An Investigation into the Analysis of the Herbicide Glyphosate in the Environment

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Thesis prepared for the degree of
Doctor of Philosophy, 1996

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ACKNOWLEGEMENTS

I am grateful to Dr. H.J. Duncan for direction and supervision during my time as a student in his department.

I wish to thank all the staff and my friends and colleagues from the AFE section for their valuable assistance, past and present.

Thanks are also due to SERC, the Reynolds and Buttles Trust and the Merchants of Glasgow, without whose financial support this work would not have been possible.

Finally, a special thank you to my family for support and assistance in the preparation of this thesis.

DEDICATION

In memory of

Robin Wilson Oliver

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ABBREVIATIONS USED IN THE TEXT

cm	centimetre
cv.	cultivar
df	degrees of freedom
ed(s).	editor(s)
et al.	and others (authors)
g	gram
GC	gas chromatography
GF	gel filtration
GPC	gel permeation chromatography
ha	hectare
HPLC	high performance liquid chromatography
i.e.	that is
kg	kilogram
l	litre
LSD	least significant difference
m	metre
M	molar
mg	milligram
min.	minute
ml	millilitre
ppm	part per million
µg	microgram (10^{-6} gram)
µl	microlitre
SEC	size exclusion chromatography
s.d.	standard deviation
SPE	solid phase extraction
%	percentage
°c	degree centigrade
~	about

SUMMARY

The aim of the work described in this thesis was to develop improved methods for the determination of glyphosate in the environment.

The introduction outlines the success of glyphosate as a herbicide and focuses principally on the physiochemical properties and generally favourable behaviour of the chemical in the environment which have significantly contributed to this success. Some of the less favourable aspects of the behaviour of the chemical in the environment are then considered, especially the deleterious effect which glyphosate can have on non-target crops. Reasons why the analysis of glyphosate in environmental and crop matrices is necessary are also outlined.

Chapter 2 investigates the analysis of glyphosate in plants. An examination of the problems inherent in the analysis of the chemical and the previously published literature regarding the analysis of glyphosate in plants is made. Methods utilising High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) appeared to be the most likely techniques to meet the sensitivity requirements of modern regulatory requirements. Publications involving these methods were therefore considered in some detail.

From the literature review, an HPLC method first published by Moye and Boning (1979) which utilised pre-column derivatisation of glyphosate with 9-fluorenylmethylchloroformate (FMOCCl) and fluorescence detection was identified as potentially able to meet the requirement for sensitivity and was also suitable for this laboratory. Furthermore, Size Exclusion Chromatography (SEC) and Solid Phase Extraction (SPE) were identified as clean-up

techniques that may provide improvements over the commonly used column chromatography methods. During preliminary work with the HPLC method, peak shape and retention time problems were encountered and a number of possible causes were investigated. The problems were eventually traced to the column packing. A number of alternative column packings and adjustments to the technique were examined to eliminate these problems. Eventually, the use of a guard column and occasional repacking of the end of the column provided a working solution.

The reproducibility of the derivatisation and the linearity of detector response were then determined and found to be satisfactory. SEC using Sephadex gel was investigated as a clean-up step. Despite many attempts using a range of mobile phases, a suitable retention volume for glyphosate could not be achieved. SPE was then considered. A range of SPE packings were screened and initally promising results were obtained with amino (NH_2), cyano (CN) and diol (2OH) phases. A more detailed examination found that only the NH_2 packing provided sufficient retention of glyphosate to be effective with plant extracts. Having shown good retention with glyphosate from a standard solution, NH_2 SPE cartridges were tried with a fortified barley grain extract. Barley was selected as the test matrix for two reasons 1) it is an economically important crop in Scotland and glyphosate has been implicated in reduced germination in seed barley, and 2) barley seed is a low moisture content matrix and therefore presents a formidable clean-up challenge. Accordingly, if a method succeeds with barley it is more likely to succeed with a range of crops. The use of NH_2 SPE cartridges alone was partially successful although the recoveries obtained were lower than ideal. This was probably due to an overloading of the sorbent capacity of the cartridges by co-extractives. Some of the cartridges that had been shown not to retain glyphosate were therefore used as pre-treatment steps. The aim of this was to remove sufficient

interferences to enable maximum retention of glyphosate on the NH_2 sorbent. Octadecylsilyl (C18) cartridges gave the best results, giving a recovery of > 80 % at a fortification level of $2\mu g g^{-1}$ (2 ppm). Finally, it was shown that, once derivatised, the glyphosate–FMOCCl complex could be retained on C18 cartridges, giving a further potentially useful clean–up step. Use of SPE cartridges has a number of advantages over manually packed ion exchange columns. Methods utilising SPE can be easily automated, allowing methods to be fully optimised and enabling high sample throughput. Greater consistency of analysis should also be obtained because the cartridges are uniformly packed. Some suggestions for further work are then made.

Chapter 3 considers the extraction and analysis of glyphosate from typical UK soils. In contrast to the analysis of glyphosate in plants, the emphasis in soil analysis was on the type of extractant rather than the clean–up stage. This is because the limiting factor in the analysis is the achievement of good extraction efficiencies across a range of soil types. Obviously, without consistent extraction efficiencies, reliable and comparable results cannot be obtained (unless an arbitrary definition of extractable glyphosate is made). Also, if an effective and selective extractant could be found it would reduce or eliminate the need for any preliminary clean–up steps. As well as reviewing the previously published methods of analysis, published work on the mechanisms of glyphosate binding to soil constituents was also reviewed. This was done in order to select extractants on a rational basis and not by a hit–or–miss procedure. Five extractants were selected and tested. Two, sodium hydroxide and potassium dihydrogen phosphate were included because they had been used successfully in previously published methods. Sodium citrate and sodium bicarbonate were selected after consideration of how glyphosate is thought to bind to soils. Finally, water was included to show that extensive binding of glyphosate had ocurred over the 24 hour incubation period.

Sodium hydroxide was found to be the most effective extractant of those tested. All of the other extractants, apart from water, released significant levels of glyphosate (> 40%). Water released < 5% of the applied glyphosate showing that significant binding had taken place. Extracts derived from sodium hydroxide produced the 'dirtiest' chromatograms and an attempt to use SPE cartridges as a clean-up step was made. The implications of these results are then discussed in depth and some ideas for future work are suggested.

Chapter 4 examines the possible use of aromatic amino acids to improve the selectivity of a bioassay for glyphosate in aqueous samples. It then investigates the use of aromatic amino acids as safeners for glyphosate on whole plants.

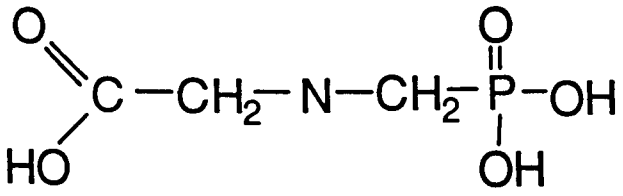
Relevant published work on bioassays for the determination of glyphosate in water are reviewed. A suitable bioassay using cress seedlings (*Lepidium sativum*) was developed and the concentration range over which glyphosate affected the seeds was determined. The effect of the aromatic amino acids, tryosine, phenylalanine and tryptophan, individually and as a mixture, on the glyphosate induced growth inhibition was examined. A statistically significant reversal of growth inhibition was observed when the mixture was used. This may enable more selective bioassays for glyphosate to be developed. The second part of the chapter looks at the possibility of harnessing this effect as a safener for crops. Beans (*Phaseolus vulgaris*) were variously treated with glyphosate only, glyphosate plus an amino acid pre-treatment and an amino acid pre-treatment only. The plant heights were regularly recorded and at the terminal harvest the plant fresh weights were measured. Statistical analysis showed no significant difference in height or fresh weight. The implications of these results are discussed and some suggestions for further work are made.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The world market for agrochemicals in 1990 was an estimated $26,400 million. Of this total market, herbicides accounted for 43.8%, insecticides 29.0% and fungicides 21.6% (British Agrochemicals Association, 1991). In the United Kingdom, agrochemical sales were worth around £413.5 million and of this amount herbicides made up £199.4 million (48.2%) (British Agrochemicals Association, 1991). Even in times of recession and with ever increasing regulatory pressure, agrochemicals are obviously still big business.



Glyphosate

Introduced in 1971, n-(phosphonomethyl) glycine or glyphosate is almost certainly the world's best selling herbicide. In 1993, this chemical alone accounted for 67% of its discoverer Monsanto's sales of agrochemicals, some $1295 million (County Nat. West, 1994). This is a remarkable amount for a single chemical. Over the next few years the amount of glyphosate sold is likely to increase, although the value of sales may decline. This apparently contradictory situation is due to four principal factors:

1) The patent on glyphosate expired in some countries in 1991. This has lead

to other manufacturers (e.g. Zeneca) taking up production.

2) Monsanto have pursued a rigorous price cutting strategy, resulting in a large increase in glyphosate sales as the range of economic applications increases.

3) Glyphosate resistant crops presently in development will begin to appear on the market.

4) The recent introduction of "set aside" in the European Community has boosted sales, since glyphosate is an excellent field clearance chemical.

The success of glyphosate is due to a range of properties that make the chemical unique. Sold in formulation as "Roundup" (also "Glyphotex", "Herbdex", "Rodeo", "Glycel" and "Greenscape" amongst others) it is a post-emergence, non-selective weedkiller. It is effective over a range of annual, biennial and perennial grassy and broadleaf weeds, including 76 of the world's 78 worst weeds (Franz, 1985). This herbicidal effectiveness is due to the chemical's relatively high water solubility, rapid uptake and translocation by plants and its low in-vivo degradation (Guinivan et al., 1982). Plants are killed by inhibition of 5-enolpyruvylshikimic acid 3-phosphate (EPSP) synthase, an enzyme of the shikimic acid pathway which produces aromatic amino acids (Amrhein et al., 1981). This site of action also explains the selective toxicity of glyphosate, as this pathway only exists in plants and micro organisms. The oral LD50 for rats was determined to be > 5,000 $mgkg^{-1}$ (Branch, 1986) and for rabbits to be 3,800 $mgkg^{-1}$ (Monsanto, 1986). A recent review of the biological activity of glyphosate (Smith and Oehme, 1992) concluded that the chemical had not been found genotoxic, mutagenic or teratogenic. As well as its efficiency and benign toxicology, glyphosate also displays favourable environmental properties. It is tightly bound on contact with soil (Sprankle et al., 1975b; Glass, 1987) and hence does not leach through the soil. It does not tend to run off the soil surface (Damanakis, 1976;

Edwards et al., 1980) and is therefore not prone to contamination of surface waters. A summary of the properties of glyphosate are given in apppendix 1.

This fortuitous combination of properties makes glyphosate both effective and "environmentally friendly", a potent mix in today's market.

1.2 REASONS FOR INVESTIGATION

Glyphosate is registered for use in a wide variety of situations including weed control in fruit, vegetables, trees, pre- and post-harvest weed control in cereals and as a clearance chemical for "set aside" areas, roads and railways (Ivens, 1991). In the future glyphosate may also be routinely used for weed control in genetically engineered, resistant crop varieties. As outlined in the background section, the low mammalian toxicity and favourable environmental behaviour of glyphosate have helped to give the compound an edge in the market place. The compound's almost ubiquitous usage, low level bioactivity and broad spectrum herbicidal activity can, however, have some less benign effects.

The toxicology of glyphosate has been reviewed by Atkinson (1985) and by Smith & Oehme (1992). Both reviews indicate that the chemical is of low toxicological concern. The U.S. Environmental Protection Agency (EPA), however, does have some reservations about the oncogenic potential of the chemical and classifies the cancer risk from glyphosate as "presently not classifiable as to its carcinogenicity to humans" (Lang & Clutterbuck, 1991). At the present time U.K. maximum residue limits (MRLs) have not been set. In the U.S. the EPA has laid down MRLs in most foods for direct consumption. These are generally around 0.2 $mgkg^{-1}$ but are lower (0.1 $mgkg^{-1}$) in grain products which are consumed in larger quanitities (Atkinson, 1985).

As would be expected, glyphosate can cause considerable damage to crops if it comes into contact with them at a vulnerable growth stage. Labelling restrictions can reduce the likelihood of misapplication but misuse and drift are much more difficult to avoid. As well as causing obvious symptoms of damage in the crop at high dose rates, there is also evidence of impairment of

seed germination at relatively low levels. Glyphosate is approved in the UK for pre-harvest desiccation and weed control in barley, oats and wheat (Ivens, 1991). Pre-harvest application was shown to give improved weed control over post-harvest application in cereals (O'Keeffe, 1981a). Further work showed that when applied between 7 and 17 days prior to harvest, glyphosate had no deleterious effect on grain yield, thousand grain weight or germination capacity (O'Keeffe, 1981b). Glyphosate did, however, significantly reduce soybean germination and vigour when applied between 2 and 4 weeks pre-harvest at rates of 1.7 and 3.4 $kgha^{-1}$ (Whigham & Stoller, 1979) and reduced grain sorghum germination at rates of 2.24 and 4.48 $kgha^{-1}$ but not at rates of 0.56 and 1.12 $kgha^{-1}$ (Bovey et al., 1975). Evidence suggests that germination suppression can occur if glyphosate is translocated to the meristems of developing seeds (Shaban et al., 1987). The timing of the application to crops is therefore critical. Glyphosate is not recommended for use on cereal or other crops grown for seed (Ivens, 1991). A recent publication by the Official Seed Testing Station for Scotland (Don et al., 1990) indicated that the use of glyphosate on crops intended for seed has occasionally taken place. The resulting seed gave a positive tetrazolium salt test, indicating viability, but actual germination rates were poor. The authors intimated that if a method of tetrazolium assessment cannot be developed for glyphosate affected seed, it would be helpful if a simple test for the detection of glyphosate could be developed. As well as the implications for crops intended for seed production, glyphosate drift can result in substantial damage to the affected crop. Accidental exposure of a tomato test plot to drift from a 4.5% solution of glyphosate (Roundup) applied at 3.4 $kg(a.i.)ha^{-1}$, prior to flowering, had a significant effect on yield. Only 38% of the resulting fruit was marketable. Subsequent greenhouse experiments suggested that the amount of glyphosate required to mimic this result was approximately 0.1 ml of a 4.5% solution (Russo, 1990). This equates to 0.45 mg per plant. A number of incidences of

drift damage have been documented by Atkinson (1985). Little information is currently available on the actual levels of glyphosate responsible for germination suppression and other symptoms of glyphosate injury. Recent work in this laboratory (Yusof, 1988) simulating the effects of glyphosate drift on potatoes, found that the performance of tubers from treated plants was significantly impaired even at the lowest treatment level of 0.09 $kgha^{-1}$. Measurement of the actual glyphosate residue present gave a value that was not significantly different from that determined for the control plants. Control plants had a germination percentage of 96%. This suggests that low levels of the chemical can have an extremely damaging effect and also illustrates the difficulty inherent in reliably determining glyphosate residues at low levels.

The problems encountered in analysing glyphosate at the residue level, which are considered in depth in section 2.1, mean that quantitative information on residue levels and their implications is scarce in relation to the usage of the chemical. As outlined above, this information and the ability to collect it, is important for a number of reasons:

1) To ensure that regulatory requirements, such as MRLs, are being adhered to.

2) To enable the screening of crops destined for seed to ensure that contamination with glyphosate has not taken place.

3) To enable irrefutable determination of whether glyphosate drift has been responsible for crop damage.

Furthermore, suitable methods of analysis are necessary to enable meaningful assessments of the impact of the chemical on the environment to be made.

CHAPTER 2

AN INVESTIGATION INTO THE ANALYSIS OF GLYPHOSATE IN FOOD CROPS WITH PARTICULAR REFERENCE TO BARLEY

2.1 INTRODUCTION AND LITERATURE REVIEW

As outlined in the introduction to this thesis, there is potential for glyphosate residues to be present in foodstuffs at their time of consumption and in seed upon which growers are dependent for the success of their crop. Although, toxicologically speaking, glyphosate is not considered a "problem" chemical, it is very bioactive and caution should always be exercised. The chemical has been shown to cause germination problems in grain (Don et al., 1990) and in potatoes (Yusof, 1988). It is imperative therefore that concerned parties have at their disposal reliable, straightforward and sufficiently sensitive analytical methods to determine the amount of glyphosate present in a particular matrix. The purpose of this chapter is to investigate existing methodologies and to develop such a method for food crops, in particular barley. As barley is a low moisture content crop any method successful with this matrix stands a good chance of working with other crops. A comprehensive review of previously published material was undertaken to examine the techniques that had been tried, how successful they had been and how suitable they were for this laboratory. Methods developed to determine glyphosate in soil are included in a separate review. This is a bit of a false division as there is a large degree of cross-fertilisation of ideas between the fields of crop and environmental analysis. Nevertheless the division was felt necessary to keep the reviews to a manageable size.

Procedures to determine glyphosate (in common with other pesticides) generally involve three stages:

1) Extraction with suitable solvents.

2) Purification to remove the bulk of the co-extracted material.

3) A final determination stage.

Although the methods of extraction and purification are important, indeed the clean-up steps are often crucial, the basis on which a method stands or falls is the success of the final determination step. In order to keep this review relevant and of a manageable size, some qualifying criteria were introduced. To be included in this review the determining step of a method would have to be sensitive enough to meet current regulatory requirements, i.e. levels of ngg^{-1}(ppb) and the instrumentation must be widely accessible if not ubiquitous in analytical laboratories. Examination of the literature revealed that the following techniques had been utilised in the detection of glyphosate:

(i) High Performance Liquid Chromatography (HPLC).

(ii) Gas Chromatography (GC).

(iii) Polarography.

(iv) Thin Layer Chromatography (TLC).

(v) Colourimetry.

(vi) Molecular Emission Cavity Analysis.

(vii) 31P Nuclear Magnetic Resonance (NMR).

(viii) Amino Acid Analysis.

Some techniques were eliminated because the equipment required was felt to be too specialised: molecular cavity analysis (Ragab, et al., 1979) 31P

nuclear magnetic resonance (Dickson et al., 1988) and polarography (Bronstad & Friestad, 1976 and 1985). In any case, even if the necessary instrumentation had been widely available, none of the methods would have been sufficiently sensitive. The most sensitive is polarography with a detection limit in water of approximately 0.7 μgml^{-1} (0.7ppm). Of the remaining techniques, the colourimetric method (Glass, 1981) and the amino acid analyser method (Ekstrom & Johansson, 1975) were insufficiently sensitive with limits of detection of 1 μgml^{-1} and 3.4 μgml^{-1} respectively. It should be noted that the colourimetric technique is not specific for glyphosate but detects orthophosphate.

Numerous methods employing thin layer chromatography have been published. Some utilise autoradiography (Rueppel et al., 1975) and others, visual detection methods (Young et al., 1977; Ragab, 1978 and Sprankle et al., 1978). A range of detection limits for the visual detection methods have been reported from 0.2 ppm in distilled water (Ragab, 1978) to 5 ppm in extracts of Canada thistle (Young et al., 1977). Without the use of radiolabelled material, TLC methods are unlikely to provide sufficient sensitivity and are also likely to be prone to interferences from co-extracted material both during the running of the plate and during the reactions necessary to visualise the glyphosate spots. Having discarded the above techniques, only those methods which utilised G.C. and H.P.L.C. remained. The published methods using these techniques are covered in detail in the following literature review.

2.1.1 THE DETERMINATION OF GLYPHOSATE RESIDUES IN FOOD CROPS BY GAS CHROMATOGRAPHY (GC)

The analysis of glyphosate by GC is hampered by a number of factors. The most obvious are that glyphosate is non-volatile and its structure does not

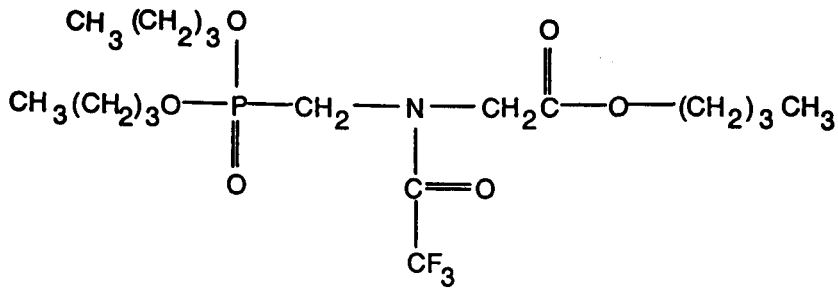
lend itself to easy detection. In order to utilise GC therefore, the compound must be derivatised to make it volatile and to enhance its detectability. Achieving suitable derivatisation of the compound is complicated by the presence of three functional groups, namely phosphonic acid, carboxylic acid and secondary amine. To obtain a structure suitable for GC analysis, most, if not all of these groups must be derivatised. As glyphosate is generally insoluble in organic solvents, achieving adequate derivatisation is difficult. This is because most conventional acylation and esterification reactions are hindered or become impossible in the presence of water (Blau and King, 1978). Derivatisation is also likely to be affected by the presence of aqueous co-extractives and adequate clean-up prior to derivatisation is imperative. A number of published methods have attempted to resolve these difficulties. A list of those published to date is given in Table 2.1. Each of the techniques is then discussed in more detail. Some methods have only been attempted with standard solutions or potable water and not with more taxing crop matrices. These have been included for the sake of completeness.

Year	Author(s)	Matrix	Extractant	Clean-up	Derivatisation	Detector	Sensitivity
1976	Rueppel et al.	Standards only			Tri-n-butyl N-trifluoroacetyl N-phosphonomethyl glycine	MS	
1977	Pesticide Analytical Manual	Wide variety	1. Organic solvent pre-extraction 2. Water	1. Cation exchange 2. Charcoal 3. Anion exchange	Methyl N-trifluoroacetyl ester	FPD	$0.1\mu gg^{-1}$
1981	Thompson et al.	Avocado	Chloroform/Water (1:2)	1Anion exchange 2 Cation exchange	Methyl N-trifluoroacetyl ester	FPD	$0.01\mu gg^{-1}$
1982	Guinivan et al.	Blueberry	Water	1 L/L Partition 2 Gel permeation 3 Cation exchange	2-chloroethyl-N-heptafluoro butyryl ester	ECD	$0.01\mu gg^{-1}$
1984	Seiber et al.	Kiwi fruit Asparagus		1Anion exchange 2 Gel permeation 3 HPLC (post derivatisation)	Methyl N-trifluoroacetyl ester	FPD	$0.1\mu gg^{-1}$ $0.05\mu gg^{-1}$
1984	Moye and Deyrup	Standards only			Dimethyl-tert-butylsilyl ester	FPD	
1985	Deyrup et al.	Standards only			Various	FPD	

Key.

L/L: Liquid,liquid.

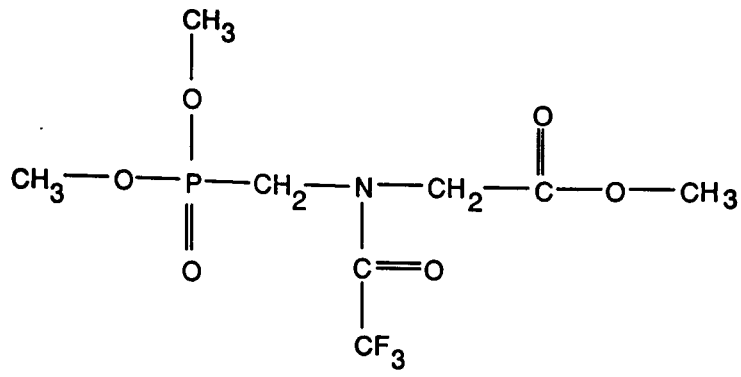
The earliest published GC method for the analysis of glyphosate was that by Rueppel, Suba & Marvel (1976). This involved the preparation of the n-Butyl-N-trifluoroacetyl ester of glyphosate and confirmation of the structure obtained (a) by mass spectrometry.



(a)

The derivatisation was carried out by solubilizing glyphosate in trifluoroacetic acid and then, on the addition of trifluoroacetic acid anhydride and warming at 40ºC for an hour, acylation takes place. After the removal of excess solvent and reagent, treatment with N-butanol and ethereal diazo-n-butane, the fully derivatised product was obtained. The main drawback with the method was the reported fall in the conversion rate to 40-50% at the 25-50 mg range. Furthermore, the technique was only used on standards.

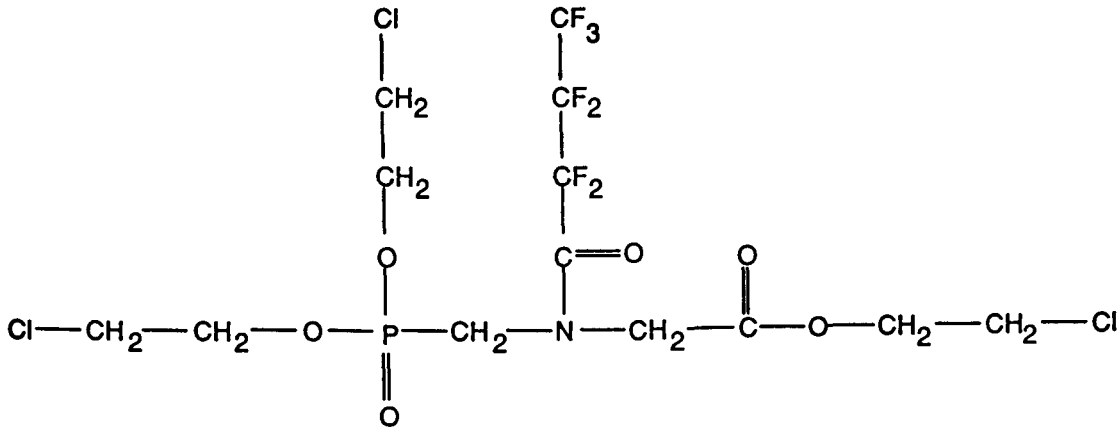
The method originally developed by Monsanto and recommended by the Environmental Protection Agency (Pesticide Analytical Manual, 1977) has been used on a wide range of crops as well as soil and water. The main steps involve pre-extraction using an organic solvent, aqueous extraction followed by cation exchange, charcoal and strong anion exchange clean-up steps. Derivatisation to the methyl N-trifluoro acetylester is then carried out using diazomethane followed by trifluoroacetic acid and trifluoroacetic acid anhydride to yield the product (b).



(b)

The obvious advantage of this method is that it has been proven on a wide range of matrices. The hindrances are the time consuming nature of the clean-up, the need for a double derivatisation and the difficulty inherent in obtaining anhydrous conditions for the derivatisation to proceed. It should also be borne in mind that diazomethane is toxic and explosive. Thompson et al., (1981) modified this method in order to analyse glyphosate residues in avocado. Chloroform/water was used as the extractant and the charcoal step was left out of the clean-up. This shortened procedure still contains the drawbacks of the full method and its success is probably more matrix specific.

A method for the analysis of glyphosate in blueberries was published by Guinivan et al. (1982a). It involved the use of gel permeation chromatography and cation exchange before derivatisation to yield the 2-chloroethyl-N-heptafluorobutyl ester (c) overleaf.

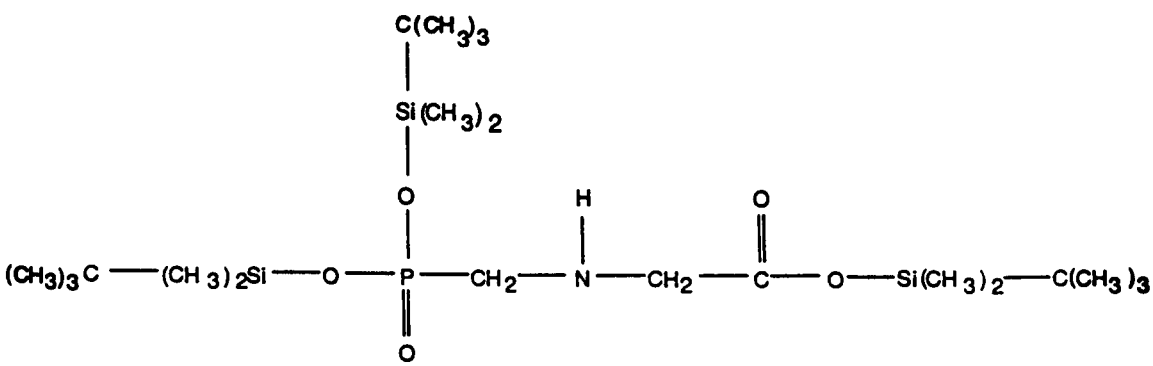


(c)

Gel permeation eliminated much of the sugar from the matrix and reduced the amount of pigment present. Cation-exchange then removed enough of the remaining sugar to allow the sample to be taken to dryness for derivatisation. The derivatisation was conducted in two stages. The first involved esterification via BCl_3-2-chloroethanol while the second resulted in acylation using heptafluoro-butyric anhydride. A subsequent paper (Guinivan et al., 1982b) confirmed the structure of the product by mass spectrometry. The advantages of this method claimed by the authors were its usefulness for crops with a high sugar content, speed, higher recoveries and a lower limit of detection.

Glyphosate residues in kiwi fruit and asparagus were investigated by Seiber et al. (1984). A novel approach was developed by the authors because analysis using the "Pesticide Analytical Manual" method and the method of Guinivan et al.(1982a), were attempted and found to be inadequate. The resulting method utilised steps from a range of previously published works. Anion exchange and gel-permeation were utilised as preliminary clean-up steps before

derivatisation as described in the "Pesticide Analytical Manual" method. Where this method departed from other work was in the use of HPLC as a clean up step post-derivatisation and prior to analysis by G.C. Although the authors claimed success in the analysis of these matrices, the use of HPLC as a clean-up and the continued reliance on a double derivatisation make this method extremely time consuming. A single step derivatisation of glyphosate for GC analysis was published by Moye & Deyrup in 1984. The authors attempted to use diazomethane amongst other reagents as the derivatising agent, but gas chromatography - mass spectrometry revealed the presence of multiple products. Successful single step derivatisation was achieved by using N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetimide (MTBSTFA) and a silylation catalyst, yielding (d) below.



(d)

Binding of glyphosate to the glass of the reaction vessel was found to be a problem. Coating of the tubes with phosphoric acid reportedly gave the best solution. A subsequent paper (Deyrup et al., 1985) gave details of a single step derivatisation using fluorinated alcohols mixed with a perfluorinated anhydride. The method was developed because the authors found that even

after coating the reaction vessel, reactions with MTBSTFA still gave unacceptably low yields at levels below 10 $ngml^{-1}$. In this publication, reactions with a range of fluorinated alcohols and perfluorinated anhydrides were carried out. The advantages cited for this reaction were the good recoveries at low levels, the ability to vary chromatographic retention by selecting various alcohols and the incorporation of halogens into the structure of the derivative allows detection by electron capture to be used, which can be very sensitive.

2.1.1.1 SUMMARY

A number of derivatisation reactions have been utilised for the determination of glyphosate by GC. Apart from the Pesticide Analytical Manual (1977) method, none have been widely applied. Many of these derivatisations (including the Pesticide Analytical Manual) utilise diazomethane which is toxic and explosive. Furthermore, the effectiveness of the preliminary purification steps and the complexity of the matrix of interest appear to be the principal determinants of the likely success of the method. Many of the published clean-ups are shortened versions of that used in the Pesticide Analytical Manual and little thought has been given to a practical universal method (if indeed one is possible). The use of GC should not be ruled out altogether. In laboratories where there is a broad experience with GC and where alternatives are limited, the technique may be the preferred choice. In the author's laboratory, Yusof (1988) attempted unsuccessfully to use the method of Deyrup et al. (1985). This was due to the appearance of impurity peaks close to the retention time of glyphosate. These were present even when standard solutions were used.

2.1.2 THE DETERMINATION OF GLYPHOSATE RESIDUES IN FOOD CROPS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) analysis of glyphosate residues has one significant advantage over GC in that the compound can be chromatographed without derivatisation. There is, however, a problem. In order to achieve a low limit of detection the chemical must be derivatised! Detection without derivatisation has been attempted but is insensitive and prone to interferences. With HPLC this derivatisation can either be carried out before chromatography (pre-column) or after chromatography (post-column). HPLC shares with GC analysis most of the problems associated with aqueous co-extractives, therefore the effectiveness of the clean-up procedure is likely to have a significant bearing on the success of a method. The development of glyphosate analysis by HPLC has followed a somewhat different path to that of GC analysis (see Table 2.2). This is because satisfactory derivatisation reactions were discovered at an early stage. Subsequent publications have therefore tended to be aimed at improvements on the original method or broadening the number of crops that the methods can be applied to.

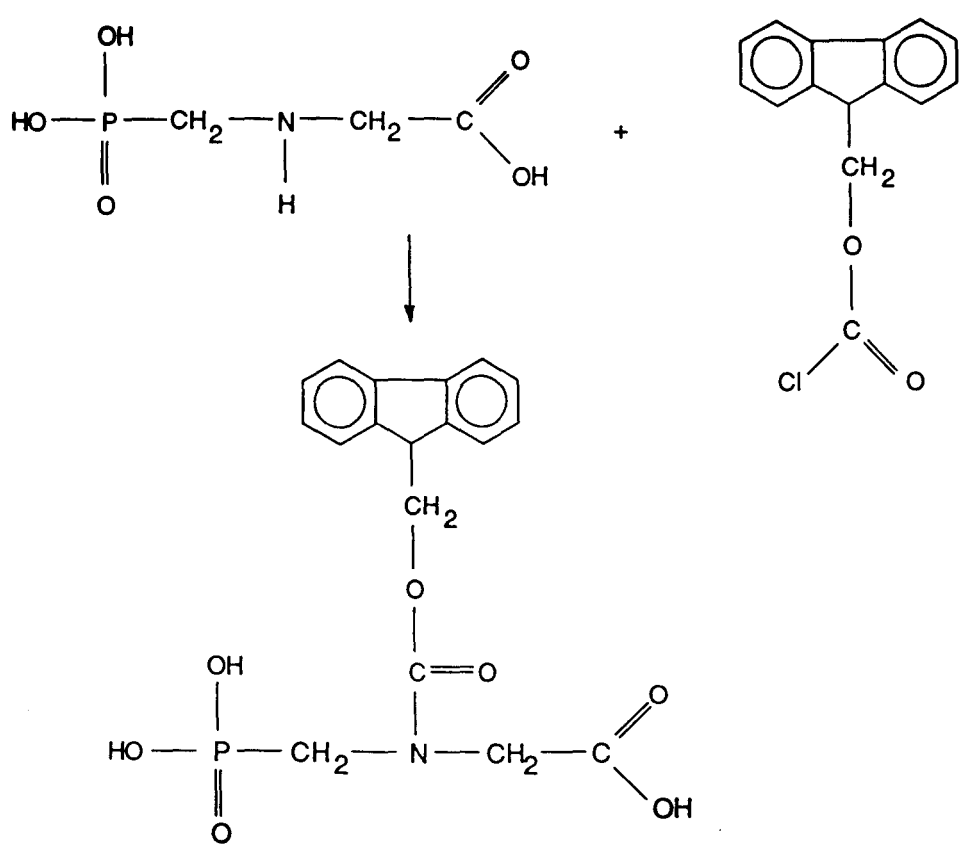
Year	Author(s)	Matrix	Extractant	Clean-up	Derivatisation	Detector
1979	Burns and Tomkins	Standards				UV
1979	Moye and Boning	Standards			FMOCCl	F
1980	Moye and St. John	Various foods	Water/chloroform (2:1)	Cation exchange	FMOCCl and OPA-MERC	F
1982	Roseboom and Berkhoff	Straw	Water/chloroform (2:1)	Cation exchange	FMOCCl	F
1983	Moye et al.	Various fruits and vegetables	Water/chloroform (2:1)	Cation exchange	OPA-MERC	F
1984	Archer and Stokes	Blackberries	Dilute HCl	1. Cation exchange 2. Anion exchange	OPA-MERC	F
1985	Huber and Calabrese	Standards	Water/chloroform (3:1)		1. Triethyl formate 2. FMOCCl	F or UV
1986	Cowell et al.	Various crops	Dilute HCl/chloroform (3:1)	1. Chelation 2. Anion exchange	OPA-MERC	F
1987	Tuinstra and Kienhuis	Cereals and vegetables	Water/chloroform (2:1)	HPLC	OPA-MERC	UV
1991	Wigfield and Lanouette	Lentils	Water/chloroform (3:1)	1. Cation exchange 2.Anion exchange	OPA-MERC	F

Key.

FMOCCl: 9-fluorenylmethylchloroformate
OPA-MERC: o-phthalicdicarboxaldehyde-mercaptoethanol
F: Fluorescence
UV: Ulra-violet

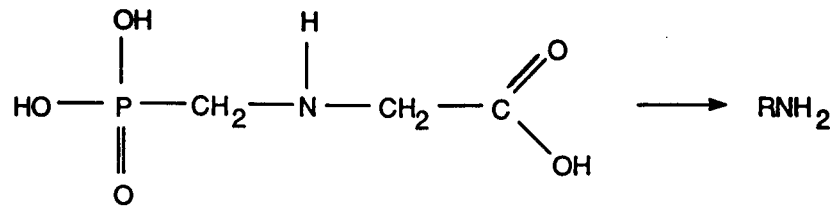
The earliest published method for the analysis of glyphosate by HPLC was by Burns & Tomkins (1979). It was intended for use on formulations and technical samples, and involved no derivatisation. The chromatography involved a strong anion exchange column (SAX) and a 96:4, 0.005M KH_2PO_4/methanol mobile phase adjusted to pH 2.1 with H_3PO_4. U.V. detection at 195 nm was used. Although the method may have been successful for the analysis of formulations, its insensitivity (limit of detection = 2.5 $ugml^{-1}$) and the likelihood of overwhelming interference from coextractives make it unlikely to be suitable for determining residues in plants. Subsequently published methods have invariably incorporated a derivatisation step to increase the sensitivity and the selectivity of detection. The vast majority of these have used one of the following two derivatisations. The first is a pre-column derivatisation reaction using 9-fluorenylmethyl chloroformate while the second involves post column derivatisation with O-pthalicdicarboxaldehyde-mercaptoethanol.

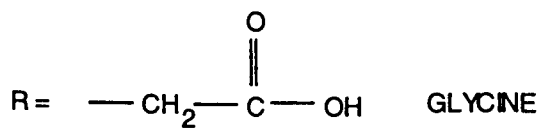
1. The derivatisation of glyphosate using 9-fluorenylmethylchloroformate (FMOCCl)

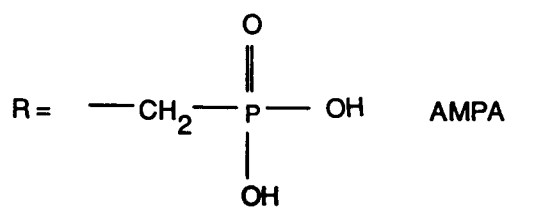


2. The derivatisation of glyphosate using O-pthalicdicarboxaldehyde-mercaptoethanol (OPA-MERC) (after Tuinstra & Kienhuis, 1987).

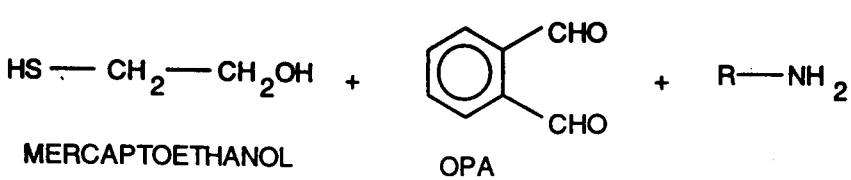
STEP 1: Oxidation

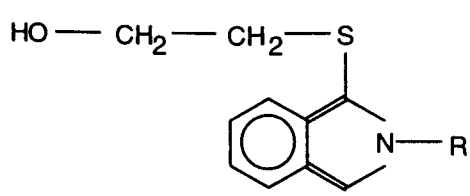






STEP 2: Derivatisation





FLUORESCENT COMPLEX

The use of these derivatisation reactions for glyphosate analysis was first described by Moye & St John (1980). They compared the performance of each derivatisation procedure for the analysis of glyphosate and aminomethylphosphoric acid (AMPA) residues in a range of crops after cation exchange clean up. The authors reported that pre-column derivatisations with FMOCCl made it impossible to determine both glyphosate and AMPA using a single set of HPLC conditions. As a result they did not report any recovery data for this technique. A limit of detection of 0.1 $\mu g g^{-1}$ was determined for both derivatisations. Chromatography of the FMOCCl derivatives involved either a Waters μ carbohydrate or μNH_2 silica particle column and the mobile phase comprised of 25:75 Acetonitrile/0.025M KH_2PO_4(pH4). OPA-MERC derivatives were chromatographed on an Aminex A-27 plastic bead column using a 0.1M H_3PO_4 mobile phase. Recoveries with the OPA-MERC system were 70-96% for glyphosate and 61-82% for AMPA.

Only one other published method since Moye & St John has used FMOCCl for the determination of glyphosate residues in plants, although FMOCCl derivatisation has also been used to determine glyphosate in soil. These methods are considered later. Roseboom & Berkhoff (1982) used FMOCCl derivatisation to determine glyphosate and AMPA in straw, again using a cation exchange clean up procedure. As with Moye & St John, the authors found that both moieties could not be determined simultaneously. A hypersil APS (NH_2) column was used. For glyphosate determination, the mobile phase used was 0.04M KH_2PO_4 and 0.01M NaH_2PO_4/methanol, 55:45 (v/v) buffered at pH8. AMPA was determined using 0.05M NaH_2PO_4/methanol 2:3 (v/v). The recoveries claimed were 75% for glyphosate and 79% for AMPA, with a detection limit for both of 0.1 $mg kg^{-1}$.

In the author's department (Yusof, 1988) FMOCCl has been used to determine

glyphosate in potatoes and barley grain. Activated carbons were successfully used as a purification step to give levels of detection down to 0.05 μgg^{-1} for potato extracts. Activated carbons also gave the best results of the techniques tested for cleaning up barley extracts. The limit of detection in this case however was 1 μgg^{-1}. Strong anion and cation exchange, solid phase extraction cartridges and chelating resins were also tried for cleaning up the extracts with little success. Again, a hypersil APS column was used to chromatograph the derivatives, using a 0.1M pH4 phosphate solution/acetonitrile, 75:25 (v/v) mobile phase.

OPA-MERC has been more widely utilised for plant residues than FMOCCl in published material. Moye et al. (1983) published an improved approach to their post-column derivatisation method (Moye & St John, 1980). This was said to offer "improvements in the amount of sample required, sample throughput, chromatographic efficiency, resolution, sensitivity and reagent consumption". These enhancements were achieved by optimising the post-column reactions, using separate columns for glyphosate and AMPA analysis and by altering the composition of the mobile phase used for glyphosate. Clean-up was again carried out using a cation exchange column. An aminex A-27 (Bio-rad) anion exchange column was used for glyphosate with a 0.09M H_3PO_4/0.01M H_2SO_4 mobile phase running at 0.5 $mlmin^{-1}$. AMPA was analysed using an HA-X10 column (Pierce Chemical Co.) with 0.02M KH_2PO_4 buffered at pH5 as the mobile phase, again running at 0.5 $mlmin^{-1}$. Recoveries for three crops (cantaloupe, cucumber and pumpkin) fortified at 1.0 μgg^{-1} were 88 to 107% for glyphosate and 88 to 89% for AMPA. Both glyphosate and AMPA were then determined in field-weathered cranberries. A similar procedure was employed by Archer & Stokes (1989) to determine glyphosate in field treated blackberries. After sample purification using both cation and anion exchange, determination of glyphosate was again achieved using an Aminex A-27 column

and a H_3PO_4 3.0 gl^{-1}/H_2SO_4 0.3 gl^{-1} mobile phase. A commercial post-column reactor was used in this instance.

A five analyst, interlaboratory study undertaken by Cowell et al. (1986) utilised advances in clean-up techniques and the availability of commercial post column reaction systems to validate a residue method for glyphosate and AMPA that was based on that of Moye et al. (1983) and Archer & Stokes (1984). Four species, soybeans, grapes, cabbage and alfalfa were chosen to represent the major crop types and were analysed along with environmental water samples. Preliminary concentration and isolation was achieved using chelation and anion exchange columns. HPLC analysis was conducted using a Bio-Rad Aminex A-9, 30 x 0.46cm i.d. column thermostated at 50°C and a 4% methanol in 0.005 M potassium dihydrogen phosphate solution adjusted to pH 1.9 with concentrated H_3PO_4. The method was validated over the concentration range from 0.05 to 5.00 μgg^{-1} with recoveries of 80.9 ± 13.8% for glyphosate and 79.2 ± 13.8% for AMPA. The authors were confident that the variation between analysts was such that the method was reliable. Wigfield & Lanouette (1991) used a modified clean-up procedure involving cation and anion exchange columns before post column derivatisation and HPLC analysis, to determine glyphosate and AMPA in lentils. The limits of detection determined were 0.08 μgg^{-1} with a mean recovery for glyphosate of 92.5% and 0.1 μgg^{-1} with a mean recovery of 92.8% for AMPA.

An elegant two-dimensional HPLC procedure to determine glyphosate in cereals and vegetables was published in 1987 by Tuinstra & Kienhuis. This involved direct injections of centrifuged extract supernatant onto a pre-column (Corasil Bondpak, anion exchange) to purify and preconcentrate the glyphosate before forward elution onto an Ionosphere A, anion exchange column. The eluent then passed through a post column reaction system before

fluorescence detection. The pre-column was regenerated with acetic acid. Phosphate buffer at pH 2.1 was used as the eluent. Recoveries with barley, wheat and rye fortified at 1 $\mu g g^{-1}$ were 80%. This method did not use a commercial post-column reaction system.

2.1.2.1 SUMMARY

When choosing between the pre- and post- column techniques for the HPLC determination of glyphosate, two questions must be considered: (1) Does AMPA have to be measured? (2) What equipment is available for the analysis? If AMPA is to be determined then the OPA post-column method is the better choice, unless further development of the FMOCCl derivative is undertaken. Post-column reaction however, requires either a multiplicity of pumps or a commercial post-column reaction system. Optimisation of the post-column system is likely to be tricky and require a high degree of analyst skill. Pre-column FMOCCl reaction is by contrast, relatively easy. Hence, where the determination of glyphosate alone is the object, the simplicity of pre-column derivatisation would make it the method of choice.

Possibly the largest factor in the success of the determining step is the effectiveness of the clean-up. Almost all the purification steps in the published literature concerning HPLC analysis of glyphosate are variations on those used in the Pesticide Analytical Manual (1977), where cation exchange, anion exchange and charcoal were used. However, Guinivan et al. (1982) and Seiber et al. (1984) used gel permeation chromatography. Seiber et al., (1984) and Tuinstra & Kienhuis (1987), used HPLC as a clean-up. Strong anion exchange and strong cation exchange solid phase extraction cartridges were used unsuccessfully by Yusof (1988).

2.1.3 CONCLUSION

The aim of this literature review was to consider which techniques could provide a suitable base for the development of a reliable, straightforward and sensitive method for the determination of glyphosate in food crops, particularly barley. Of the G.C. methods published, only the P.A.M. (1977) method has been widely applied and no single derivatisation procedure predominates. In addition, a previous attempt to use G.C. in this laboratory was unsuccessful (Yusof, 1988), so although a range of G.C. equipment was available, it was felt that the prospect of success with G.C. was low. Conversely, two techniques for H.P.L.C. dominate the literature. These were both first reported by Moye & Boning (1979 & 1980). One requires pre-column derivatisation with FMOCCl, whilst the other utilises post-column derivatisation with OPA-MERC. Post-column derivatisation has been the most widely used probably because it also enables AMPA to be determined (although not simultaneously). Unfortunately, no post-column reaction system was available to utilise this technique, leaving the pre-column derivatisation method. This appeared to be relatively simple to execute, suitably sensitive and required no specialised equipment beyond a fluorescence detector for the HPLC system. Although there will be some occasions when the determination of AMPA will be important, in many cases analysis of glyphosate alone will suffice. Another factor in favour of this method was its previously successful use in this laboratory (Yusof, 1988).

Having decided to base the determining step on pre-column derivatisation with FMOCCl, some consideration must also be given to suitable purification strategies, with particular emphasis on simplicity and their likely utility over a range of matrices. The majority of published methods use the clean-up from the P.A.M. method. This involves charcoal and anion and cation exchange

chromatography. Some authors have used shortened versions to reduce the amount of time required per analysis. The success of these truncated methods appears to depend on the complexity of the matrix to be analysed. Little thought seems to have gone into developing a quick, straightforward and widely applicable clean-up. In this laboratory an attempt was made to modernise the P.A.M. method by using 'Bond Elut' anion exchange (SAX) and strong cation exchange (SCX) solid phase extraction cartridges (Yusof, 1988). Although these were effective at retaining glyphosate in standard solutions, they were poor at extracting glyphosate from plant extracts. However, a wide range of bonded phases are available and are worthy of investigation. Gel permeation chromatography (GPC) has been used prior to GC analysis (Guinivan et al., 1982; Seiber et al., 1984) but not prior to HPLC analysis. Since GPC lends itself to samples in an aqueous medium this technique is also worthy of further investigation. SEC separates molecules on the basis of size and a range of gels are available that are suitable for use with aqueous mobile phases. This makes the technique ideal as a first purification step since glyphosate is a small molecule and can therefore be separated from the larger co-extractives such as proteins and starch. Although ion exchange cartridges were used unsuccessfully by Yusof (1988), a wide range of cartridges is now available. These cover a range of polarities and even if a cartridge that retains glyphosate cannot be found, it is likely to retain some co-extractives. A further advantage of both SPE and SEC is that they can be easily automated.

2.2 TROUBLESHOOTING

2.2.1 INTRODUCTION

As mentioned in the introduction to the preceding literature review, the aim of this chapter was to develop a reliable, straightforward and sensitive method for the analysis of glyphosate in barley. A review of the literature indicated that the HPLC based, pre-column derivisation method of Moye & Boning (1979) was the most promising for use in this laboratory and that solid phase extraction and gel permeation chromatography were worthy of investigation as purification steps for extracts prior to HPLC. Before determining the usefulness of these clean-up techniques, the chromatographic method was examined to ensure that the optimum conditions were being used and provided suitable sensitivity, reliability, reproducibility and gave a linear detector response. During preliminary invesigations, however, a number of problems with the system had to be remedied. An overview of this investigation is given below.

2.2.2 EXPERIMENTAL

2.2.2.1 HPLC SYSTEM

A fluorometric HPLC system was constructed from a Waters model 6000A pump, a Rheodyne sample injection valve fitted with a 20 µl loop and a Shimadzu fluorescence detector model RF-530. Chromatograms were variously recorded on a Servoscribe model RE511.2 chart recorder, a Shimadzu CR-16 integrator or a Spectra Physics SP4290 integrator. The detector was set with excitation at 270 nm and emission at 315 nm. The column used initially was a Shandon 250mm x 4.6mm i.d., 5µm Aminopropyl (NH2) bonded silica column.

2.2.2.2 REAGENTS

Deionised water was used throughout. All solvents were analytical grade except for acetonitrile which was HPLC grade (Rathburns, Walkerburn, Scotland). A 1 $mgml^{-1}$ glyphosate (Greyhound, U.K.) stock solution was prepared by dissolving 1.034 g in a 95:5 (V/V) mixture of water and methanol. (The methanol was included as an anti-microbial). A series of glyphosate standards, 1 μgml^{-1} to 0.1 ηgml^{-1} were then prepared by dilution. These were refrigerated when not in use. 9-fluorenylmethyl chlorformate (FMOCCl), (Aldrich) 0.01M was prepared by dissolving 0.0065 g in 25 ml of acetone. Sodium borate (BDH) was prepared by dissolving 2.38 g in 250 ml of deionised water.

The HPLC mobile phase comprised of a pH4 phosphate solution modified with 25% acetonitrile. This was prepared by dissolving 13.609 g of potassium dihydrogen orthophosphate in 1 litre of deionised water. The pH of the resulting solution was then adjusted to pH4 with orthophosphoric acid. The pH was monitored using a pH meter while the acid was added. 333 ml of acetonitrite were then added to the solution. The mobile phase was filtered through a 0.45 µm Millipore membrane filter prior to use. This performed the dual functions of removing any particulate material which could damage the HPLC system and also degassed the solution. The mobile phase was gently sparged with helium while in use to ensure that no air redissolved in the mobile phase.

2.2.2.3 DERIVATISATION PROCEDURE

Glyphosate standards were derivatised using the method of Moye & St John (1980).

0.1 ml of each standard was placed with 0.9 ml of 0.025M sodium borate solution, 0.9 ml of acetone and 0.01M FMOCCl in a quickfit test tube. The mixture was incubated at room temperature for twenty minutes. Three 1 ml portions of diethyl ether were then used to extract unreacted reagent. The resultant solution was placed under a stream of nitrogen to remove any remaining ether prior to HPLC analysis.

2.2.3 DISCUSSION 1

As mentioned in section 2.2.1, a number of problems were encountered with the system. These manifested themselves as badly tailing and split/shouldered peaks on the chromatograms.

Investigations into these problems focused initially on the components of the derivatisation reaction and the glassware in which the reaction was conducted. Glyphosate can complex metal ions and one possibility investigated was that the split/shouldered peaks were caused by resolving complexed and uncomplexed glyphosate. To eliminate this, all glassware was washed in detergent, rinsed with tap water prior to soaking in a dilute solution of Decon 90 (Decon Laboratories) for 24 hours. After soaking, glassware was rinsed with deionised water and dried in an oven prior to use. All reagents were freshly prepared prior to use to reduce the possibility of degradation. These precautions however did not eliminate the poor peak shapes. The purity of the glyphosate standard was then investigated by TLC. If a breakdown product was present and contained the amino functional group it could, once derivatised with the large FMOCCl group, be close enough in structure to elute close to the glyphosate peak. The TLC method of Sprankle et al. (1978) was used to assess the purity of the glyphosate. Microcrystalline cellulose plates were prepared by mixing 30g with 60 ml deionised water. The cellulose

suspension was applied to 20 x 20cm or 5 x 20cm glass plates using a thin layer applicator. The coated plates were dried for an hour in an oven at 100°C, then stored in a dessicator prior to use. Glyphosate from two sources (Monsanto and Greyhound), AMPA and Glycine were applied as spots 2cm from the base of the plate. The plate was then developed using ethanol / water / 18N NH_4OH / trichloroacetic acid / 17N acetic acid (55 : 35 : 2 : 3.5g : 2, v/v/v/w/v). Visualisation was achieved by spraying with 0.5% ninhydrin in butanol and heating in an oven at 100°C to develop colour. Each compound run gave rise to only one spot. The rf values calculated were 0.82, 0.82, 0.86 and 0.94 for Monsanto glyphosate, Greyhound glyphosate, AMPA and glycine respectively. This result suggested that glyphosate from both sources was pure. Having ruled out derivatisation of the standard glyphosate and as far as possible the possibility of glyphosate complexing with metal ions, suspicion turned to the derivatising agent: 9-fluorenylmethylchloroformate. Preparing the reagents immediately prior to derivatisation had failed to improve the peak shape and therefore a new batch of FMOCCl was purchased (Aldrich). Again, although all glassware was scrupulously clean and the reagents freshly prepared, the problem was still not apparent. Having exhausted the possibilities in the reaction mixture, attention turned to the HPLC equipment.

Tailing and split/shouldered peaks with unacceptably short retention times, are often symptoms of poor column condition (Snyder & Kirkland, 1979). To examine this possibility a column test mixture was run. By calculating the number of theoretical plates from this, an impression of the column efficiency could be gained. The test mixture used was a mixture of toluene and nitrobenzene run using a mobile phase of hexane/ethylacetate 98:2, UV detection was conducted at 245nm. From the resultant chromatogram the number of theoretical plates (N) was calculated using equation 1.

Equation 1:

$$N = 5.54 \left(\frac{ta}{W\frac{1}{2}}\right)^2$$

Where ta = The retention time of the peak of interest,

W½ = The width of the peak at half peak height.

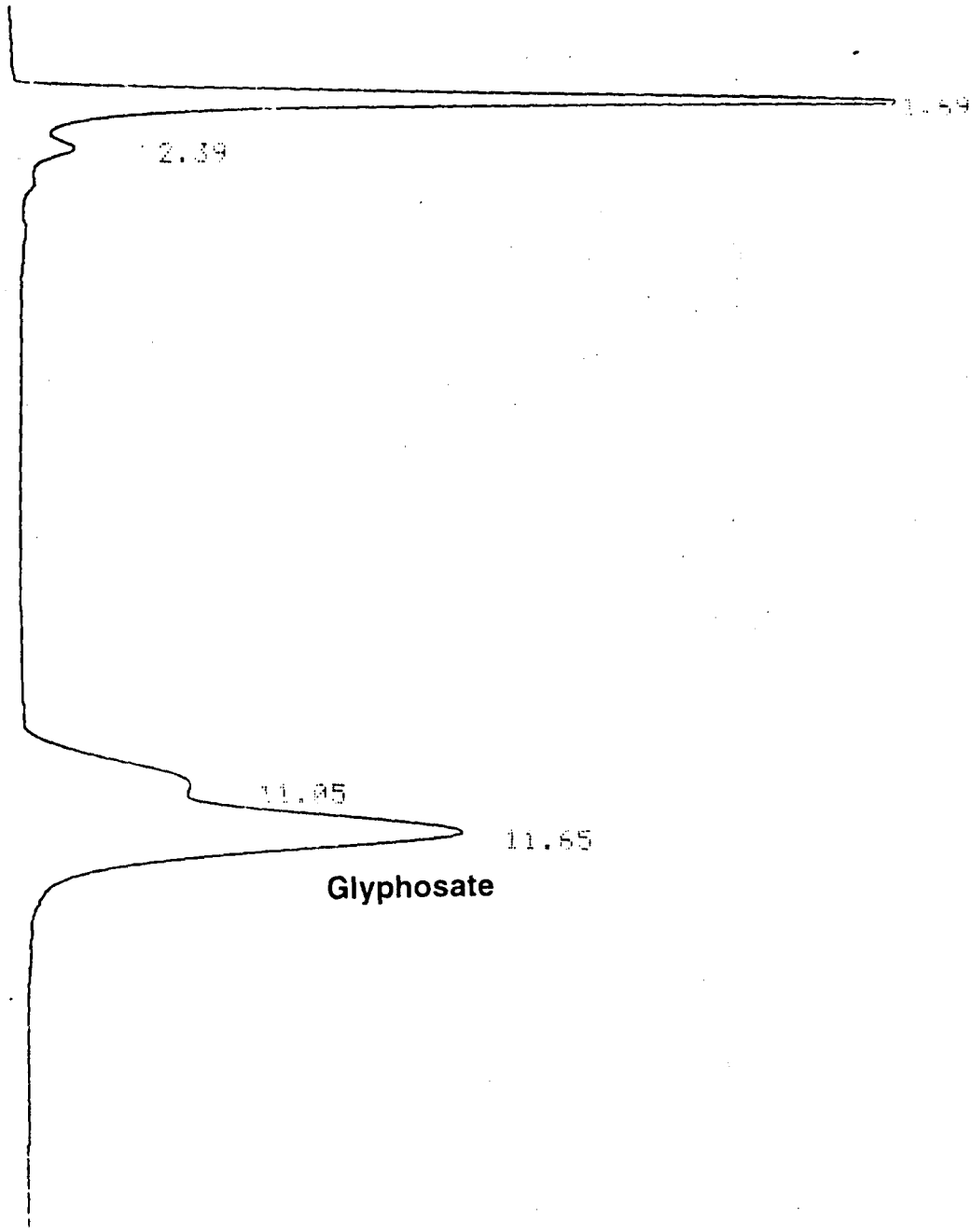
A theoretical plate value of approximately 500 was calculated for the column. Since a well packed column in good condition should possess several thousand theoretical plates, this was an indication that the column was in poor condition and was likely to be the cause of the peak shape and retention time problems. Accordingly the column was dismantled, emptied and thoroughly cleaned. New seals and frits were fitted and the column repacked with 5 µm Hypersil Aminopropyl (APS) bonded silica using a Shandon column packer.

The repacked column was tested as previously and a theoretical plate number (N) of approximately 5000 was obtained. On running a derivatised glyphosate sample a much longer retention time and a much improved peak shape were obtained. However, when an attempt was made to measure the linearity of the detector response, the intermittent peak splitting problem resurfaced (see figure 2.1). Since the repacked column had given a satisfactory result for N, suspicion turned to the injection valve; scarred or pitted surfaces in the injector can cause dead volumes and these may distort the peak shape. A Waters model injector was substituted for the Rheodyne system and this initially seemed to solve the problem, however it soon returned. Again, suspicion returned to the column. Since the column had functioned well initially, consideration was given to the possibility that the column was being damaged by some means. Since the mobile phase was not unusual and was

prepared as recommended by Rabel (1979) for ion exchange packing materials, and the column was rinsed with water followed by methanol at the end of each working day, it was thought unlikely that this was causing any degradation. The other obvious source of possible damage to the column was the sample being injected. Since traces of acetone are likely to remain in the sample after the derivatisation reaction and acetone can react with the amino groups of the packing material to form Schifts bases (Meyer, 1988) this seemed a likely source of damage to the column. To avoid this, an attempt was made to eliminate acetone from the derivatisation reaction by substituting isopropanol. This did not appear to interfere with the derivatisation of glyphosate, but gave rise to much larger artefact peaks on the chromatogram. In order to investigate the problem further, the column was disconnected from the system and the condition of the packing material at the end of the column was checked. Two things were apparent, a void in the packing material had developed and the packing material was discoloured. Either or both of these features may have been the cause of the peak splitting. The discoloured material may have had different chromatographic properties to the intact packing material leading to distortions in the chromatography, while the void would create a "dilution space", allowing the injected sample to be diluted many-fold before coming into contact with the column, resulting in decreased chromatographic efficiency (Rabel, 1979). The void at the column end was probably due to compaction of the packing material. This problem is likely to have been exacerbated by the self-packed nature of the columns. Discolouration of the packing material may well have been the result of the production of Schifts bases, as mentioned earlier. To get around these problems two partial solutions were implemented. A guard column packed with the same material as the analytical column was placed on line betweeen the injector and the column. This was designed to intercept any acetone before it reached the analytical column. Since it was not economically feasible to

purchase pre-packed columns, which should be less prone to compaction of the packing material, the voids were hand filled when they occurred with fresh packing material. These measures eliminated the peak shape problems long enough to make the method usable.

Figure 2.1 Chromatogram Showing Shouldered Glyphosate-FMOCCl Peak



2.3 OPTIMISATION OF THE CHROMATOGRAPHY

2.3.1 INTRODUCTION

In HPLC the chromatographic conditions can be optimised by altering both the stationary phase (column packing) and by altering the components of the mobile phase. This gives a tremendous range of variables. Moye & St John (1980) used Waters Association μCarbohydrate and μNH_2 columns for the analysis of FMOCCl derivatised glyphosate, while Roseboom & Berkoff (1982) used a Shandon Hypersil APS (NH_2) column. Yusof (1988) tested a range of anion exchange packing materials including Vydex and Ionosphere anion exchange columns but found the most suitable column to be the Shandon Hypersil APS column. Since problems had been encountered with this type of column, it was decided to examine the suitability of more robust packing materials. Huber & Calabrese (1985) used a C-18 column (generally regarded as the most reliable packing) for the analysis of glyphosate after esterification of the phosphoric acid moiety and reaction with FMOCCl. Accordingly, the use of a C-18 column with glyphosate derivatised with FMOCCl only, appeared to be worth trying. A cyanopropyl (CN) column was also tried since CN is the next most polar packing material after NH_2. The replacement of the NH_2 column can only be legitimately considered if the alternative packings match its performance, since resolving power is likely to be of paramount importance when analysing plant extracts.

The mobile phase used by Moye & St John (1980) consisting of 0.1M pH4 phosphate solution containing 25% acetonitrile had been used in the initial work with the method. This mobile phase was also used by Yusof (1988). Roseboom & Berkoff used 0.05M NaH_2PO_4 in 3:2 methanol/water adjusted to pH5 while Huber & Calabrese used a gradient from 20 to 100% acetonitrile

with 0.1% trifluoroacetic acid with the C-18 column. Little information has been given in the literature on the rationale behind these mobile phases. To address this, some of the possible permutations in the composition of the mobile phase were examined. There are two reasons why this is worth doing. First, to determine which mobile phase gives the best combination of peak shape and retention time and, second, by observing the effect that varying the components of the mobile phase has on the retention time it may be possible to move the peak of interest away from interferences when necessary. There is some debate as to the best way to examine the effects of mobile phase variations. Some workers (Deming et al., 1984) advocate a "full factorial" technique which involves studying the effect of each variable at different values of all the other variables. Since at this stage only one component is present in the sample this was thought to be too time consuming, therefore the simple strategy of altering one component of the mobile phase at a time, while the others were held constant, was adopted. Thus the effect of the percentage of acetonitrile was examined with 0.1M phosphate solution at pH4. The effect of ionic strength was examined at 30% acetonitrile and pH4 and the effect of pH was examined at 0.1M phosphate and 30% acetonitrile. Using methanol in place of acetonitrile was also considered, since methanol is cheaper and less toxic. Finally, because phosphate does not buffer at pH4 (Dolan, 1990) the use of acetate buffer at pH4 was examined.

2.3.2 EXPERIMENTAL

The HPLC system and derivatisation procedure outlined in Section 2.2.2 was used. Two Shandon 250mm x 4.6mm i.d. HPLC columns were thoroughly cleaned and fitted with new seals and frits. One was packed with Shandon Hypersil octadecylsilane ODS-2 (C-18) material while the other was packed

with Shandon Hypersil cyanopropyl (CN) packing material. A suitable mobile phase for the C-18 column was developed following the scheme of Snyder & Kirkland (1979). This involved starting with 100% acetonitrile and working down to 20% acetronile, 80% water in 20% steps. No mixture of acetonitrile/water alone gave sufficient retention and resolution of the glyphosate peak. The same 0.1M pH4 phosphate solution as used in 2.2.2.2 was then used with various percentages of acetonitrile.

The mobile phases for use with the NH_2 column were prepared as follows. To examine the effect of the percentage of acetonitrile, 0.1M pH4 phosphate solution was prepared as in section 2.2.2.2, then amended with acetonitrile to give mobile phases containing 10, 20, 25, 30 and 40% (V/V) acetonitrile. To study the effect of ionic strength, 1.36, 6.80, 13.61 and 20.91 g of potassium dihydrogen orthophosphate were dissolved in 1 litre of deionised water to give 0.01, 0.05, 0.10 and 0.15M phosphate solutions respectively. These were then adjusted to pH 4 with orthophosphoric acid, before acetonitrile was added to each to give a 30% (V/V) solution. Mobile phases with a range of pH values were prepared by amending a 0.1M phosphate solution as follows. To give pH 3.5 and pH 4.0 orthophosphoric acid was added, for pH 4.5 no pH adjustment was necessary and for pH 5.0 1M NaOH was added. These were again amended with acetonitrile to give a 30% (V/V) solution. The methanol amended mobile phase was prepared by adding methanol to a 0.1M pH 4 phosphate solution to give a 30% (V/V) methanol solution. Finally the acetate mobile phase was prepared by mixing 900 ml of 0.2M sodium acetate with 410 ml of 2M glacial acetic acid, to give a pH of 4, this was then amended to give a 30% (V/V) acetonitrile solution.

2.3.3 RESULTS AND DISCUSSION

2.3.3.1 ALTERNATIVE COLUMN PACKINGS

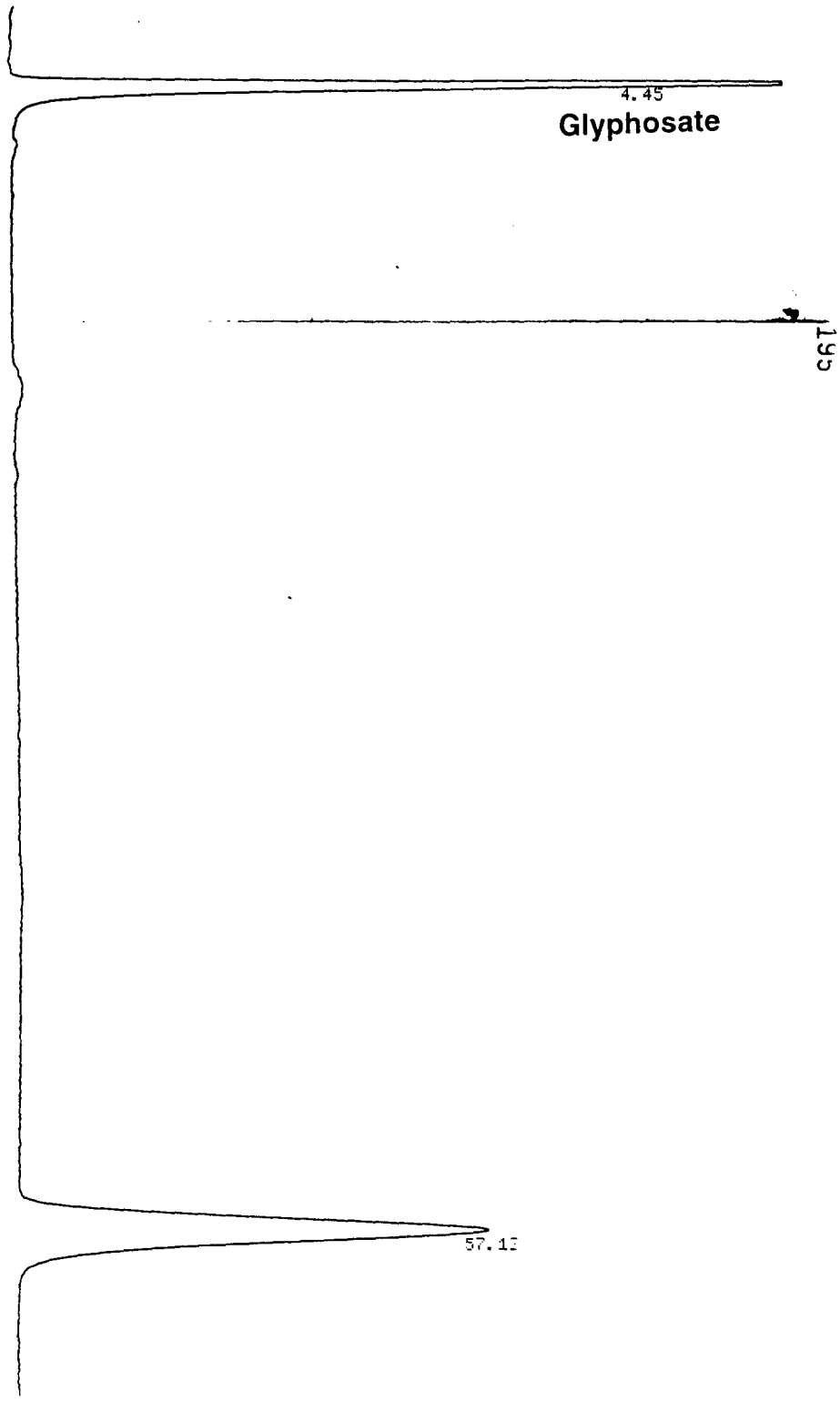
Table 2.3 gives details of how the retention time of glyphosate altered with the percentage of acetonitrile on the C-18 column.

Table 2.3 Effect of acetonitrile concentration on retention time

% Acetonitrile	RT (minutes)
10	>60
20	31.0
25	8.8
27	5.2
30	4.3
40	unretained

Although the glyphosate - FMOCCl peak could be retained on the C-18 packing material, the chromatography was extremely sensitive to the acetonitrile content of the mobile phase, and a large late eluting artefact peak was present on the chromatogram (see fig. 2.2). This artefact peak could have interfered with subsequent analyses. Although it was possible to circumvent this problem by using a gradient programme to flush off the interfering peak, because the chromatography is very sensitive to the organic modifier content, consistent results were unlikely to be obtained from a gradient system. The cyanopropyl column gave a similar elution pattern but with much shorter retention times for both glyphosate and the artefact peak. Neither C-18 nor CN column packings offered any meaningful improvement in the chromatography obtained with an NH_2 column.

Figure 2.2 Chromatogram Showing Late Eluting Artefact Peak When C-18 Columns Were Used



2.3.3.2 OPTIMISATION OF THE MOBILE PHASE FOR USE WITH A NH_2 COLUMN

The results obtained with the various mobile phase compositions are given in Tables 2.4 - 2.7.

Table 2.4 The effect of increasing acetonitrile concentration on retention time (Acetonitrile/0.1M phosphate solution at pH4)

% Acetonitrile	Retention Time (mins)
10	40.0
20	21.6
25	17.7
30	12.2
40	5.1

Table 2.5 The effect of increasing ionic strength on retention time (30% acetonitrile phosphate solution at pH 4)

Molarity	Retention Time (mins)
0.01	> 60.0
0.05	27.5
0.10	12.6
0.15	8.3

Table 2.6 The effect of pH on retention time

(30% acetonitrile/0.1M phosphate solution)

pH	Retention Time (mins)
3.5	10.0
4.0	11.8
4.5	10.8
5.0	9.0

Increases in the percentage of acetonitrile and in the ionic strength of the mobile phase both resulted in reduced retention of the glyphosate peak. This was expected since increasing the ionic strength increases the competition for binding sites between the mobile phase components and glyphosate, while increasing the percentage of organic modifier increases the affinity of the derivatised glyphosate for the mobile phase. (Snyder & Kirkland, 1979). Increasing the pH had the effect of increasing the retention time up until pH 4, then at higher values of pH the retention time decreased. This is somewhat different to what was theoretically predicted. Generally with anion exchange systems increasing the pH results in a decrease in solvent strength and an increase in retention time. However there is a complex equilibrium in the system between the pH of the mobile phase, the charge on the stationary phase and the charge on the analyte. An additional complication in this case was the difference in preparation of the mobile phase at the various pHs, which resulted in variations in the ionic strength. In any case pH 4 seemed to be the optimum pH. For much of the preliminary work with standard solutions, an acetonitrile content of 30% and a molarity of 0.1 offered a good compromise between analysis time and resolution. Neither the mobile phase amended with methanol in place of acetonitrile or the acetate buffered mobile phase were able to elute glyphosate in under an hour and this was unsatisfactory.

As previously demonstrated, the NH_2 stationary phase is prone to degradation and as a result the mobile phase composition may have to be varied to produce acceptable chromatography. Columns in good condition may require stronger mobile phases in order to keep the analysis time to a reasonable length. Conversely, older columns may require the use of progressively weaker mobile phases to achieve sufficient retention.

2.4 REPRODUCIBILITY OF DERIVATISATION AND LINEARITY OF THE DETECTOR RESPONSE

2.4.1 INTRODUCTION

In order to utilise the external standard method to quantify glyphosate in extracts, the derivatisation reaction must give reproducible results and the response of the detector to the derivatised molecule must be linear over the working range of concentrations.

2.4.2 EXPERIMENTAL

A 1000 $\mu g ml^{-1}$ glyphosate stock solution was prepared by dissolving 0.1034 g of 96.7% glyphosate (Greyhound, UK) in 100ml of deionised water. This stock solution was then serially diluted to give 100, 50, 10, 5, 2, 1, 0.5 and 0.25 $\mu g ml^{-1}$ standards. In order to determine the linearity of the detector response duplicate 0.1ml aliquots of each standard were derivatised as outlined in section 2.2.2.3. To determine the reproducibility of the detector response 10 x 0.1 ml aliquots of the 1$\mu g ml^{-1}$ standard were also derivatised. Due to the irrepairable breakdown of the HPLC system outlined in section 2.2.2.1 a second fluorometric HPLC system was constructed. This comprised of a Perkin Elmer 400 series pump and autosampler, and a Shimadzu model RF-530 fluorescence detector. Chromatograms were variously recorded using a Perkin Elmer computing integrator or a Spectra Physics SP4290 integrator.

2.4.3 RESULTS AND DISCUSSION

The linearity of the detector response was determined by plotting the concentration of each standard against the mean area measured for the replicate analyses, the plot obtained is shown in Figure 2.3. The r^2 value

calculated on an Excel spreadsheet was 0.9998, illustrating that the detector response is linear over almost 3 orders of magnitude. The areas obtained for the 10 replicate analyses of the 1 μgml^{-1} standard analysed to determine the reproducibility of the derivatisation reaction are shown in Table 2.7. The coefficient of variation was calculated to be 6.0%. Since this value also included the variability of the HPLC determination and integration, it was well within acceptable limits.

Table 2.7 Reproducibility of the determination

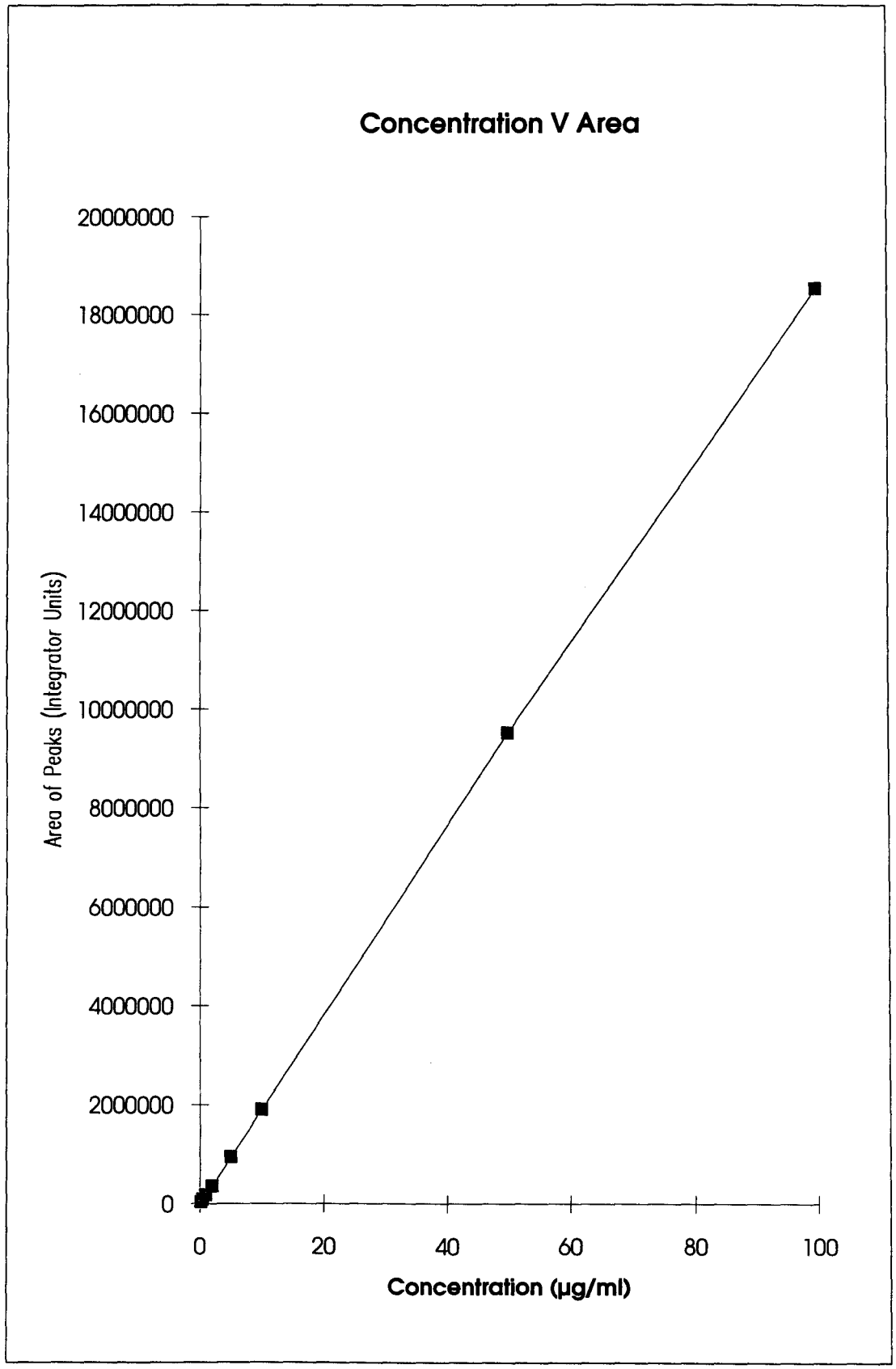
Replicate	Peak Area	Mean	Standard Deviation
1	4,304,909	3,959,686	237,153
2	3,997,835		
3	4,092,507		
4	3,589,450		
5	3,841,621		
6	3,645,698		
7	4,269,508		
8	4,071,900		
9	3,876,251		
10	3,907,181		

$$\text{Coefficient of Variation} = \frac{\text{Standard Deviation}}{\text{Mean}} \times \frac{100}{1} \%$$

$$= \frac{237153}{3959686} \times \frac{100}{1} \%$$

$$= 6.0\%$$

Figure 2.3 Plot of Glyphosate Concentration vs Peak Area



2.5 INVESTIGATION INTO THE SUITABILITY OF SIZE EXCLUSION CHROMATOGRAPHY AS A PURIFICATION STEP IN THE ANALYSIS OF GLYPHOSATE RESIDUES

2.5.1 INTRODUCTION

Size exclusion chromatography (SEC) is the preferred name for the two independently evolved techniques of gel filtration (GF) and gel permeation chromatography (GPC). These techniques essentially differ only in terminology; gel filtration indicates the use of aqueous eluents to fractionate biological molecules, whilst gel permeation chromatography indicates the use of organic solvents. GF first became practicable following the introduction of "Sephadex" cross link dextrans, by Porath & Flodin in 1959.

SEC is a form of chromatography where (ideally) separation is solely governed by molecular "size". Generally when discussing SEC molecular weights are used. It must be remembered, however, that the over-riding factor is molecular size and that the two parameters do not always vary in unison. The stationary phase for SEC is an inert porous polymer which contains a large volume of solvent held in pores. Solvent within the pores is normally considered to be stagnant and to exchange with the mobile phase only by diffusion. Solvent flow within the pores may become important where the polymer particle sizes are small and pore cross-sections large (Groh & Halasz, 1981).

Thus the total column volume (Vt) is comprised of three components; the volume occupied by the polymer (Vg), the volume occupied by eluent within the polymer pores (Vi) and the volume occupied by eluent outwith the polymer pores (Vo).

The basis of the separation mechanism revolves around the access of solute

molecules to the pores of the polymer. The gels are manufactured with a maximum pore size (the exclusion limit) and solute molecules above this size will be unable to enter the pores. Such solutes will therefore traverse a relatively uninterrupted path through the gel, and will emerge within the "void volume" of the column which equates with Vo mentioned earlier. Obviously this mechanism alone would only allow the sorting of molecules into those above or below a certain size, depending on the pore radius. The mechanism by which fractionation of molecules below this exclusion size occurs can be explained by more detailed consideration of the processes occurring within the pores. Because solute molecules themselves occupy a finite space, any solute molecule has only limited access to the volume of the stagnant eluent, larger solutes will therefore have a lower pore volume available to them and will as a result reside in the pores for a shorter duration.

Theoretically, because the technique utilises the ubiquitous property of size as the mechanism for separation, it should be applicable to the initial purification of any matrix extract for pesticide analysis. Since there are a wide range of "Sephadex" gels available with various exclusion limits, which utilise aqueous eluents, the technique should prove a useful purification step for the analysis of glyphosate.

To date the use of SEC as a clean-up step in the analysis of glyphosate has been reported by two groups. Guinivan et al. (1982) used SEC chromatography as part of their clean-up procedure for the determination of glyphosate in blueberries by GC with electron capture detection. Bio-gel P-2, a polyacrylamide gel with a fractionation range of 100 - 1800 daltons, was used with pH 2.1 water as the eluent. The gel was calibrated with ^{14}C-glyphosate prior to use. Use of SEC was designed to eliminate most of the sugar present. Pigments were also eliminated to some extent by adsorption. Sieber et al.

(1984) used the same gel and eluent as the second stage of a purification procedure for glyphosate in kiwi fruit and asparagus extracts. Determination was again by GC, this time with a flame photometric detector. The authors found that the elution volume of glyphosate from the column was apt to vary. To date none of the published methods using HPLC have utilised SEC as part of the clean-up strategy. This is a little puzzling. Since glyphosate is extracted by aqueous solvents, a range of high molecular weight co-extractives such as proteins and starch are likely to be present. SEC would therefore appear to be the ideal technique to remove these components prior to the use of adsorption based techniques which such co-extractives may interfere with. Calibration of the gel is important, not only to give an indication of where glyphosate will elute but also to determine if any interactions with the gel are taking place. Although in theory no interactions between the gel and the solute should occur, this is not always the situation in practice. If the elution volume of glyphosate indicates that undesirable interactions are occurring, it may be possible to counteract them with changes in the mobile phase composition.

2.5.2 EXPERIMENTAL

2.5.2.1 COLUMN AND MOBILE PHASE PREPARATION

Sephadex G-25 and G-75 gels (Pharmacia) were prepared for packing by boiling in water for approximately three hours. Once the gel returned to room temperature, excess water was decanted and the slurry poured into a glass column (100cm x 2.4cm i.d.) plugged with glass wool. The tap on the column was then opened to ensure even packing of the gel. Once the gel had settled, a disc of filter paper (Whatman No. 1) was placed on the surface of the gel to minimise disturbance of the bed during sample application. The void volume

(Vo) of each column was determined by measuring the volume of mobile phase required to elute "Blue Dextran", a high molecular weight polysaccharide that is excluded by the gel pores.

In addition to deionised water two other mobile phases were used. Acidified water (pH 2) was prepared by adding 1.2 ml of concentrated hydrochloric acid to two litres of deionised water. Potassium dihydrogen orthophosphate was prepared by dissolving 6.8 g in a litre of deionised water.

2.5.2.2 CALIBRATION WITH GLYPHOSATE STANDARDS

1 ml of a 10 $\mu g ml^{-1}$ glyphosate standard solution was applied to the top of the column using a pasteur pipette. The column tap was opened and the glyphosate solution allowed to run into the column bed. The top of the column was then rinsed twice with approximately 1 ml of mobile phase. After the second rinse had been applied to the column, the reservoir was filled and the flow rate adjusted to approximately 1 $ml min^{-1}$. The eluent was collected in 20ml fractions. Aliquots of each fraction were then derivatised as outlined in section 2.2.2.3 and glyphosate was determined using the HPLC system described in section 2.4.2.

2.5.2.3 RESULTS AND DISCUSSION

The glyphosate elution profiles obtained with each gel are shown in tables 2.8 to 2.12 below.

Table 2.8 Column 1 : Run 1

Sephadex G-25 gel, void volume = 30ml, total volume = 60 ml, deionised water eluent.

Fraction (ml)	% Recovered Glyphosate
0 - 20	0.3
20 - 40	92.1
40 - 60	1.4
60 - 80	0.2

Table 2.9 Column 1 : Run 2

Sephadex G-25 gel, void volume = 30 ml, total volume = 60 ml, pH 2 water eluent.

Fraction (ml)	% Recovered Glyphosate
0 - 20	0.4
20 - 40	20.7
40 - 60	76.0
60 - 80	2.3
80 - 100	0.6

Table 2.10 Column 2 : Run 1

Sephadex G-75 gel, void volume = 30ml. total volume = 58 ml, deionised water eluent.

Fraction (ml)	% Recovered Glyphosate
0 - 20	0.2
20 - 40	84.9
40 - 60	13.4
60 - 80	1.1
80 - 100	0.5

Table 2.11 Column 2 : Run 2

Sephadex G-75 gel, void volume = 30 ml, total volume = 58 ml, 0.05M KH_2PO_4 eluent.

Fraction (ml)	% Recovered Glyphosate
0 - 20	0.2
20 - 40	75.6
40 - 60	21.3
60 - 80	2.7

Table 2.12 Column 3 : Run 1

Sephadex G-25 gel, void volume = 41ml, total volume = 80 ml, deionised water eluent.

Fraction (ml)	% Recovered Glyphosate
0 - 20	0.2
20 - 40	81.0
40 - 60	18.6
60 - 80	0.1

As gels with a higher degree of cross linking (i.e. smaller pores such as G-10 and G-15) were unavailable, G-25 and G-75 gels were used. Since glyphosate is a small molecule and should be able to fully permeate the pores of both gels, the larger pore sizes of these gels were not envisioned to be a problem. The G-25 and G-75 gels packed initially had total volumes of 60ml and 58ml respectively. Assuming that the volume occupied by the molecules of the gel is negligible, glyphosate, as a fully included molecule, would be expected to elute from these columns in a volume close to the total volume. Both columns were run initially using deionised water as the mobile phase and the majority of glyphosate was recovered in the 20-40ml fractions from each column. Elution of glyphosate so early would be unlikely to give good resolution of the compound from matrix material in an extract sample. After some consideration three possible explanations for the poor performance of the gels were examined:

1) The gels may have been damaged during packing or by the weight of eluent above the column. This would result in the blocking or crushing of

pores in the gel, denying access to glyphosate molecules.

2) At pH7, the majority of glyphosate molecules should be present as the dianion. If the gel itself possessed a negative charge from the presence of residual carboxylic acid groups (Braithwaite & Smith, 1985), glyphosate may be electrostatically repulsed from the gel.

3)The use of G-25 and G-75 gels, which possess larger than ideal pores, may have meant that exchange of solvent into and out of the pores was significant. This would have resulted in faster transport of glyphosate down the column.

Although gels with a lower degree of cross-linking were unavailable, preventing investigation of the effect of pore size (3), it was possible to examine the other two possibilities. The original G-25 and G-75 gels were run using pH2 water and 0.05M KH_2PO_4 as the respective eluents. Lowering of the eluent pH will reduce the negative charge on both glyphosate and the gel and should therefore eliminate any electrostatic repulsion. Using 0.05M KH_2PO_4 was intended to have the same effect by introducing a counter ion into the system to balance the effect of any carboxylic acid groups in the gel. Also a second G-25 column was packed, on running this column the weight of eluent above the packing material was kept to a minimum to prevent damage to the column. This column was eluted with deionised water as previously and gave the same elution pattern with the majority of glyphosate eluting in the 20-40 ml fraction. Using 0.05M KH_2PO_4 as the eluent did not significantly alter the elution pattern. However, the use of pH2 water resulted in increased retention of glyphosate, with the majority eluting in the 40-60 ml fraction. This was a significant improvement. The elution of glyphosate with pH2 water gives some scope for the use of SEC to clean up extracts. This finding is in agreement with the published methods of Guinivan et al. (1982) and Seiber et al. (1984), although neither group published any justification for their mobile phase selection.

2.6 INVESTIGATION INTO THE SUITABILITY OF SOLID PHASE EXTRACTION CARTRIDGES AS A PURIFICATION STEP IN THE ANALYSIS OF GLYPHOSATE RESIDUES.

2.6.1. INTRODUCTION

Solid phase extraction (SPE) cartridges resemble small liquid chromatography columns. They generally comprise of a plastic tube (most often a polypropylene syringe barrel) with porous metal or plastic frits at both ends and 100–500 mg of 40µm stationary phase particles in the middle. This is the most common configuration since the sample sizes, sample volumes and elution volumes are appropriate for subsequent analysis by gas or liquid chromatography (Markell et al., 1991). The use of solid sorbents has been common for many years. These materials have generally been used in large, manually packed glass columns. SPE cartridges in the modern format were first marketed by Waters Associates in 1978, and they offer a number of advantages over traditional open column chromatography, including:

1) The availability of a wide range of sorbent species
2) The cartridges are reproducibly packed
3) Reduced solvent consumption
4) Methods utilising SPE cartridges are easy to automate
5) Cost

The sorbents used in SPE cartridges are manufactured from a silica backbone to which the required functional group is covalently bonded (see Appendix II). The functional groups available range from non-polar species such as octadecyl (C18) to polar species such as diol (2OH) and also include cation and anion exchangers. Unreacted silanol groups in the sorbent mean that the sorbents are also capable of secondary reactions with analytes that may be quite different from the primary interaction. The wide range of sorbents

provides the analyst with a powerful and selective tool. In order to get the best results from SPE, methods are generally developed and optimised with standards prior to use on samples. Method optimisation can minimise the risk that interferences from the sample matrix will interfere with the clean-up. A typical SPE procedure involves six steps (Calverley, 1993):

1) Sample pre-treatment
2) Column solvation
3) Column pre-equilibration
4) Sample application
5) Elution of interferences
6) Analyte elution

The principal factors that required optimisation in the development of a method for glyphosate isolation were determining the best sorbent, optimising sample pre-treatment and determining the best rinsing and elution solvents.

To date, SPE cartridges have not been used in a published method for glyphosate analysis. Most authors have used one or both of cation and anion exchange resins manually packed in the traditional manner. In this laboratory, strong cation (SCX) and strong anion exchange (SAX) SPE cartridges were evaluated for the retention of glyphosate from standard solutions and from a potato extract (Yusof, 1988). SCX cartridges did not retain glyphosate from standard solutions adjusted to pH2. SAX cartridges retained glyphosate from standard solutions adjusted to pH12 but not from potato extract at pH12. Since 100mg SPE cartridges (the smallest size available) were used it is possible that the ionic strength of the potato extract was too great to allow retention of glyphosate. Citric acid was identified as the matrix component most likely to interfere with glyphosate retention. Citrate anions have a high selectivity

for anion exchange SPE cartridges (Van Horne, 1985). SAX, SCX, C18 (octadecyl silyl) and NH_2 (aminopropyl) cartridges were then evaluated to see if they would retain matrix interferences without retaining glyphosate, and thus give cleaner chromatograms. None of the cartridges tested were successful in this aim.

A range of cartridges were included in a preliminary screen to determine which types of sorbent material could retain glyphosate and were thus suitable for optimisation. Since SCX and SAX cartridges had been unsuccessful before, these sorbents were omitted from the screen. The preliminary screen also ensured that cartridges were not eliminated on a purely theoretical basis, as the possibility of secondary interactions makes precise prediction of the behaviour of the sorbents difficult.

2.6.2 EXPERIMENTAL 1

The following SPE cartridges (100g Bond Elut: Varian) were tested to see if they would retain glyphosate from a standard solution : C18, C8, C2, CH, 20H, PH, CN and NH_2. Each cartridge was solvated by passing 0.5 ml of methanol through the column, then rinsed with 0.5 ml of deionised water, 0.1 ml of a 10 $\mu g ml^{-1}$ glyphosate standard solution was then applied to the column and the column was rinsed with 1 ml of deionised water. Aliquots of the rinse were then derivatised using the method outlined in section 2.2.2.3 and assayed for glyphosate by HPLC.

2.6.3 RESULTS AND DISCUSSION 1

The percentage of applied glyphosate recovered in the 1 ml deionised water rinse from each column is given in table 2.13 below. The presence of

glyphosate in the column rinse indicates that the compound is not being retained on the sorbent.

Table 2.13 Percentage of glyphosate recovered from SPE cartridges after elution with water

Cartridge Type	% Applied Glyphosate in 1 ml rinse
C18	77
C8	77
C2	71
CH	90
2OH	60
PH	90
CN	0
NH_2	0

The most successful cartridges in retaining glyphosate were NH_2 and CN. These did not allow any glyphosate to elute in the aqueous rinse and were therefore automatic choices for further development. Of the other sorbents the 20H material retained slightly more glyphosate than the others. Although 60% of the applied glyphosate was unretained, this sorbent was included for further investigation because simple sample pre-treatments, such as lowering the pH, could result in improved retention. The remaining cartridges were considered unlikely to retain sufficient glyphosate to be viable, no matter what sample pre-treatment was conducted. In order to produce a viable method for glyphosate isolation, the best sorbent, optimum sample pH for retention and the most effective rinsing and elution solvents had to be determined. Since

the compound is extracted by aqueous solvents, water is likely to be the most effective solvent for the removal of interfering species. As glyphosate is insoluble in organic solvents, the eluting solvent will also have to be an aqueous solvent of some description. The ionic nature of glyphosate means that pH is likely to play a significant part in the effectiveness of the eluting solvent, especially in the case of the NH_2 sorbent which can act as an anion exchanger. The pka of the NH_2 sorbent is 9.8, above this pH therefore the majority of the functional groups will be neutral and unable to retain glyphosate by ionic bonding. Phosphate buffer at pH11 was therefore prepared as the elution solvent for this experiment.

2.6.4 EXPERIMENTAL 2

Duplicate NH_2, CN and 20H SPE cartridges (1g, Bond Elut, Varian) were solvated with 0.5 ml of methanol and rinsed with 1 ml of water. 1 ml of a 1 $\mu g ml^{-1}$ glyphosate standard solution was applied to the top of the cartridges. The glyphosate solution applied to each type of cartridge was acidified to pH2 with orthophosphoric acid while the others were left untreated (giving a pH of approximately 6). The glyphosate solution was then pushed through the cartridge under pressure from a syringe. The cartridges were subsequently rinsed with 2 x 1 ml aliquots of water and eluted with 2 x 1 ml aliquots of pH 11 phosphate buffer, again under pressure from a syringe. The various rinses and eluates were collected and aliquots derivatised as outlined in section 2.2.2.3, then assayed for glyphosate by HPLC.

2.6.5 RESULTS AND DISCUSSION 2

The percentage of recovered glyphosate determined in each fraction is shown in table 2.14, overleaf.

Table 2.14 Glyphosate retention by SPE cartridges

Cartridge Type	pH of Applied Standard	% Unret.	% in Aqueous Rinses		% in Eluate	
			0-1 ml	1-2 ml	0-1 ml	1-2 ml
NH_2	2	79.7		20.3*	0	0
NH_2	6	0	0	0	95.5	4.5
CN	2	67.1		29.5*	2.0	1.3
CN	6	83.6	13.5	2.9	0	0
2OH	2	34.4		65.6*	0	0
2OH	6	76.9	13.8	3.1	6.2	0

*The aqueous rinses were combined in these cases.

Applying glyphosate at pH6 to an NH_2 SPE cartridge gave the best retention of the compound. At pH2 the majority of the applied glyphosate was unretained on the NH_2 sorbent. Since the pka of the NH_2 sorbent is 9.8, at any pH below 9.8 the sorbent will be positively charged (Van Horn, 1985). Maximum positive charge will be reached at approximately pH7.8. At pH6 the majority of glyphosate will be present as the dianion (pKa_2 = 5.58) while at pH2 the majority will be in the mono-anion form. Hence, better retention at pH6 would be expected. However, the almost complete lack of retention at pH2 is surprising. It may be due to strong competition for binding sites from the orthophosphoric acid used to acidify the standard solution or may be due to the low pH damaging the sorbent. In any case, the pH of sample extracts applied to NH_2 sorbents is likely to be of great importance. Both the CN and 20H sorbents gave very poor results. Only the 20H at pH2 retained a significant amount of glyphosate and this was eluted in the aqueous rinse.

These results suggest that only the NH_2 sorbent is likely to retain glyphosate sufficiently to be effective in isolating glyphosate from crop extracts. Since aqueous salt solutions are difficult to concentrate by evaporation it is important that the compound is eluted from the sorbent in the minimum volume possible. Two additional elution solvents were therefore examined, 0.1M potassium dihydrogen phosphate adjusted to pH4 with orthophosphoric acid, which is the aqueous component of the HPLC mobile phase, and 0.2M sodium citrate. As previously mentioned, citrate ions are very selectively retained by anion exchange SPE cartridges and should therefore make an effective elution solvent.

2.6.6 EXPERIMENTAL 3

Two NH_2 SPE cartridges (5g) were solvated with 5 ml of methanol and rinsed with 5 ml of deionised water. 5 ml of a 1 $\mu g ml^{-1}$ glyphosate standard solution were applied to each cartridge and eluted. Each cartridge was then rinsed with 2 ml of deionised water. One cartridge was eluted with 4 x 1 ml aliquots of phosphate solution and the other with 4 x 1 ml aliquots of citrate solution. All the solutions were run through the cartridge under pressure applied by a syringe. The applied eluent, water rinse and the elution fractions were collected and aliquots derivatised as outlined in section 2.2.2.3 and assayed for glyphosate by HPLC.

2.6.7 RESULTS AND DISCUSSION 3

The percentage of the recovered glyphosate present in each fraction is given in table 2.15, overleaf, along with the recovery obtained for each cartridge

Table 2.15 Comparison of phosphate and citrate as elution solvents

Eluent	% Rec.	% Unret.	% H_2O Rinse	% 0-1ml Eluent	% 1-2ml Eluent	% 2-3ml Eluent	% 3-4ml Eluent
0.1M Phosphate	105.6	0.1	3.0	8.0	49.2	34.1	5.7
0.2M Citrate	112.2	0.0	0.43	12.6	80.2	6.2	0.7

Rec. = Recovery; Unret. = Unretained

Both cartridges successfully retained glyphosate from the standard solution. A small amount was detected in the unretained fraction from the phosphate eluted cartridge and a small amount was present in the aqueous rinses of the cartridges. More glyphosate was rinsed off from the phosphate cartridge. The breakthrough of glyphosate from this cartridge may indicate that the sorbent was not fully solvated prior to use. Despite this, these results were enouraging because the glyphosate was applied in 5 ml of water rather than 1 ml as used previously, and the results indicate that little or no elution from the sorbent occurs when water is the eluent. Overall recoveries were acceptable for both cartridges, although the recovery for the citrate cartridge was slightly high (112.2%). This may have been due to the errors inherent in subdividing the citrate eluent into four fractions. Citrate was the more effective eluent removing 92.8% of the glyphosate in the first 2 ml. Phosphate solution gave reasonable results with 91.3% eluted in 3 ml. Phosphate may be a useful alternative if it elutes glyphosate more selectively. Since citrate has a much higher affinity for the sorbent it may elute more interfering compounds from the cartridges.

2.6.8 SUMMARY

NH_2 SPE cartridges are able to retain glyphosate from standard solutions. This

retention is stronger when the glyphosate solution is applied at pH6 and is strong enough to allow H_2O to be used as the rinsing solvent. Glyphosate can be eluted off the cartridge with a variety of salt solutions, phosphate buffer at pH11 was less effective than 0.2M sodium citrate as an elution solvent. Having determined suitable conditions with glyphosate standard solution, this technique was then applied to barley extracts.

2.7 THE ANALYSIS OF GLYPHOSATE IN FORTIFIED BARLEY GRAIN USING NH_2 SPE CARTRIDGES AS THE PRINCIPAL CLEAN-UP STEP

2.7.1 INTRODUCTION

Barley was chosen as the test crop in this investigation for a number of reasons. It is an economically important crop in Scotland with 311,269 ha planted in 1992 (Anon., 1992). Barley for malting is the staple crop of the whisky industry and reliable germination of barley intended for malt is vital. At the time of writing, no figures were available on the proportion of the Scottish barley crop that is treated with glyphosate, however 236,107 ha of barley in England and Wales were treated with 174.83 tonnes of glyphosate in 1990 (Anon., 1990). The use of glyphosate on barley can, as outlined previously, have a detrimental effect on the germination of the grain produced. Since glyphosate affected seed can give a positive tetrazolium salt test (Don et al., 1990), analytical procedures that are sensitive enough to detect low levels (ppb) of glyphosate in grain would be a benefit in industries dependent upon reliable grain germination. Analysis of pesticide residues in grain is generally recognised as being difficult because of the low moisture content of the matrix. With glyphosate residue analysis, this problem is compounded by the polar nature of the herbicide. Aqueous solvents are necessary to extract glyphosate from the crop and the unique solvating properties of water means that these extracts will contain high levels of co-extractives. The taxing nature of this matrix means that if a suitable clean-up procedure for this crop can be developed, then it stands a good chance of being effective with a range of species.

2.7.2 EXPERIMENTAL

2.7.2.1 EXTRACTION AND FORTIFICATION OF BARLEY GRAIN

Barley grain that was untreated with pesticides was obtained from Scottish Agricultural Industries. Approximately 50 g of grain were homogenised in a Waring commercial blender with 200 ml deionised water and 100 ml of chloroform for 1 minute. The homogenate was transferred to 200 ml centrifuge bottles and spun at 4,300 rpm for 35 minutes. The supernatant was carefully decanted into a beaker and fortified with 5 ml of a 20 μgml^{-1} glyphosate solution. The extract was then partitioned, once against an equal volume of chloroform and once against an equal volume of diethyl ether. Trichloroacetic acid (5M) was added (to precipitate proteins) until no further increase in the cloudiness of the solution was apparent. The extract was centrifuged and the supernatant again decanted into a beaker. The pellet was re-suspended in a minimum volume of water and re-centrifuged. The supernatants were combined. An equal volume of acetone was added to precipitate starch and the solution filtered through a Whatman No.40 filter paper under vacuum. The organic layers were discarded. The resulting solution was reduced to less than 100 ml on a rotary evaporator and the pH was adjusted to between 6 and 7. The solution was transferred quantitatively into a 100 ml volumetric flask and diluted to volume with deionised water.

2.7.2.2 CLEAN-UP USING NH_2 SPE CARTRIDGES

Three NH_2 SPE cartridges (500 mg) were each solvated with 3 ml of methanol and rinsed with 2 ml of deionised water, using a syringe to control the flow rate. 5 ml of the fortified barley were applied to each cartridge. The first cartridge was rinsed with 2 ml deionised water and eluted with 3 ml of a 0.2M

sodium citrate solution. The second was eluted with 3 ml of citrate solution, without any prior rinse, and the third cartridge was rinsed with 2 ml of methanol and eluted with 3 ml of citrate solution. Duplicate aliquots of citrate eluate from each cartridge were derivatised and assayed for glyphosate by HPLC.

2.7.3 RESULTS AND DISCUSSION

Recoveries of glyphosate were calculated to be 40.2% for the first cartridge, 145.0% for the second and 0.0% for the third. Specimen chromatograms are shown in figures 2.4 to 2.6. By inspection of the chromatograms it was clear that rinsing the cartridge with water reduced the level of interference in the chromatograms and also reduced the amount of glyphosate retained by the cartridge. The recovery of 145% calculated for the unrinsed cartridge is a result of the error inherent in integrating "noisy" chromatograms. Methanol was entirely unsuccessful as a rinsing solvent since no glyphosate peak was obtained in the resulting eluent fraction. This absence of a glyphosate peak is difficult to explain. However, given that glyphosate is insoluble in methanol, this may be at the root of the problem. Although glyphosate applied in aqueous standard solution was not removed from the cartridge by rinsing with water, this is not the situation in this case. Competition for binding sites from co-extracted material in the extract was probably the principal reason for the low recovery. A preliminary step to remove some co-extracted material prior to application of the extract to the NH_2 SPE cartridge may produce better results. Since an array of SPE sorbents had already been shown not to retain glyphosate, some were examined for their suitability as a preliminary clean-up.

Figure 2.4 Chromatogram obtained without water rinse of NH_2 SPE cartridge

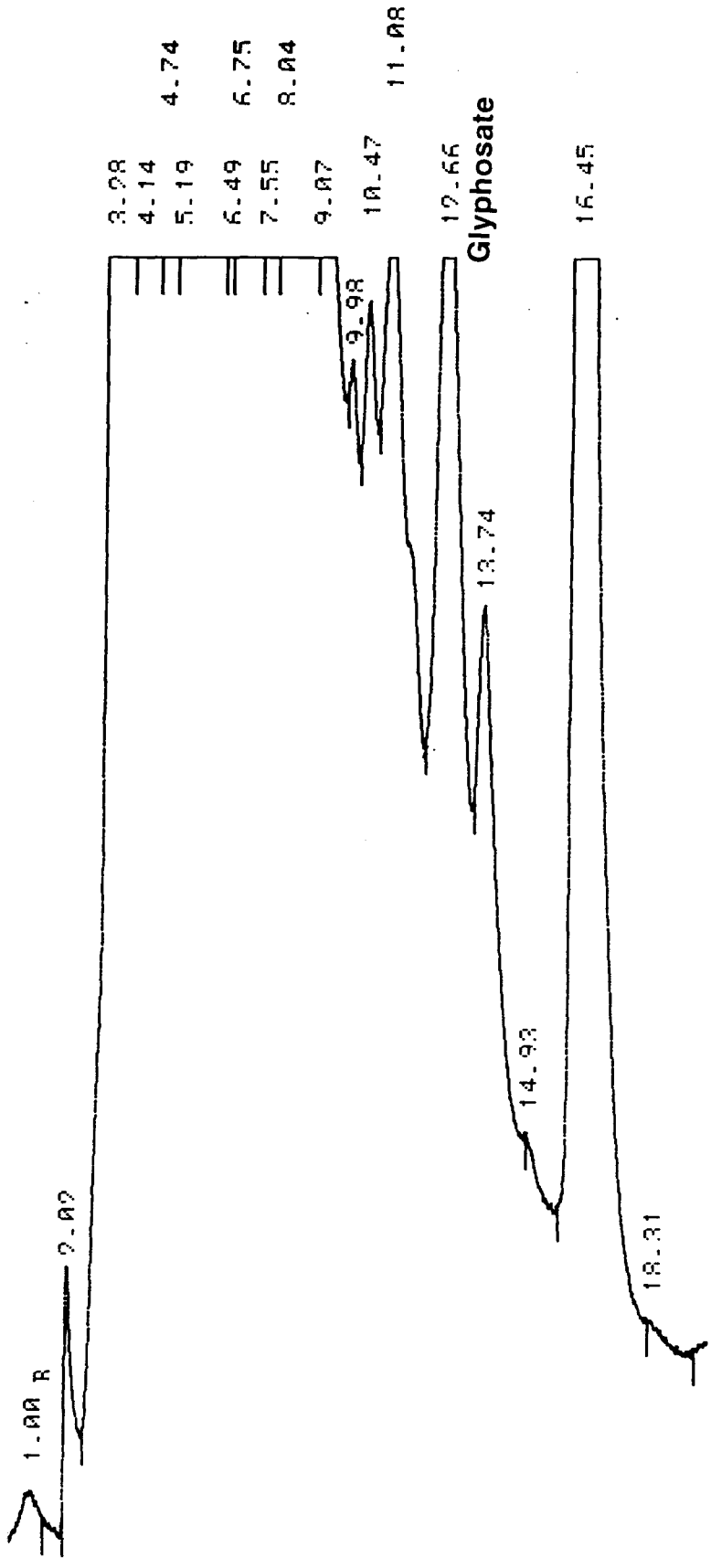


Figure 2.5 Chromatogram obtained with water rinse of NH_2 SPE cartridge

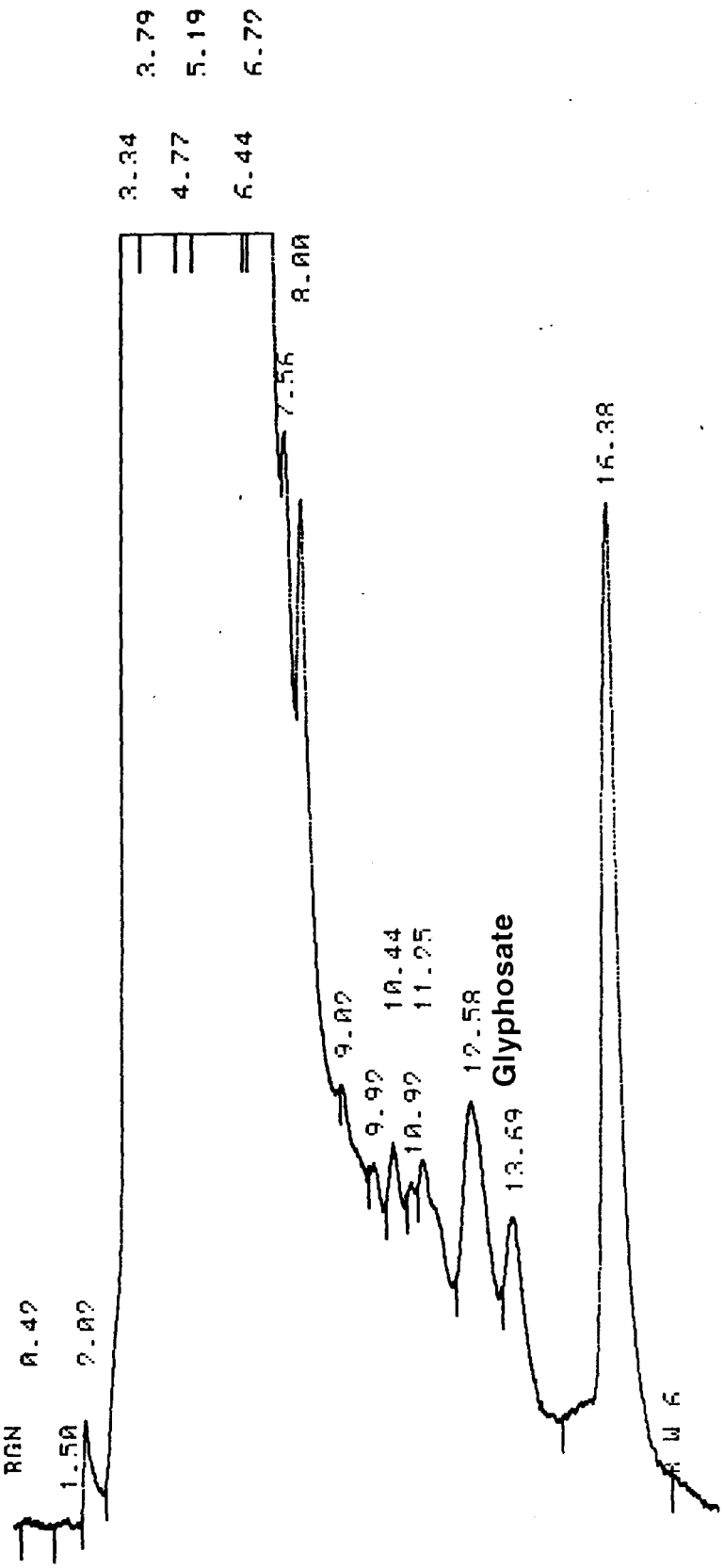
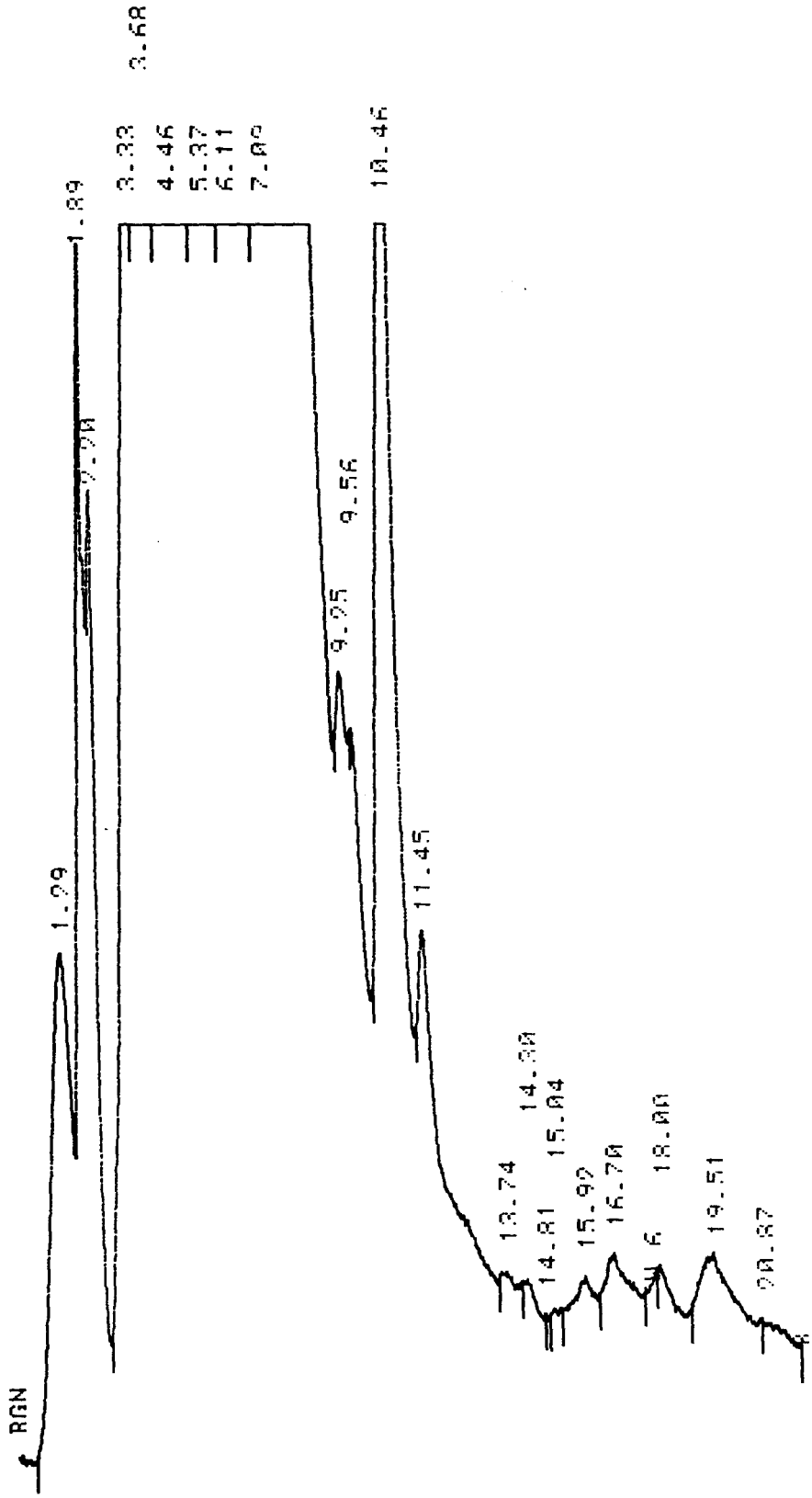


Figure 2.6 Chromatogram obtained with methanol rinse of NH_2 SPE cartridge



2.7.4 THE USE OF VARIOUS SPE CARTRIDGES AS PRE-TREATMENTS

Duplicate 2OH, CH and C-18 SPE cartridges were solvated with methanol and then rinsed with deionised water. The 2OH cartridge contained 3g of sorbent and the others contained 1g. A portion of the barley extract prepared in section 2.7.2.1 was acidified to about pH 2, to maximise the retention of co-extractives. 5 ml of the acidified extract were applied to each 2OH cartridge and 2 ml to each CH and C-18 cartridge.

The extract was eluted through the cartridge using a syringe to control the flow rate. The cartridge was then rinsed with 2 ml deionised water and the extracts combined and adjusted to pH 6. This solution was then applied to an NH_2 SPE cartridge and eluted with two portions of 0.2M citrate solution (2 ml and 1 ml respectively). Duplicate aliquots of the citrate eluents were then derivatised and assayed for glyphosate by HPLC.

2.7.5 RESULTS AND DISCUSSION

The recoveries calculated for each system are given in table 2.16 and specimen chromatograms are shown in figures 2.7 to 2.9.

Table 2.16 Use of various SPE cartridges as a preliminary clean-up

Cartridge	Eluent	µg Glyphosate	Overall Recovery%
2OH a	1^{ST} 2 ml	4.24	51.3
	2^{ND} 1 ml	0.89	
2OH b	1^{ST} 2 ml	4.58	50.6
	2^{ND} 1 ml	0.48	
CH a	1^{ST} 2 ml	2.41	66.3
	2^{ND} 1 ml	0.24	
CH b	1^{ST} 2 ml	3.52	96.2
	2^{ND} 1 ml	0.33	
C-18 a	1^{ST} 2 ml	3.09	81.3
	2^{ND} 1 ml	0.16	
C-18 b	1^{ST} 2 ml	3.30	87.0
	2^{ND} 1 ml	0.18	

Inspection of the chromatograms reveals that there is little to choose between the various cartridges used as a preliminary clean-up step. However, they all improved the chromatography in comparison with the unrinsed NH_2 cartridge only. Good, consistent recoveries were obtained with the C-18 cartridges whilst the CH cartridges gave good, but inconsistent, recoveries. The 2OH gave relatively poor recoveries. 2OH cartridges loosely hold glyphosate when applied at pH 2 (section 2.6.5), although the use of the 2 ml water rinse was intended to elute any retained glyphosate from the cartridge. Perhaps the use of 300 mg of sorbent rather than 100 mg resulted in more glyphosate being retained on the sorbent. The C-18 cartridges gave overall recoveries of 81.3% and 87.0%, which are acceptable at this fortification level (2 $\mu g g^{-1}$ or 2 ppm).

Figure 2.7 Chromatogram of barley extract after clean-up using 2OH SPE cartridge prior to NH_2 SPE cartridge

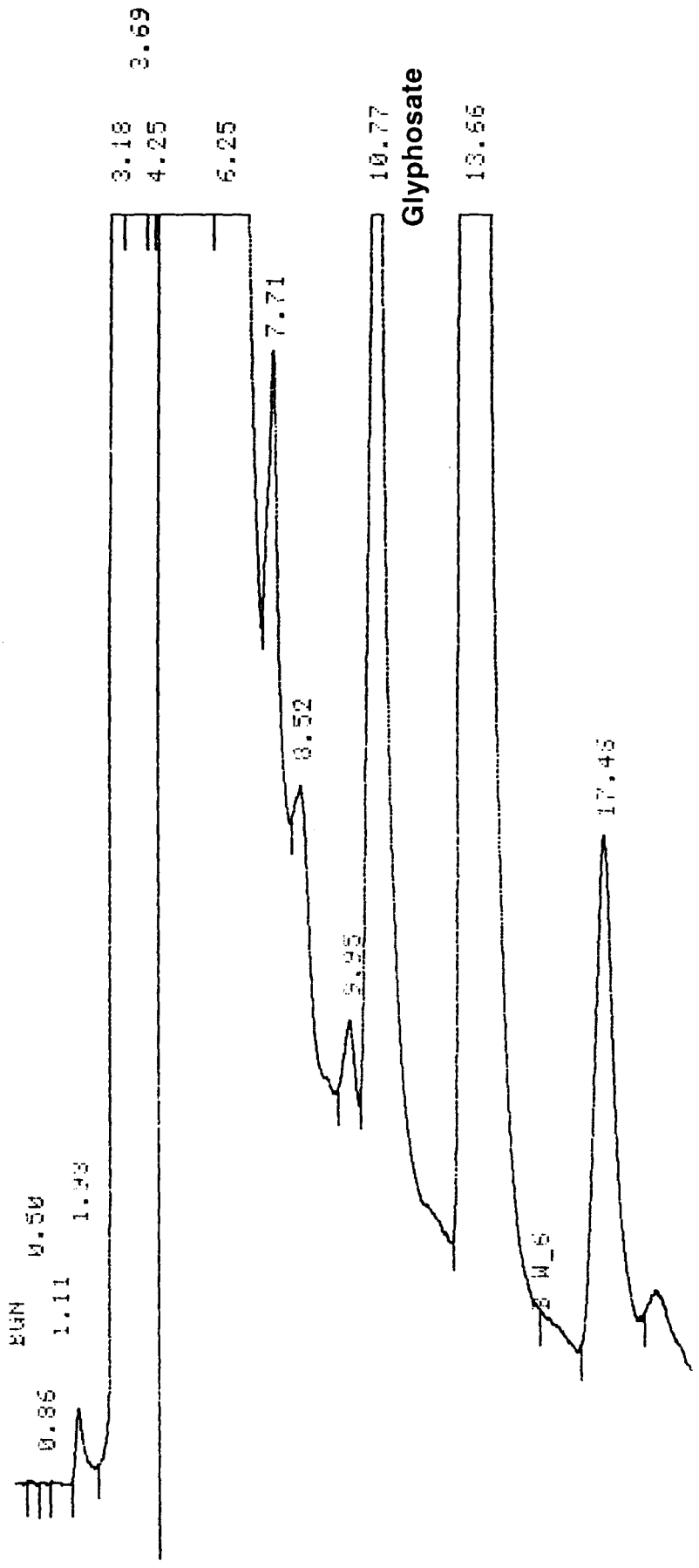


Figure 2.8 Chromatogram of barley extract after clean-up using C-H SPE cartridge prior to NH_2 SPE cartridge

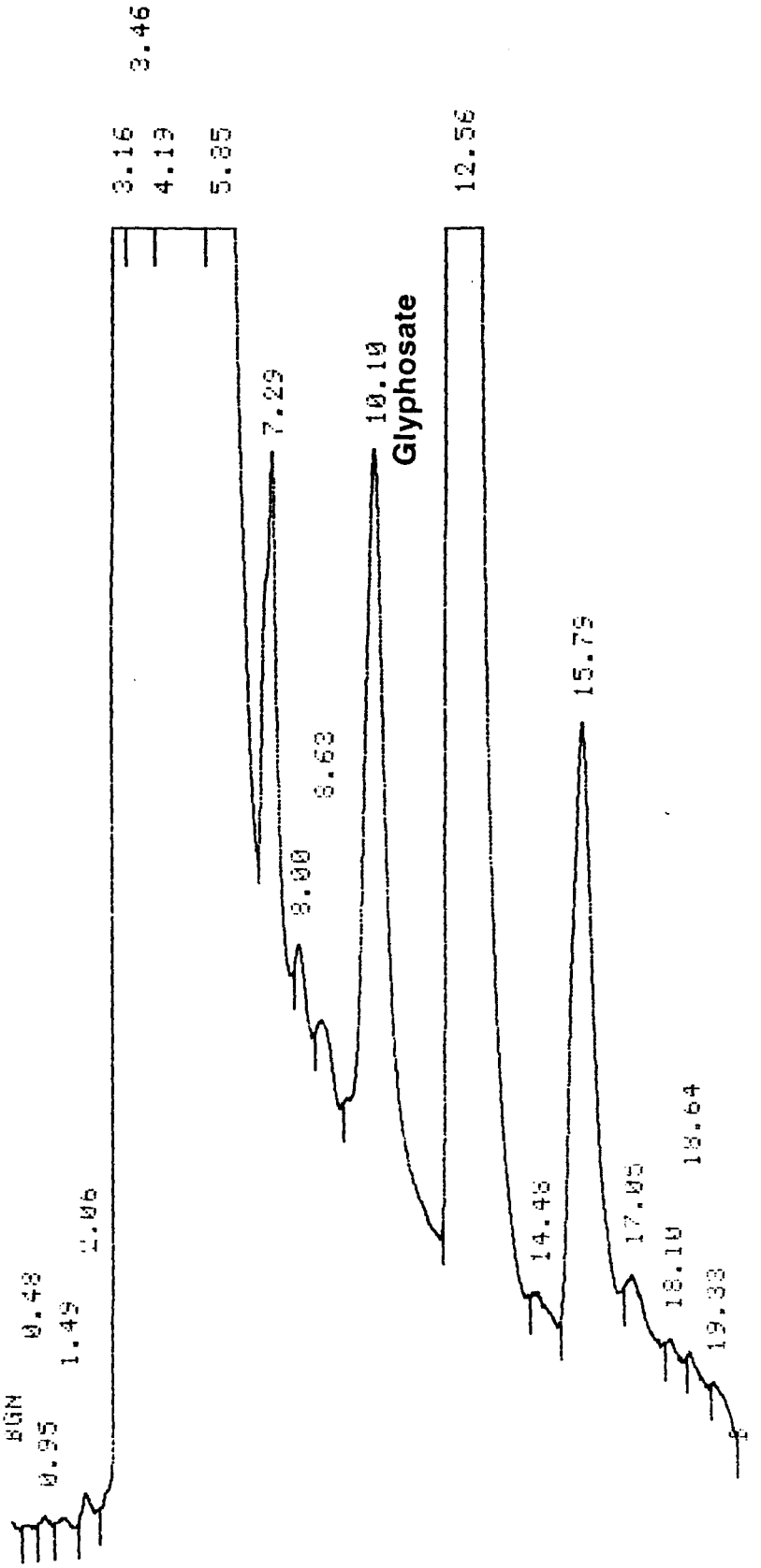
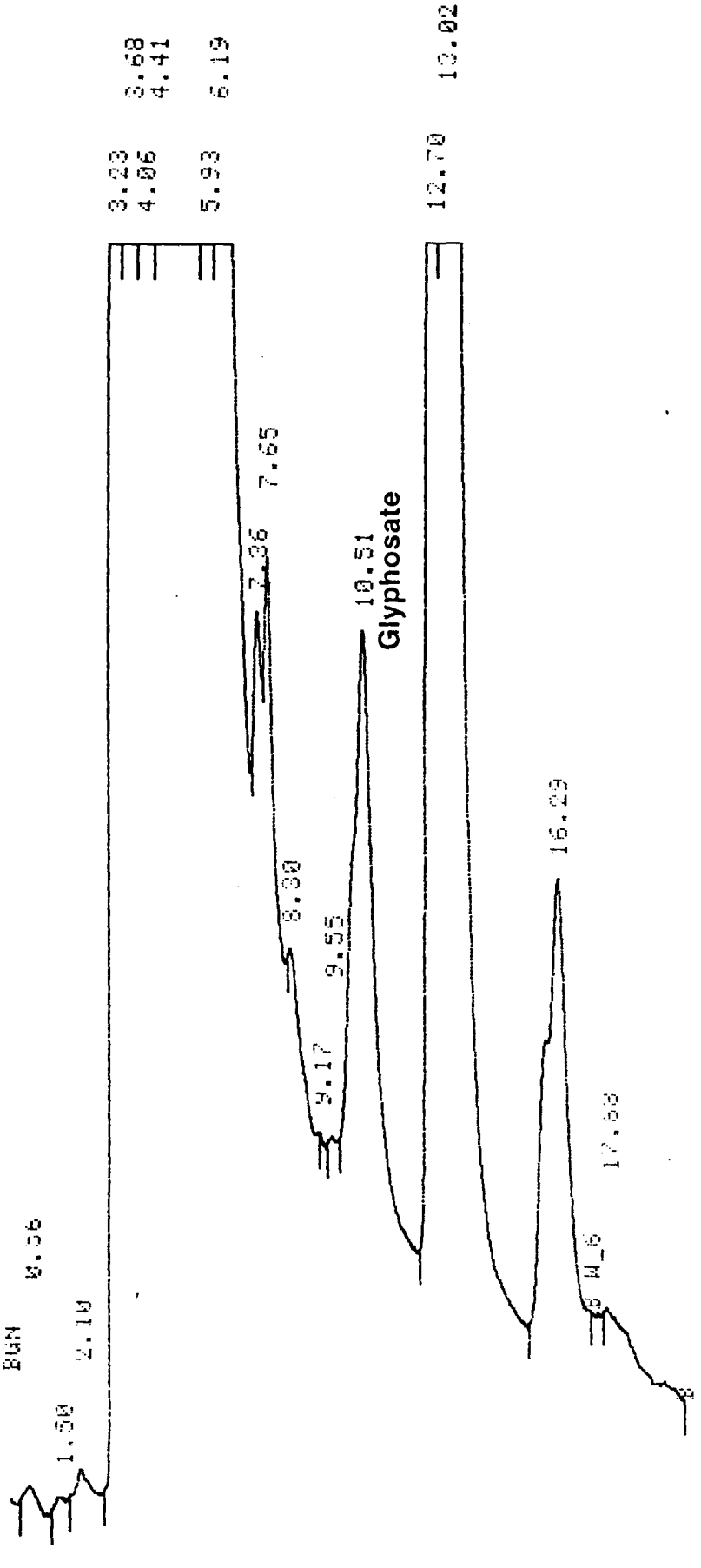


Figure 2.9 Chromatogram of barley extract after clean-up using C-18 SPE cartridge prior to NH_2 SPE cartridge



2.8 RETENTION OF THE GLYPHOSATE-FMOCCl COMPLEX USING C18 SPE CARTRIDGES

2.8.1 INTRODUCTION

As has been outlined previously in Chapter 1, many of the analytical problems associated with glyphosate arise from its polar nature. In particular, the need to use aqueous based extractants results in particularly "dirty" extracts. Also, the chemical's lack of solubility in organic solvents hinders the use of many classical clean-up methodologies and also makes concentration of extracts difficult. Although promising methods were obtained using C18 and NH_2 SPE cartridges to clean up an aqueous barley extract, it would be useful to have other methods available that may improve the sensitivity of the analysis or enable a broader range of matricies to be analysed. Since derivatisation of glyphosate with FMOCCl results in a much less polar product, there is scope to use stationary phases to retain the derivatised complex that are very different in nature to those used to retain glyphosate alone. To this end, C18 SPE cartridges which are among the most widely used and available were again examined. This time to retain glyphosate after derivatisation, rather than to retain interferences.

2.8.2 EXPERIMENTAL

A pH 4 phosphate buffer solution was prepared as previously outlined in section 2.2.2.2. This was then amended with acetonitrile to produce 10, 20, 40 and 50% acetonitrile solutions. 4 x 0.1mL aliquots of a $1 mgml^{-1}$ glyphosate standard solution were derivatised with FMOCCl using the method outlined in section 2.2.2.3.

Two of the derivatised glyphosate solutions were each applied to a solvated C18 cartridge. Each cartridge was then sequentially eluted with 2ml of 0, 10, 20, 30, 40 and 50% acetonitrile in phosphate buffer solution. Each eluate was then directly analysed by HPLC using the system and mobile phase outlined in section 2.2.2.1. The other aliquots were analysed directly.

2.8.3 RESULTS AND DISCUSSION

The percentage of the glyphosate-FMOCCl complex in each eluent fraction is summarised bleow in table 2.17.

Table 2.17 Elution profile of Glyphosate-FMOCCl from C18 SPE cartridge

Fraction	Mean % of Complex
0% Acetonitrile	0
10% Acetonitrile	0
20% Acetonitrile	0
30% Acetonitrile	19.9
40% Acetonitrile	80.1
50% Acetonitrile	0

These results clearly demonstrate that the glyphosate-FMOCCl complex can be retained on reverse phase cartridges from standard solution. Unfortunately, there was insufficient time to establish if this technique would work with extracts. However, this result raises a number of other possibilities. The glyphosate-FMOCCl complex could possibly be cleaned up using some of the more classical techniques such as liquid-liquid partition and normal phase techniques e.g. silica columns. Many published methods have concentrated on isolating underivatised glyphosate prior to analysis using post-column on line derivatisations. Utilisation of the very different properties of the derivatised complex may produce successful, quicker and easier clean-up procedures.

2.9 CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

The aim of the work outlined in this chapter was to develop a reliable, straightforward and sensitive method for the analysis of glyphosate residues in plants. A review of the existing literature in this area indicated that analysis of glyphosate by HPLC after pre-column derivatisation with FMOCCl, first described by Moye and Boning in 1979, should be a suitable determining step for such a method. In order to obtain a suitable level of sensitivity however, some form of clean-up procedure was needed. Most published methods used truncated versions of the purification procedure outlined in the Pesticide Analytical Manual (1977). This method utilised cation and anion exchange resins and charcoal. Two other techniques: Size Exclusion Chromatography (SEC), which has been used as a clean-up step prior to gas chromatographic determination (Guinivan et al., 1982) and Solid Phase Extraction (SPE), which had been previously tried unsuccessfully in the author's department (Yusof, 1988), appeared to have potential as clean-up steps. During preliminary work, the already difficult analysis of glyphosate was further complicated by problems with the glyphosate-FMOCCl peak shape. These were eventually traced to two likely sources:

1 Residual traces of acetone in the injected sample which could have damaged the column packing

2 Voids at the end of the column which resulted from the self-packed nature of the columns

A number of possible solutions were attempted without permanent success. However, two adaptations, placing a guard column on line and repacking the end of the HPLC column with fresh stationary phase when the peak shape

deteriorated, enabled good results to be obtained with the method. Commercially purchased columns may be more robust. The linearity of the detector response and reproducibility of the derivatisation and analysis procedure were then examined. Derivatised glyphosate gave a linear detector response over the range of concentrations tested (0.25 μg ml^{-1} to 100 μg ml^{-1}). Reproducibility of analysis was good with a coefficient of variation of 6.0% for 10 replicate analyses.

Having developed a working method of determination, attention switched to an assessment of SEC and SPE as potential clean-up methods.

Theoretically, SEC is an ideal first purification step for small polar molecules such as glyphosate. This is because the technique can be used with water as the mobile phase. Particularly convenient in the case of glyphosate which is not soluble in organic solvents. SEC should eliminate high molecular weight species which may interfere with adsorption based techniques. Two pore sizes of Sephadex gel and three different mobile phases; deionised water, pH 2 water and 0.05M KH_2PO_4, were tried with a standard glyphosate solution. In each case glyphosate eluted in a lower volume than was theoretically predicted. The small size of the molecule should have resulted in elution close to the total volume of the gel. Of the systems investigated, the best results were obtained with the use of pH 2 water as the mobile phase. This tends to indicate that the poorer than expected performance of the gels was due to residual carboxylic acid groups (Braithwaite and Smith, 1985) electrostatically repelling glyphosate, thus reducing the ability of the compound to penetrate the pores of the gel. The disappointing performance of the SEC columns with a standard solution of glyphosate suggested that the technique, as it was being used, would be of little use as a clean-up step. However, a more exhaustive investigation of the technique covering a range of different gel types from

various manufacturers may turn up a more suitable packing material. The use of ^{14}C-glyphosate would eliminate the need to determine the glyphosate content of the eluent portions by HPLC, thus dramatically reducing the time taken to optimise the method and improving the accuracy of the investigation.

Solid Phase Extraction (SPE) cartridges have been gaining in popularity with analytical chemists since their introduction by Waters Associates in 1978. To date however, they have not been used in a published method for glyphosate analysis. A previous worker in this department (Yusof, 1988) attempted to use strong anion and strong cation exchange cartridges for the analysis of glyphosate with little success. As a first step in the investigation, a range of SPE cartridges was tested to see if they would retain glyphosate at all. The most successful cartridges in the initial screen were those with an aminopropyl (NH_2) or cyano (CN) packing. A limited degree of retention was also noted with diol (OH) cartridges and these were included in the optimisation process. During the optimisation work, the effect of pH on the retention of glyphosate was investigated and best results were obtained with NH_2 cartridges when the pH of the applied glyphosate solution was approximately 6. Since sensitivity is a principal consideration when developing an analytical method, it is a bonus if a clean-up step also concentrates the analyte. To achieve this with SPE cartridges it is necessary to find a solvent that will elute the analyte in the minimum volume. Of those tested, 0.2M sodium citrate was the most successful. Promising results were obtained with the NH_2 SPE cartrdiges with a standard solution of glyphosate, and this technique was therefore put to the test of cleaning up a fortified barley extract.

Barley was extracted using a water/chloroform 2:1 v/v mixture. This extract was then pre-treated with trichloroacetic acid to precipitate proteins and

acetone to reduce the starch content. After partitioning against chloroform and diethyl ether to remove non-polar interferences, the extract was concentrated using a rotary evaporator. Initially, the extract was cleaned-up using NH_2 SPE cartridges alone. The effect of excluding a rinsing step and the use of methanol as a rinsing solvent were also studied. A glyphosate recovery of 40.2% was obtained when the cartridge was rinsed with water. Rinsing with water, as expected, also gave cleaner chromatograms. Methanol was of no use as a rinsing solvent, possibly due to solubility problems. Including a rinsing step appeared to reduce the recovery of glyphosate. This may have been due to competition for binding sites from co-extracted material. Although the recovery obtained after rinsing was on the low side, this result demonstrates that NH_2 SPE cartridges do have potential to clean-up "real" samples. There are two possible ways to circumvent the problem of competition for sorbent binding sites. These are to use larger capacity cartridges or to eliminate more of the interferences prior to application on the NH_2 cartridge. As the 5g cartridges used were the largest available at the time, the latter of these strategies was attempted. With the introduction of increasingly higher capacity cartridges the former course of action would be worthy of investigation. The 2OH, CH and C-18 cartridges that had been shown not to retain glyphosate at pH 6 were then examined for their usefulness as pre-treatments. All the cartridges gave better recoveries than those obtained with NH2 cartridges alone. However, 2OH cartridges gave only marginally better recoveries, this may have been due to competition from co-extracted material as before, or may be due to some retention of glyphosate by 2OH cartridges as previous results have indicated 2OH cartridges do seem to be able to retain glyphosate to a limited extent under some conditions. Use of ^{14}C labelled material would allow easier tracking of losses throughout the procedural development process, this in turn would enable more effective changes to be made in less time. CH cartridges can give inconsistent recoveries for reasons which are

unclear. C18 cartridges however, gave both high (81.3 and 87.0%) and consistent recoveries at the fortification level used of 2 $\mu g g^{-1}$ (2ppm). This is a very encouraging result. There is no doubt that the quality of the chromatography could be further improved. The C18 SPE cartridges used in this instance contained only 5g of packing material, testing of this method using larger capacity cartridges (both C18 and NH2) may improve the sensitivity of the method. This method has the great advantages of reducing the requirements for solvent and materials and the scope that the use of SPE cartridges gives for automation. A final "quick look" experiment indicated that once derivatised, the glyphosate-FMOCCl molecule could be retained by C18 cartridges. This provides the analyst with yet another potential purification step and one that uses yet another retention mechanism. Further work is required to ensure that all components of the method from the clean-up to the determination step are reliable, reproducible and robust. As has been mentioned throughout this conclusion, the use of ^{14}C labelled glyphosate would enable fast and accurate assessment of losses. Once a method had been fine-tuned with ^{14}C-glyphosate however, it would require full validation with unlabelled material to ensure that the method functioned with conventional instrumentation.

CHAPTER 3

AN INVESTIGATION INTO THE ANALYSIS OF GLYPHOSATE IN SOILS

3.1 INTRODUCTION

The first question to answer when considering the extraction and analysis of glyphosate from soil is "Why bother ?". After all, the chemical has low mammalian toxicity (Smith & Oehme, 1992). It is rapidly bound to the soil (Glass, 1987) and is therefore bioavailable for a comparatively short time. It does not leach through soil (Malik et al., 1989) nor does it run off the soil surface (Damanakis, 1976; Edwards et al., 1980) and so is unlikely to cause gross contamination of water systems. Furthermore, it has been stated (Malik et al., 1989) that "glyphosate has been shown to have no drastic effect on the populations of soil microbes when used at its recommended rates.". However the situation is not that straightforward. As outlined in the introduction to this thesis, the use of glyphosate has risen steadily since its introduction and the increasing levels of use currently show no sign of abating. Additionally, since glyphosate tolerant crop varieties have now been developed there is the prospect of multiple glyphosate applications during the growing season. With the use of the chemical on such a massive scale world wide, the appearance of subtle effects on environments and ecosystems cannot be ruled out. Although there is no direct evidence of such effects as yet, some published results are not so favourable. The binding of glyphosate to the soil is reversible, and some soil activity has been reported. Germination and growth of alfalfa and red clover were reduced when seeds were distributed on a sprayed soil surface up to 24 hours after glyphosate application, whilst bentgrass growth was reduced up to 5 days after application (Salazar &

Appleby, 1982). In a sandy soil, glyphosate reduced wheat shoot length up to ten days after application at treatment rates of 2.5 and 5.0 $kgha^{-1}$ (Devlin et al., 1986). Growth inhibition of a bacterium (*E. coli*), an alga (*chlamydomas reinhardi*), plant cell cultures (carrot and soybean) and roots of whole plants (*Arabidopsis thaliana*) were noted by Gresshoff (1979), although apart from plant roots these effects were transitory. On balance, the evidence would suggest that glyphosate is unlikely to cause problems in the environment but there is the possibility of difficulties occurring, especially if users and manufacturers become blasé about the use of what is an extremely active chemical. Glyphosate adsorption in several soils has been correlated with the unoccupied phosphate sorption capacity (Hance, 1976) and it may be necessary for growers who intend to use glyphosate tolerant crop species to pay attention to the timing of fertiliser applications to avoid a reduction in the soil's ability to absorb glyphosate.

To enable meaningful long-term environmental impact assessments to be carried out there is an obvious need for straightforward and sensitive methods for determining glyphosate in soil. Another more prosaic need for such methods is to help settle disputes between parties when cases of drift or misapplication damage occur. Since the half life of glyphosate in soil can vary from less than than 1 week to several months, (Tortensson, 1985) it may be easier to detect glyphosate from contaminated soil than from the crop, both because of the potentially longer half life in soil and also because soils, in general, present an easier matrix clean-up challenge than plants.

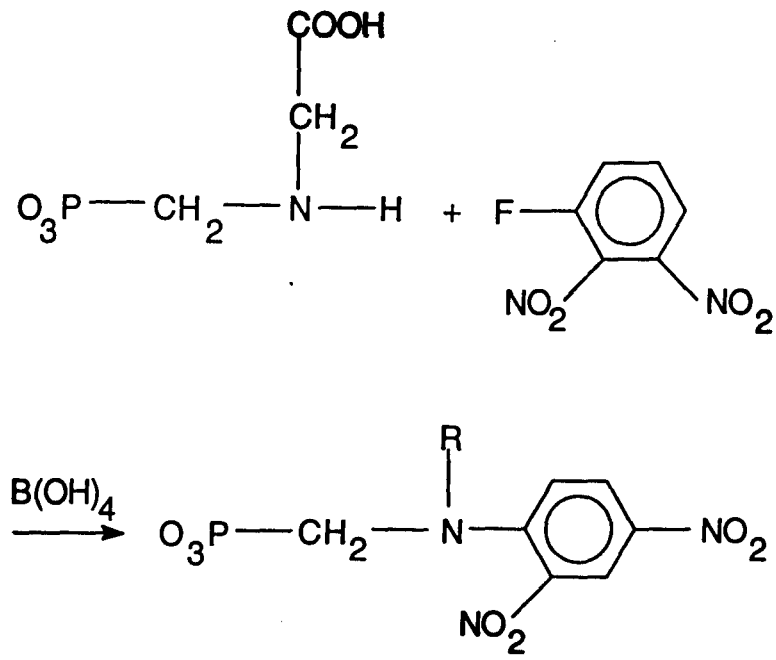
In contrast to the relatively large number and diversity of techniques that have been adopted to determine glyphosate in plants, few have been published relating to soil residues. In general the published methods are extensions of those previously attempted on plant residues. It is as a natural

result of this that more attention has been paid in these publications to the method of extraction than to the clean-up and final determination steps. Since some success has been achieved in Chapter 2 using the pre-column derivatisation HPLC method of Moye and Boning (1979) as a determining step the principal emphasis of this Chapter (as has been the trend in the literature) was on finding a selective and effective extractant for glyphosate, thus allowing determination of residues in UK soils with minimal clean-up. Prior to the commencement of experimental work several candidate extractants were identified not only by consideration of previously published analytical methods but also by consideration of literature on the binding of glyphosate to soils.

The Pesticide Analytical Manual method (1977) used 0.5 M ammonium hydroxide as an extractant followed by purification using anion and cation exchange resins prior to a two-step derivatisation and determination by GC. Some authors (Glass, 1983; Miles & Moye, 1988 and Lundgren, 1986) have criticised this method because of its complexity and time consuming nature. Pre-column derivatisation with 9-fluorenylmethylchloroformate after extraction with 0.1 M NaOH and an anion-exchange column clean-up was used by Glass (1983) to determine glyphosate in three soils: a silt loam, a sandy loam and a clay loam. Determination was by HPLC using an amine bonded phase column. At fortification levels of 50 ppm for the clay loam and 25 ppm for each of the sandy loam and the silt loam, the recoveries obtained were 25.8%, 35.8% and 30.7% respectively. A different pre-column derivatisation reaction using 1-fluoro-2,4 dinitrobenzene (Fig. 1.3) was used by Lundgren (1986) to analyse glyphosate in three soils; a clay loam pH 6.4, a sand pH 6.6 and an organogenic soil pH 6.4. The soils were extracted by shaking with aqueous 0.1M triethylamine for 15 minutes. Extracts were cleaned-up prior to derivatisation using anion exchange resin batchwise. Once the derivatisation was complete the extract was further purified by liquid-liquid partition. HPLC

analysis was carried out by ion-pair chromatography on a reverse phase (C-18) column. UV detection of the peak of interest was at 405 nm.

Figure 3.1 Derivatisation of glyphosate with 1-fluoro-2,4-dinitrobenzene



Using N-(phosphonomethyl)-B-alanine as an internal standard, recoveries were 90%, 93% and 56% for the clay loam, sand and organogenic soil respectively. These recoveries, however, are normalised against control samples put through the same work-up and derivatisation procedure and do not represent the actual amounts recovered from the soil samples. Miles & Moye (1988) used ^{14}C-glyphosate to investigate extraction of the compound from clay minerals and two soils. They also used pre-column derivatisation with 9-fluorenylmethyl chloroformate to determine unlabelled glyphosate residues in soils. This study had the two-fold objective of "determining the extent of sorption/desorption of glyphosate to several soils and clay minerals" and "developing an analytical method for the determination of glyphosate in

soils with high amounts of clay". From the sorption/desorption work using ^{14}C-glyphosate two general conclusions were drawn.

1) Extraction efficiency increases with increasing pH.

2) Extraction efficiency increases with increasing ionic strength of solvent.

Extraction of two contrasting Calvin silt loam samples by shaking for 3 x 15 minutes with 0.1M KOH and determination by HPLC following FMOCCl derivatisation gave recoveries of 119 ± 18% at 1.0 ppm and 93.0 ± 0.8% at 10 ppm for soil 1 and 108 ± 13.9% at 1.0 ppm and 86 ± 12.8% at 10 ppm for soil 2. Soil 1 contained significantly more organic matter and clay than soil 2. Extracts using 0.1M H_3PO_4 formed a gel and those of K_2HPO_4 gave unacceptably high backgrounds. Extraction with 0.1M KH_2PO_4 and 30 fold concentration gave acceptable recoveries at the 0.5 ppm level with low to moderate clay content soils.

A novel ion exchange HPLC method utilising post-column derivatisation with ninhydrin was used by Thompson et al. (1989) to determine glyphosate in a range of forest matrices including organic and mineral soils. Soil samples were extracted by shaking three times with 0.5M NH_4OH. Purification and pre-concentration was carried out with anion-exchange and cation exchange resins prior to analysis on an Aminex A-9 cation exchange column. Mean recovery values obtained for the soil analysis were 79% for organic soils fortified between 4 and 30 μgg^{-1} and 73% for mineral soils fortified between 4 and 8 μgg^{-1}. Extraction efficiencies using 0.5M NH_4OH were determined using ^{14}C-glyphosate, and found to be 89.7 to 95.4% for the organic soils and 90.7 to 96.0% for the mineral soils. The losses were attributed to the clean-up steps.

Determination of glyphosate in soils with moderate to high clay contents was studied by Spann and Hargreaves (1994). Six soils with clay contents of 25–87% were fortified with glyphosate, incubated for 24 hours, then extracted with 0.1M KOH by shaking for 16 hours on a horizontal reciprocating shaker. Clean-up of the extracts was by cation exchange and quantification was carried out by HPLC with post-column derivatisation with OPA-MERC. All recoveries were better than 80% and a detection limit of 0.04 $mgkg^{-1}$ was claimed, although the lowest fortification level reported was 0.56 $mgkg^{-1}$.

Analysis of soil extracts using gas chromatography has not been as popular as the analysis of plant extracts using the technique. This may be due to the difficulty of eliminating the salt solutions required as extractants prior to derivatisation. The method of Deyrup et al. (1985) was however adapted for use with soil extracts by Roy and Konar (1989). Two soil types, a clay and a sand, and their organic matter components were used for recovery experiments. These were extracted with phosphoric acid then cleaned-up by liquid-liquid partition and charcoal prior to derivatisation. A reasonable mean recovery of 75% was obtained for the organic matter, however the mean recoveries for the clay and sand fortified at 1 μgg^{-1} were 54.3% and 50.2% respectively. A detection limit of 0.05 μgg^{-1} was claimed.

Eberbach and Douglas (1991) describe a method for determining glyphosate in soil using GC. Extraction with triethylamine was followed by cation and anion exchange clean-up steps and single step derivatisation with trifluoroacetic anhydride and trifluoroethanol. Where extraction of the soil was conducted immediately after fortification, the recoveries ranged from 88 to 104%. However, after incubation for 13 hours, the recoveries fell to between 48 and 67%.

The effectiveness of various extraction solvents, after soils had been fortified and incubated for 14 days, was investigated using ^{14}C-glyphosate by Aubin and Smith (1992). Good recoveries of 72.8 to 90.7% were obtained with phosphoric acid, sodium hydroxide and ammonium hydroxide. Two shaking procedures were compared; a 1 hour shake and an extended procedure involving a 1 hour shake, overnight incubation and a further 1 hour shake. Various soil/solution ratios were examined. The authors concluded that for the soils chosen, 0.1M NaOH at a soil solution ratio of 1:2.5 using the extended shaking procedure was the extraction method of choice. Interestingly, after incubation for 56 days, good extraction recoveries of 79%, 76% and 85% for the three soils were still obtained. This would tend to suggest that under certain conditions glyphosate is quite resistant to breakdown in soil.

As previously mentioned, much of the emphasis in the development of methods has been devoted to determining the best extractant. To date these include 0.5M ammonium hydroxide, 0.1M NaOH, 0.1M KOH, 0.1M triethylamine and phosphoric acid. The use of various soil types and methods of determination make comparison of the effectiveness of the extractants problematical. In general, however, extractants that produce a high pH environment seem to be most effective. In order to gain some insight into why those particular extractants have been successful, and to postulate other extractants that may give selective extraction and good recoveries, some consideration of how glyphosate is thought to bind to soil is worthwhile.

Using a bioassay technique, Sprankle et al. (1975a) found that a clay loam and a muck soil both inactivated glyphosate applied at 56 $kgha^{-1}$. Addition of phosphate to the soil surface decreased the inactivation of glyphosate. Further work by the same authors (1975b), again using a bioassay system, indicated that organic matter and clay minerals deactivated glyphosate most in the

presence of Fe^{3+} and Al^{3+}. The authors postulated that glyphosate may form an adsorbent-cation-herbicide complex with clay and organic matter. Small quantities of glyphosate also bound to iron and aluminium hydroxides. Binding seemed to be related to the cation on the clay rather than to the cation exchange capacity. In whole soils, the phosphate level appeared to have some importance in determining the quantity of glyphosate absorbed. Unoccupied phosphate sorption capacity was determined by Hance (1976) to have a greater correlation with glyphosate adsorption than Tamms Oxalate extractable aluminium and iron. Adsorption was higher than, but of the same magnitude as, that of diuron, a soil acting herbicide. Because of this the author postulated that the lack of soil acting activity is due to low intrinsic activity through the root system. A root biossay using grain sorghum was used by Hensley et al. (1978) to study the inactivation of glyphosate by various soils and metal salts. The results of this study reinforce those of Sprankle et al. (1975a) with glyphosate inactivation showing no correlation with cation exchange capacity. However $FeCl_3$, $FeCl_2$ and $AlCl_3$ significantly reduced the activity of glyphosate. One muck soil inactivated glyphosate but another and bentonite clay had little effect. From their results the authors hypothesised that glyphosate may be inactivated in the soil by iron and aluminium.

The relationship of system pH with glyphosate adsorption in two smectites saturated with Al^{3+}, Ca^{2+} and Na^{+}; NaCl washed kaolinite, haematite and goethite was examined by McConnell and Hossner (1985). Glyphosate adsorption to kaolinite, haematite and goethite was found to be pH dependent. Since the charge on the mineral surface is also pH dependent, with each mineral being positively charged below its point of zero charge and negatively charged above this point, decreasing adsorption with increasing pH would be expected as glyphosate will also become increasingly negatively charged as the pH is raised and increasing repulsion will result.

Interaction of glyphosate with a pure iron-humic acid complex was investigated by Piccolo et al. (1992). Adsorption isotherms were constructed at different shaking times. Maximum adsorption values showed that glyphosate was adsorbed on the iron-humic acid complex to an extent similiar to that found for whole soils. The S-type adsorption isotherms determined through the application of the Freundlich equation indicate that, at low concentrations, glyphosate is adsorbed by a ligand exchange mechanism involving the phosphono group of glyphosate and the iron hydration sphere in the humic-acid complex, whereas at higher concentrations, more glyphosate may be bound by hydrogen bonding to previously sorbed molecules.

In summary: the available evidence suggests that glyphosate adsorption in soils will be governed by a variety of factors. The clay and organic matter content may be important, but the presence of sufficient iron and aluminium as the trivalent species is likely to be more important as they allow the postulated adsorbent-cation-glyphosate complex to form. The quantity and type of minerals present may also be of some importance but the scale of their role will be governed by the pH of the soil. Finally, the rather poorly defined concept of unoccupied phosphate adsorption capacity is reported to be correlated to the binding capacity of soils for glyphosate.

In terms of the search for a solvent that will give clean glyphosate extracts and will be compatible with the pre-column derivatisation reaction with 9-fluorenylmethyl chloroformate, NaOH or KOH and KH_2PO_4 must be tried on the basis of previous successes (Miles and Moye, 1988; Aubin and Smith, 1992, and Spann and Hargreaves 1994). Unfortunately, 0.5M ammonium hydroxide (P.A.M., 1977) and 0.1M triethylamine (Lundgren, 1986) cannot be used as they interfere with the derivatisation reaction. Bicarbonate (sodium hydrogen carbonate) was attempted on the basis of its alkalinity and because,

theoretically, it will disperse organic matter and clays, but will not complex iron or aluminium. Citrate (sodium citrate dihydride) should have some complexing ability and may displace glyphosate from complexes. The presence of Na^+ in both reagents will have a dispersing effect on clays. Finally, water was included to gauge the effectiveness of the incubation period in allowing the binding of glyphosate to take place.

3.2 EXPERIMENTAL

3.2.1 DETERMINATION OF THE EFFECTIVENESS OF EXTRACTANTS

3.2.1.1 SOIL DETAILS

A brief description of the sites from which the soils were sourced is given below.

3.2.1.2 SOIL NO. 1 MIDELNEY (FEN ARABLE)

The site is located at Bank Farm, Norfolk, England. Grid reference No. TF 588022. The soil is used for intensive arable crop production. It belongs to the Midelney series which is developed from calcareous alluvial clay parent material. The series has been classed as a ground water gley. The properties of the soil are listed in Table 3.1.

3.2.1.3 SOIL NO. 2 DARVEL

The site is located at Westerton Farm, Lennoxtown, Scotland. Grid reference No. TF 635 773. The soil is laid out to grass for rough grazing and belongs to the Darvel association formed from fluvioglacial sands and gravels derived from carboniferous, igneous and sedimentary rocks. The properties of the soil are listed in Table 3.1, overleaf, and appear courtesy of Quasim.

Table 3.1 Soil Properties

Soil Property	Midelney	Darvel
% Coarse Sand	1.5	33.5
% Fine Sand	7.4	20.0
% Silt	50.8	22.8
% Clay	40.4	24.4
Textural Class	Silty Clay	Sandy Clay Loam
Total C %	4.4	3.5
LOI	14.7	9.1
pH (water)	7.4	5.6
pH (CaCl)	7.0	4.7
Total N %	0.4	0.25

3.2.2 PREPARATION OF EXTRACTANTS

A) Sodium Hydroxide (0.1M) - 4.9g of sodium hydroxide were dissolved in one litre of distilled and deionised water.

B) Sodium Citrate (0.1M) - 29.4g of tri-sodium citrate anhydride were dissolved in one litre of distilled and deionised water.

C) Potassium Phosphate (0.1M) - 13.6 g of potassium dihydrogen orthophosphate were dissolved in approximately 950 ml of water, the pH was adjusted to 8.5 with 5M sodium hydroxide and the solution made up to 1 litre with distilled and deionised water.

D) Sodium Bicarbonate - 42.0 g of sodium hydrogen carbonate were dissolved in approximately 900 ml water, the pH was then adjusted to 8.5 with 5M sodium hydroxide and the solution made up to 1 litre with distilled and deionised water.

E) Water - Distilled and deionised water was used.

3.2.3 FORTIFICATION AND EXTRACTION OF SOILS

Triplicate subsamples of each soil (10 g oven dry equivalent) were placed in 8 oz glass bottles. Fortification with 1 ml of 100 $\mu g ml^{-1}$ glyphosate in water was then carried out, to give a concentration of 10 $\mu g g^{-1}$ (10 ppm). The soils were left to incubate for twenty-four hours at room temperature. Each sample was then extracted with 50 ml of extractant by shaking for thirty minutes on an 'end-over' shaker. The resulting slurry was decanted into a centrifuge tube, the bottle rinsed three times with a small volume of fresh extractant and the rinses added to the centrifuge tube. After centrifugation at 4,500 rpm for 25 minutes the supernatant was decanted and filtered through a Whatman number 1 filter paper into a 100 ml round bottom flask. The pellet was resuspended in a small volume of fresh extractant, recentrifuged and the supernatant filtered and bulked in the round bottom flask. The extract was then reduced to approximately 5 ml on a rotary evaporator. The residue was quantitatively transferred to a 10 ml centrifuge tube and spun at 4,500 rpm for 30 minutes, the supernatant decanted into a 10 ml volumetric flask and the pellet resuspended in a small amount of water and recentrifuged. The supernatants were bulked in the volumetric flask and diluted to volume with water. Unfortified soil blank extracts and no soil control extracts were also prepared and analysed. Derivatisation and HPLC analysis was conducted using the procedure and apparatus outlined in section 2.2.2.3.

3.3 RESULTS AND DISCUSSION

The percentage recovery obtained using each extractant is shown in Table 3.2, along with the mean recoveries calculated for the no-soil control samples and the recovery for each extractant corrected for the control recovery value. Coefficients of variation are also given for each set of triplicate analyses. No glyphosate was detected in any of the blank (unfortified) soil extracts, indicating that the soils were not contaminated with glyphosate prior to fortification. Nor were any co-eluting interferences apparent. The most effective extractant was 0.1M NaOH which gave a mean corrected recovery of 60.5% for Midelney soil and 99.3% for the Darvel soil. Apart from extraction with water which (as expected) removed very little glyphosate (5% from Midelney and 3.1% from Darvel) there was little to choose between the other three extactants. The low level of glyphosate extraction by water also indicates that significant binding of glyphosate had taken place over the incubation period. The calculated coefficients of variation (CV = (standard deviation / mean) x 100 %) illustrate that good reproducibility was obtained between the triplicate samples analysed. The only high value (27.0%) was obtained for the extraction of Darvel soil with water and is a product of the low values used to calculate the CV. Extraction with 0.1M sodim citrate gave corrected mean recoveries of 43.4% for Midelney and 42.9% for Darvel, while 0.1M KH_2PO_4 gave corrected mean recoveries of 43.7% for Midelney and 43.4% for Darvel. 0.1M sodium bicarbonate gave corrected mean recoveries of 41.0% and 49.5% for Midelney and Darvel soils respectively. The similarity in the recoveries may be in part due to the fact that the KH_2PO_4 and sodium bicarbonate extracts were adjusted to pH 8.5 with sodium hydroxide.

Table 3.2 Extractant Recoveries for Darvel and Midelney Soils

Soil Type	Extractant	Replicate	Recovery %	CV %	Mean Recovery	Control Recovery	Corrected Recovery
Midelney	NaOH	1	68.1	1.65	67.07	110.9	60.47
		2	67.2				
		3	65.9				
Darvel	NaOH	1	97.1	10.27	110.07	110.9	99.25
		2	117.8				
		3	115.3				
Midelney	Citrate	1	42.6	2.95	41.20	94.9	43.41
		2	40.4				
		3	40.6				
Darvel	Citrate	1	40.6	0.57	40.73	94.9	42.92
		2	40.6				
		3	41				
Midelney	KH_2PO_4	1	40.6	7.22	41.47	95	43.65
		2	44.8				
		3	39				
Darvel	KH_2PO_4	1	45.1	8.27	41.20	95	43.37
		2	39.7				
		3	38.8				
Midelney	Bicarb.	1	35.7	12.38	31.30	76.3	41.02
		2	29.8				
		3	28.4				
Darvel	Bicarb.	1	36.6	9.62	37.77	76.3	49.5
		2	35				
		3	41.7				
Midelney	Water	1	4.3	8.91	4.53	89.5	5.07
		2	5				
		3	4.3				
Darvel	Water	1	3	26.96	2.83	89.5	3.17
		2	2				
		3	3.5				

Noticeably lower extraction efficiencies were obtained for the Midelney soil compared to Darvel when 0.1M NaOH and 0.5M sodium bicarbonate were used as extractants. This differential was not apparent when 0.1M sodium citrate or 0.1M KH_2PO_4 were used even though the 0.1M KH_2PO_4 was adjusted to pH 8.5. This mirrors the trend in the literature where alkaline extractants have been less successful at extracting glyphosate from soils with high clay content then from those with low to moderate clay content. From the available evidence it is impossible to tell whether this is due to a fraction of the glyphosate residue being held more tightly in soils with higher clay contents, or if it arises as a result of an interaction between the high pH of the extractant and the high clay content. The fact that there was little difference in the extraction efficiency between the two soils when sodium citrate and KH_2PO_4 were used does not necessarily mean that the clay content is immaterial when these extractants are used, because the overall recoveries are so much lower than those obtained with NaOH. It may be that NaOH can extract a certain amount of more tightly bound glyphosate that the other extractants cannot reach. In order to investigate the possibility of extractants selectively removing glyphosate "pools", it would be necessary to use the extractants in sequence, something that does not seem to have been attempted to date. However, some evidence for glyphosate being bound by more than one mechanism could be inferred from the fact that extractants with widely differing properties such as citrate and bicarbonate, extract significant amounts of glyphosate. It is unlikely that this effect is due solely to the presence of Na^+, especially since KH_2PO_4 is just as successful. Although NaOH was the most effective extractant it also, perhaps unsurprisingly because of its effect on humic material, produced chromatograms with the greatest level of interferences. Specimen chromatograms are shown in figures 3.2 - 3.4. The high level of interferences means that 0.1M NaOH is unlikely to be of use with residue levels below about 0.5 ppm without the use of a clean-

up. It may be possible to go below this level with one of the other extractants because they give much cleaner chromatograms, however the lower extraction efficiency must be borne in mind. Furthermore there is no guarantee that at lower levels of residue, if glyphosate is bound by a variety of mechanisms, that the tighter binding sites would not be occupied preferentially, resulting in a drop in extraction efficiency as the level of residue declines. Using 0.1M NaOH with a single thirty minute shake gave recoveries at the 10 ppm level as good or better than those achieved by Glass (1983); Lundgren (1986) and Miles & Moye (1988). This puts some doubt on the need for three successive shakes. Also the soils used are normal agricultural soils not sands (which have been used by some previous workers) which generally do not bind glyphosate as tightly. Although more vigorous extraction may be required to investigate low level residues, this method does offer a straightforward and simple screen for levels of glyphosate down to 0.5 ppm. This is more than sufficient for many cases where confirmation of the compound's presence is all that is required. The control recovery values were all within an acceptable range with the possible exception of that obtained using 0.5M sodium bicarbonate at 76.5%. Initially this was thought to have arisen as a result of the high salt concentration in the sample interfering with the derivatisation reaction. To examine this possibility the extraction solvents were prepared at five times the concentration used for extraction, in order to mimic the concentration step. These were then spiked with 1 $\mu g ml^{-1}$ of glyphosate and aliquots removed, derivatised and determined as outlined in section 2.2.2.3. Table 3.3 shows the results obtained.

Table 3.3 Derivatisation recoveries in high salt concentrations

Extractant	Recovery
0.5 M NaOH	103 %
0.5 M KH_2PO_4	111 %
0.5 M $C_6H_5Na_3O_7$	101 %
2.5 M $NaHCO_3$	102 %

Figure 3.2 Sodium Hydroxide Extract of Midelney Soil

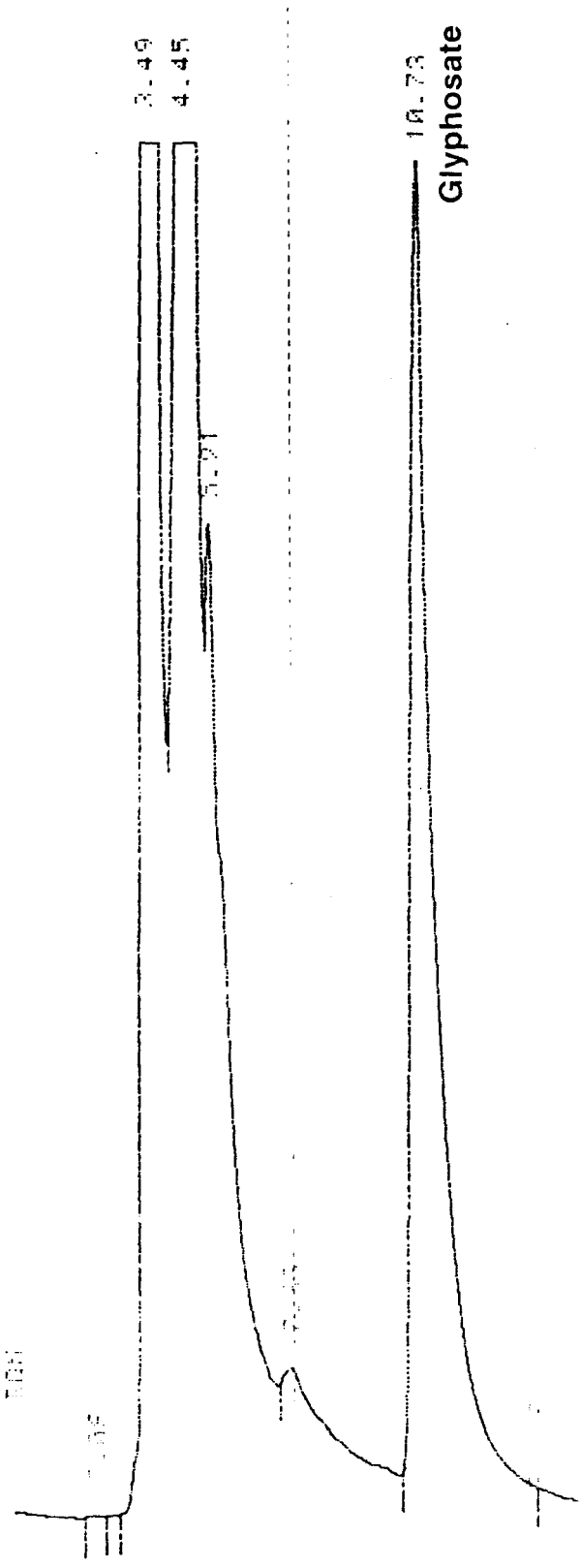


Figure 3.3 Sodium Citrate Extract of Midelney Soil

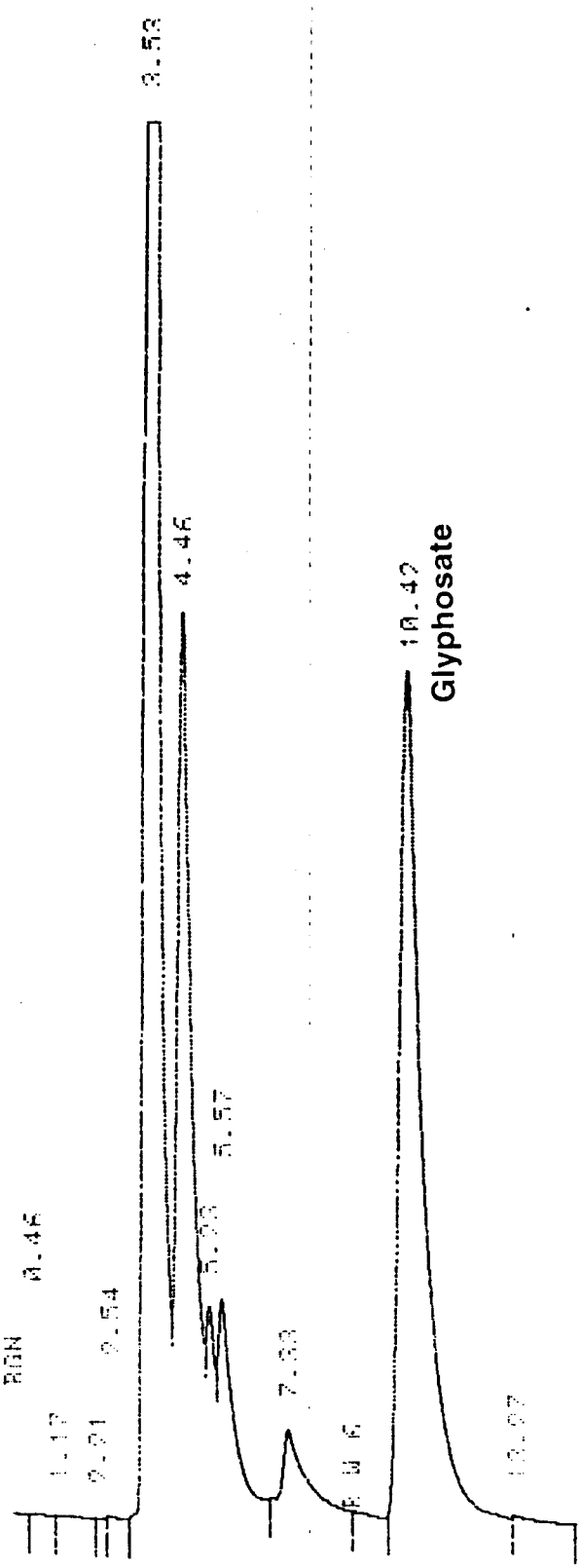


Figure 3.4 Potassium Dihydrogen Orthophosphate Extract of Midelney Soil

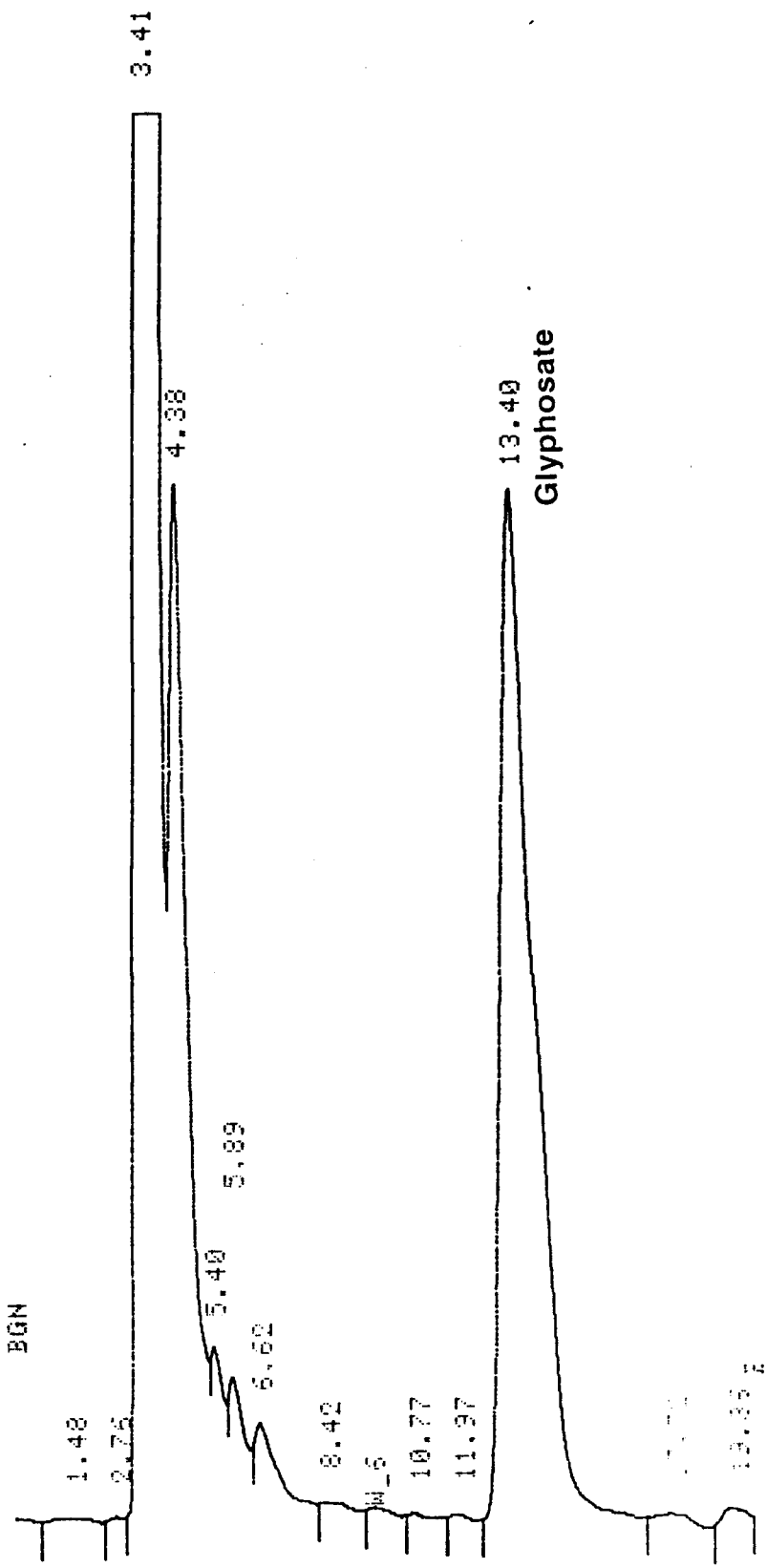
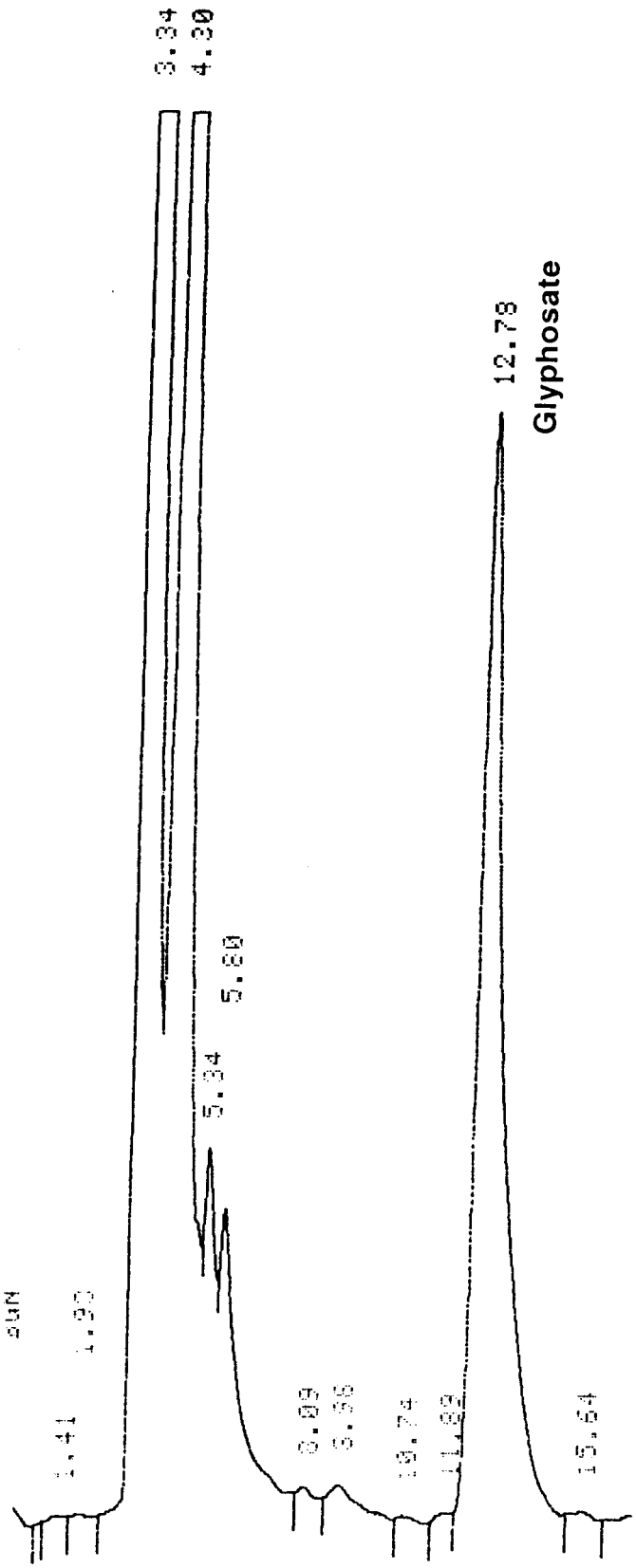


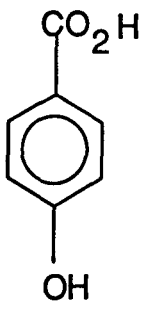
Figure 3.5 Sodium Bicarbonate Extract of Midelney Soil



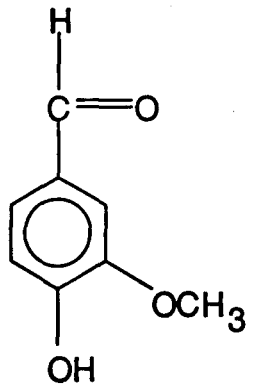
The recovery is expressed as the mean percentage recovery of two replicate values, with the value obtained from conducting the derivatisation in water as the baseline (100%). This result suggests that the derivatisation reaction with 9-FMOCCl is relatively immune to the salt concentration in which the reaction is conducted. This probably holds true until the buffering capacity of the sodium tetraborate, which comprises part of the reaction mixture, is exceeded. As the reaction requires an alkaline medium to proceed, alkaline extractants are likely to be less of a problem than acidic ones. The low control recovery may be due to glyphosate binding to glass, which was reported to be a problem by Deyrup et al. (1985).

As outlined in the introduction, most of the published methods require clean-up steps that involve either or both anion and cation exchange resins. One group (Thompson et al. 1989) attributed most of their losses to this step. To date 0.1M NaOH is the best extraction solvent that is compatible with pre-column derivatisation with FMOCCl. However, as previously mentioned, this extractant also gives a larger amount of interferences on the chromatogram. These co-extracted interferences are most likely to arise from the degradation of humic substances. Hydrolysis of humic material produces components such as vanillin, vanillic acid, B-hydroxy benzoic acid etc. whose structures are shown in Figure 3.6. If these are indeed the type of compounds that are interfering in the chromatograms it should be possible to use reverse phase, solid phase extraction cartridges to remove them. The advantage of this method is that the extracts could be eluted straight through the cartridge with retention of the interferences but without retaining glyphosate. This would give a quick and straightforward clean-up procedure.

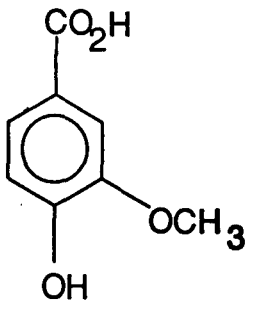
Figure 3.6 Structures of Vanillin, Vanillic Acid and B-hydroxy benzoic acid



B-hydroxy benzoic acid



Vanillin



Vanillic Acid

3.4 EXPERIMENTAL 2

The NaOH blank extract of Darvel soil was fortified with glyphosate to give a 0.2 μgml^{-1} solution. Four C-18, CN and 20H solid phase extraction cartridges were solvated with 1 ml of methanol followed by 1 ml of deionised water. Duplicate 1 ml aliquots of the fortified and unfortified blank extract were then eluted through each cartridge type, and the eluent collected. A 0.1 ml aliquot of each was then derivatised and determined by HPLC as previously outlined (2.2.2.3.). A 0.2 μgml^{-1} standard was derivatised and determined simultaneously for quantification purposes. The blank extract was also analysed without any clean-up to provide a comparison.

3.5 RESULTS AND DISCUSSION 2

Only the 20H SPE cartridge was partially successful in cleaning-up the fortified extract. Figure 3.7 shows a chromatogram derived from the intact blank extract and Figure 3.8 shows a chromatogram of the fortified extract cleaned-up using a 20H cartridge. There is still far too high a level of interferences to allow accurate determination of glyphosate. Comparison of the two chromatograms does show that a significant amount of interfering material has been removed from the extract.

The ineffectiveness of the cartridges could be due to the low capacity of the cartridges available and/or the high pH of the NaOH extract. The low capacity of the 1cc cartridges may have resulted in saturation of the bonded phase hence allowing interfering compounds to be eluted unretained. High pH results in the ionisation of any compounds possessing an acidic functional group, these would then be more polar and unlikely to be retained on reverse phase cartridges used. Evaluation of larger SPE cartridges at different pHs or perhaps combinations of bonded phases may produce better results.

Figure 3.7 Blank NaOH Extract of Darvel Soil

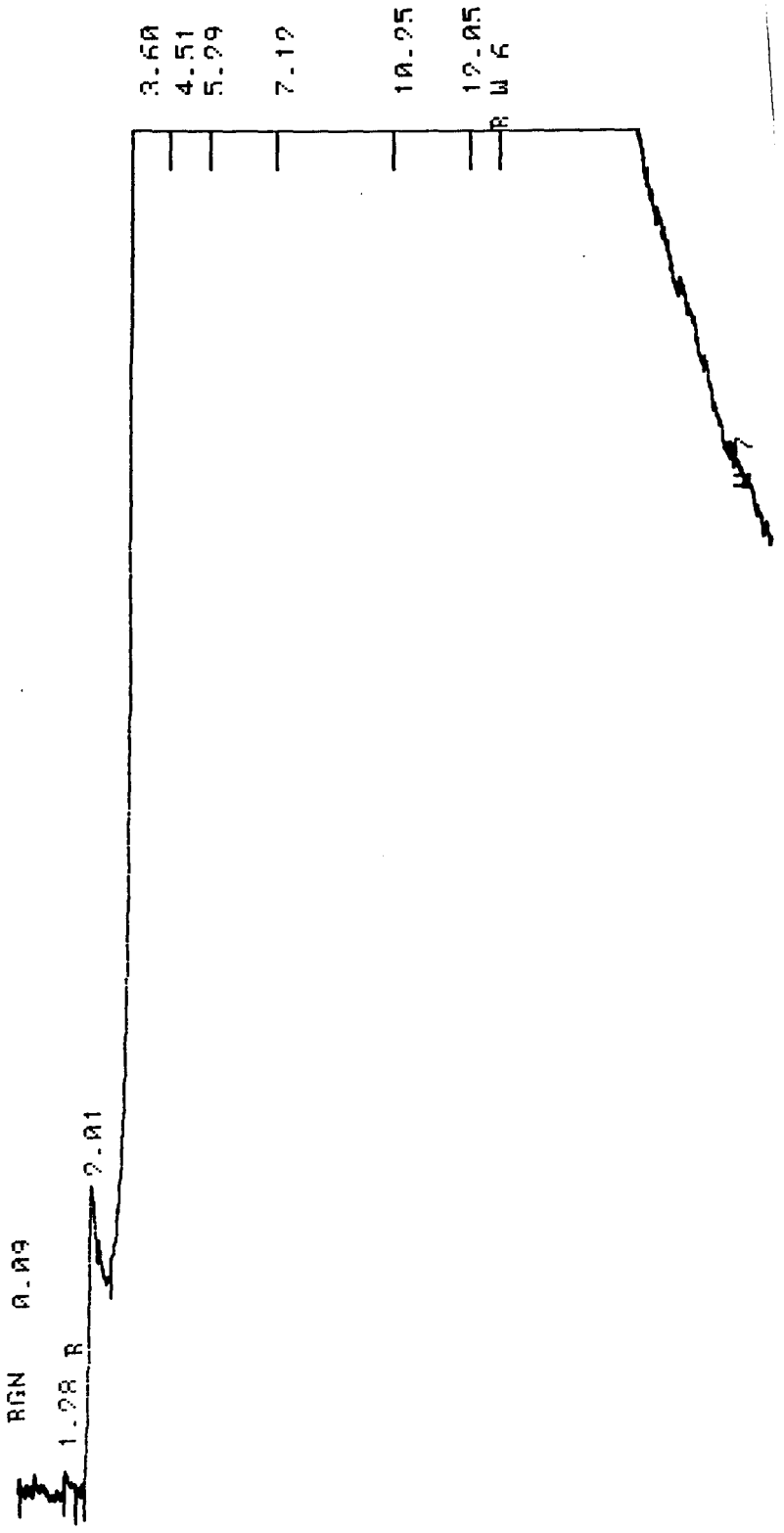
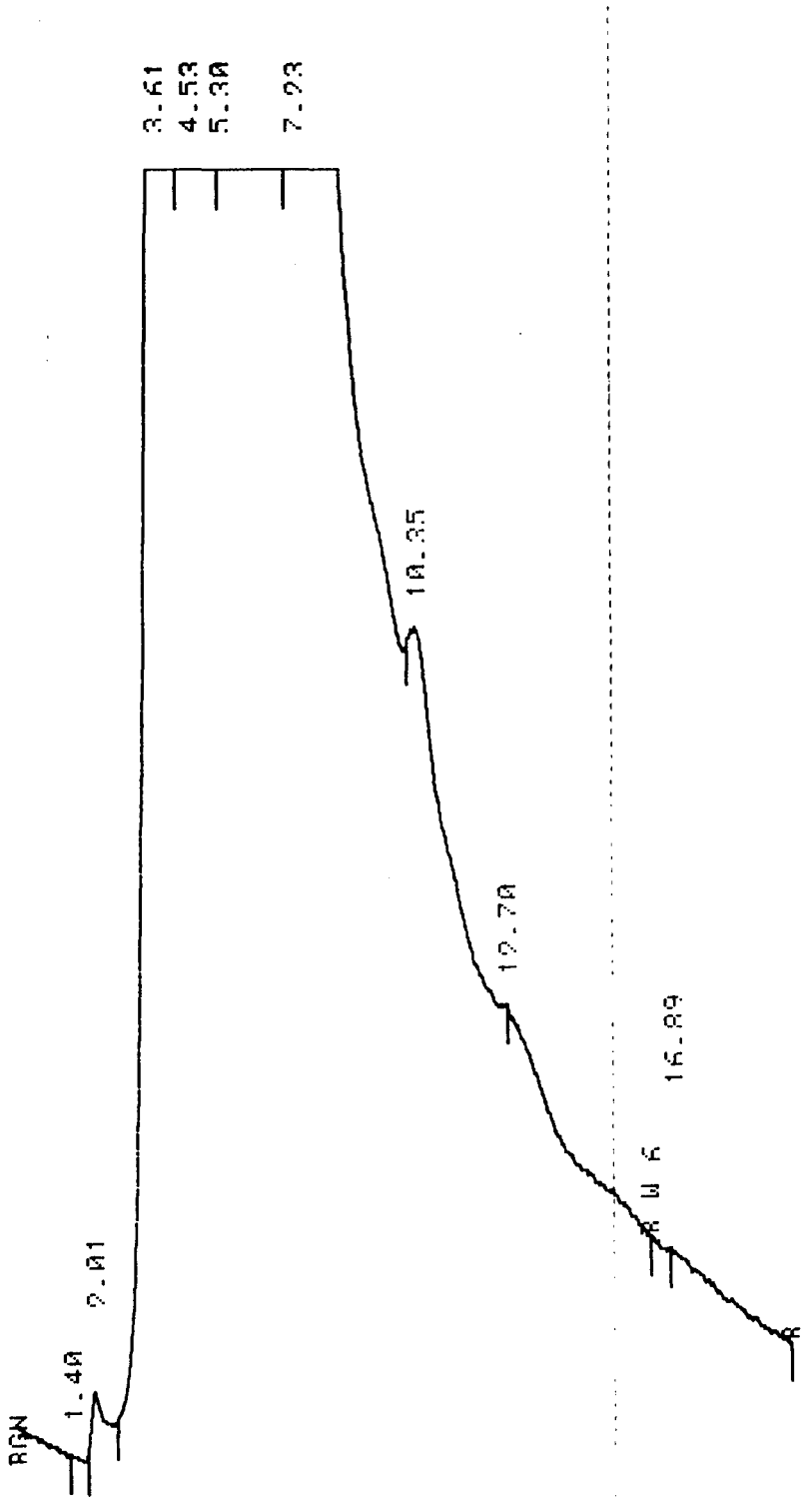


Figure 3.8 Fortified NaOH Extract of Darvel Soil After 2OH SPE Clean-Up



3.6 CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

In contrast to the analysis of glyphosate in plants, the principal problem encountered with soils is the extraction step, not the clean-up procedure. The principal purpose of this chapter was to examine various extractants in order to determine which gave the best recoveries and cleanest chromatograms and hence the greatest sensitivity for glyphosate analysis in soils. Of the extractants used, sodium hydroxide and potassium dihydrogen orthophosphate were selected because of their previous successful use in the literature, while sodium citrate and sodium bicarbonate had not been previously used. Sodium citrate was chosen because of its complexing ability while sodium bicarbonate was chosen because of its alkalinity. Two contrasting soil types were selected: Midelney, a silty clay with 40.4% clay, and Darvel, a sandy clay loam with 24.4% clay. Of the extractants used, sodium hydroxide was the most successful, giving a corrected mean recovery of 99.3% for the Darvel soil and 60.5% for Midelney soil. However, it also gave the 'dirtiest' chromatograms. An attempt was therefore made to clean-up the sodium hydroxide extract using solid-phase extraction cartridges. This was unsuccessful at producing chromatograms from which glyphosate could be quantified at 0.2 $\mu g ml^{-1}$ (0.2 ppm), but the 2OH (diol) SPE cartridge did reduce the level of interferences. An examination of cartridges with larger capacities than those used and the effect of adjusting the pH of the extract prior to SPE clean-up would be worthwhile. It is interesting to note that both bicarbonate and citrate, extractants with different properties, released significant amounts of glyphosate. The success of sodium hydroxide is in agreement with the findings of previous workers (Miles and Moye, 1988) and with work done after the completion of this study (Spann and Hargreaves, 1994, and Aubin and Smith, 1992). Spann and Hargreaves' methodology required two shakes with 0.1M potassium hydroxide, the first for 16 hours and the second for 6 hours.

Recoveries for soils with > 25% clay ranged from 79.4 to 85.7%. A limited improvement on the 60.5% mean recovery obtained for Midelney soil with 40.4% clay in this study with a 30 minute shake and no clean-up. None of the soils used by Spann and Hargreaves contained > 2.2% organic carbon and most contained < 1.6% and are therefore generally unrepresentative soil types. The failure of sodium hydroxide (or KOH) to liberate > 80% of the applied glyphosate from soils with high clay contents implies that the remaining glyphosate is either irreversibly bound or is unavailable to NaOH. As mentioned previously, to date, different extractants have not been used in series, nor have treated soils been subjected to acid or alkali hydrolysis. In order to speed up the screening process and to reduce the experimental error inherent in the use of derivatisation reactions, the use of ^{14} C radiolabelled glyphosate would be helpful. This would also assist in the development of subsequent clean-up method development (if a clean-up was required). A tentative explanation of the failure of NaOH in high clay soils may be inferred from the work of Piccolo et al. (1992). This suggests that glyphosate is held on humic acid-polyvalent cation complexes by two mechanisms. At low concentration by a ligand exchange mechanism and at high concentration by hydrogen bonding to already sorbed molecules. A similar two (or more) tier binding system with clays may be responsible for the limitations of NaOH. Furthermore, the existence of two or more binding mechanisms would explain why citrate and bicarbonate both extracted significant amounts of glyphosate although they are quite different in nature.

Finally, the presence of significant amounts of glyphosate in the citrate and bicarbonate extracts may have some interesting implications. The soil solution contains appreciable amounts of bicarbonate (Russell, 1988) and citrate/citric acid may be released from decaying plant material in the soil or excluded from plant roots. Given that the soil solution may contain citrate and

bicarbonate and possibly other potential glyphosate extractants, more glyphosate may be free in solution than is generally believed. If this is the case, crops such as the potato, (where glyphosate has been shown to impair germination in seed tubers from treated plants (Yusof, 1988)) may be vulnerable if glyphosate in the soil solution can reach the tubers. Phytotoxicity through the soil is also a possibility but requires a relatively high concentration (Hance, 1976). Further work involving direct analysis of the soil solution for glyphosate may produce some interesting results.

CHAPTER 4

INVESTIGATION INTO THE USE OF BIOASSAYS TO STUDY GLYPHOSATE IN THE ENVIRONMENT

4.1 DEVELOPMENT OF A MORE SELECTIVE BIOASSAY FOR THE ANALYSIS OF GLYPHOSATE IN WATER

4.1.1 INTRODUCTION

Thus far, this thesis has been principally concerned with analysing glyphosate using modern chromatographic techniques. As outlined in the introductions to Chapters 2 and 3, the analysis of glyphosate residues in some matricies can be problematical and time consuming. Furthermore, there is some evidence (Yusof, 1988, Don et al., 1990 and Shuma et al., 1995) that glyphosate can have damaging physiological effects on plants, often at levels below those detectable by HPLC and GC. An alternative approach that can often give valuable information on the quantity of a chemical residue and its effects is a bioassay. This is the measurement of the response of a biological material to a chemical or other agent, and can be quantitative or qualitative. Bioassays have been used in a number of ways to study the fate and behaviour of glyphosate in the environment and these have been reveiwed by Richardson (1985).

Where bioassays can be used, they are generally straightforward to set up and give results relatively quickly. The obvious drawbacks of this method are the difficulty in obtaining glyphosate extracts from plants and soils in a suitably clean form and ensuring that any observed effects are due to the presence of glyphosate. Furthermore, it has been shown (Hensley et al., 1978) that glyphosate can be inactivated by a variety of materials including Cu^{2+}, Fe^{2+},

Fe^{3+} etc. The chemistry of this inactivation is not yet fully understood and it is unknown whether the inactivated forms of glyphosate can be easily converted back to 'free' or active glyphosate. Bioassays which will only be sensitive to the active species may underestimate the total quantity of glyphosate present. Paradoxically, this could also be perceived as an advantage since glyphosate residues in the environment are often of interest because of their potential effects on crop species. By definition, bioassays provide this information directly, whereas instrumental methods provide a value for the concentration of the chemical, the possible effects of which must be extrapolated from observations on plants.

One application where bioassays for glyphosate may be of particular use is for its detection in water, particularly in irrigation water where the chemical will come into contact with a growing crop. There, the sample is already in an ideal form for a bioassay and requires little in the way of preparation. A bioassay to measure residue concentrations in irrigation water was developed by Bowmer (1982a, 1982b). Results obtained from the bioassay were compared to those determined by polarography and radiolabelled glyphosate studies. The method involved planting 15-20 safflower (*Saffola* 208) or sunflower seeds (*Helianthus annuuss husun* 30) about 3cm from the edge of a strip of adsorbent paper and spaced every 5cm. The paper was then rolled into a coil, fastened and placed in a standard solution of glyphosate or drainage water contained in 500ml beakers, with the seeds uppermost. Each beaker was covered with a watch glass and incubated for 6-7 days at 25°C. After incubation the lengths of the roots were measured. A 50% reduction in growth was observed with a glyphosate concentration of 0.4 - $0.9gm^{-3}$ ($4-9\mu gml^{-1}$) for safflower and $2.4gm^{-3}$ for sunflower.

A petri-dish bioassay was developed by the Research Group on Weed Control at

the Institute of Plant Physiology in Shanghai, China, using flax as the test plant (Anon., 1978). The useful concentration range of the method was 0.01 to 10 ppm. Seeds were germinated in water for 16-18 hours at 25°C ± 1°C. Ten seeds with uniform radicles were selected and transferred to a 6cm petri-dish containing 8 layers of filter paper, moistened with 5ml test solution. Pre-germinating the plants removed the potential problem of variable germination and distilled water was used for the control. Comparisons of the root and shoot lengths of plants grown in the dark and in light were made. Increases in root growth were produced by the two lowest glyphosate treatment rates (0.01 and 0.1 ppm). Above these rates roots were found to be more sensitive than shoots. Roots were longer when grown in the dark than in the light. The authors maintained that the test was simple, efficient and reproducible.

A significant drawback of both methods is that with a sample of unknown composition there is no way to check that the inhibition of growth is due to glyphosate. However, it has been known for some time that the inhibition of growth due to glyphosate can be ameliorated by certain combinations of aromatic amino acids (Jaworski 1972, Haderlie 1977 and Gresshoff 1979). Currently, glyphosate is the only herbicide on the market with a mode of action that disrupts the Shikimic acid pathway. Therefore, if the inhibition of growth is suspected to be due to glyphosate, amelioration of this growth reduction by certain amino acids would lend weight to the hypothesis that the solution contained glyphosate.

4.1.2 MATERIALS AND METHODS 1

Cress (*Lepidium sativum*) seeds were selected as the plant species for this work as they are inexpensive and readily available. Some preliminary experiments were conducted to assess the best format for the bioassay and the following

set-up was chosen.

Two Whatman No.1 filter papers were placed in the bottom of a 6cm plastic petri dish (Sterilin). Water (1ml) and the test solution (1ml) were then used to soak the papers and fifteen cress seeds were placed on the surface. (It was easier to obtain an even distribution of the seeds if the papers were wetted first). The petri dishes were placed in an incubator in the dark at 25°C for 72 hours, after which time the lengths of the roots and shoots were measured. In some early measurements, the entire length of the seedling was recorded (1).

The initial work was aimed at determining the sensitivity and useful range of cress as a bioassay material. Attention later focused on whether the ameliorating effect of amino acids reported on a range of species in the literature could be repeated in the bioassay.

4.1.3 RESULTS AND DISCUSSION 1

The effect of glyphosate concentrations over the range 0.1 μgml^{-1} to 100 μgml^{-1} on the total length of cress seedlings was investigated. Two parallel tests were set up, one covering the concentrations 0.1, 1.0, 10.0 and 100 μgml^{-1}, the other 0.05, 0.5, 5.0 and 50 μgml^{-1}, each with a distilled and deionised water control. Triplicate petri dishes were set up for each concentration. Tables 4.1 and 4.2 show the mean lengths for each replicate in tests.

Table 4.1 0.01 to 100 μgml^{-1} Glyphosate

Treatment	Rep. 1	Rep. 2	Rep. 3	Mean
Control	54.3	59.4	56.2	56.3
0.1μg glyphosate	54.2	61.4	54.8	56.8
1.0μg glyphosate	55.2	55.4	61.0	57.2
10μg glyphosate	41.0	51.1	48.1	46.7
100μg glyphosate	32.8	30.4	34.0	32.4

Table 4.2 0.05μgml^{-1} to 50μgml^{-1}

Treatment	Rep. 1	Rep. 2	Rep. 3	Mean
Control	60.7	60.5	53.8	58.4
0.05μg glyphosate	55.6	61.5	46.4	54.5
0.5μg glyphosate	56.1	44.6	62.2	54.8
5.0μg glyphosate	53.0	55.3	53.5	53.9
50μg glyphosate	37.0	38.6	39.8	38.5

Analysis of variance followed by pair comparison using the least significant difference (LSD) test shows that glyphosate reduces growth very significantly (1% level) at a concentration of 10.0μgml^{-1}. Since the effect is very significant at this level and no significant effect was observed at 5.0μgml^{-1}, it is reasonable to assume that the lowest concentration necessary to cause an observable effect lies between these two concentrations. Although an ED_{50} (Effective Dose for 50% length reduction) can only be extrapolated from this data it is likely to be in the region of 120μgml^{-1}.

4.1.4 MATERIALS AND METHODS 2

Having determined the concentration range over which glyphosate had a significant effect on the growth of cress seedlings, the potential of the aromatic amino acids; phenylalanine, tryosine and tryptophan; and an organic foliar feed (Stimufol) to reverse the growth inhibition was investigated. The foliar feed was included to determine whether growth inhibition could be reversed by any plant nutrients that are likely to be present in natural water samples. Each petri dish was set up as outlined in section 4.1.2. Individual amino acids were tested at a concentration of 1mmol while the mixture of all three was tested at two levels, 100 and 400μg^{-1} respectively. The foliar feed was also tested at at two levels of 0.5 and 5.0mgml^{-1}. In these tests, both roots and shoots were measured.

4.1.5 RESULTS AND DISCUSSION 2

As was found previously by the Weed Control Research Group at Shanghai (Anon., 1978), roots were more sensitive than shoots. No single amino acid was able to reverse the inhibitory effect of 100$\mu g ml^{-1}$ glyphosate. However, using a mixture of amino acids was successful. The results from the tests using the mixture are shown below in tables 4.3 and 4.4.

Table 4.3a Effect of a mixture of Amino Acids at 100μgml^{-1} each and Foliar Feed at 0.5mgml^{-1}

Treatment	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Trt. Total	Trt. Mean
Control	42.4	46.23	43.1	50.0	37.3	219.0	43.8
Stim	38.3	39.7	36.1	37.4	32.3	183.8	36.8
AA's	44.3	38.8	47.5	35.7	46.6	212.9	42.6
Gly	12.9	13.2	13.0	15.8	13.1	68.0	13.6
Gly + AA	27.4	25.0	26.4	22.2	32.6	133.6	26.7
Gly + Stim	10.3	8.6	10.3	10.8	11.2	51.2	10.2

Table 4.3b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	5	5289.8	1057.96	86.32	2.62	3.90
Experimental Error	24	294.2	12.26			
Total	29					

Table 4.3c Least Significant Difference

		Gly+Stim	Gly	Gly+AA	Stim	AA's	Control
		10.2	13.6	26.7	36.8	42.6	43.8
Gly+Stim	10.2	0	3.4	16.5	26.6	32.4	33.6
Gly	13.6		0	13.1	23.2	29.0	30.2
Gly+AA	26.7			0	10.1	15.9	17.1
Stim	36.8				0	5.8	7.0
AA's	42.6					0	
Control	43.8						0

Tabular t values: 1% = 4.38, 5% = 3.23

Table 4.4a Effect of a mixture of Amino Acids at 400μgml^{-1} each and Foliar Feed at 5.0mgml^{-1} on Cress Roots

Treatment	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Trt. Total	Trt. Mean
Control	42.6	43.8	37.8	45.0	46.3	215.46	43.1
Stim	24.8	26.1	27.2	21.7	20.8	119.99	24.0
AA's	19.3	28.4	26.3	29.2	28.8	130.13	26.0
Gly	13.9	14.1	14.9	14.5	14.7	71.93	14.4
Gly + Stim	10.3	9.8	10.7	10.4	11.5	52.43	10.5
Gly + AA	21.9	27.7	27.1	23.2	25.0	125.9	25.0

Table 4.4b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	5	3222.2	644.45	90.25	2.62	3.90
Experimental Error	24	171.4	7.14			
Total	29					

Table 4.4c Least Significant Difference

		Gly+Stim	Gly	Stim	Gly+AA	AA	Control
		10.49	14.4	24.0	25.0	26.0	43.1
Gly+Stim	10.5	0	3.9	13.5	14.5	15.5	32.6
Gly	14.4		0	9.6	10.6	11.6	28.7
Stim	24.0			0	1.0	2.0	19.1
Gly+AA	25.0				0	1.0	18.1
AA's	26.0					0	17.1
Control	43.8						0

Tabular t values: 1% = 4.38, 5% = 3.23

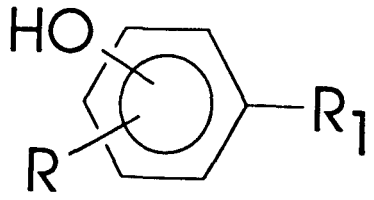
These results show that at both levels used a mixture of phenylalanine, tryosine and tryptophan gave statistically very significant (1% level) reversal of glyphosate induced growth reduction in cress roots. This suggests that the inclusion of a mixture of these amino acids in a bioassay to qualitatively determine glyphosate may make the assay more specific for glyphosate. The failure of the organic foliar feed to ameliorate the effect of glyphosate suggests that such a method may be relatively resistant to the presence of nutrients in test water samples. It is interesting to note that at the higher treatment level, the mixture of amino acids alone had a significant depression of root growth, but still gave a significant reversal of inhibition when glyphosate was present. The degree of growth inhibition reversal displayed by both levels of amino acids was very similar. This may suggest that the lower level of the amino acid mixture ($100\mu gml^{-1}$) was already an excess. It would be interesting to discover if lower levels of amino acids would give a quantitative response.

4.2 POTENTIAL OF A MIXTURE OF AROMATIC AMINO ACIDS AS A SAFENER FOR GLYPHOSATE

4.2.1 INTRODUCTION

In section 4.1, a significant reduction of glyphosate induced growth reduction was demonstrated by applying a mixture of tryosine, phenylalanine and tryptophan. This result was consistent with those results obtained by Jaworski (1972), Haderlie et al. (1977) and Gresshoff (1979) in investigations into the mode of action of glyphosate. Although the reversal of cress seedling growth inhibition was partial, the action of the aromatic amino acids may form the basis of a 'safener' for glyphosate. A safener is a chemical which increases the tolerance of a crop to a herbicide. In the bioassay described in section 4.1, the seed was in continuous contact with a solution of glyphosate, whereas in normal practice a plant would only be exposed to the chemical for a short period. This may make complete reversal of the effects of glyphosate a possibility. Very little work has been published in relation to safeners for glyphosate. This is probably due to the almost complete lack of selectivity exhibited by the chemical. Hence, there is likely to be little scope for any safening effects to favour the crop over the weed species. Nevertheless, the widespread and increasing usage of glyphosate means that if a safener of even limited utility can be found, there may be significant interest. Only three publications concerning the use of safeners for glyphosate were found. Hatzios (1987) evaluated ferrocene, fertilysin, 1,2-Bis(diphenylphosphino)ethane, 1,2-Bis-(dimethylphoshphino)aniline and N,N'-Bis (2,2-diethoxyethyl)methylamine, as potential safeners in glyphosate studies using maize. Candidate compounds were applied at various rates either as seed dressings or post-emergence sprays. Glyphosate was then sprayed on the pre-treated plants at a range of rates from 0.28 to 1.12Kgha^{-1}. None of these materials showed any safening

action. Some success was reported using a family of molecules patented by Lee and Starratt (1987). These were based on the molecules below:



R = H, OH, OMe

R' = COMe, $CHCHCO_2H$

The evidence cited was the mitigation of buckwheat growth inhibition by 0.1mmol glyphosate after pre-treatment with caffeic acid. Finally, and most relevantly, Shaban et al. (1987) reported the recovery of Faba bean (*Vicia faba L)* plants in the field after glyphosate treatment, if they were pre-treated with a variety of growth regulators, amino acids and plant nutrients. These included gibberelic acid, cytokinin, tryptophan, phenylalanine, boric acid, zinc sulphate and urea. These results contradict those of Gresshoff (1979), who found that only a mixture of amino acids acting synergistically could reverse the effect of glyphosate on the growth of a range of organisms. A finding that is reinforced by the results of section 4.1 of this thesis. As the mixture of aromatic amino acids was sussesful in reversing glyphosate induced growth reduction in cress seedlings, it was decided to see if a similar effect could be obtained with whole plants.

4.2.2 MATERIALS AND METHODS

Vermiculite growth medium was used to fill approximately 140 paper cups. Bean seeds (*Phaseolus vulgaris*) were pre-germinated in the dark for 72 hours, then one seedling was placed in each cup. The plants were then grown under glass for 3 weeks. The position of each plant was changed daily in order to ensure even growth across the batch. After three weeks, the plants were divided into three replicates of ten plants for each of four treatments. These were controls, 100μgml^{-1} glyphosate only, 100μgml^{-1} glyphosate plus 400μgml^{-1} amino acid mixture and 400μgml^{-1} amino acid mixture only. Prior to the treatment applications, the shoot height of each plant was recorded. The application of the amino acids was then made by spraying 30 ml of the 100μgml^{-1} solution (with respect to each amino acid) as evenly as possible over each of the triplicate batches using a carbon dioxide driven TLC plate sprayer. The plant height was measured 24 hours after the amino acid spray and the batches nominated to receive glyphosate treatment were sprayed in the same manner as those treated with amino acids. Plant height was recorded after 24 hours, 4 days, 7 days, 10 days, 13 days, 19 days and 23 days. On the 23rd day after glyphosate treatment, the number of leaves and the fresh weight of each plant was recorded, along with any relevant observations.

4.2.3 RESULTS AND DISCUSSION

The mean plant heights recorded for each replicate at each time point are shown in tables 4.5 to 4.13, along with the results of the analysis of variance conducted according to Gomez and Gomez (1984). The mean plant fresh weights and the corresponding analysis of variance and least significant difference figures are shown in table 4.14.

Table 4.5a Plant height 24 hours before first treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	16.03	15.27	14.95	46.25	15.42
AA's only	15.32	16.85	13.83	46.00	15.33
Gly only	13.10	16.35	16.29	45.74	15.25
Gly + AA	16.11	17.26	17.05	50.42	16.81

Table 4.5b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	4.93	1.64	1.02	3.48	5.99
Experimental Error	8	12.84	1.61			
Total	11					

Table 4.6a Plant height 24 hours after amino acid treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	16.27	15.60	15.18	47.05	15.68
AA's only	16.59	17.47	15.74	49.80	16.60
Gly only	13.65	15.10	17.66	46.41	15.47
Gly + AA	16.49	17.30	17.25	51.04	17.01

Table 4.6b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	4.86	1.62	1.21	3.48	5.99
Experimental Error	8	10.76	1.34			
Total	11					

Table 4.7a Plant height 24 hours after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	16.19	15.78	15.58	47.55	15.85
AA's only	14.56	17.08	16.02	47.66	15.89
Gly only	13.23	15.42	17.77	46.42	15.47
Gly + AA	16.33	17.80	17.12	51.25	17.08

Table 4.7b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	4.39	1.46	0.79	3.48	5.99
Experimental Error	8	14.79	1.84			
Total	11					

Table 4.8a Plant height 4 days after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	16.72	17.20	15.95	49.87	16.62
AA's only	15.14	17.98	16.56	49.68	16.56
Gly only	16.61	17.93	17.25	51.79	17.26
Gly + AA	18.17	15.82	13.21	47.20	15.73

Table 4.8b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	3.54	1.18	0.52	3.48	5.99
Experimental Error	8	18.01	2.25			
Total	11					

Table 4.9a Plant height 6 days after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	17.20	17.34	15.88	50.42	16.81
AA's only	16.97	18.06	15.26	50.29	16.76
Gly only	13.68	15.89	18.48	48.05	16.02
Gly + AA	16.72	18.20	17.07	51.98	17.33

Table 4.9b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	2.62	0.87	0.39	3.48	5.99
Experimental Error	8	18.03	2.25			
Total	11					

Table 4.10a Plant height 10 days after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	17.76	17.78	16.01	51.55	17.18
AA's only	17.40	18.32	15.85	51.57	16.60
Gly only	13.38	16.41	18.64	48.43	15.47
Gly + AA	16.97	18.65	17.76	53.38	17.01

Table 4.10b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	4.23	1.41	0.55	3.48	5.99
Experimental Error	8	20.54	2.57			
Total	11					

Table 4.11a Plant height 13 days after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	18.15	18.11	16.16	52.42	17.47
AA's only	15.78	18.50	17.47	51.75	17.25
Gly only	12.70	16.40	18.82	47.92	15.97
Gly + AA	16.94	18.60	17.55	53.09	17.69

Table 4.11b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	5.36	1.79	0.53	3.48	5.99
Experimental Error	8	26.77	3.35			
Total	11					

Table 4.12a Plant height 19 days after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	18.37	18.39	16.66	53.42	17.82
AA's only	17.84	18.88	18.59	55.31	18.44
Gly only	13.40	16.63	19.23	49.26	16.42
Gly + AA	17.20	18.77	17.97	53.94	17.98

Table 4.12b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	6.79	2.26	0.87	3.48	5.99
Experimental Error	8	20.84	2.61			
Total	11					

Table 4.13a Plant height 23 days after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	18.27	18.66	17.62	54.55	18.18
AA's only	19.44	20.53	18.44	58.41	19.47
Gly only	13.20	16.79	19.38	49.37	16.46
Gly + AA	18.18	19.14	17.79	55.11	18.37

Table 4.13b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	13.97	4.66	1.62	3.48	5.99
Experimental Error	8	22.97	2.87			
Total	11					

Table 4.14a Plant fresh weight 23 days after glyphosate treatment

Treatment	Rep. 1	Rep. 2	Rep. 3	Treat. Total	Treat. Mean
Control	75.71	67.27	68.25	211.23	70.41
AA's only	79.03	82.12	77.49	238.64	79.55
Gly only	33.53	48.71	57.45	139.69	46.56
Gly + AA	54.97	59.40	43.17	157.54	52.51

Table 4.14b Analysis of Variance

Variation Source	Deg.Free	Sum Sq.	Mean Sq.	Calc. F	Tab.F 5%	Tab.F 1%
Treatment	3	2119.90	706.63	11.60	3.48	5.99
Experimental Error	8	487.49	60.94			
Total	11					

Table 4.14c Least Significant Difference

		Glyphosate	Gly + AA's	Control	AA's
		46.56	52.51	70.41	79.55
Glyphosate	46.56		5.95^{ns}	23.85**	32.99**
Gly + AA's	52.51			17.90**	27.04**
Control	70.91				9.14^{ns}
AA's	79.55				

Tabular t values: 1% = 14.28, 5% = 10.28

* denotes significant at 5% level

** denotes significant at 1% level

ns denotes not significant

At no time point was there any significant difference in the plant height between the treatment groups. Despite this, there were clear signs of glyphosate injury in the batches treated with both glyphosate only and glyphosate plus amino acids. The glyphosate treated plants tended to have a large number of small (< 2cm across) deformed (crinkly looking) leaves (see photographs, figures 4.1 to 4.4). At the terminal harvest, a very significant difference in the fresh weights between the glyphosate treated and untreated groups was apparent. However, although the glyphosate only treated group was the lightest, the difference between this group and the group pre-treated with amino acids was not statistically significant. Although there is little statistical evidence to support the viewpoint, in appearance the amino acid pre-treated plants did not seem to be as badly affected as those treated with glyphosate alone (See Figure 4.4). These results indicate that there may be differences in the behaviour of whole plants and seeds, which could arise through the

different routes of entry in the two experiments. Use of surfactants or other additives to assist the penetration of the amino acids through the plant cuticle may enhance their performance. The reversal of glyphosate growth inhibition obtained by Shaban et al. (1987) using L-tryptophan and L-phenylalanine singly is difficult to reconcile with the results of this experiment. Both Jaworski (1972) and Gresshoff (1979) reported that reversal of growth inhibition could only be obtained by mixtures of phenylalanine and tyrosine acting synergistically. No significant reversal by tryptophan alone was reported by either author. A larger experiment using a range of amino acid concentrations may give a better indication of the potential of aromatic amino acids to reverse the effects of glyphosate.

Figure 4.1 Photograph showing control plants at harvest

Figure 4.2 Photograph showing glyphosate treated plants at harvest

Figure 4.3 Photograph showing amino acid plants at harvest

Figure 4.4 Photograph showing amino acid plus glyphosate treated plants at harvest

4.3 CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

This chapter was principally concerned with investigating and utilising the ability of aromatic amino acids to reverse glyphosate-induced growth inhibition. Two areas where this phenomenon could be of use were considered: 1) to increase the selectivity of bioassays for glyphosate and 2) to use amino acids as a safener for glyphosate.

A petri-dish bioassay for glyphosate was developed. This consisted of a 6 cm petri-dish with two Whatman No.1 filter papers in the base. Test solutions were applied in a total volume of 2 ml and cress *(Lepidium sativum)* were used as the test species. An observable effect limit for the bioassay was determined to be between 5.0 and 10.0μgml^{-1}. The effect of the aromatic amino acids, phenylalanine, tryosine and tryptophan individually and as a mixture, was investigated. At the two concentrations examined (100μgml^{-1} and 400μgml^{-1}) the mixture of amino acids gave a statistically very significant reversal of glyphosate induced growth inhibition. Neither the individual acids nor an organic foliar feed gave any reversal. The failure of the foliar feed to reverse the effects of the glyphosate suggests that plant nutrients likely to be found in "real" samples will not interfere with the test. These results provide the basis for a bioassay that can more selectively determine glyphosate. Further work to validate the method with environmental water samples would be useful. The degree of growth inhibition reversal at both concentrations of the amino acid mixture was similar. This suggests that the maximum effect was obtained with the lower concentration. It would be interesting to see if a quantitative correlation between the degree of growth inhibition reversal, amino acid concentration and glyphosate concentration could be established.

The potential of the mixture of aromatic amino acids to act as a safener for

whole plants was then investigated. Beans were variously treated with glyphosate only, amino acids only and an amino acid pre-treatment followed by a glyphosate treatment. Plant heights were measured before treatment and at various times up to 23 days after treatment. No statistically significant difference in the plant heights between any of the treatments was found. However, measurement of the fresh weights at harvest showed that those plants treated with any glyphosate were statistically significantly lighter than those untreated (control plants and those treated only with amino acids). No significant difference was found between those plants treated with glyphosate only and those pre-treated with amino acids prior to treatment with glyphosate. These results contradict those of Shaban et al. (1987), who obtained significant reversal of the effects of glyphosate on a variety of physiological measurements. Gibberelic acid (GA_3) alone, or in a mixture with cytokinin, reversed the effect of glyphosate on plant height. Cytokinin, phenylalanine, tryptophan and GA_3 each reversed the effect of glyphosate on plant dry weight. The results obtained with single amino acids contradict those of Jaworski (1972) and Gresshoff (1979) who found that only a mixture of amino acids acting synergistically could reverse the effects of glyphosate. The growth inhibition reversal caused by cytokinin and gibberelic acid may be explained by the fact that glyphosate can cause increased rates of indole-3-acetic acid (IAA) metabolism (Lee, 1982). Other plant growth regulators may compensate for this.

The confusion in this area would benefit from further work to elucidate the effects of various growth promoting substances on glyphosate-induced growth inhibition. This work may also provide information on the effect glyphosate is reported to have in increasing the the rate of IAA metabolism. Further work on whether safening effects can be obtained on whole plants using amino acids, the potential of plant hormones and the degree of selectivity between crop and

weed species would be valuable.

APPENDIX I

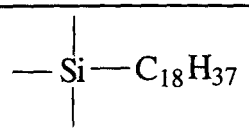
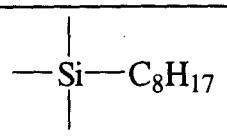
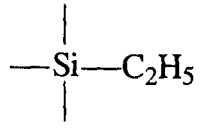
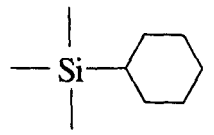
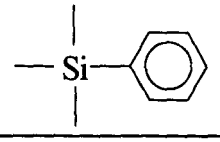
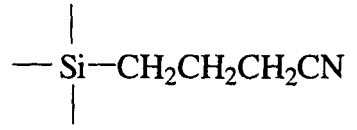
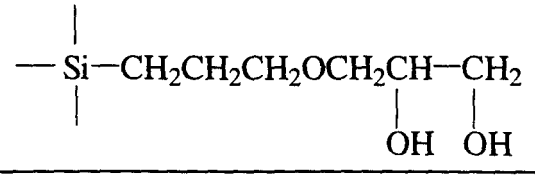
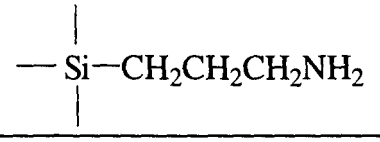
PROPERTIES OF THE HERBICIDE GLYPHOSATE

Molecular Formula:	$C_3H_8NO_5P$
Formula Weight:	169.08
Colour:	Colourless to white.
Physical Appearance:	Crystaline or powdery solid.
Melting Point:	230°C(decomposes)
log K_{oc}:	3.43-3.69
log K_{ow}:	-1.60
Solubility (Organic Solvents):	Insoluble in most organic solvents.
Solubility (Water):	$12gl^{-1}$ at 25°C.
Vapour Pressure:	7.50×10^{-6} mmHg at 25°C.

Source: Agrochemicals Desk Reference (Anon., 1993)

APPENDIX II

STATIONARY PHASE STRUCTURES OF SOLID PHASE EXTRACTION CARTRIDGES

C18	Octadecyl	
C8	Octyl	
C2	Ethyl	
CH	Cyclohexyl	
PH	Phenyl	
CN	Cyanopropyl	
20H	Diol	
NH2	Aminopropyl	

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