and Deyl 1972). With excess reagent available under these conditions, the yield of 2,4-DNP derivatives levels off at 30 min.

In my research, detailed kinetic studies were not carried out, but the effect of temperature was investigated. The effect of temperature was evaluated based on the measured area of the peaks, from injection of standard solutions derivatized for 30 min at 20°C, 40°C, 60°C, and 80°C. The chromatographic results indicated that the yield of DNP-GLYPH increased from 20°C to 60°C. A subsequent reduction in yield was observed at 80°C. Therefore, derivatization at 60°C was chosen as the optimum condition for maximum chromatographic response based on the experimental conditions used here. These results are consistent with other published studies involving this derivatizing reagent (McIntire et al. 1953; Day et al. 1966). Samples derivatized at room temperature would have probably required a much longer reaction period to yield an adequate

chromatographic response. Derivatization at 80°C may have resulted in the decomposition of the product.

For consistent chromatographic response, it was necessary that the derivatized product of DNP-GLYPH be protected from the light at all times. Initial sample processing in the light resulted in an unidentified peak eluting just after DNP-GLYPH. Integration of the DNP-GLYPH and unidentified peak areas was not reliable as these peaks were not completely resolved from one another. Standards chromatographed at the beginning and end of the day indicated that the size of the unidentified peak had increased, and the size of the DNP-GLYPH peak had decreased over the course of the day. The unidentified peak was not observed in subsequent analyses of standards derivatized and kept completely protected from the light. The peak was not observed in AMPA standards or water controls. Therefore, it was concluded that photodegradation of the DNP-GLYPH product was occurring and resulting in the appearance of the peak. It was postulated that the peak could have resulted from the decarboxylation of DNP-GLYPH producing DNP-methyl aminomethylphosphonic acid, which is a compound having intermediate polarity between GLYPH and AMPA.

Initially, the random observation of peaks interfering with GLYPH and AMPA chromatography was thought to be due to an organic contaminant (isobutanol was suspected) still present

in the aqueous solution. The samples in which this problem was occurring were adjusted to pH 9 and re-heated to 40°C in an attempt to remove the suspected organic contaminant. Subsequent chromatography was worse than the initial analyses, therefore re-heating of samples is not recommended. However, it was later determined that this random observation of ghost or artifact peaks was due to the buildup of contaminants in the injection port. This was corrected by flushing the injection loop with phosphate buffer.

Other essential components of the method, which contributed significantly to consistent results, were the vortexing and centrifugation steps. The vortexing of each extraction step for one min was done to speed equilibration of the chemicals in the aqueous/organic phases. It was also important to centrifuge after each of these extractions to remove any organic micro-droplets suspended throughout the aqueous sample and to speed phase separation. These micro-droplets, if not removed from the aqueous phase, could adversely affect both partitioning and chromatographic response.

Sensitivity of the method presented here could potentially be increased with respect to the 0.1 M sodium tetraborate step. Instead of using 5 mL of 0.1 M sodium tetraborate, a smaller volume could be used to extract the chemicals out of the isobutanol phase. Replicate standards of 0.1, 0.5, and 1 ppm

GLYPH and AMPA were prepared and derivatized as usual. The sodium tetraborate step was reduced to 2.5 mL for one of the replicates at each concentration. Comparison of peak area counts for these standards consistently indicated that, when DNP-GLYPH and DNP-AMPA were extracted into 2.5 mL of sodium tetraborate, the sensitivity was increased by 100%. Further investigation would be needed to determine whether volume reduction of the sodium tetraborate step would yield consistent recoveries at other concentration levels. This was not investigated with respect to samples involving plant tissues. However, it would be expected that plant tissue co-extractives may limit the degree to which sensitivity may be successfully increased by manipulating the volume of this step, as this would also serve to increase the concentration of potential interferences.

In the initial stages of method development, organic phase extractions with dichloromethane were performed on acidified aqueous solutions (pH 1). Subsequent difficulties with the peak shape of DNP-AMPA led to the attempt to remove interferences with a third dichloromethane extraction of the acidic aqueous solution. Results indicated that, although the DNP-AMPA peak shape had improved somewhat, a significant reduction in peak size had occurred. The movement of some yellow colour into the organic phase was observed during these extractions, indicating that the DNP chromophore (possibly

including DNP-GLYPH and DNP-AMPA) were being lost to this phase. Therefore, it was decided to carry out dichloromethane extractions on the aqueous basic solutions to ensure that no product would be lost during organic extractions. Peak shape of DNP-AMPA was consistently good following the adoption of dichloromethane extractions of the aqueous solution at pH 9.

Method development using 2,4-DNFB as a derivatizing reagent for GLYPH and AMPA analysis was well established at the time when Lundgren (1986) published a similar method applicable to soil samples. Lundgren's method also used the 2,4-DNFB reagent to derivatize GLYPH and AMPA, but it was evident that a contradiction existed with the scheme presented in my research. Lundgren's method claimed to extract the derivatized product from the acidified aqueous solution (pH 1) into ethyl acetate, allowing subsequent concentration and re-dissolving of the product in a small amount of buffer.

To ascertain the degree to which DNP-GLYPH and DNP-AMPA partitioned into this organic phase at pH 1, four replicate samples of a 50 ppm standard solution of GLYPH and AMPA were processed according to Lundgren's method (1986). Each step was analyzed and results are shown in Table 2. An overview of Lundgren's scheme (1986) is shown in Figure 6, and may be compared to the overview of my scheme shown previously (Figure 1). Ethyl acetate fractions were evaporated to dryness and



**Table 2. Recoveries of GLYPH and AMPA using the derivatization scheme of Lundgren (1986).**

50 ppm Standard <sup>2</sup> (250 ug)	ug Recovered <sup>1</sup>			
	First Organic <sup>3</sup>	Aqueous Solution <sup>4</sup>	Final Organic <sup>4</sup>	Total
<b>GLYPH:</b>				
1	ND <sup>5</sup>	124	135	259
2	0.4	91	148	239
3	ND	128	131	259
4	ND	122	138	260
Average	0.2%	46%	55%	254
<b>AMPA:</b>				
1	0.5	82	163	245
2	1.1	57	182	240
3	0.7	79	154	234
4	1.1	78	169	248
Average	0.3%	30%	67%	242

<sup>1</sup> GLYPH and AMPA recoveries were determined in each extraction step using an external standard.

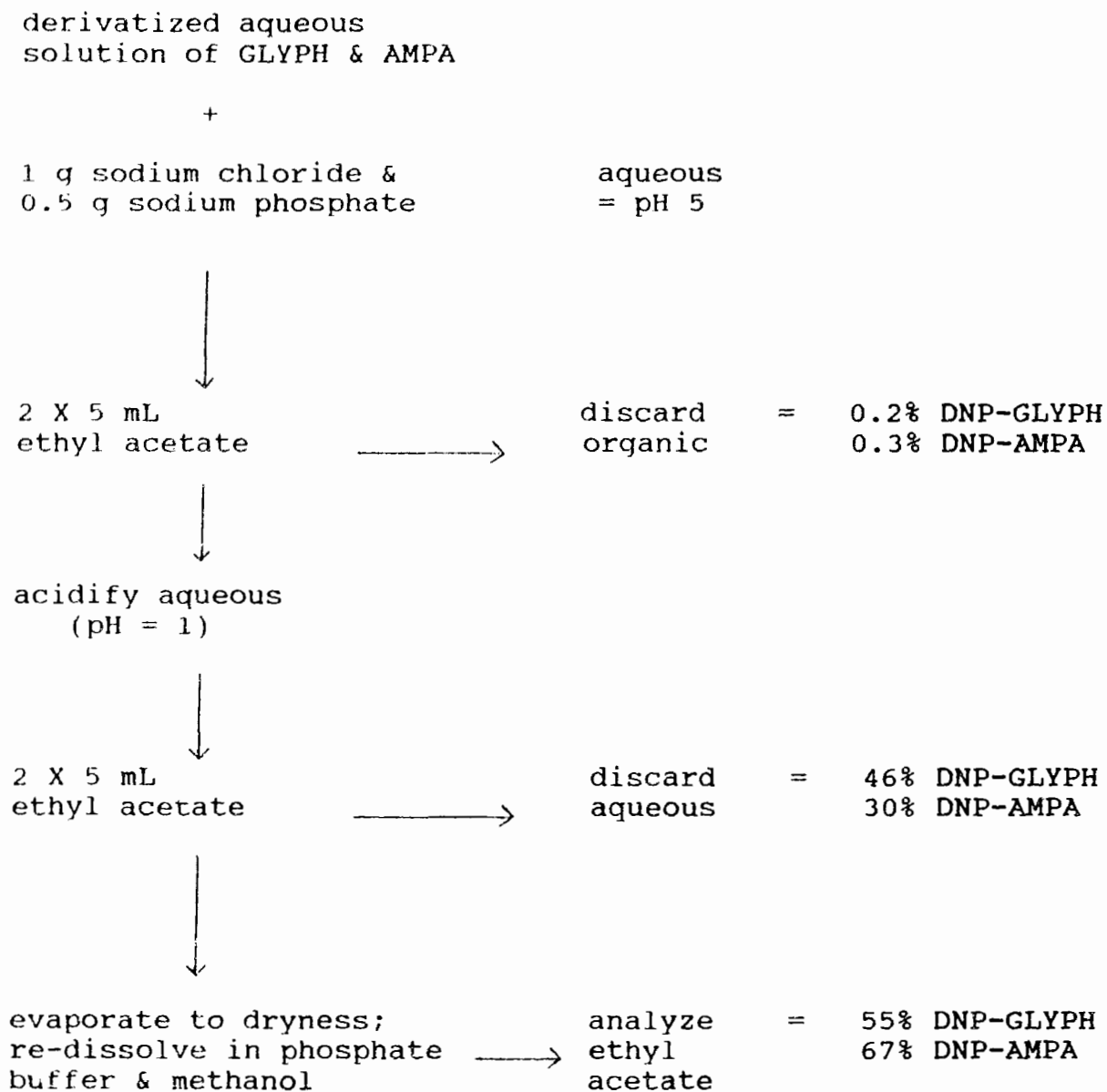
<sup>2</sup> Four 5 mL-replicates were prepared containing 50 ppm of GLYPH and AMPA (250 ug each).

<sup>3</sup> Two x 5 mL of ethyl acetate were used to extract excess derivatizing reagent from the aqueous standard at pH 5. This organic phase was discarded during Lundgren's scheme.

<sup>4</sup> The 5 mL aqueous standard was acidified to pH 1 and extracted with 2 x 5 mL of ethyl acetate. The aqueous fraction was discarded and the ethyl acetate fraction was analyzed for quantification of DNP-GLYPH and DNP-AMPA according to Lundgren (1986).

<sup>5</sup> ND = not detected.

Figure 6. Flow diagram of the derivatization scheme according to Lundgren's method (1986) and recoveries of DNP-GLYPH and DNP-AMPA for each fraction.



re-dissolved in methanol and phosphate buffer. The first 2 x 5 mL ethyl acetate extraction step at pH 5, performed to remove excess reagent, contained negligible amounts of the derivatized products. The aqueous solution was then acidified to pH 1 and extracted with 2 x 5 mL of ethyl acetate. The aqueous fraction, which was analyzed here, would be discarded according to Lundgren (1986). Results indicate that 46% DNP-GLYPH and 30% DNP-AMPA remain in the aqueous phase and would be subsequently discarded. The final ethyl acetate fraction contained 55% DNP-GLYPH and 67% DNP-AMPA. According to the method developed here, dichloromethane extraction of the aqueous solution at pH 9 would prevent the derivatized products from partitioning into the organic phase. Therefore, the method presented here is more efficient than that presented by Lundgren (1986) for extracting derivatized GLYPH and AMPA for HPLC analysis and quantification.

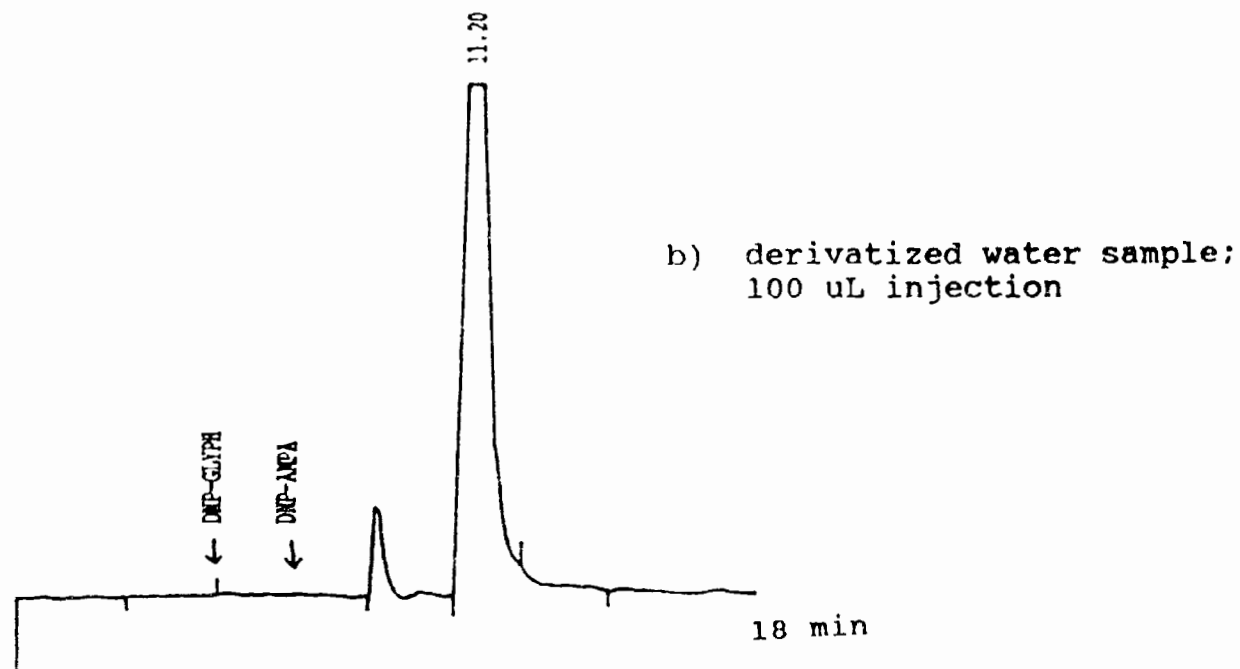
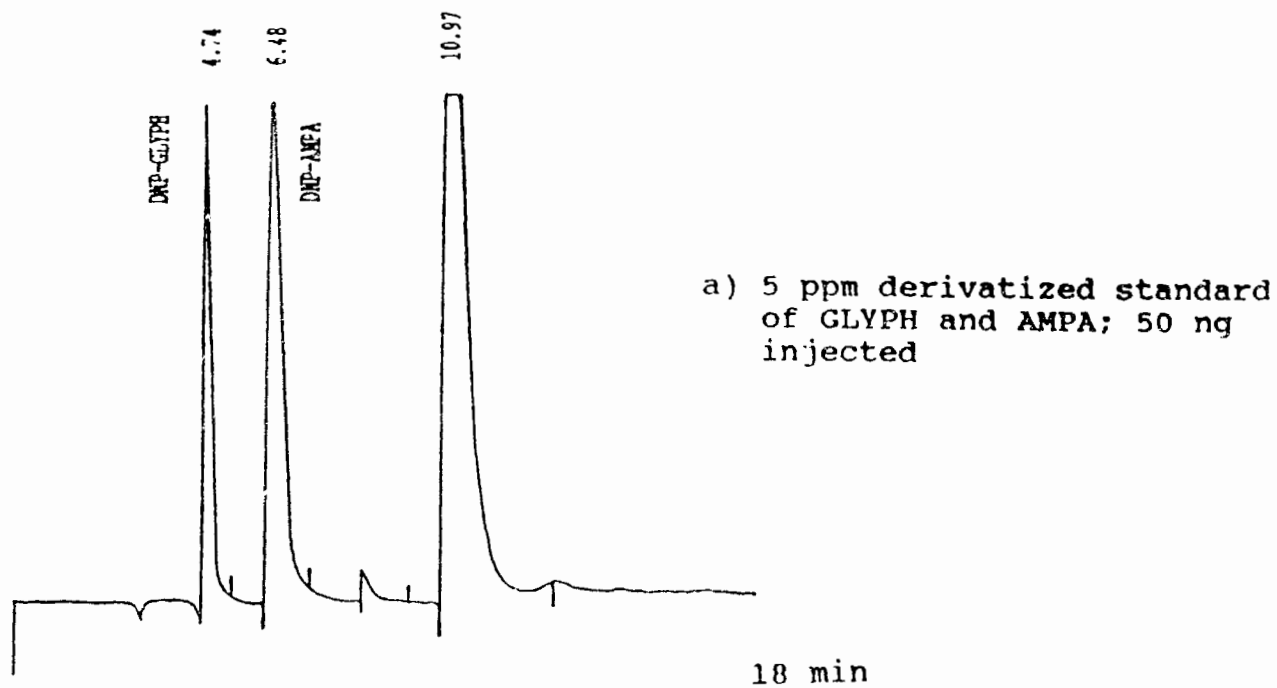
#### 4.2 Chromatography of GLYPH and AMPA Standards

The 0.05 M phosphate buffer at pH 2.1 was used in HPLC to suppress ionization of the DNP-GLYPH and DNP-AMPA molecules so that they could be chromatographed using a reverse-phase column. Although running the system at low pH (such as 2.1) is not particularly desirable for the longevity of the column,

washing the column with lots of water at the end of the day prevented any significant adverse effects on column performance. The ionic strength of the buffer was not increased above 0.05 M as this would have caused precipitation and clogging of the HPLC pump when the methanol was added. Methanol was chosen for the organic solvent as buffer salts are relatively more soluble in methanol than in acetonitrile (Burdick and Jackson Laboratories 1985), and methanol tends to be less harsh on the pump parts. Before the first injection of the day, the injection loop was flushed out with phosphate buffer to ensure that no methanol was left in the sample loop. The complete filling method was decided to be the most reliable method of sample injection. This involved injecting a volume of the sample that was greater than the size of the attached sample loop.

A reverse-phase  $C_{18}$  column was used for chromatography. In reverse-phase chromatography, compared with normal phase, the more polar compounds are eluted first. Peaks of DNP-GLYPH and DNP-AMPA were well resolved and retention times approximated 4.7 min and 6.5 min, respectively. Examples of chromatograms obtained for an aqueous standard of GLYPH and AMPA, and a water sample (no GLYPH and AMPA) are shown in Figure 7. The peak observed at about 11 min was suspected as being dinitrophenol, however the actual identity of this peak was not determined.

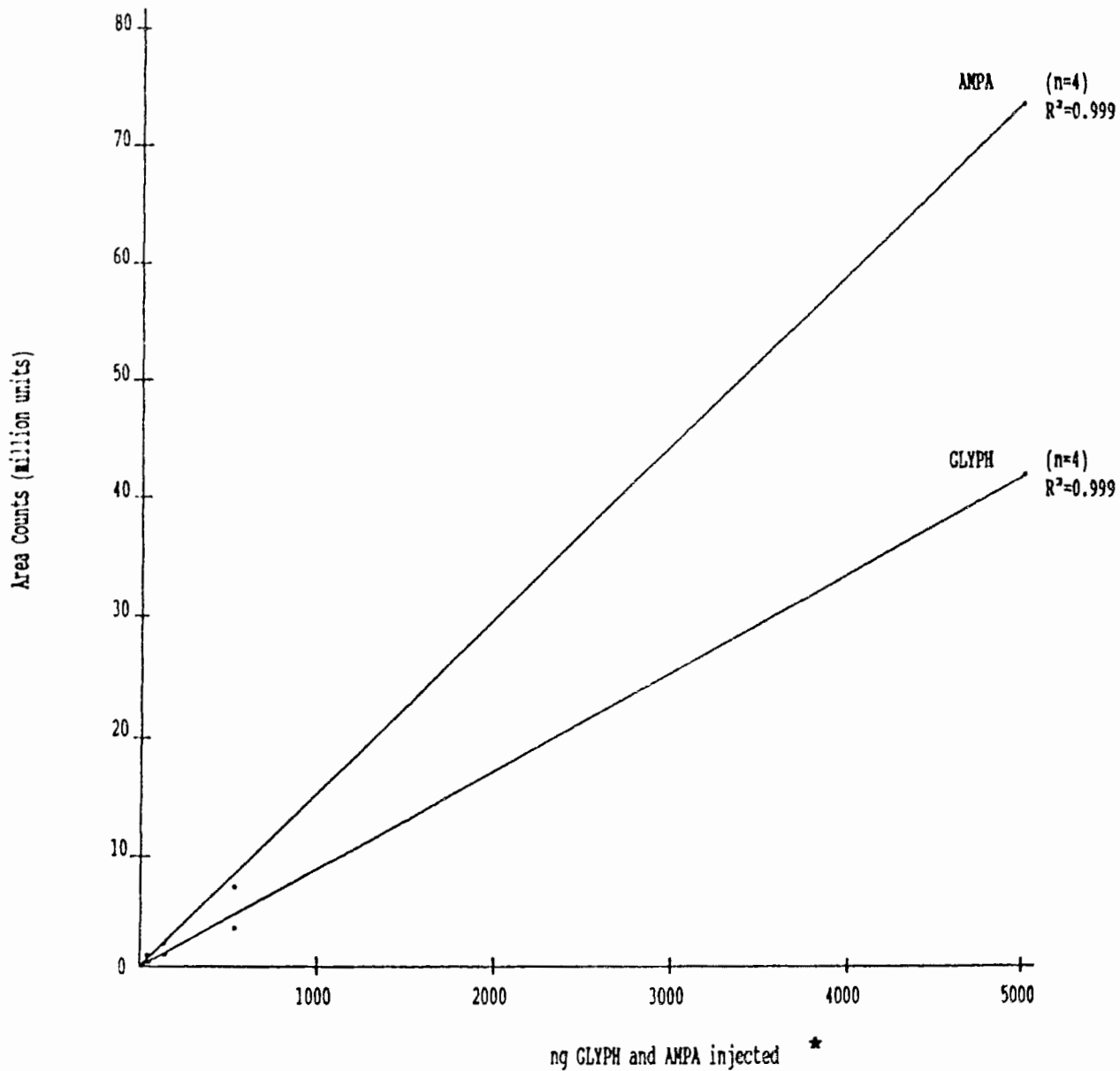
Figure 7. Chromatograms for an aqueous standard containing DNP-GLYPH and DNP-AMPA, and a derivatized water sample containing no GLYPH or AMPA.



A constant volume of a derivatized GLYPH and AMPA standard was injected into the HPLC over the course of a day and evaluated for consistency of response. Both the retention time and the peak area counts for both chemicals were reproducible. This indicated good precision with respect to injection technique as well. Similar samples injected over the course of one month were also reproducible (coefficients of variation or C.V.'s < 10%) as long as running parameters were kept constant. This indicated that derivatized standards, which were kept in the freezer, were stable during this storage period.

The precision of the method was evaluated using analytical curves prepared with 0.5, 1, 5, and 50 ppm standards containing both GLYPH and AMPA. Peak area counts were plotted against the amount of GLYPH and AMPA injected (representing a 100 uL injection volume) and the relationship was found to be linear with  $R^2 = 0.999$  ( $n=4$ ) for both GLYPH and AMPA (Figure 8). This indicated that the reaction was reaching equilibrium and that the derivatizing reagent was not a limiting factor in the reaction. The 5 and 50 ppm samples were diluted prior to injection, therefore, the results shown (Figure 8 and Table 3) represent the actual peak area counts obtained for the diluted samples multiplied by the dilution factor. Precision of the method is indicated by the standard deviations (SD) and coefficients of variation in Table 3. Coefficients of

Figure 8. Peak area counts of DNP-GLYPH and DNP-AMPA vs. ng of each compound injected.



\* Note: Results for the 5 and 50 ppm samples represent actual peak area counts obtained for the diluted samples (performed for HPLC analysis) multiplied by the dilution factor.

Table 3: Peak area counts for derivatized standard solutions (5 mL each) ranging from 0.5 to 50 ppm GLYPH and AMPA.

Sample	Mean Area Counts <sup>1</sup>	SD	C.V.
GLYPH:			
50 ppm	42,372,300	1,153,600	2.7%
5 ppm	3,500,030	89,330	2.6%
1 ppm	588,820	15,642	2.7%
0.5 ppm	209,965	34,980	16.7%
AMPA:			
50 ppm	73,873,500	2,133,600	2.9%
5 ppm	6,546,850	256,990	3.9%
1 ppm	1,345,890	18,964	1.4%
0.5 ppm	670,875	20,769	3.1%

<sup>1</sup> Peak area counts represent the mean of 4 replicate samples for each concentration level. Sample volume injected was 100 uL. Corrections were made for dilutions of the 5 and 50 ppm standards performed prior to HPLC injection. Therefore, results shown here are the actual peak area counts obtained for the diluted samples multiplied by the dilution factor.



variation for GLYPH were all less than 3% with the exception of the 0.5 ppm concentration. Two additional repeats of this concentration level (each consisting of four replicate samples) were performed at a later date and produced similar results. Precision of the method for AMPA was consistently good for all concentration levels, with coefficients of variation being less than 4%. Replicates of the four concentration levels were prepared over the course of a month. Therefore, the coefficients of variation include variability over time with respect to my performance, method reproducibility, and chromatographic conditions. Detector response was linear over the range tested (25 to 250 ng GLYPH and AMPA injected).

The minimum level of quantitation in aqueous standards was 5 ng injected for GLYPH and 3 ng injected for AMPA. A substantially lower chromatographic response was observed for DNP-GLYPH compared with DNP-AMPA for the same amount injected. This observation might be explained by the possibility of two dinitrophenyl groups reacting with AMPA (see Figure 4), thus resulting in a higher chromatographic response for this derivative. A lower response for DNP-AMPA as compared with DNP-GLYPH was reported by Lundgren (1986). This was attributed to the relatively lower sensitivity of DNP-AMPA compared with DNP-GLYPH at the detection wavelength used (405 nm).

### 4.3 Anion Exchange Calibration

In the ion exchange process, the counterions bound to the functional groups of the resin are replaced by sample ions having the same charge (Day and Underwood 1967). Neutral molecules and molecules having the same charge as the functional group flow through the column without being sorbed. Thus, GLYPH and AMPA were sorbed onto the resin column through the replacement of the bicarbonate counterion, made possible by the relatively stronger affinity of these molecules for the resin bed.

The AG 1-X8 anion exchange resin allows for a certain degree of flexibility in eluant pH and ionic strength used without deionization of the active groups (BioRad 1990). The percentage of crosslinking agent of 8% gives an approximate molecular weight exclusion limit for globular molecules of 1000. The choice of the 50 to 100 dry mesh size provided for a relatively fast flow rate. The exchange capacity or the number of ion exchange sites per unit weight or volume of resin is expressed as milliequivalents per dry gram of resin, or mL of packed resin bed. A bed volume of 12 mL (7 g of resin) proved sufficient for my purposes, but a larger bed volume may be required depending on the sample size and type.

Consistency in column recoveries required complete resin conversion from the chloride to the bicarbonate form. Conversion, using a 1 M sodium bicarbonate solution, was carried out according to the manufacture's suggestions (BioRad 1990). With the AG types of anion exchange resins, there is known to be a potential for aliphatic amines to increase slowly with time due to slight resin decomposition (BioRad 1990). Therefore, the resin was allowed to equilibrate in water for about an hour, then the resin columns were conditioned for use the same day. Regeneration of used columns produced inconsistencies in chromatographic results of GLYPH and AMPA put through the column, therefore the resin was used only once and then discarded.

The pH of the sample loaded onto the column was crucial to the success of GLYPH and AMPA being retained on the resin, due to their zwitterionic characteristics. Preparation of standards using a 0.3 M sodium hydroxide solution resulted in the premature elution of GLYPH and AMPA from the column during loading of sample volumes greater than 75 mL. Subsequent analyses showed that GLYPH and AMPA were successfully retained on the resin with samples prepared in water and adjusted to pH 8.0 with 1 M sodium hydroxide. A sample volume of 100 mL did not result in an overload of the column with GLYPH and AMPA under the conditions described. Analyses of the resin washes, consisting of 25 mL of water passed through the columns,

indicated that no GLYPH or AMPA was being displaced from the resin column. Much larger resin washes with water have been used with no elution of these chemicals (Archer and Stokes 1984; Seiber et al. 1984).

The ultimate purpose of the anion exchange process is to separate the compounds of interest from the many other compounds that may exist in the sample solution. This is partially achieved with the sorption process described. Further separation may be achieved with various elution methods to displace the sorbed ions differentially (Day and Underwood 1967). Such methods may include increasing the ionic strength of the eluant or using a counterion solution of greater selectivity. A 1 M solution of potassium nitrate, with a higher relative selectivity for the anion exchange resin (BioRad 1990), was compared with the present scheme described using 1 M sodium chloride. However, no differences were observed in the elution patterns of GLYPH and AMPA with respect to 25 mL fractions of a 100 mL total volume used for elution. This would seem to suggest that ion exchange is not the only process involved.

Recoveries of GLYPH and AMPA from the anion exchange column were determined by comparison with results of the standards analyzed without column chromatography (see Table 3). Recoveries from the anion exchange columns are presented in

Table 4. Reproducibility among replicates was not as good as that achieved for the standards. However, the level of variation among the replicates was still acceptable. Coefficients of variation were higher for the anion exchange samples, with the exception of GLYPH at 0.5 ppm which was similar to that obtained for its standard. Higher coefficients of variation were observed for the lowest concentration. Peak area counts were plotted against the concentrations of aqueous solutions passed through the anion exchange columns and the relationship was found to be linear, with  $R^2 = 0.998$  for GLYPH ( $n = 4$ ) and  $R^2 = 0.999$  for AMPA ( $n = 4$ ).

Recoveries from the anion exchange columns represent that achieved with 50 mL of 1 M sodium chloride used to elute the chemicals. Results in Table 4 suggest that some loss of GLYPH and AMPA occurred during the anion exchange process for the 5 and 50 ppm concentrations, as well as the 0.5 ppm concentration for AMPA only. This loss represents the amount of these chemicals still retained on the columns. The 134% recovery of GLYPH at 0.5 ppm would suggest that interference had occurred. However, a 75 mL water sample passed through the anion exchange column showed no interference from the column in the GLYPH and AMPA retention region (Figure 9). No explanation can be offered for this observed interference.

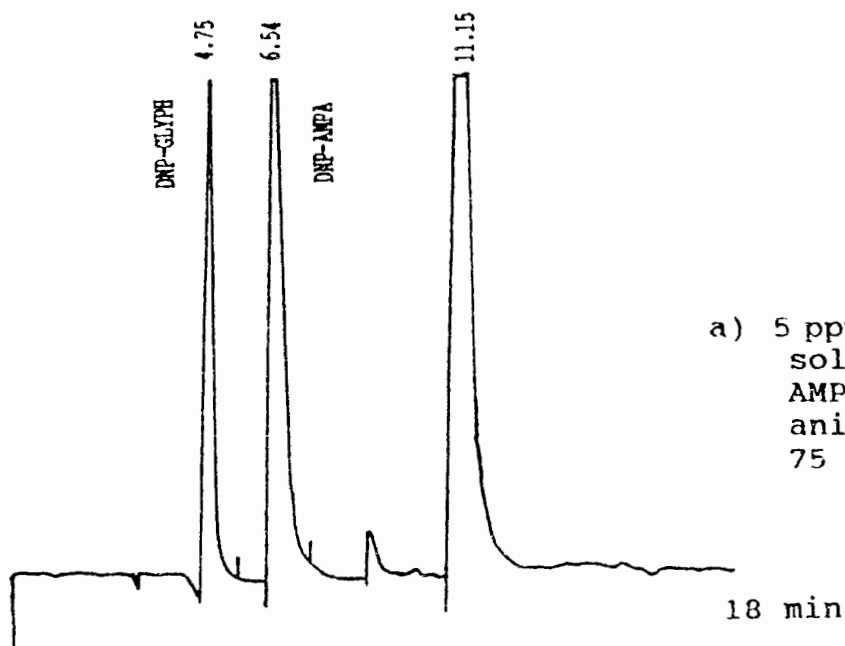
Table 4. Peak area counts for derivatized 5 mL aliquots of anion exchange column eluates containing GLYPH and AMPA.

Sample	Mean Area Counts <sup>1</sup>	SD	C.V.	Recovery <sup>2</sup>
GLYPH:				
50 ppm	36,643,000	1,883,500	5.1%	87%
5 ppm	2,360,900	268,530	11.4%	68%
1 ppm	607,454	27,376	4.5%	103%
0.5 ppm	281,647	44,799	15.9%	134%
AMPA:				
50 ppm	66,078,200	3,737,700	5.7%	90%
5 ppm	5,164,850	500,960	9.7%	79%
1 ppm	1,331,214	52,952	4.0%	99%
0.5 ppm	567,463	76,419	13.5%	85%

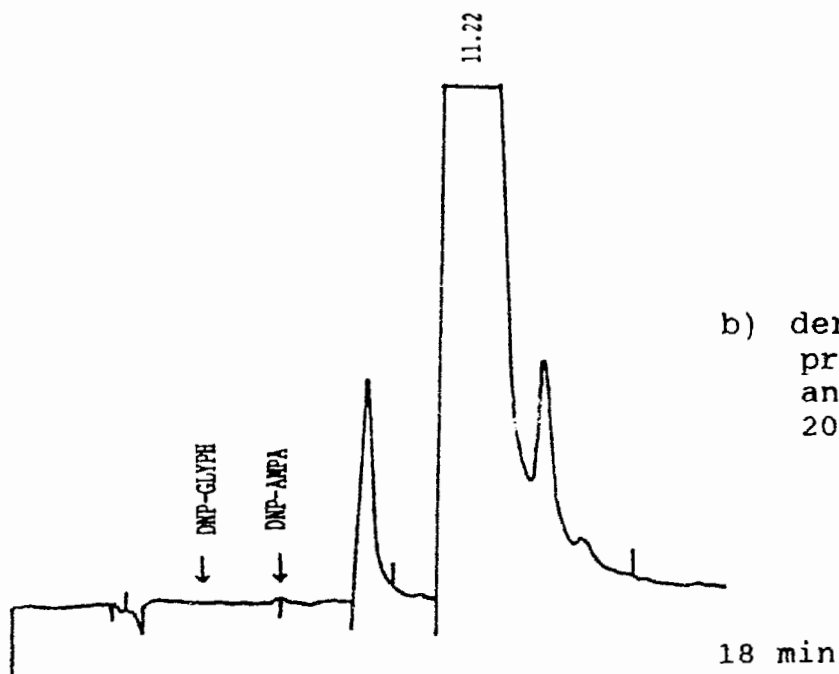
<sup>1</sup> Peak area counts represent the mean of 4 replicate samples, 75 mL each loaded onto the anion exchange column. Data represent 100 uL injected, and the 5 and 50 ppm samples were corrected for dilution required for HPLC injection. Therefore, results shown here are the actual peak area counts obtained for the diluted samples multiplied by the dilution factor.

<sup>2</sup> Recovery is expressed as a percentage of the peak area of the eluant sample and a similar sample not passed through the anion exchange resin column. Recovery represents the first 50 mL volume of the 1 M sodium chloride eluate.

Figure 9. Chromatograms for a derivatized aqueous solution of GLYPH and AMPA and a derivatized water sample containing no GLYPH or AMPA, both of which were processed through the anion exchange column.



a) 5 ppm derivatized aqueous solution of GLYPH and AMPA processed through anion exchange column; 75 ng injected



b) derivatized water sample processed through the anion exchange column; 200 uL injection

Most GLYPH and AMPA was eluted using 50 mL of a 1 M solution of sodium chloride. An additional 50 mL of sodium chloride was used for the elution of 1 ppm GLYPH and AMPA aqueous solutions loaded onto the resin column (two repeated analyses consisting of four replicate samples each). This fraction was found to contain significant amounts of these chemicals as well (Table 5). The mean recoveries of these duplicate analyses of the 50-100 mL eluate fraction were 17% for GLYPH and 10% for AMPA. Recoveries among replicates in this fraction were not as consistent as those achieved among replicates of the 0-50 mL fraction. Therefore, a 50 mL volume was decided as being more efficient for the elution of GLYPH and AMPA from the column; increasing the eluate volume to 100 mL would only reduce sensitivity. It would be impractical to try and retrieve all of the GLYPH and AMPA sorbed onto the column, because large volumes would be necessary for their elution. A compromise is also necessary for plant tissue samples, since larger volumes of eluate tend to elute more plant constituents, thus increasing the possibility of chromatographic interference with GLYPH and AMPA.



Table 5. Analysis of the 50-100 mL anion exchange eluate fraction for 75 mL replicate samples of 1 ppm GLYPH and AMPA (75 ug of each) loaded onto the column.

50-100 mL Fraction <sup>1</sup>	GLYPH Recovered (ug)	AMPA Recovered (ug)	% of Total ug Applied <sup>2</sup>	
			GLYPH	AMPA
1 A	19	9	25	12
B	12	9	16	12
2 A	14	6	19	8
B	9	5	12	7
3 A	7	7	9	9
B	11	7	15	9
4 A	19	10	25	13
B	9	6	12	8
Mean	13	7	17%	10%
C.V.	37%	25%		

<sup>1</sup> Samples A and B consisted of separate analyses. Each sample consisted of 4 replicates. The 50-100mL fraction represented an additional 50 mL of 1 M sodium chloride used to elute the resin columns for determination of GLYPH and AMPA.

<sup>2</sup> The percentage recovery was determined using the ug of GLYPH or AMPA recovered in the 50-100 mL eluate and the total 75 ug of each chemical loaded onto the column.

#### 4.4 Plant Tissue Recovery

Mixtures of biological or environmental samples are often too complex, too dilute, or not compatible with the chromatographic system to permit analysis by direct injection (Poole and Schuette 1984). Therefore, efficient sample cleanup is needed to enable the detection and quantification of the compounds of interest. The main difficulties with sample cleanup for the analysis of GLYPH and AMPA lie in their unusual physicochemical properties and consequently, the incompatibility of most organic solvents normally used during this process (Bardalaye et al. 1985). Therefore, a variety of cleanup procedures for these compounds exist in the literature. Many authors have stated that their attempts to adopt previously published methods met with little success for their particular purpose (Guinivan et al. 1982a; Moye et al. 1983; Seiber et al. 1984; Roy and Konar 1989; Glass 1983).

Plant tissue cleanup using anion exchange chromatography was essential in the work presented here for separating GLYPH and AMPA from many plant co-extractives. Plant substances of different polarity or size would have been removed during this step. Potential plant co-extractives may include sugars, nucleic acids, amino acids, proteins, vitamins, etc., and some species of plants may be easier than others with respect to sample cleanup. This was demonstrated by Seiber et al.

(1984), as different limits of quantitation were found for asparagus and kiwi fruit. The calibration of the cleanup column for plant tissue analysis was necessary to determine its capacity for the separation of plant substances that may interact with the resin. Analyses of plant sample loadings and 25 mL water washes of the resin indicated that GLYPH and AMPA were successfully retained on the column.

Gradient solvent programming for HPLC analysis consisted of increasing the percentage of methanol with sample analysis time. This was used to elute the plant co-extractives, appearing later in the chromatograms, from the HPLC column. During the HPLC analysis of samples containing plant co-extractives, it was especially important to regularly flush the injection loop with phosphate buffer to prevent the buildup of contaminants in the injection port.

Plant samples that had been acidified and analyzed by HPLC were kept in the freezer for future re-analysis. Subsequent HPLC analyses of these samples, over a period of about one month, were reproducible indicating that the samples were stable during this storage period.

Leaf, stem, and root samples were fortified with a desired amount of GLYPH and AMPA ( $\mu\text{g/g}$ ), with four replicates per fortification level. For all replicates, the 50 mL of eluate

collected from the anion exchange column was equivalent to 1 g of tissue (fresh weight), with the exception of the 938 ppm leaf sample which was equivalent to 4 g (fresh weight). One g of tissue was chosen to estimate recoveries of GLYPH and AMPA from plant tissues because control samples showed no interference with the chromatography of GLYPH and AMPA (Figures 10 and 11). DNP-GLYPH and DNP-AMPA eluted from the HPLC column ahead of plant co-extractives. However, a 50 mL eluate representing a 4 g tissue sample required a 1:10 dilution in the final derivatized product in order to remove interfering peaks in the GLYPH and AMPA retention region. Dilution in the final product would be needed for higher tissue concentrations, but the ratio of GLYPH and AMPA to the tissue interferences will be very important in determining whether dilution will allow successful chromatography and quantitation of these chemicals.

Mean recoveries and coefficients of variation for each plant tissue sample and fortification level are shown in Tables 6 and 7. Recoveries from plant tissue samples were determined by comparison with a standard of similar concentration also passed through the anion exchange column. Results in Table 6 indicate that GLYPH interference occurred for the 75 and 375 ppm leaf fortifications, as recoveries ranged from 109% to 131%. No interference with GLYPH and AMPA was observed in

Figure 10. Chromatograms for derivatized eluates of leaf and stem tissue samples (1 g fresh weight) containing no GLYPH or AMPA.

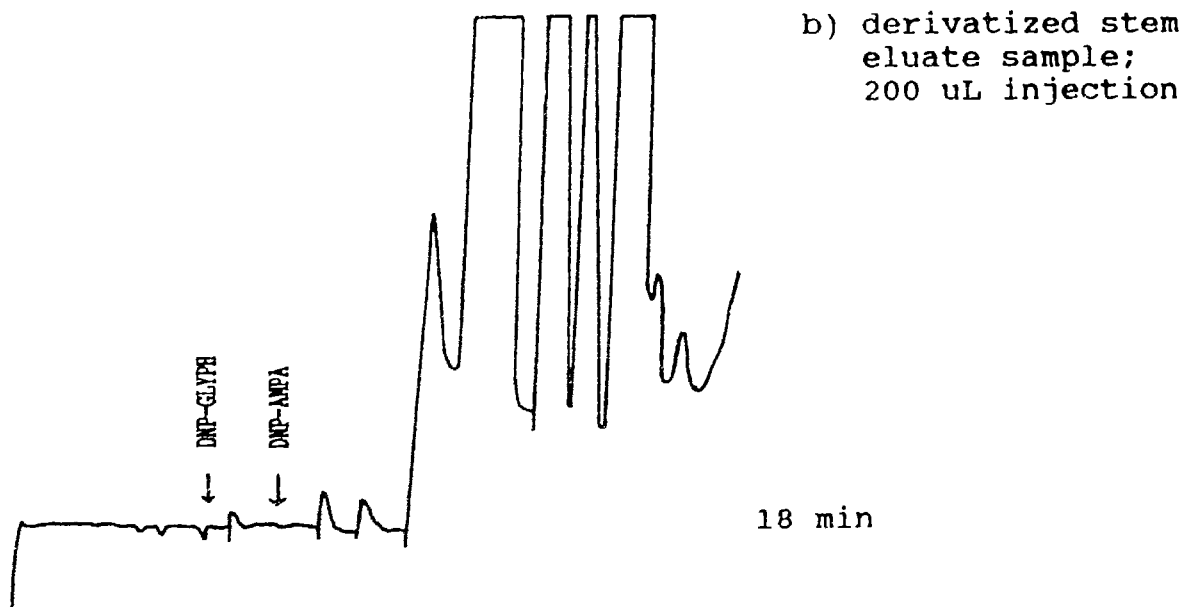
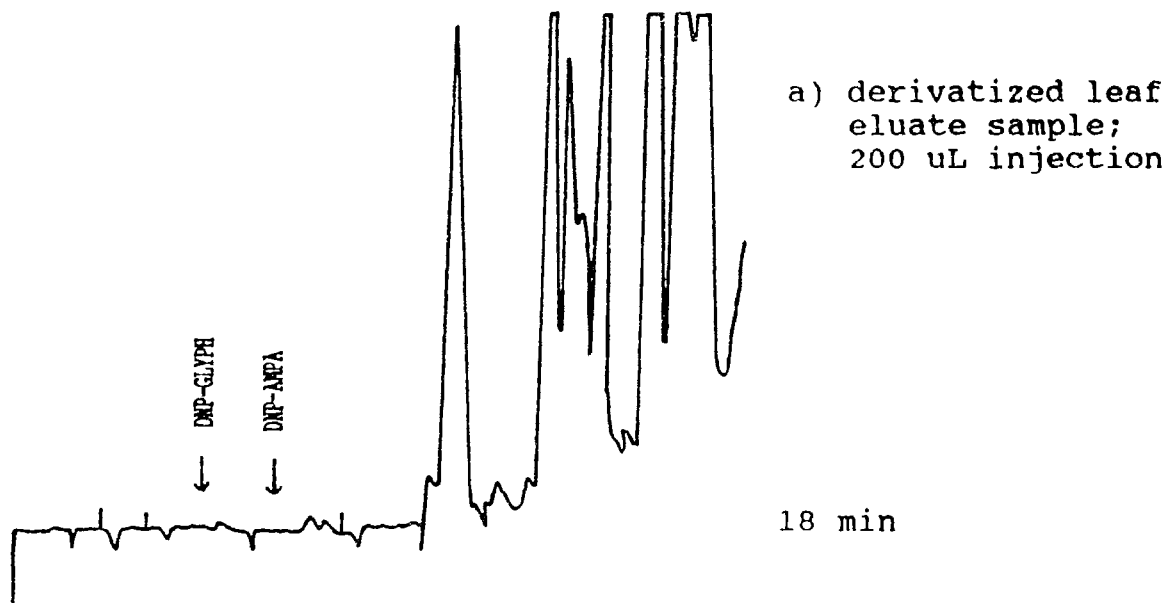


Figure 11. Chromatograms for derivatized eluates of a root tissue sample containing no GLYPH or AMPA, and a root tissue sample spiked with GLYPH and AMPA prior to the anion exchange column (1 g fresh weight).

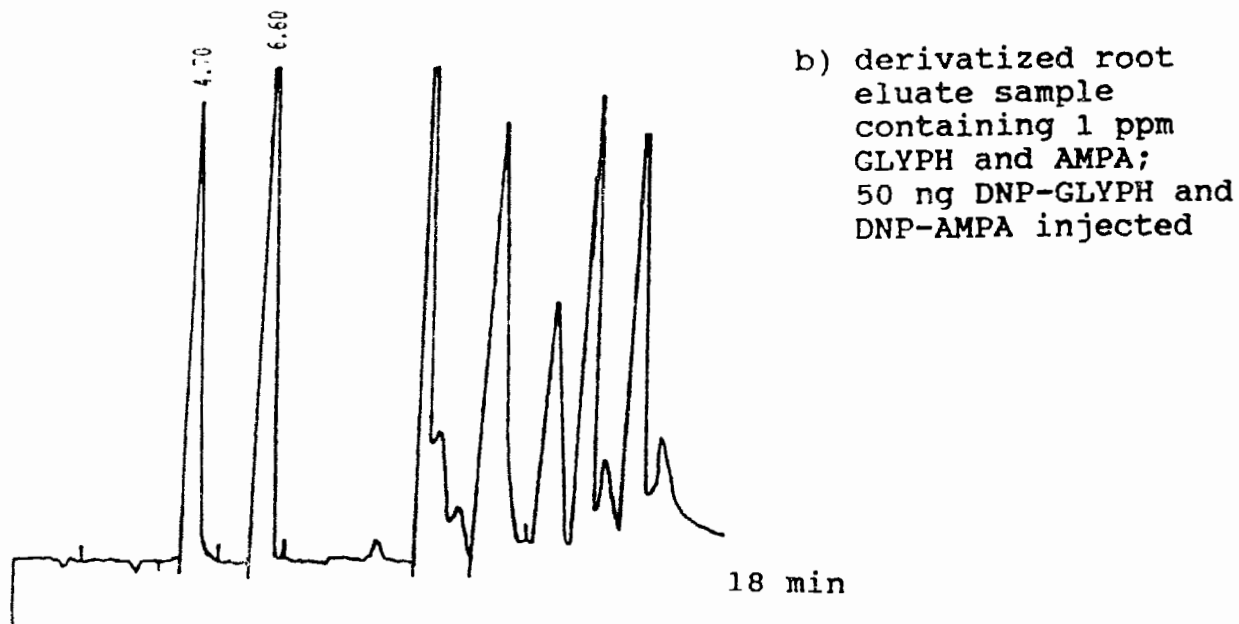
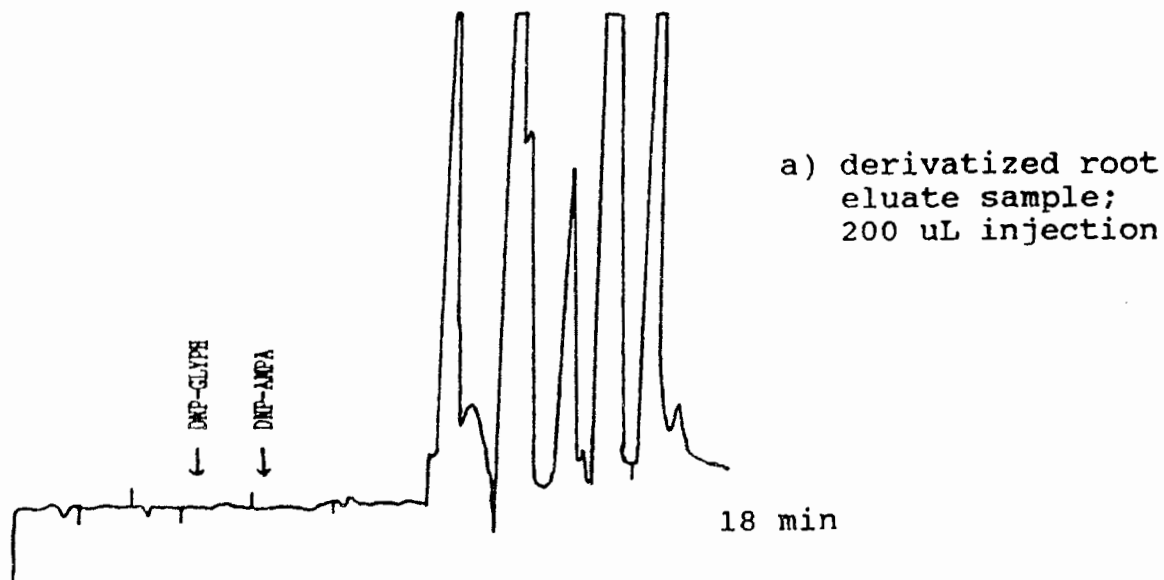


Table 6. Recoveries of GLYPH and AMPA from fortified leaf tissue.

Concentration (ug/g)	Mean Recovery <sup>1</sup> (ug)	C.V. (%)	Recovery (%)
GLYPH:			
938	853	6.6	91
375	491	3.3	131
375 (frozen) <sup>2</sup>	484	7.0	129
75	82	8.0	109
AMPA:			
938	844	7.5	90
375	263	2.1	70
375 (frozen) <sup>2</sup>	274	4.4	73
75	61	7.2	81

<sup>1</sup> Mean recoveries were determined for four replicate plant samples prepared at each concentration. Recoveries represent 50 mL of 1 M sodium chloride used to elute the anion exchange column and are determined by comparison with a standard also passed through an anion exchange column.

<sup>2</sup> Leaf tissues were fortified prior to storage in the freezer at -20°C. The freezer storage period was 3 months.

plant tissue control samples (Figure 10), thus the source of this interference is not known. Recoveries of less than 100% for GLYPH and AMPA indicated that these chemicals may have been adsorbed to the tissue matrix, thus resulting in GLYPH and AMPA loss during sample preparation. Precision of GLYPH and AMPA recoveries were acceptable (C.V.'s < 8%).

Stem and root tissue recoveries for GLYPH and AMPA are shown in Table 7. GLYPH recoveries, ranging from 117% to 136%, indicated that interference occurred for these plant tissue fortifications. For these samples as well, no plausible explanation for this interference can be offered. AMPA recoveries ranged from 91 to 100% for stem and root tissues. Higher recoveries for these tissues, compared with leaf tissues, suggest that less adsorption of AMPA to the tissue matrix may have occurred for the stem and root samples. Precision of GLYPH and AMPA recoveries for these tissues were also acceptable (C.V.'s < 9.3%).

To determine the effects of freezer storage prior to analysis, four replicates of a leaf sample fortified with 375 ug of GLYPH and AMPA were stored at -20°C for 3 months. Recoveries were similar for fresh and frozen leaf tissue samples (131% and 129% for GLYPH, 70% and 73% for AMPA, respectively) fortified at this level. This indicated that no breakdown or increased sorption of the chemicals to the sample matrix took



Table 7. Recoveries of GLYPH and AMPA from fortified stem and root tissues.

Concentration (ug/g)	Mean Recovery <sup>1</sup> (ug)	C.V. (%)	Total Recovery (%)
GLYPH:			
Stem: 75	98	9.2	131
38	44	2.0	118
Root: 375	510	5.5	136
38	44	2.2	117
AMPA:			
Stem: 75	72	9.3	96
38	34	3.7	91
Root: 375	349	4.4	93
38	38	2.0	100

<sup>1</sup> Mean recoveries were determined for four replicate plant samples prepared at each concentration. Recoveries represent 50 mL of 1 M sodium chloride used to elute the anion exchange column and are determined by comparison with a standard also passed through an anion exchange column.

place during this time. Therefore, samples could be stored in the freezer if analysis was not immediately possible. Other studies have reported that no apparent breakdown of GLYPH had occurred after prolonged freezer storage of samples prior to analysis (Miles et al. 1986; Seiber et al. 1984). Storage in the dark at sub-zero temperatures would inhibit the chemical processes that may result in a change in the sample composition (Poole and Schuette 1984).

Analyses of all extraction steps according to Lundgren's scheme (1986) (Table 2) showed that negligible amounts of the derivatized products were lost to the first organic extraction, which was carried out at pH 5. Dichloromethane extractions were performed on derivatized water samples at pH 4, and subsequently processed for HPLC analysis according to my scheme. Movement of the yellow colour to the organic phase was observed during dichloromethane extraction at this pH. Chromatograms indicated that the suspected dinitrophenol peak, which was consistently observed at about 11 min in all other samples, was partitioned into the organic phase at this pH as no peak at 11 min was observed for these water samples. To ascertain whether the dichloromethane extractions would reduce plant co-extractives when done at pH 4, recoveries were determined at pH 9 and pH 4 for plant tissue samples (1 g fresh weight) fortified with 100 ug of GLYPH and AMPA. Five mL aliquots of the anion exchange eluates were derivatized

then extracted with 2 x 5 mL of dichloromethane. Four replicates of the aqueous solution were extracted at pH 9, and four replicates were extracted at pH 4. Subsequent extraction steps followed my usual scheme. Recoveries were determined using a GLYPH and AMPA standard of similar concentration.

Results in Table 8 show that extraction at pH 4, compared with extraction at pH 9, resulted in lower recoveries of DNP-GLYPH and DNP-AMPA; recoveries being reduced by 15% and 17%, respectively. Chromatograms showed that peaks of plant co-extractives, eluting much later than DNP-GLYPH and DNP-AMPA, were only slightly reduced at the pH 4 extraction. Samples extracted at either pH 4 or pH 9 were equally as good with respect to chromatography in the retention times of DNP-GLYPH and DNP-AMPA. Since only 1 g of tissue was loaded onto the anion exchange column, it is not known whether this scheme would prove beneficial to removing GLYPH and AMPA interferences that would occur at higher tissue concentrations. Precision of GLYPH and AMPA recoveries were acceptable for both pH extractions (C.V.'s < 4.4%).

Other cleanup procedures described in the literature for GLYPH and AMPA analysis involve many different combinations of techniques, and almost always include more than one rotary evaporation step. Examples include gel permeation followed by cation exchange chromatography (Guinivan et al. 1982a); anion

Table 8. Recoveries of 100 ug each of GLYPH and AMPA from fortified plant tissue after derivatization and extraction with dichloromethane at pH 9 or pH 4.

Sample Replicate <sup>1</sup>	GLYPH (ug)	Mean Recovery	C.V.	AMPA (ug)	Mean Recovery	C.V.
pH 9:		108	8.4%		89	3.6%
1	113			86		
2	113			92		
3	108			91		
4	95			85		
pH 4:		92	1.6%		77	4.4%
5	94			73		
6	94			75		
7	91			78		
8	91			81		

<sup>1</sup> At each pH, 4 x 5 mL aliquots of the 50 mL plant sample eluate (representing 1 g of plant tissue and 100 ug of GLYPH and AMPA eluted from the anion exchange column with 1 M sodium chloride) were taken for derivatization. The 2 x 5 mL dichloromethane extraction following derivatization was done on the aqueous solution at pH 9 for four of the replicates, and at pH 4 for another four replicates. Recoveries were determined using a 2 ppm GLYPH and AMPA standard.

exchange followed by gel permeation and an HPLC cleanup step (Seiber et al. 1984); anion and cation exchange chromatography (Friestad and Bronstad 1985; PAM 1977; Thompson et al. 1989); post-derivatization cleanup on Florisil and alumina columns (PAM 1977); acid refluxing of plant homogenates with cation and anion exchange chromatography (Archer and Stokes 1984); and ligand exchange followed by anion exchange chromatography (Cowell et al. 1986). Most methods using cation exchange chromatography resulted in the elution of GLYPH and AMPA in separate fractions, thus requiring separate treatment of these samples following cleanup procedures (PAM 1977; Moye and St. John 1980; Moye et al. 1983). Although this may have contributed to the lower limits of quantitation achieved by these methods, it increased analysis time as well. The method presented here is relatively simple and allows for the analysis of both GLYPH and AMPA in one run. The entire analysis requires 2 days, and one person can easily process 6-8 samples at a time.

The detection limit required for the analysis of GLYPH and AMPA will ultimately be determined by the end-use of the method. Recommended application rates for commercial products containing GLYPH range from about 1 to 6 kg ai per ha. Therefore, there is the potential for a plant with a leaf area of 100 cm<sup>2</sup> to receive about 1-6 mg of GLYPH. Assuming that a plant of this size weighs 10 g, the amount of GLYPH present in

a treated plant sample might range from 100 to 600 ppm or more, depending on the plant part analyzed and length of time between treatment and analysis. The method presented here would be applicable to the analysis of GLYPH and AMPA at these levels in treated plants, assuming these plants are equal to the cleanup challenge of bean tissues. The ability to determine low levels for residue studies may be required for regulatory purposes or for applications to register GLYPH for use in certain crops as directed sprays or as a dessicant (Atkinson 1985; Seiber et al. 1984). The method presented here has the potential to be applicable to residue studies if a more complex cleanup procedure was adopted.

Traditionally, radio-labelled compounds have been used to study the movement and degradation of GLYPH in soil, water (Sprankle et al. 1975; Rueppel et al 1977) and plants (Gottrup et al. 1976; Marshall et al. 1987). My method may be applicable to translocation studies in plants depending on the levels of GLYPH treatment, the amounts translocated or metabolized, and the experimental conditions. Certain soil microbes have been found to cause a significant decrease in the amount of GLYPH needed to kill a plant (as low as 1-10 ug per plant; Rahe et al. 1990). Quantitation of GLYPH or AMPA in plant tissues at these levels may be impossible with any method other than the use of the radio-labelled compound. The HPLC method presented here could be used in combination with

radio-labelled GLYPH for detection at low levels. Other field workers concerned with the fate of GLYPH in the environment have used GC and HPLC methods as published in the literature to analyze samples; in most cases the methods used were those developed in the workers' own laboratories (Roy et al. 1989; Muller et al. 1981; Feng et al. 1990; Feng and Thompson 1990). In a few other cases, samples were sent to Monsanto for analysis, and thus, the exact protocols were not described for proprietary reasons (Comes et al. 1976; Newton et al. 1984; Edwards et al. 1980). The method presented here has the potential for use in studies of the fate of GLYPH in the environment, and seems to be much simpler and less time consuming than other GC and HPLC methods published. Depending on the matrix being analyzed, more complex cleanup procedures may be needed. For environmental studies, general trends in the distribution of GLYPH, are often more important than being able to determine extremely low residue levels.

## CHAPTER 5

### CONCLUSIONS

Chemical derivatization of GLYPH and AMPA is needed to enhance the sensitivity of their detection to acceptable levels. Procedures for GC analysis are generally more difficult than those for HPLC, and often involve a double derivatization scheme because of the need to impart volatility to these compounds. In comparing my method with other HPLC methods for analysis of GLYPH and AMPA, the only other reported method to have employed the 2,4-DNFB derivatizing reagent and UV detection is that of Lundgren (1986). This reagent is a common UV-tag for amino compounds, and has a high molar extinction coefficient (Jupille 1979). The reactivity of 2,4-DNFB for GLYPH and AMPA is similar to that of FMOCCl, a pre-column derivatizing reagent used with fluorescence detection, in that it reacts with both primary and secondary amines. OPA-MERC has also been used as a derivatizing agent, but it is specific to primary amines and requires that a cleaving reagent be used for post-column derivatization in order that GLYPH may be derivatized and then detected by fluorescence. Post-column derivatization methods for HPLC analysis are relatively more automated than pre-column



methods, but they require fast reaction times, equipment modifications and expertise (Lawrence 1979).

The derivatization procedure described in my research was shown to be highly reproducible. Although DNP-GLYPH was light sensitive, the precautions needed to avoid its degradation were simple. The minimum level for quantitation in aqueous standards was 5 ng injected for GLYPH and 3 ng injected for AMPA. Dichloromethane extraction of the aqueous solution at pH 9 prevented DNP-GLYPH and DNP-AMPA from partitioning into the organic phase, which was subsequently discarded. The scheme presented here is more efficient for removing co-extractives from the derivatized products than that of Lundgren (1986), since Lundgren's method involved a significant loss of GLYPH and AMPA in the aqueous phase that was discarded.

A reverse-phase column was used for HPLC analysis of GLYPH and AMPA. Most other methods used ion exchange columns, which are relatively less stable than the bonded reverse-phase columns (Gilbert 1987). The method presented here also uses UV detection for the quantitation of the DNP derivatives. The variable wavelength detector used in my work enabled the optimization of sample response by choice of an appropriate wavelength for DNP-GLYPH and DNP-AMPA detection, and scanning capabilities permitted absorption spectra of sample peaks when

desired. Most other methods used fluorescence detectors, with the exception of Lundgren (1986) and Thompson et al. (1989), which used UV and UV-visible detectors, respectively.

The variety and number of plant co-extractives that may interfere with subsequent GLYPH and AMPA analysis dictates the need for efficient sample cleanup. The degree to which this is carried out will be determined by the level of sensitivity required, and will become more important as levels of detection are lowered (Touchstone and Dobbins 1983). The method presented here uses a very simple cleanup step for plant tissues with an anion exchange resin column. Other cleanup procedures described in the literature were much more complex, but low limits of quantitation were realized.

The limit of quantitation in plant tissues for the method presented here was not determined. However, a detection limit in plant tissues comparable to other methods might be realized with my method if one or more cleanup steps, such as those described in other published methods, were added. Other cleanup procedures described in the literature for GLYPH and AMPA analysis involve many different combinations of techniques, which were described in Chapter 4. If sample cleanup procedures were used to sufficiently remove GLYPH and AMPA interferences, which were observed with plant tissue samples weighing 4 g, then a larger amount of plant tissue

could be processed and sensitivity increased. A larger volume of the anion exchange resin or a larger mesh size may increase the column capacity, but may not effect a greater sensitivity if a larger volume of eluant is required to recover GLYPH and AMPA. It is also recommended that rotary evaporation of the aqueous anion exchange eluate be attempted to further concentrate GLYPH and AMPA and potentially increase sensitivity. Injection volumes larger than 200 uL may be tried as well, although at some point peak shape may be significantly degraded and unacceptable.

The research presented here provides a simple, alternative method for the analysis of GLYPH and AMPA in plant tissues. This analytical procedure may be used to further study the distribution of GLYPH and AMPA in treated plants, and has the potential for use in studies concerning the fate of these chemicals in the environment.

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