THE FATE OF CHLORPROPHAM WITH PARTICULAR REFERENCE TO ITS USE AS A POTATO SPROUT SUPPRESSANT

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CONTENTS

A list of abbreviations for names used in this thesis

cm	centimetre
cpm	counts per minute
Cv.	cultivar
dpm	disintegration per minute
dps	distintegration per second
ECD	electron capture detector
EPA	environmental protection agency
FDA	food and drug administration
FID	flame ionisation detector
g	gram
GC	gas chromatograph(y) (ic)
h	hour
HPLC	high performance liquid chromatograph(y) (ic)
i.d.	internal diameter
IR	infra-red
l	litre
LSC	liquid scintillation counter/liquid scintillation counting
m	metre
M	molar unit
mg	milligram
min	minute
ml	millilitre
mm	millimetre
N	normal unit (concentration)

nm	nanometre
ng	nanogram
OD	optical density
ppm	parts per million
psi	pounds per square inch
sec	second
TLC	thin-layer chromatography
UV	ultra-violet
°C	degrees Celsius
<	less than
>	more than
µg	microgram
µℓ	microlitre

Note: abbreviations for the name of units are the same for singular and plural.

SUMMARY

This thesis examines the fate of chlorpropham when applied to stored potatoes to suppress sprouting. Throughout this project the work concentrated on the use of chlorpropham in potato processors' stores and in particular crisp manufacturers' stores since these are operated in a consistent manner and the largest proportion of the sprout suppressant formulation of chlorpropham is used in these stores.

The fate of chlorpropham was investigated in two distinct areas. The portion of the applied chlorpropham that ends up in the crisps as a residue is the first area, while investigation of the possible biotransformation products of chlorpropham produced by three potato pathogens is the second.

A sensitive analytical method for the determination of chlorpropham residues in crisps was developed using acetonitrile as extracting solvent followed by an oil reducing step which was essentially a counter-current separation of chlorpropham and co-extracted oil with final quantification by gas chromatography (GC) or high performance liquid chromatography (HPLC). Recovery from crisp samples spiked with chlorpropham was 93.2% ± 6.37 and the minimum detectable amount was 0.035 μg/g when the GC was used. When the HPLC was the instrument used to quantify the residues then a minimum of 0.02 μg/g could be detected and quantified.

A chlorpropham residue of 0.45 μg/g was detected in crisps produced from slices originally containing a 0.18 μg/g residue.

Chlorpropham contamination of untreated material resulted after frying in friers which had been previously processing chlorpropham treated material.

The existing literature concerning the transformation of chlorpropham is reviewed in Chapter 2, with particular reference to potatoes and potato storage situations although little information was available on the fate of chlorpropham that is applied to this staple food.

Radiolabelled chlorpropham that had been synthesised by Isabel Boyd in this department was purified using an alumina clean-up column and made ready for use in the biotransformation studies.

Erwinia carotovora var. atroseptica (bacterial soft rot), Polyscytalum pustulans (skin spot) and Phoma exigua var. foveata (gangrene) were grown on standard growth media apart from the fact that labelled chlorpropham had been incorporated into the media.

The E. carotovora var. atroseptica, a facultative anaerobe, was cultured under both aerobic and anaerobic conditions. Complete recovery of the medium incorporated radiolabel as unaltered chlorpropham was observed.

In the P. pustulans study complete recovery of the radiolabel was not achieved. Unaltered chlorpropham contained all of the label that was extracted, but since only incomplete recovery of the applied radiolabel was achieved then it is invalid to state that no metabolites were produced.

In the metabolism study of the P. exigua var. foveata, more complete recoveries of the radiolabel from the cultures were accomplished and a water soluble metabolite was detected. Characterisation of this

metabolite was not possible but some solvent partitioning characteristics were identified as were its chromatographic behaviour on HPLC.

CHAPTER 1

INTRODUCTION AND THESIS OBJECTIVES

1.1 INTRODUCTION

Nowadays a sizeable proportion of the United Kingdom potato crop is stored for up to nine months. The pattern of consumption of domestically prepared potato products shows a disproportionately high consumption during the traditional harvest period of maincrop varieties (Anon, 1981) and these potatoes do not need to be treated with a sprout suppressant. The processing industry, for reasons of economy and shelf life of their products, requires a more uniform supply of potatoes throughout the year and, in addition, does not switch to using early varieties until some time after domestic users of potatoes have been utilising the new crop. In order to produce a bright product, the processors and, in particular, those processors engaged in crisp manufacture require their potato supplies to be of a low reducing sugar content to keep the browning reactions that occur during frying to a minimum.

The method used to minimise the accumulation of reducing sugars in the tubers is to store the potatoes at 8-10°C. Potatoes that are stored at temperatures of 3-4°C for long periods result in minimal sprout growth over periods of up to one year, while in stores that are at higher temperatures, sprout growth is rapid and without the application of sprout suppressants, storage at temperatures of 8-10°C would be impossible (Burton, 1978).

In Britain the chemical sprout suppressants available at present are tecnazene (1,2,4,5-tetrachloro-3-nitrobenzene), chlorpropham (isopropyl N-(3-chlorophenyl)carbamate), chlorpropham/propham (isopropyl N-phenyl carbamate) mixtures and maleic hydrazide (6-hydroxy-2H-pyridazin-3-one). Of these four options only the first three are approved on a basis of safety and efficiency under the Agriculture Chemical Approval Scheme (ACAS). Maleic hydrazide had limited clearance on a restricted acreage of potatoes in 1985 on the basis of safety.

The use of maleic hydrazide to control sprouting of onions in the United Kingdom has been approved for many years. Despite its widespread use in the United States on the potato crop, it was only first given limited Pesticides Safety Precaution Scheme (PSPS) clearance for application to the United Kingdom potato crop in 1985. Maleic hydrazide is the only commercial sprout suppressant that is applied pre-harvest to the potato crop and is required to be translocated to the tuber to be effective as a sprout suppressant. It will be interesting to see if full PSPS clearance is obtained for the 1986 season and whether it will become extensively used in the United Kingdom.

Propham in combination with chlorpropham is used widely in continental Europe for the suppression of potato sprouting, but it is not as widely used in the United Kingdom as the chlorpropham formulations. The two main sprout suppressants used in the United Kingdom then are tecnazene and chlorpropham, with the chlorpropham accounting for the majority of the processors market.

The advantages of chlorpropham over tecnazene include low cost, ease of application and reapplication and its potency as a sprout

inhibitor. Chlorpropham is "fogged on" to stored material as an aerosol and this is usually achieved by placing the applicator between the passageway in a box store or at the fan intake of the ventilating system of a bulk store. The ventilating system of a box store does not utilise outside air during and typically for 24 hours after the aerosol application as this helps to reduce losses of the sprout suppressant from the store. The application can be easily repeated with the frequency of applications being dependent on the application rate used and the intensity of ventilation. A typical application rate would be 20g chlorpropham/tonne of potatoes and this would be repeated every 8-12 weeks (Anon, 1985).

In the case of tecnazene no foggable formulation is at present available and this coupled with the inability of tecnazene to control sprouting after the break of dormancy and its relatively high cost results in limited use of tecnazene in the processing industry. Tecnazene is, however, often used in processors stores to control sprouting during the wound healing period since it does not appear to inhibit wound healing of damaged tubers (McGee, 1984).

It is perhaps worth mentioning that although tecnazene is not as suited to large store use as foggable chlorpropham, it is still widely used in smaller on-farm stores where sophisticated ventilating systems are rarely a feature of the storage building. The absence of a requirement for sophisticated applicators is often considered an advantage by the individual grower. The ability to treat batches and its approved use on seed potatoes adds to its flexibility on the farm. More importantly, however, the storage of seed grain and other crops which will be required to germinate at a later date is not precluded from buildings that were

used to store tecnazene treated material. Cereal seed stored in a building that had been previously used to treat potatoes with chlorpropham has become contaminated and subsequent field emergence has been poor (Anon, 1975a).

1.2 Thesis Objectives

Today, before a new chemical receives clearance to be used in agriculture, a massive research effort is required to produce the necessary data relating to the toxicology, metabolism and residue chemistry. In the past, however, agrochemicals were introduced without as great a research effort into these aspects as would be required today, although upgrading of the information relating to these so-called backlog chemicals is requested, from time to time, by the Ministry of Agriculture, Food and Fisheries (MAFF).

The objectives of this thesis were to provide additional information and insight into the behaviour of chlorpropham in potato stores. This, however, did not include the study of the distribution of chlorpropham within the store and various tuber residues that result from the application of chlorpropham or the headspace concentration analyses which were part of the work of Isabel Boyd and William D. Boyd, respectively. The work described here was to centre on the likely sites of metabolism of chlorpropham and the loss of chlorpropham during the frying process.

At the beginning of this work, residue levels in the crisp products had only been reported as less than the limit of sensitivity of the analytical method employed (0.05 μg/g) by Martens et al (1971) and

Rudd (1959). Rudd (1959) had reported one residue figure which was marginally above the 0.05 μg/g limit of sensitivity of their analytical method, but this was in crisps produced from tubers which had a larger than normal application of chlorpropham and were only stored for a short period. Clearly, if less than this value was present in the crisp, then significant amounts of the chlorpropham residue must have been lost during the frying process and the mechanisms of these losses was in need of elucidation.

As regards the metabolism of chlorpropham by potato tubers and in the situation of the chemical's use, i.e. a potato store, little specific work has been carried out on these aspects. Much of the reported work centred on improving the distribution and efficacy of the sprout suppressant. There has of course been a great deal of metabolism studies of chlorpropham but omission of the case where the application of this agrochemical was directly onto the edible tuber seems surprising. Although only relatively small doses are applied to the tuber, relatively large residues remain in comparison to the other uses of chlorpropham. Other approved uses include spraying onto bare ground as a pre-emergence herbicide or onto the small exposed plant parts when used as an early post-emergence herbicide, and its use in these circumstances results in very small crop residues. Much of the early metabolism work was to determine the mechanism of action and to explain the tolerance and susceptibility that exists between plant species.

The sites of transformation of the chlorpropham could be divided into biological and non-biological. The non-biological transformation sites were not considered in this study since it was thought that

investigations by others would have identified any major non-biologically implicated transformations. In the case of biological sites of transformation, it is important to try and maintain a degree of authenticity in such studies and relevance to the potato store situation. Obviously the most realistic system to employ would be that of the store itself. However, without previous knowledge of what transformation would occur, the operation of such a system would be impossible. Under such a system only quantification of previously identified metabolites could be undertaken.

Identification of metabolites is a first step and radiolabelling of chlorpropham is an invaluable analytical tool in this respect but, at the same time, would prevent such a study from being carried out in a large commercial store. The biological systems had to be simplified into single component systems which it was thought might transform chlorpropham. Although this simplistic approach would not be able to elucidate interactions between the many biologically active areas which are potentially sites of pesticide transformation, it was thought to be more manageable since growth regimes, extraction systems and identification of metabolites would be less complex.

CHAPTER 2: Metabolism of Chlorpropham

Reviews the literature on the metabolic fate of chlorpropham, not only when used as a sprout suppressant, but also its metabolism by micro-organisms, animals and plants, since the type of transformations that occur are often not unique to any one organism or class or organism.

CHAPTER 3: The Determination of Chlorpropham Residues in Crisps

The initial aim of this study was to develop a method for the determination of chlorpropham residues at low levels in crisps and crisp frying oil and to determine the mechanism of loss of the chlorpropham residue from potatoes during the crisp manufacturing process.

CHAPTER 4: High Pressure Liquid Chromatography

This chapter describes the chromatograph, column packing techniques, operation and use of the chromatograph, as well as some background theory.

CHAPTER 5: The Synthesis and Purification of Radiolabelled Chlorpropham

The object of this work was to purify and quantify the total amount of radiolabelled chlorpropham available. The activity of the radiolabelled chlorpropham that had been previously synthesised by Isabel Boyd was also determined.

CHAPTER 6: The Metabolism of Chlorpropham by Erwinia carotovora var. atroseptica

The object of this study was to identify the metabolites of chlorpropham produced by the causal agent of bacterial soft rot of potatoes.

CHAPTER 7: The Metabolism of Chlorpropham by Polyscytalum pustulans

The aim of this investigation was to identify the metabolites of chlorpropham produced by the fungus responsible for the development of skin spot on potatoes.

CHAPTER 8: The Metabolism of Chlorpropham by Phoma exigua var. foveata

The purpose of this study was to identify the metabolites of chlorpropham produced by the fungus responsible for the development of gangrene in potatoes.

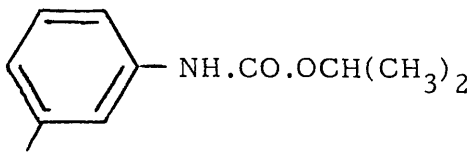
CHAPTER 9: Conclusions

The role of this chapter was to discuss the findings of this work, draw some conclusions and make some suggestions for further work.

CHAPTER 2

METABOLISM OF CHLORPROPHAM

Chlorpropham is the common name used for isopropyl N-(3-chlorophenyl)carbamate. Isopropyl 3-chlorocarbanilate and isopropyl m-chlorocarbanilate have also been used and the chemical abstracting service of the American Chemical Society (ACS) identify chlorpropham by its carbamic acid parent compound and the N-3-chlorophenyl derivative and the 1-methylethyl ester. The chemical abstracts (CA) registration number of chlorpropham is [101-21-3]. Trivial names of Chloro-IPC and CIPC are also used.



Chlorpropham

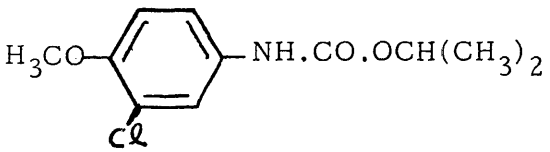
Chlorpropham is one of the phenylcarbamate group of pesticides. Propham is a closely related analogue of chlorpropham with the only difference in the chemical structure being that no chlorine atom is attached to the phenyl ring.

The range of agrochemical uses of propham are similar to chlorpropham including the use of both as sprout suppressants on potatoes although chlorpropham is usually the main constituent of the active ingredients in potato sprout suppressant formulations (Vliet and Sparenberg, 1970) and in the United Kingdom only a small amount of the propham containing sprout suppressant formulation is used (French, 1976).

The metabolism of chlorpropham directly related to its use as a sprout suppressant has been investigated by Coxon and Filmer (1985), Jumar and Sieber (1964) and Heikes (1985). Jumar and Seiber (1964) were primarily concerned with determining the effects of storage conditions on the residue of chlorpropham remaining in or on the potatoes. They used chlorine-36-labelled chlorpropham to monitor the residue of chlorpropham remaining in and on potatoes during the storage season and could detect no chlorpropham metabolites in dichloromethane extracts of potatoes. The investigations of Jumar and Seiber (1964), were essentially paralleled by Steinbeiss et al. (1972) and Peisker et al. (1972) who used carbon-14-labelled (in the isopropyl moiety) chlorpropham but no attempt was made to identify any metabolites which may have been produced. Coxon and Filmer (1985) used dual-labelled chlorpropham containing both a chlorine-36-label, and a carbon-14-label (in the isopropyl moiety) in what was essentially a distribution and volatilisation study within containers of potatoes. Coxon and Filmer used thin layer chromatography (TLC) radioscanning of methanolic extracts of potatoes and detected a metabolite more polar than chlorpropham but only 0.1% of the amount applied to the potatoes had been metabolised after a storage period of 34 weeks.

The only potato metabolite of chlorpropham to have been identified and characterised was that observed by Heikes (1985). Heikes is a member of the Total Diet Program Team which monitors residues of pesticides, among other things, in the United States national food supply under the direction of the United States Food and Drug Administration.

During their routine analysis of food samples it was noticed that unidentified analytical responses had been associated with potato products which also contained a residue of chlorpropham. When unidentified analytical responses could not be shown to be attributable to a known pesticide or contaminating industrial pollutant they became candidates for analysis by mass spectrometry. It was subsequently shown that the unidentified analytical response associated with the chlorpropham residues of potatoes was due to the presence of isopropyl N-(3-chloro-4-methoxyphenyl)carbamate in the extract of potatoes.



isopropyl N-(3-chloro-4-methoxyphenyl)carbamate

The biotransformation of chlorpropham to its methoxy substituted analogue was confirmed by treating potatoes with chlorpropham and monitoring the appearance of the metabolite. The concentration of the methoxylated chlorpropham built up so that after a storage period of six weeks 1% of the chlorpropham initially applied had been metabolised.

Apart from the work of the above research workers no others have published work relating to the transformation of chlorpropham when used in a potato storage situation. That is not to say however that none of the other work concerning the fate of chlorpropham is not relevant to potato storage since pathways of biotransformation are often not unique to one situation or organism.

The fate of chlorpropham in the environment can be divided into biological and non-biological implicated "loss mechanisms". The physical characteristics of an agrochemical influence; its rate of removal from the immediate environment of the application; its segregation by compartmentalism or occlusion from the site of action; and the rate of inactivation of the agrochemical. The rate of removal of a field applied chemical is affected by its water solubility, its sorption-desorption characteristics in the soil and the volatility of the agrochemical. Although rainfall is not a consideration in potato store uses of chlorpropham its adsorption to the soil particles which adhere to the surface of the potatoes should be considered. Volatility of the chlorpropham is an important factor when it is used as a sprout suppressant.

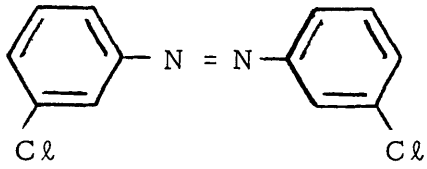
Losses of chlorpropham in the ventilating air and redistribution within the potato stocks has been shown (Boyd, 1984; Coxon and Filmer, 1985; Peisker et al., 1972; Steinbeiss, 1972; Corsini et al., 1979). Adsorption onto soil particles can affect the availability for degradation (Hurle, 1980) just as the adsorption of chlorpropham by soil particles in aqueous suspension can affect its availability for microbial transformations (Steen et al., 1980). Since photolysis of chlorpropham has been demonstrated in aqueous media to yield an hydroxylated biphenyl (Tanka et al., 1984) then it is likely that volatility as well as water solubility will influence the proportion which is transformed in this way.

The stability of the agrochemical towards attack by acid and alkali and its stability in oxidation/reduction systems may affect the time period that it persists at physiologically effective concentrations.

The partition coefficient of a pesticide displayed in an octan-1-ol/water system can be used as an indication of the likelihood of bioaccumulation of a chemical and may result in it being concentrated in parts of the body which are less exposed to the xenobiotic metabolising apparatus of an animal. Although the octanol/water partition coefficient of chlorpropham would give a figure that would suggest that bioaccumulation was possible, the excretary system of animals generally results in rapid removal of chlorpropham from body tissues. Bioaccumulation of chlorpropham has not been demonstrated in mammals but it has been observed in fish and crustaceans in model ecosystems (Erb et al., 1980).

In the case of metabolism of chlorpropham in the soil environment hydrolysis of the chlorpropham molecule yields 3-chloroaniline, propan-2-ol and carbon dioxide. Soils, pure microbial cultures and isolated enzyme systems have all been shown to produce these products from chlorpropham (Kaufman, 1967; Kaufman and Kearney, 1965; Kearney and Kaufman, 1964). The production of these products follows the hydrolysis of the ester or amide bond of the carbamate linkage. Hydrolysis of the ester bond yields propan-2-ol and 3-chlorophenylcarbamic acid which spontaneously decomposes to 3-chloroaniline and carbon dioxide. Amide hydrolysis on the other hand yields 3-chloroaniline and isopropyl carbonate which spontaneously decomposes to propan-2-ol and carbon dioxide. On the basis of the products, therefore, it is not possible to say which mechanism of hydrolysis of the carbamate linkage operates.

During the late 1960's and early 1970's there was considerable concern over the formation of azobenzenes from pesticide derived anilines. Small amounts of 3,3'-dichloroazobenzene have been shown to have been produced in soil when 0.05g or 0.1g of 3-chloroaniline was added to 50g of soil. This however represents an aniline application of the equivalent of 1,000 or 2,000 kg/ha for each 10 cm depth of soil (Bartha et al., 1968; Kearney et al., 1969). 3,3'-Dichloroazobenzene has not been detected in commercial fields where chlorpropham has been applied as a herbicide.



3,3'-dichloroazobenzene

However, traces of 3,3',4,4'-tetrachloroazobenzene have been detected in rice fields treated with the herbicide propanil (N-(3,4-dichlorophenyl) propionamide) which yields the 3,4-dichloroaniline on hydrolysis. Electronegative substituents in the 3 position of the ring reduces the likelihood of azobenzene production in isolated enzyme studies and therefore chlorpropham derived 3-chloroaniline is unlikely to yield 3,3'-dichloroazobenzene under field conditions (Bordeleau and Bartha, 1972).

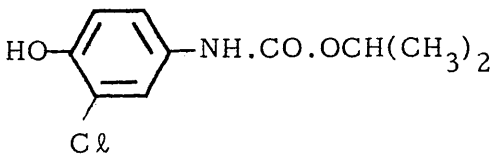
A microbial involvement in soil metabolism of chlorpropham has been shown by the reduction in metabolism following soil incorporation of a microbial inhibitor (Fletcher and Kirkwood, 1982) and when incubation conditions are conducive to microbial activity chlorpropham transformation is rapid (Herret, 1969). Incorporation of ring labelled

chlorpropham (and/or its metabolites) into the soil organic matter has been noted (Kaufman, 1967). Chloroaminophenols have been detected as metabolites of chlorpropham in soil by Fletcher and Kaufman (1979) and they suggest that once formed they are quickly incorporated into the soil organic matter. Cultures of algae too have been shown to hydrolyse chlorpropham to yield 3-chloroaniline (Wright and Maule, 1981). It is likely that the activity of the soil inhabiting microbial population on which chlorpropham transformation in soil depends would be low in potato stores due to the small amounts of the adhering soil being relatively dry and the storage temperatures being cool.

The research effort on the metabolism of chlorpropham in plants has been extensive. During the late 1960's and through the 1970's much of this effort concentrated on finding a role for a differential toxicity mechanism of chlorpropham. The idea was that if a rapid rate of metabolism of chlorpropham was observed in tolerant plant species and if these metabolites were of lower herbicidal activity then this could be used to explain the selectivity of chlorpropham as a herbicide.

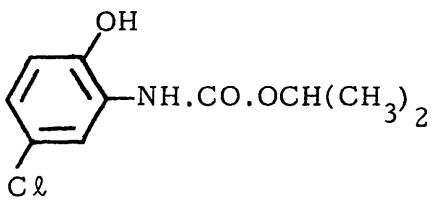
Prendeville et al. (1968) studied the metabolism of chlorpropham in three plant species of differing susceptibilities to chlorpropham and observed the formation of water soluble metabolites in all three species. This was followed by James and Prendeville (1969) who identified the plant metabolites as being β-glucosides. They reported that no aryl hydroxylation had occurred but that the alkyl moiety had been modified. The United States Department of Agriculture Metabolism and Radiation Research Laboratory in Fargo, North Dakota then continued

with the efforts to identify if metabolic inactivation was responsible for the differential toxicity of chlorpropham. Still and Mansager (1971) reported that polar water soluble metabolites were first formed and then conjugation with plant components rendered them insoluble and that the propyl moiety was unchanged by soybeans. Still and Mansager (1972) reported the polar water soluble metabolite as isopropyl N-(3-chloro-4-hydroxyphenyl)carbamate.



isopropyl N-(3-chloro-4-hydroxyphenyl)carbamate [4-hydroxy-chlorpropham]

Later, Still and Mansager (1973a) reported that isopropyl N-(5-chloro-2-hydroxyphenyl)carbamate was also a metabolite of chlorpropham in soybeans.



isopropyl N-(5-chloro-2-hydroxyphenyl)carbamate [2-hydroxy-chlorpropham]

Cucumber plants could also metabolise chlorpropham to yield 4-hydroxy-chlorpropham (Still and Mansager, 1973b). It was subsequently suggested that no simple differential phytotoxic mechanism was operating (Still et al., 1974a).

The formation of an S-cysteinyl conjugate of chlorpropham (as an aryl thioether) without the loss of the chlorine or the hydroxyl group from 4-hydroxychlorpropham was shown to be effected by excised

oat seedling shoot tips or root systems (Still and Rusness, 1977) and by a partially purified enzyme from oat (Rusness and Still, 1977).

A straightforward explanation of selective toxicity based on differential rates of metabolism of chlorpropham to metabolites of different toxicities is yet to be put forward. It may be that the early ideas of these workers that the chlorpropham conversion to polar metabolites and then to insoluble residues may be partially responsible for the selectivity of chlorpropham.

Plant metabolism of chlorpropham can be summed up as follows:

1. Cleavage of the carbamate linkage is not observed.
2. Aryl hydroxylation is widespread and depending on the plant species can be rapid.
3. Conjugation of the hydroxylated chlorpropham often occurs most commonly with glucose or cysteine.
4. Alkyl hydroxylation also occurs.
5. A large proportion of applied chlorpropham is subsequently detected as in insoluble residue.

Animal metabolism is outwith the scope of this thesis but metabolites which have been reported are worthy of examination because some of the degradative pathways are similar to non-mammalian systems. Animal metabolism of chlorpropham has been reviewed by Ryan (1971) and Menzie (1978).

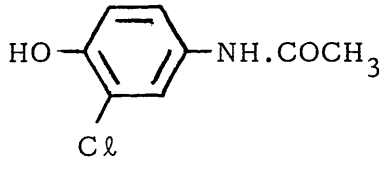
Rapid metabolism of chlorpropham occurs in animals. Excretion of the radiolabel from rats orally dosed with phenyl (ring) labelled chlorpropham showed more than 80% of the administered label

appeared in the urine and respirational gases accounted for 5% (Fang et al., 1975) and the faeces only contained 3-4% (Bobik et al., 1972) over a period of 2-3 days. When isopropyl labelled chlorpropham was administered the respired air contained 35% of the total dose and the urine contained only 56% (Fang et al., 1974). Therefore hydrolytic cleavage of the carbamate linkage was effected in the rat. In the case of propham, the closely related analogue of chlorpropham only about 5% of the total radioactivity administered as isopropyl labelled propham was lost through respired air (Fang et al., 1974). A hydrolytic role for the gut microflora is not likely since prior elimination of the microflora in the gut resulted in no major change in the metabolic products of chlorpropham (Bobik et al., 1972).

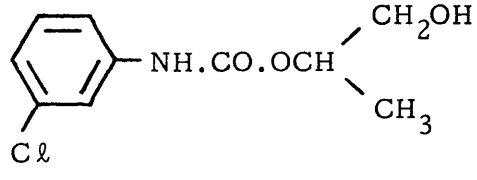
Aryl hydroxylation in the 4 position of the ring is the most extensive transformation occurring in animals. Hydroxylation at this position occurs with both intact chlorpropham and its hydrolysed products (Grunow et al., 1970). Aryl hydroxylation in the 2 position is less favoured with only 6-15% of the total administered carbon-14-labelled chlorpropham displaying substitution at this position in comparison to 55-70% being hydroxylated at the 4 position (Ryan, 1971).

Once the chlorpropham has been hydroxylated sulphate and glucuronide conjugates are formed and excreted in the urine with the sulphate ester being the major metabolite. The anilines produced by hydrolysis of the carbamate linkage undergo N-acetylation to 3'-chloro-4'-hydroxyacetanilide and 5'-chloro-2'-hydroxyacetaniline which are again excreted as glucuronide or sulphate conjugates (Grunow et al., 1970).

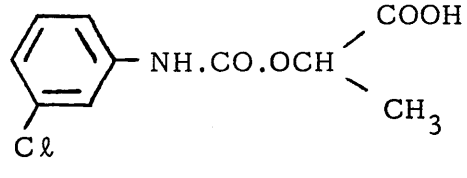
Oxidation of the alkyl moiety also occurs to give 1-hydroxy-2-propyl N-(3-chlorophenyl)carbamate and further oxidised to the 1 carboxyl-1-ethyl analogue of chlorpropham (Grunow et al., 1970). 1,3-Dihydroxy-2-propyl derivative of chlorpropham is also formed but only to the extent of about a quarter of the monohydroxylated derivative (Fang et al., 1974).



3'-chloro-4'-hydroxyacetanilide



1-hydroxy-2-propyl N-(3-chlorophenyl)carbamate



1-carboxy-1-ethyl N-(3-chlorophenyl)carbamate

In the case of animals dosed with radiolabelled chlorpropham the situation could be summed up as follows:-

1. The radiolabel was excreted rapidly.
2. The carbamate linkage of the chlorpropham and its metabolites was cleaved in animals.
3. Further metabolism included aryl and alkyl hydroxylation, N-acetylation and conjugation with sulphate or glucuronic acid.
4. No intact chlorpropham appeared to have been excreted.

The potential transformation sites for chlorpropham in a potato store include the soil, the potatoes themselves and potato pathogens. The metabolism of chlorpropham in the soil situation has been extensively studied (Herret, 1969). For the reasons stated above i.e., the small amount of soil present, its water content and temperature, significant chlorpropham transformation in store soil is unlikely. Potato pathogens are often active in a potato store and these could participate in chlorpropham biotransformations. The potatoes themselves, despite being treated with what is essentially a herbicide (chlorpropham), respire throughout the storage season and transformation of chlorpropham within the potato tubers may occur. The one metabolite observed by Heikes (1985) (see above) is the only metabolite to have been identified in a potato storage situation. It may be the case that significant metabolism of chlorpropham occurs within potato tissue and the high activity of the non-extractable radiolabel in the potato peel fraction observed by Coxon and Filmer (1985) may not be entirely due to unaltered radiolabelled chlorpropham. The characterisation and fate of this fraction is in need of elucidation.

The metabolism of insoluble residues of propham contained in propham treated alfalfa was studied by Paulson et al. (1975). These workers were concerned not just with the metabolism of the propham residue in the alfalfa feed but also with the residues of the metabolites of propham which result from propham herbicide treatment of an alfalfa crop.

Using sheep and rats as test animals they were able to show rapid excretion of the radioactivity contained in the alfalfa feed which had been treated with ring labelled propham during the growth of the crop. In the rats a greater proportion of the radiolabel initially present in the alfalfa feed appeared in the faeces (44%) (Paulson et al., 1975) compared to the case when isopropyl-labelled propham was orally administered (9%) (Bend et al., 1971). Sheep on the same alfalfa diet as the rats only excreted 32% of the radiolabel in the faeces over the same 4 day period. This presumably reflected the different digestive system of the sheep in that much of the "fibre" in the feed could be digested in the ruminant but not by the simple stomached rat. Despite greater absorption of radioactivity from the gastrointestinal tract in the sheep a smaller proportion of that fed was retained in the sheep's body than was the case in the rat.

Paulson et al. (1975) also prepared alfalfa which had been extracted to remove the soluble propham residue and this extracted alfalfa was fed together with the insoluble residue contained therein to rats. The radioactivity excreted in the faeces amounted to 93% of total intake, and this again highlights the inability of the rat to digest feeds high in fibre. The amount of radiolabel excreted in the urine was very

low and the radioactivity retained in the bodies of the rats was low, but that which was retained represented a greater proportion of the total absorbed from the gut.

To investigate the possibility of selective retention of a component of the insoluble material absorbed from the gut, rats were fed for six consecutive days on alfalfa containing the insoluble radiolabel which could not be extracted from the feed. At the end of the feeding programme the rats' bodies had retained a smaller amount of radiolabel than rats that had received only a single meal of the alfalfa containing the propham residues. Paulson et al. (1975) therefore concluded that the insoluble propham residues passed through the rat gut with little or no modification.

The same research team also conducted similar feeding trials using chlorpropham treated alfalfa as a feed for rats and a sheep. Although their results were only reported in a shortened form (Still et al., 1974b), no major differences in excretory or metabolic routes were reported between propham and chlorpropham.

While it can not be assumed that any unextractable chlorpropham residue of potatoes would have been metabolised and deposited in a similar way to the chlorpropham or propham added to the hydroponic nutrient medium of the growing alfalfa, a similarly high proportion of this residue in potatoes may not be absorbed but pass through the gastrointestinal tract of simple stomached animals.

CHAPTER 3

THE DETERMINATION OF CHLORPROPHAM RESIDUES IN CRISPS

3.1 INTRODUCTION

The requirements of the potato processing industry for a continuous supply of potatoes necessitates the storage of the industry's raw material for long periods of time. While the potatoes processed during the harvesting season need not be treated with a sprout suppressant, those which are intended for use outwith this period usually receive at least one application of sprout suppressant.

When sprout suppressant treated tubers are processed, the initial processing operations of washing and peeling considerably reduce the residues of the sprout suppressant tecnazene (Dalziel and Duncan, 1974; Dalziel and Duncan, 1980). Chlorpropham residues are also reduced by washing and peeling (Martens et al., 1971; Gard, 1959; Vliet and Sparenberg, 1970).

In the case of the frying operation, Dalziel and Duncan (1980) report an increase in the tecnazene residue in the crisp after frying, while Martens et al (1971) state that the frying process reduces the chlorpropham residue to less than the limit of sensitivity for their analytical method. Gard (1959), while using the same analytical method as Martens et al, carried out similar residue studies on crisps and found that all but the highest application rates of chlorpropham to the potato

tubers resulted in residues of chlorpropham in the crisp which were below the limit of sensitivity.

Despite the widespread use of chlorpropham in sprout suppressant formulations, the increased public awareness of pesticide use and the requirement of regulatory authorities for more detailed information, the only published method for the analysis of chlorpropham residues in crisps is that of Gard and Rudd (1953) and, prior to the work described in this chapter being carried out, only two authors reported residues in crisps (Martens et al., 1971 and Gard, 1959). After the studies described below had been completed and published (Ritchie et al., 1983), residue values for chlorpropham in crisps and chips were reported (Heikes, 1985). This chapter describes the development of a method for determining chlorpropham in crisps and crisp frying oil and the analysis of commercial samples.

3.2 Development of the Analytical Technique

3.2.1 Possibilities

A major obstruction to the development of any method which aims to accurately quantify the residue of chlorpropham in an oily medium is that of the difficulty of extracting this lipophilic pesticide from the oil in which it is present.

Previous work in this department (Dalziel and Duncan, 1980) succeeded in quantifying the residues of the sprout suppressant, tecnazene, in crisps and crisp frying oil. These workers used solvent extraction to remove the tecnazene, but large amounts of co-extractives contaminated the extract, resulting in considerable interferences in the residue

determination. The overall success of the method relied heavily on the fact that sensitive instrumentation in the form of a gas liquid chromatograph (GC) fitted with an electron capture detector (ECD) was available. The responsiveness of the electron capture detector to tecnazene, relative to that of the co-extractives which had similar retention times, dispensed with the necessity for a laborious clean up of the solvent extract.

However, an electron capture detector cannot be used for chlorpropham determinations, due to the low responsiveness of the detector to chlorpropham, although derivatisation to yield an electron capture detector sensitive derivative has been reported (Gutenmann and Lisk, 1964; Bradway and Shafik, 1977). However, the wide spectrum of compounds likely to be present in the co-extractives fraction of the sample could have produced derivatives also suited to electron capture detection resulting in a complex chromatogram, or even saturation of the detector if high concentrations of derivatised material are present in the detector. Moreover, it was considered that a requirement of this analytical method should be its ability to be used in laboratories where only standard analytical instrumentation was available and since an electron capture detector equipped GC could not be considered part of the equipment in a general purpose laboratory, then, for this reason, together with the problems mentioned above, it was decided to develop a method which did not rely on the use of an electron capture detector.

In the method of Gard and Rudd (1953) for determining chlorpropham residue in agricultural materials, chlorpropham is extracted with dichloromethane and the non-volatile material including the chlorpropham remaining after solvent evaporation is hydrolysed to produce

3-chloroaniline which is determined colorimetrically. When the sample material contains high levels of oil, for example, cottonseed (Gard and Rudd, 1953), and peanuts (Gard, Pray and Rudd, 1954), an additional solvent partitioning step is introduced.

The material is extracted with the dichloromethane and the solvent is evaporated as with materials low in oil, but in the case of cottonseed and peanuts, this leaves a significant quantity of oil. The chlorpropham is extracted from the oil by four acetonitrile partitionings which are then bulked, reduced in volume by evaporation of the solvent with the residue then being hydrolysed and the aniline determined colorimetrically in the same way as when little oil was present.

The use of acetonitrile extractions of the oil led to a concentrating of the chlorpropham in the hydrolysis flask rather than the chlorpropham being present in a large volume of oil and if large amounts of oil were extracted by the acetonitrile, a saponification is in effect carried out.

Saponification of an oil containing chlorpropham to yield 3-chloroaniline was considered, but the interferences and recoveries reported by Gard (1959), when determining 3-chloroaniline levels in a hydrolysate of a crisp extract, dampened enthusiasm for this technique. While a wide range of products may be co-distilled from the saponification flask production of an azo dye from the aniline and the separation of this dye by high pressure liquid chromatography may be a technique worthy of appraisal. Fluorogenic labelling of the aniline moiety of chlorpropham produced in this way could also result in a sensitive high pressure liquid chromatographic method being developed.

Dalziel (1978) also investigated the potential of solvent partitioning procedures, in this case to remove tecnazene from crisps. He blended crisps with ethanol and hexane then added water to form a two-phase system with the tecnazene in the organic phase. The tecnazene was extracted from this hexane fraction by two dimethyl-formamide (DMF) extractions. These DMF extractions were then combined and had hexane, water and a little acetic acid (to speed partitioning) added to give a hexane extract containing the majority of the tecnazene but, unfortunately, this extract still contained much of the oil originally co-extracted with the tecnazene from the crisps.

Although Dalziel had little success with solvent partitioning to obtain an extract containing most of the tecnazene with only a minimal amount of co-extracted oil components, it was hoped that solvent partitioning as a technique may be able to be used in the extraction of chlorpropham from oily materials. The main reason for this optimism was due to the difference in lipophilicity that exists between tecnazene and chlorpropham. This can be demonstrated by the different solubilities of the two compounds in various solvents. The solubility of tecnazene in water, ethanol and hexane, is about 1 mg/ℓ, 40 g/ℓ and 60 g/ℓ respectively. The solubility of chlorpropham in water is 89 mg/ℓ, and it is miscible with the lower alcohols and only soluble in hexane to the extent of about 45g/ℓ.

These different properties should allow the choice of solvent to be made on the basis of its polarity, which influences the efficiency of extraction of chlorpropham from the oil and the degree to which it is miscible with the oil. It was hoped that a solvent immiscible with the oil

would also extract minimal amounts of oil components along with the chlorpropham.

3.2.2. Development work leading to the final method

The primary requirements of this method of analysis was that it had to be capable of determining the very low levels of chlorpropham that were thought to be present in crisps. The method of Gard and Rudd (1953) used by Gard (1959) and Martens et al. (1971) in their analysis of crisps for chlorpropham residues, has a reported limit of sensitivity of 0.05 μg/g and the recovery rates are often low and variable. A new method, capable of determining residues of chlorpropham below 0.05 μg/g, would be needed to trace the loss of chlorpropham which must occur during the frying of the potatoes if the residue in the crisps is in fact less than the reported 0.05 μg/g.

It was considered that to use the method of Gard and Rudd merely to conduct further studies of an already researched area, was of no challenge. However, with hindsight, it is now considered that aspects of this method are not without merit and when combined with the final residue method (Section 3.3) could be of use.

The early work on the development of the residue method was confined to dealing with the frying oil and the effort expended to produce a method for quantifying the chlorpropham residue in this material. The determination of chlorpropham residue of the oil in fact represents the same problems as determining the residue in the crisps. This is due to the high oil content of crisps (30-40%) and, while the primary objective must be to obtain a residue concentration of this agrochemical in the

material consumed by the public at large, the main problems arising from these studies invariably emanate from the oil component which is of course easily extracted from the crisps. Moreover, the extraction procedure used to remove the oil would also extract the chlorpropham.

At the start of this work, frying oil which had been used by a commercial processor, was not at hand, so locally purchased cooking oil was used. This allowed some partition studies to be carried out on a material free, it was hoped, of chlorpropham. It was also considered a "clean" material which contained none of the innumerable products of the frying process. This allowed some information to be obtained before tackling the more difficult problems which may have arisen from the presence of these by-products. "Mazola" domestic frying oil was used in the initial work.

The choice of extracting solvent was made with regard to the work of Gard and Rudd (1953) and with reference to the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) (Horwitz, 1975). The official first action recommended by the AOAC in the extraction of the lipophilic pesticide DDT from fat would be to dissolve the fat in hexane and extract the DDT with acetonitrile. Chlorpropham spiked Mazola oil was also extracted with acetonitrile. Since it was not known how much hexane would have to be added to the commercial frier oil in order to dissolve any precipitated fat, a range of hexane additions was used.

In these distribution studies a series of separating funnels was set up, into which was placed various weights of oil from zero to the equivalent of 25 ml. (The weighing of oil proved more accurate and convenient than working on a basis of volume). To these separating

funnels hexane was added to give a total volume of 25 ml in each flask. 25 ml of acetonitrile containing 1 mg/ml of chlorpropham was then added to the flasks which were shaken and allowed to settle. Unfortunately emulsions were formed in some of the flasks and these took many hours to revert to a two-phase system. The acetonitrile was removed from the separating funnels and the change in volume noted. The volume of the oil/hexane phase was obtained using a measuring cylinder. The chlorpropham remaining in the acetonitrile phase was determined by GC using the chromatographic conditions described in the final method section (3.3.3).

The results are presented in graphical form (Figure 3.I) and show that a partition coefficient of around 1.5 is possible when the hexane concentration is greater than 30% of the total hexane/oil phase and represents 60% extraction of chlorpropham from the oil by the acetonitrile in one extraction and gives more than 90% removal in three sequential extractions when a 1:1 volume ratio is used.

The main disadvantage with the partitioning was the slow separation of the phases. The next set of partitions used acetonitrile to which 2% water had been added. The addition of water to the system did speed the separation of the phases but not as much as was hoped, although it did not have any great effect on the partition coefficient (see Figure 3.II). In the next set of partitions the acetonitrile extractant contained an addition of 1% dilute sodium chloride solution, the salt concentration of which was 2.5%. The partition coefficient up to 50% hexane remained similar (see Figure 3.III) but the speed of separation of the phases was considerably faster.

The acetonitrile phases of these partitionings contained considerable amounts of yellow pigmentation. The amounts present only became apparent when several of these acetonitrile phases were run down on the rotary evaporator in order to estimate the quantity of oil removed by the acetonitrile. The analysis of these grossly contaminated acetonitrile phases was only possible due to the high concentrations of chlorpropham used in the acetonitrile added to the separating funnels (1 mg/ml). This allowed the GC to be run at low sensitivity and small injection volumes minimised contamination of the instrument.

The official first action of the AOAC in the analysis of fat for DDT residues recommends washing of the acetonitrile phase with hexane in order to remove some of the co-extracted fatty materials. In view of the large partition coefficient for chlorpropham in a hexane acetonitrile partitioning sytem, it was decided that four hexane washes should not result in large losses of chlorpropham from the acetonitrile extract of the oil.

The extraction system decided on at this point, although the volumes used were reviewed at a later stage, was to first extract the oil with four lots of acetonitrile which contained a 1% addition of 2.5% aqueous sodium chloride. The oil would have hexane added to give between 10% and 50% hexane concentration, depending on the consistency of the commercial samples. These acetonitrile extracts were to be washed with four hexane aliquots. The first acetonitrile extract was passed through each of the four separating funnels containing hexane and the order of passage maintained by the second, third and fourth acetonitrile extracts. The four acetonitrile extracts were then bulked. To minimise losses of

chlorpropham in the hexane washes, three lots of fresh acetonitrile were passed down the series of hexane separating funnels, again in the same order as the four acetonitrile oil extracts. These three lots of acetonitrile were combined with the bulked acetonitrile oil extracts and evaporated by rotary evaporator at reduced pressure and at a temperature not exceeding 40°C.

It was initially considered that one batch of fresh acetonitrile would be sufficient to remove most of the chlorpropham from the hexane washings but there were considerable amounts of pigmentation present in the first hexane containing separating funnel. The second separating funnel also contained a little pigmentation but the third and fourth funnels displayed none. In view of this apparent efficacy of hexane in removing oil components from the crude acetonitrile oil extracts and the significant effect on the partition coefficient of chlorpropham in an acetonitrile:hexane system, when even a small quantity of oil is present, it was decided that three lots of fresh acetonitrile would be used, rather than just the one. This should minimise the loss of chlorpropham to the hexane without contributing to the impurities in the bulked acetonitrile extract.

When the above extraction procedure was used to extract samples of Mazola oil which had been fortified with chlorpropham by the addition of 2 ml of a 1 mg/ml chlorpropham in hexane solution, about 0.5-1 ml of oil remained after the acetonitrile had been removed by rotary evaporation. Clearly this did not constitute a sample suitable for analysis using GC, and some further clean-up would be required.

The clean-up procedure first tried was that used in this department for the removal of interfering co-extractives from hexane extracts of chlorpropham treated fresh potato tubers (Boyd et al., 1982). In this procedure the co-extractives were removed by the use of a 15 cm by 0.9 cm i.d. column of alumina (neutral Grade V, Woelm). Hexane was used as the eluant and the 80-120 ml fraction contained the chlorpropham applied to the column in the crude hexane extract.

When the first extracts of Mazola oil were run in these columns no chlorpropham was eluted in the first 250 ml of eluant, nor was there much movement of the oil away from the top of the column. Clearly, the co-extractives were having an effect on the chromatography of the chlorpropham. This can often be the case when significant quantities of impurities are present (Heftman, 1975).

In subsequent investigations of column clean-ups, various combinations of activity grade and solvent polarity were studied. The investigations did not use neutral alumina, manufactured by Woelm, since stocks of this particular material were low, but used the more readily available material, manufactured by Hopkins and Williams.

The column clean-up system that was found to give high recoveries of chlorpropham in the presence of Mazola oil co-extractives was one which differed from the above system, in that the neutral alumina was less deactivated and the eluant used was the more polar 5% diethyl-ether in hexane. This resulted in some movement of the pigmented co-extractives away from the top of the column and the chlorpropham was eluted in the first 80 ml of eluant. Monitoring of the eluant after the first 80 ml became routine to ensure all the chlorpropham was, in fact,

being eluted in the first fraction. Table 3.I shows recoveries of chlorpropham from the column when applied together with an oil extract (B) and when applied to the column on its own in a hexane solution (A).

In the overall recovery determination, the Mazola oil was again spiked with 2 ml of 1 mg/ml chlorpropham in hexane solution and the extraction procedure repeated. After removal of the acetonitrile by rotary evaporation, a sub-sample was taken before the remainder of the sample was cleaned-up on the alumina column. By analysing these two samples, independent recovery values for the two steps could be obtained and kept laborious and tedious solvent extractions to a minimum. Table 3.I (C) shows results for the recovery of chlorpropham from the column only. Table 3.II shows the recovery of the chlorpropham from the Mazola oil before column clean-up (A) and the overall recovery from the oil including the recoveries from the column (B).

From the frequent and numerous extractions that were being carried out, it became apparent that heavy solvent consumption was a feature of this analysis technique and the result of large volumes of solvent being evaporated off would be to concentrate impurities in the small sample extract. A solvent blank extraction was used to assess the effect of these impurities on the GC analysis and this showed that considerable quantities of impurities were detectable. The total quantity of impurities was greatly reduced by the column clean-up step. The chlorpropham detected in this sample would be the sum of contamination of the solvent and apparatus by chlorpropham and any co-chromatographing impurities. The amount of chlorpropham detected in these duplicate blank extracts was less than 0.5 µg. This is equivalent to a residue of 0.007 µg/g of oil, (BASED ON FINAL METHOD VOLUMES).

TABLE 3.I. RECOVERY OF CHLORPROPHAM FROM THE ALUMINA COLUMN

	Total Quantity of Chlorpropham Applied to column (mg)	% Recovered from Column
A Chlorpropham in Hexane;		
REPLICATE I	2	107.6
REPLICATE II	2	101.7
B Chlorpropham + Oil Extract;		
REPLICATE I	2	95.0
REPLICATE II	2	95.8
C Chlorpropham Extracted from Oil;		
REPLICATE I	1.83	104.2
REPLICATE II	1.75	104.8

TABLE 3.II. RECOVERY OF CHLORPROPHAM FROM MAZOLA OIL

	Chlorpropham Fortification Level (mg)	% Recovery
A Solvent Extraction only;		
REPLICATE I	2	92.8
REPLICATE II	2	89.0
B Solvent Extraction and Column Clean-up;		
REPLICATE I	2	96.6
REPLICATE II	2	93.3

As to what proportion of this small concentration is attributable to contamination from a working environment where chlorpropham is continuously used and studied, it would be impossible to say.

The efforts made to keep this contamination to a minimum included thorough washing of all glassware with hot detergent, rinsing with distilled water and a final rinse with AR Grade acetone (Hopkins and Williams). The glassware was then baked in an oven at more than 100°C before use. A silicon grease, Apiezon N (Apiezon Products Ltd., England) was used on the ground glass joints in preference to petroleum greases.

Analysis of unspiked Mazola oil showed that chlorpropham and co-chromatographing components were less than the equivalent of 1.3 µg of chlorpropham. This represents a residue of 0.02 µg/g of oil EXTRACTED.

The conclusion of the work on Mazola oil was to have been the extraction of an oil sample which had received only a low level of fortification to verify if the same high recovery rate of the much higher 2 mg fortification level could be maintained. However, this was not carried out, due to commercial material becoming available and the results from such a study would not have been entirely relevant to the commercial samples.

3.3 The Final Method for the Determination of Chlorpropham Residues in Oil

As mentioned previously, it is a simple matter to extract the oil from crisps. This section deals with the method used to determine the chlorpropham residues in oil. The extraction of oil from crisps is described in Section 3.4.4.

3.3.1 Materials and methods

All solvents were high pressure liquid chromatography grade, unless otherwise stated, and were supplied by Rathburn Chemical Co., Walkerburn, Peeblesshire, Scotland.

Extraction of chlorpropham from oil:

a. 15 ml hexane was added to 60g of oil obtained from crisps, or in the case of frier oil, from a sample which was taken directly from the frier. Hexane reduced the viscosity of the oil and ensured that all components remained liquid at room temperature.

b. 75 ml of acetonitrile extractant was added to a separating funnel containing the oil and hexane, and shaken vigorously for 1 minute. The acetonitrile extractant consisted of 1ℓ of acetonitrile, saturated with hexane, to which was added 10 ml of 2.5% aqueous sodium chloride (A.R. Grade, Hopkins and Williams, Romford, England). Any precipitated sodium chloride was drawn off.

c. The acetonitrile extract obtained in (b) was vigorously shaken in sequence in a series of four separating funnels, each containing 30 ml of hexane. Washing with hexane reduced the quantity of oil in the acetonitrile extract.

d. The oil in (b) was extracted with a further 75 ml of acetonitrile extractant, which was then washed in the same lots of hexane contained in the same funnels and in the same sequence as the first acetonitrile extract.

e. Step (d) was repeated twice more to give four lots of acetonitrile extract which was then bulked.

f. To reduce losses of chlorpropham to the hexane, 30 ml of fresh acetonitrile extractant was passed through these funnels with vigorous shaking in the same sequence as before. The procedure was repeated with a further two lots of 30 ml of fresh extractant.

g. The acetonitrile (90 ml) from (f) was combined with the bulk obtained at step (e).

h. The solvent was removed under partial vacuum in a rotary evaporator at a temperature not exceeding 40°C. The small quantity of oil (about 1 ml) that remained was removed and the sample cleaned-up using an alumina column.

3.3.2 Alumina column clean-up

To 85g of neutral alumina, 15 ml of water was added and shaken overnight on a reciprocating shaker (Griffin and George Ltd.). A 25 cm by 0.9 cm i.d. glass column with a sintered glass base was filled to within 5 cm of the top with hexane. The deactivated alumina was slowly added to the column and a flow rate of 1 ml/min of hexane was maintained throughout the packing of the column. Gentle tapping on the side of the column during packing produced a more dense bed. It was important not to add the alumina too quickly as air bubbles which were carried into the hexane by the alumina had to be given sufficient time and space to come to the surface. If the alumina was added too quickly the dense band of descending alumina carried the air bubbles down and entrapped them in

the alumina bed which would lower the performance of the column. Any alumina which adhered to the wall of the column was washed down with hexane. When an alumina bed of 15 cm had been built up, a 1 cm deep layer of anhydrous sodium sulphate was added. The column then had 30 ml of 5% diethylether in hexane run through before the concentrate from (h) above was dissolved in approximately 2 ml of 5% diethylether in hexane and applied on top of the column. Subsequent washings of the flask which contained this concentrate were also applied to the column to ensure quantitative transfer. The flow rate was adjusted to less than 1 ml/min and the first 80 ml of eluant was collected, reduced in volume at a temperature not exceeding 40°C under partial vacuum in a rotary evaporator and then made to a volume of 1 ml with hexane for GC analysis.

3.3.2 Determination of chlorpropham by GC

The instrument used was a Packard 419 series GC equipped with flame ionisation detectors (FID).

GC conditions were as follows:

Column: 2 m x 4 mm i.d. glass column packed with 15% OV 101 on 100/120 mesh Gas Chrom Q

Carrier Gas: Nitrogen at a flow rate of 40 ml/min

Oven temp.: 170°C

Detector temperature: 285°C

FID flame gas: Hydrogen at 40 ml/min

Oxidant Gas: Oxygen at 110 ml/min

The FID signal was recorded on a flat bed potentiometric recorder (Servoscribe Model 15 RE541.20).

Linear response of the FID was checked by the injection of 0.05 µg-1 µg chlorpropham. The FID response was calculated by the method of triangulation. A standard curve was constructed and a linear response was observed over this range (Figure 3.IV). During analyses runs on the GC injections of a standard of similar concentration to the samples as well as standards of higher and lower concentrations than the samples being analysed were made and if the unit response from the standards were similar, the average unit response was used to determine the chlorpropham concentration in the sample flasks without reference to a standard curve.

Hamilton syringes were used for all GC injections. For samples of up to 1 µl, a 7000 series syringe was used. These syringes hold the sample in the needle and the plunger is a fine wire which travels to the end of the needle to expel the sample. For injections of more than 1 µl, a 700 series Hamilton syringe was used. The sample in this type of syringe is held in the glass barrel and the plunger only extends to the top of the needle, but does not enter it. The volume of sample capable of being held in the needle is about 0.8 µl. During sample injection, some of this 0.8 µl is lost from the needle through vaporisation and adds to the volume injected. This makes little difference when only one injection volume is used for standards and samples alike, provided the needle remains in the injection port for the same period of time at each injection, and does not affect the accuracy of the determination of the sample concentration. However, when varying injection volumes were used, non-linear standard curves were obtained.

To overcome this problem a bubble of air was drawn up into the syringe prior to the sample being loaded. The chromatography

resulting from the use of this technique should be improved since the sample is injected into the column in a more discrete band with only solvent being slowly volatilised from the needle but, in fact, only marginal improvement in the chromatography of short retention time substances could be observed. Although the 7000 series syringes would inject all of the sample carried, and are available in 5 µl form, the needle diameter is considerably greater than its 1 µl equivalent and, as a consequence, greatly reduces septum life.

3.4.1 Analysis of commercial samples

The help of United Biscuits Foods staff is gratefully acknowledged.

In order to understand at what points in the production process the samples were drawn, it would be advantageous to give a short description of the crisping process.

Whole potatoes contained in 1 tonne pallet boxes are placed at the beginning of the production line and these are tipped into machines which wash and abrasively peel the tubers. The peeled tubers are then sliced (1 mm-2 mm in thickness) and these are then washed again. Hot water may be used to wash these slices if the tubers have shown a high reducing sugar concentration. Starch is often removed from the washings of the sliced tubers. The slices are then conveyed to the deep fat friers which use a vegetable oil to fry the slices at 180°C to produce the crisps. The frying process takes about 5-6 minutes, with the whole process taking about 10 minutes. The high rate of oil removal from the frier by the crisps necessitates frequent additions of fresh oil. The oil is completely drained from the friers when an unacceptably high acid number is

is observed in samples routinely drawn from the frier. This drained oil can then be added in small quantities to the oil used to replenish that removed from the friers in the crisps. After the crisps are elevated out of the friers, the excess oil is allowed to drip onto collecting trays to be passed back into the friers before the crisps are passed under a bank of infra-red (IR) heaters to reduce the oil content. The final operations of cooling and addition of flavourings takes place before packaging. The output of the production line is about one tonne crisps/hour, corresponding to about 4-5 tonne/hour of fresh potatoes.

Samples were obtained from United Biscuits crisp factory in Grimsby. The samples obtained were:

1. Sliced potatoes which had received hot water washing
2. Oil from the frier
3. Crisps after they emerged from the frier and the excess oil had dripped off them but before passing under the infra-red heaters.

While sampling technique is an important consideration in any analysis, it was felt that the degree to which individual tubers had been divided and mixed prior to washing and frying, would result in a uniform and easily sampled material. This is not so with chlorpropham treated whole tubers, where large variations of chlorpropham application have been noted (Boyd, 1980). These large application variations have resulted in a wide range of chlorpropham residues for whole tubers. However, peeling results in a narrowing of the range of residues values (Boyd, 1980).

The sampling of slices and crisps involved merely the picking of a representative sample of crisps and slices from the conveyers.

The oil from the frier was obtained from a drain valve. The oil discharged from this valve was collected in a stainless steel beaker and allowed to cool before being poured into empty solvent winchesters.

3.4.2 Analysis of slices

The chlorpropham residue in the potato slices was determined using a similar method to that used in this department by Isabel Boyd. The modified method of Cerny and Blumenthal (1972) as described by Boyd et al. (1982) was subject to only minor modification. The procedure used was as follows:

To a top drive macerator (Thomson and Mercer, Croydon, England), 75g of sliced potatoes (cv. Record), 140g of anhydrous sodium sulphate and 125 ml of glass distilled grade hexane (Rathburn Chemical Co.) was added. This was blended for 2½ minutes and then the contents transferred quantitatively by the use of hexane washings to a screw-capped aluminium bottle and placed on the reciprocating shaker (Griffin and George Ltd.) overnight. The contents of the bottle were then filtered through Whatman No. 1 paper in a Büchner assembly with suction and the filter pad washed with hexane several times. The filtrate was then reduced to about 1 ml by rotary evaporation under reduced pressure at a temperature not exceeding 40°C.

This concentrated hexane extract was cleaned up on an alumina column, as described in Section 3.3.2. The column was eluted with 5% diethylether in hexane and the first 80 ml collected was reduced in volume to 1 ml by rotary evaporation. The chlorpropham concentration in this cleaned up extract was determined using the GC conditions described

in Section 3.3.3. An example of a chromatogram produced is shown in Figure 3.V. The 80-120 ml fraction from the column was also analysed for chlorpropham but it was found to contain none.

The above method was used to recover 50 µg of chlorpropham which had been used to spike duplicate samples of potatoes. The potatoes (cv. Record) were grown in a departmental field trial and therefore were of known history. The tubers were washed, diced, and placed in the macerator prior to addition of the chlorpropham in 1 ml of hexane. The recovery of the applied chlorpropham averaged 88.3% and all residues of washed and peeled tubers, unwashed tubers and slices have been corrected for this factor.

3.4.3 Analysis of frier oil

The oil from the crisp frier at Grimsby was analysed to determine the residue of chlorpropham present. The method used was that described in Section 3.3.1, with the standard 60g oil sample size being used. Before this 60g sample was drawn from the winchester containing the oil, it was heated to about 40°C in order to produce a uniform consistency of oil. This prevented the possibility of concentrating the chlorpropham in fractions which had segregated from each other when components of the oil solidified at room temperature.

Due to the non-availability of a similar oil which could be assumed to be free of chlorpropham, recovery factors had to be determined by adding chlorpropham to the same samples as were used for residue analysis. The recovery factors were calculated by taking account of the residue already present. The use of small fortifications of

chlorpropham (20μg/60g oil) to samples that already contained chlorpropham resulted in inconsistent recovery factors.

However, crisps produced from untreated potatoes became available at a later date (see Section 3.7) and they showed low chlorpropham residues. The recovery factor was determined using these crisps and found to be 93.2% S.D. ± 6.37 and all residues in oil and crisps have been corrected for this recovery factor. Several oil extracts were analysed by GC, using a Carbowax 20M-TPA stationary phase in addition to the routine OV 101 stationary phase, in order to positively identify the chlorpropham. Chlorpropham was often added to the oil extracts following analysis on the OV 101 column to ensure that this additional chlorpropham co-chromatographed with the peak tentatively identified on a retention time basis.

The GC conditions for the Carbowax column were:

Column: 2m x 4mm i.d. glass column packed with 2% Carbowax 20M-TPA on 100/120 mesh Gas Chrom Q

Carrier Gas: Nitrogen at 40 ml/min

Oven Temp.: 180°C

Detector Temp.: 285°C

Injector Port Temp.: 225°C

FID Flame Gas: Hydrogen at 40 ml/min

Oxidant Gas: Oxygen at 110 ml/min

Figure 3.VI is a chromatogram of an alumina column cleaned-up crisp extract on a Carbowax 20M-TPA column.

3.4.4 Analysis of crisps

The oil and chlorpropham were extracted from the crisps by solvent extraction. 150g-175g of crisps (sufficient to yield at least 60g of oil) were blended in a top drive macerator (Thomson and Mercer, Croydon, England) with 200 ml hexane. The mixture was filtered through Whatman No. 1 Paper in a Büchner assembly with suction and the filtrate retained. The residual solids were then blended with a mixture of 150 ml hexane and 15 ml diethylether, and the mixture filtered as above; the filtrate was retained. The residue was finally blended with 100 ml hexane and 15 ml diethylether, and the mixture filtered. The three filtrates were combined and the solvents removed under partial vacuum in a rotary evaporator at a temperature not exceeding 40°C.

The chlorpropham residues in the oil removed from the crisps in this way was determined using the same method as that used in the case of the oil from the frier (Section 3.4.3). Figure 3.VII is a chromatogram from a GC equipped with an OV 101 column. The sample was an alumina cleaned-up crisp extract.

The analytical results for the crisp oil and slices samples collected from Grimsby are shown in Table 3.III. The crisp and oil figures are corrected for a recovery factor of 93.2% and the slices residues are corrected for a recovery factor of 88.3%.

TABLE 3.III. CHLORPROPHAM RESIDUES IN COMMERCIAL SAMPLES

Sample	Chlorpropham Residue µg/g
SLICED POTATOES FOLLOWING PEELING AND HOT WATER WASHING	0.18[a]
FRIER OIL	0.40[b]
CRISPS[d]	0.45[c]

[a] Mean of two replicates

[b] Mean of five replicates

[c] Mean of three replicates

[d] Sampling point - after frying and excess oil had dripped off (see text)

3.5 Contamination of Untreated Material

A batch of samples was obtained from the Grimsby factory at the time when the last of the old season potatoes were processed. The old season potatoes, which had been treated with chlorpropham over their 9-10 month storage period, were then replaced by the new season Cyprus potatoes which had not been treated with chlorpropham. At this change-over, samples of product from the old season potatoes were taken. The samples comprised of:

1. Washed and peeled tubers
2. Crisps after they had passed under the IR heater bank to reduce the oil content and had been cooled.

The samples of product from the Cyprus potatoes comprised of:

1. Whole unwashed potato tubers
2. Crisps after they had emerged from the frier and the excess oil had dripped off them but before passing under IR heaters
3. Crisps after they had passed under the IR heater and had been cooled.

The chlorpropham residue in the crisps was determined using the method described in Section 3.4.4. The washed and peeled old season potatoes (cv. Record) were diced and subsampled prior to being analysed for chlorpropham in the same way as described for potato slices (Section 3.4.2). The Cyprus potatoes (cv. Arran Banner) were not washed although the small amount of adhering soil was removed by hand. These intact tubers were diced and subsampled prior to analysis using

the same method as that employed for potato slices (Section 3.4.2). The results of these analyses are given in Table 3.IV.

3.6 Analysis of Chlorpropham Untreated Material

Some weeks later a further batch of samples arrived in Glasgow. These samples had been collected from a line which had not processed chlorpropham treated potatoes for some three weeks. Product samples from these untreated Cyprus potatoes (cv. Arran Banner) comprised:

1. Whole unwashed tubers
2. Crisps after they had passed under the IR heater bank and had been cooled.

Again the whole tubers were not washed and were analysed in the same way as the Cyprus tubers above (Section 3.5). The crisps were analysed using the method described above (Section 3.4.4).

As can be seen from the results in Table 3.V, these crisps produced from untreated Cyprus potatoes from the line which had been using Cyprus for some time, contain only a very small chlorpropham residue. These crisps were then used to test the recovery of chlorpropham from spiked samples of crisps.

3.7 Recovery of Chlorpropham from Crisps

This study utilised crisps which had shown a low chlorpropham residue (see Section 3.6 and Table 3.V). 1 ml of 0.1 mg/ml chlorpropham in hexane was added dropwise to crisps contained in a blender. The solvent was allowed to evaporate prior to the oil being extracted with

hexane and analysed in the normal manner (see Section 3.4.4).

Table 3.VI shows the results of the chlorpropham recovery determinations. The mean recovery value of 93.2% is used in correcting all crisp and frying oil chlorpropham residue values.

3.8 Discussion

Many of the important points noted during the development of the analytical method and during the residue determinations have been mentioned in previous sections since it makes the procedures easier to follow.

The results given in Table 3.III show that the majority of the chlorpropham which enters the frier as a residue in the slices, ends up in the crisps, bearing in mind that the slices are reduced in weight, through dehydration, by a factor of more than three as they are fried to produce crisps. These residues are not insignificant but, in view of the high acute oral LD_{50} for rats, 5000-7500 mg chlorpropham/kg body weight and the absence of any data showing harmful effects resulting from long term feeding studies (Anon, 1978) and since crisps would only constitute a small part of the diet, there would seem little cause for concern over these residue figures.

These results are in contrast to those of Martens et al. (1971) and Gard (1959). Gard used one batch of potatoes which was sprayed with a 0.5% chlorpropham emulsion and stored for several months before being used to produce crisps. The residue of chlorpropham in these crisps was less than the 0.05 μg/g limit of sensitivity for their technique. A further batch of potatoes was treated by spraying with a

TABLE 3.IV. CONTAMINATION OF FRIED PRODUCTS PRODUCED FROM UNTREATED TUBERS

Sampling Point	Starting Material	Chlorpropham Residue µg/g [e]
WASHED AND PEELED TUBERS	CHLORPROPHAM TREATED	0.26
CRISPS AFTER FRYING BUT BEFORE PASSING UNDER I.R. HEATERS	CHLORPROPHAM TREATED	0.67
UNWASHED CYPRUS TUBERS	CHLORPROPHAM UNTREATED	0.028
CRISPS AFTER FRYING BUT BEFORE PASSING UNDER I.R. HEATERS (FROM CYPRUS TUBERS)	CHLORPROPHAM UNTREATED	0.27
CRISPS AFTER FRYING AND AFTER PASSING UNDER I.R. HEATERS AND AFTER COOLING (FROM CYPRUS TUBERS)	CHLORPROPHAM UNTREATED	0.36

[e] Mean of two replicates

1% chlorpropham emulsion. After these tubers were stored for a shorter period of time than the first batch, a residue of 0.85 μg/g in the peeled tuber and 0.075 μg/g in the crisps was reported. It may be worth pointing out to the reader that what in North America are referred to as chips, we in Britain call potato crisps.

Martens et al. (1971), using the same analytical method as Gard (1959) i.e. the method of Gard and Rudd (1953), reports that manually peeled potatoes with a chlorpropham residue of 0.55 μg/g and a propham residue of 0.36 μg/g had these residues reduced to 0.25 μg/g of both propham and chlorpropham after slicing to a thickness of 1.25 mm and frying in peanut oil at 145°C for 5-6 minutes. A second frying for 2-3 minutes at 180°C in a hydrogenated vegetable oil further reduced the chlorpropham and propham to less than 0.05 μg/g of each.

In both of these studies, high residues of chlorpropham were detected in the peeled tubers compared to the material obtained from Grimsby, yet very much lower residues in the crisps were observed. However, these authors failed to state whether or not fresh new oil was used to fry the slices. This is an important omission since it is feasible that frying in fresh oil, which has not been previously used to fry chlorpropham treated tubers, would itself be free of chlorpropham and, when slices were fried in this oil, the chlorpropham could have been dissipated throughout the oil and the chlorpropham residue in the crisps thereby lowered to a level less than that of the limit of sensitivity for their method. The samples collected from Grimsby, on the other hand, had come from a frier which had been processing chlorpropham treated potatoes for some time and the oil had not been changed for several weeks.

This had allowed the concentration of chlorpropham in the oil to build up to the extent that a concentration of 0.4 μg of chlorpropham/g oil was detected.

It is apparent, however, from Table 3.III that the residue in the crisp is not a result of its oil content, since the residue of chlorpropham in the crisp is greater than that in the frier oil and the oil content of the crisps is only about 35%. The slices must therefore retain much of the residue during the frying process or the slices concentrate chlorpropham from the frier oil as they are processed. Some indication as to which mechanism is operating would have been provided had frier oil samples been analysed at the time of the contamination study. Although this study shows that untreated material does become contaminated with chlorpropham when fried in the same oil as treated material, it is impossible to say, without having a chlorpropham concentration for the frier oil, if the residue in the crisps produced from untreated tubers is a result of chlorpropham having been concentrated from the frier oil or that the oil component of the crisps merely reflects the chlorpropham concentration in the frier oil.

Table 3.IV, as well as showing that untreated material can become contaminated with chlorpropham if it is fried in the same oil as treated material, shows the effect of the oil reducing step in the manufacturing process. The residue of chlorpropham increases from 0.27 μg/g to 0.36 μg/g after the crisps have passed under the infra-red heater bank and have been cooled. It was thought that this increase in residue was probably a result of the reduction in the oil content, although for such an increase in residue to have occurred, a large

reduction in the oil content would have been necessary. In actual fact, when the weight of oil extracted from the crisps is considered, only a small reduction in oil content (ca. 5%) is apparent. Therefore, all that can be concluded from the figures available is that the chlorpropham residue is not reduced by the infra-red heater bank.

As mentioned previously, the recoveries of chlorpropham were conducted on crisps which had shown a low chlorpropham residue of 0.035 µg/g (see Table 3.V). The recoveries of 100 µg fortification of chlorpropham are shown in Table 3.VI (93.2% average) and they were more consistent than when recoveries of 20 µg amounts of chlorpropham were used to spike the frier oil in the initial analyses of commercial samples (see Section 3.4.3). The recoveries from the 20 µg spiked samples had first to be corrected for the residue already present and, consequently, the recovery of spiked material ranged from under 70% to over 135%.

The recovery factor of 93.2% obtained from crisps was used to correct the amounts of chlorpropham extracted from both crisps and oil. No oil was obtained with the dispatch of material containing the crisps with very low chlorpropham residue and therefore this same 93.2% recovery factor was also used for the correction of the quantities of chlorpropham actually extracted from the frier oil to give the residue values quoted.

The residue values are in general agreement with those obtained by Dalziel and Duncan (1980), when working with the sprout suppressant tecnazene. They found that slices with residues of 0.14 µg/g of slice produced crisps with 0.55 µg tecnazene/g fried product immediately after frying. The final product after the oil reduction step

TABLE 3.V. RESIDUES OF CHLORPROPHAM IN PRODUCTS PRODUCED FROM CHLORPROPHAM UNTREATED CYPRUS POTATOES

Sample	Chlorpropham[a] Residue µg/g
WHOLE UNWASHED TUBERS	0.05
CRISPS AFTER FRYING AND AFTER PASSING UNDER I.R. HEATERS AND AFTER COOLING	0.038

[a] Mean of duplicates

TABLE 3.VI. RECOVERY OF CHLORPROPHAM FROM CRISPS

SAMPLE FORTIFICATION	% RECOVERY[b]	MEAN
100 µg	87.1	
100 µg	96.2	
100 µg	96.0	93.2±S.D. 6.37
100 µg	100.8	
100 µg	86.1	

[b] Corrected for an initial residue of 0.038 µg/g

had a residue of only 0.25 μg/g. A contamination experiment was also conducted by Dalziel and Duncan (1980) and they found that slices with a tecnazene residue of 0.00 μg/g had a final residue of 0.34 μg/g after frying. Since the frier oil before the processing of the untreated crisps had a residue of only 0.07 μg/g (Dalziel, 1978), then obviously the slices must concentrate the tecnazene from the frier oil.

It is however surprising that such a large proportion of these sprout suppressants which enter the frier appear as residues in the crisps and are not lost from the frier oil when frying at high temperatures. The oven temperature of the GC when analysing samples for chlorpropham is around only 170°C and even at this temperature chlorpropham has a sufficiently high vapour pressure to achieve a favourable equilibrium with the chlorpropham retained by the stationary phase to result in short retention times.

A similar situation must exist in the friers unless the oil used to fry the slices, in this case an 80% palm oil and 20% soya oil combination, is capable of retaining most of the chlorpropham in the oil and little is lost from the oil through volatilisation. It may be the case that the chlorpropham vapour concentration is significant but condensation of this vapour on the sides and top of the frier may result in its return to the bulk of the oil. The quantities of water entering the frier (in the slices) may steam distil some of the chlorpropham out of the oil. If a significant concentration of chlorpropham vapour exists above the frier oil at 180°C then some scope for reducing the residue in the crisps exists if the chlorpropham can be prevented from returning to the frier. Deeper peelings and ensuring that all of the peel is removed would also reduce

the chlorpropham residue in the slices and therefore that appearing in the crisps.

The starch which is recovered from the washing of the slices may contain significant residues of chlorpropham and depending on the use to which this is put may warrant some study of the residues present in this material.

Towards the conclusion of this work a high pressure liquid chromatograph (HPLC) became available and the chlorpropham concentration in several of the final residue samples were quantified using this instrument in addition to the GC method. The HPLC showed particular promise in being able to quantify the chlorpropham in the crude acetonitrile extract. The analysis procedure merely involved transferring the small amount of the residual oil (that which was left after evaporation of the solvent from the crude acetonitrile extract; see step H, Section 3.3.1) to a 10 ml volumetric flask which was then made to volume with acetonitrile. Direct injection of aliquots from this 10 ml flask were made into the HPLC equipped with an ODS-Hypersil (Shandon) column.

The chromatographic conditions used to determine the chlorpropham concentration in the crude acetonitrile extract were as follows:

Column: 25 cm x 0.5 cm

Packing: 5 μm ODS-Hypersil

Eluant: 70% methanol in water

Flow Rate: 1.0 ml/min

Pressure Prop.: 2,500 p.s.i.

Detector (U.V. Spectrometer): 0.1 AUFS, 237 nm

Figure 3.VIII is a chromatogram of a crude acetonitrile extract of crisps.

During this work, an attempt was made to reduce the content of oil in this 10 ml acetonitrile extract, by partitioning with hexane. It was thought that the high concentration of interfering co-extractives present in this 10 ml would allow some to be removed by hexane partitioning. Unfortunately no reduction was possible, but, as can be seen in Figure 3.VIII, this relatively "clean" chromatogram of the crude acetonitrile extract may provide a rapid routine method for monitoring chlorpropham residues in crisps.

Figure 3.I. Distribution of chlorpropham between acetonitrile and oil containing various proportions of hexane.

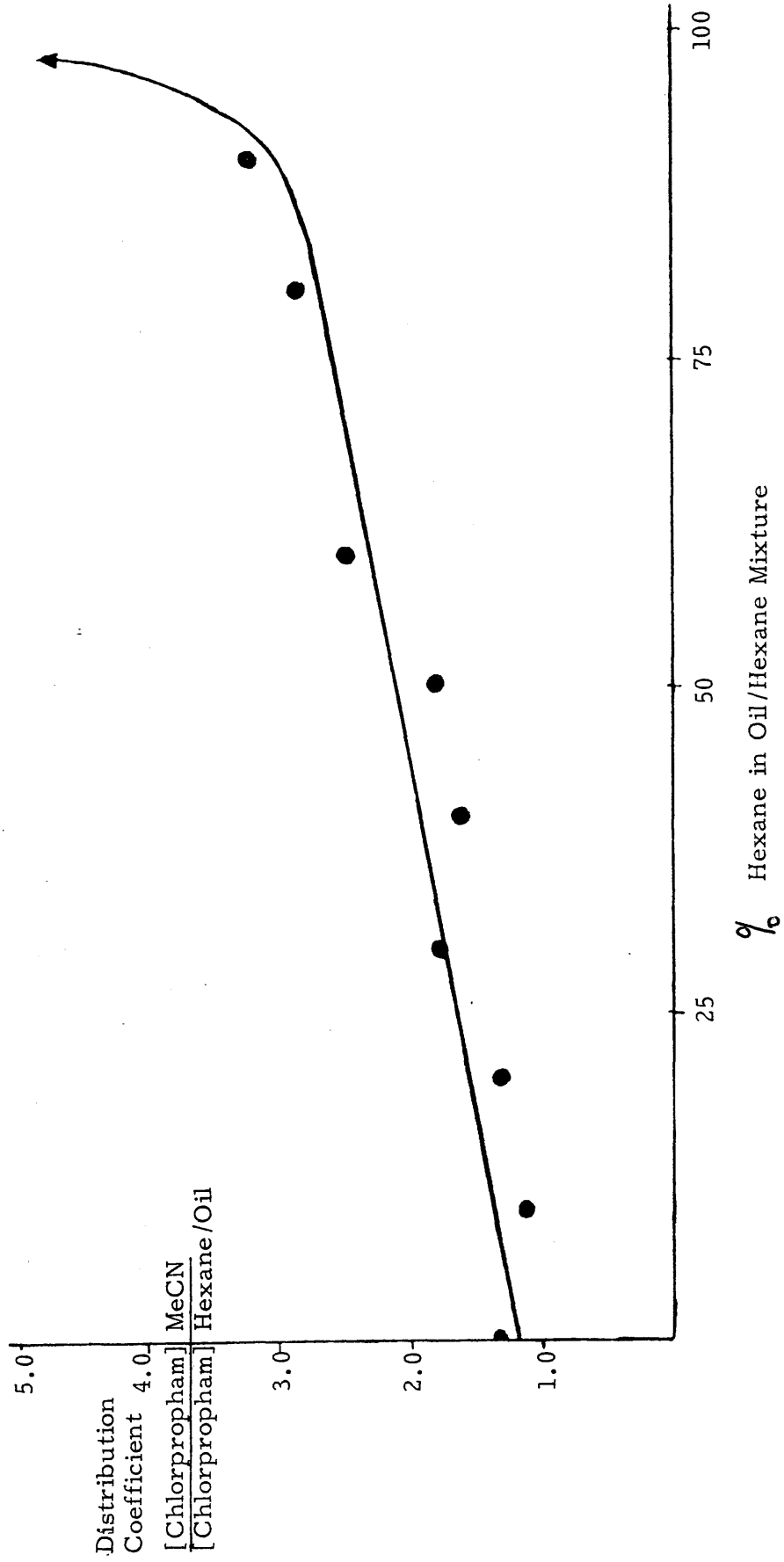


Figure 3.II. Distribution of chlorpropham between acetonitrile extractant (acetonitrile + 2% water) and oil containing various proportions of hexane.

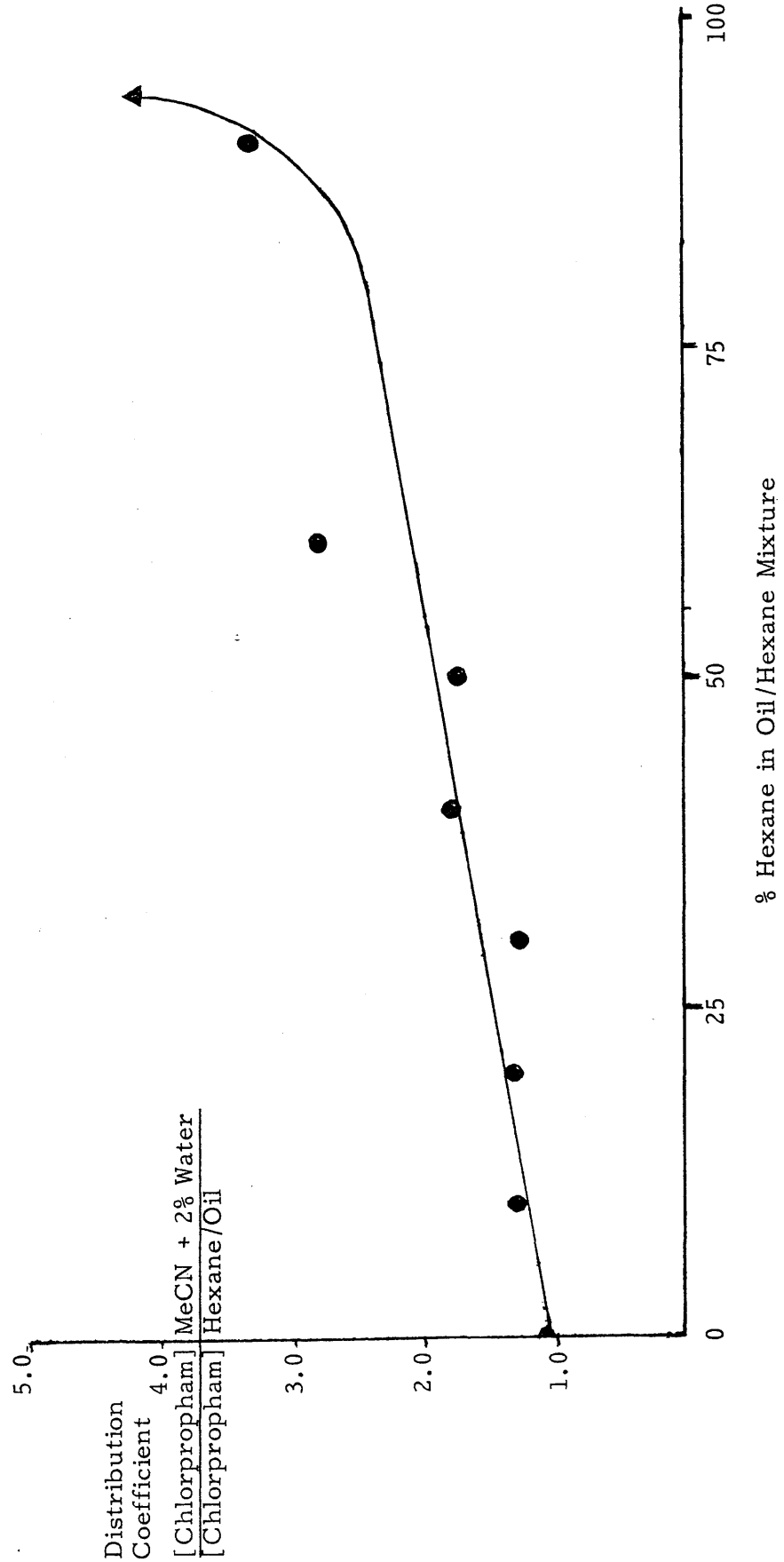


Figure 3.III. Distribution of chlorpropham between acetonitrile extractant (acetonitrile + 1% aqueous sodium chloride) and oil containing various proportions of hexane.

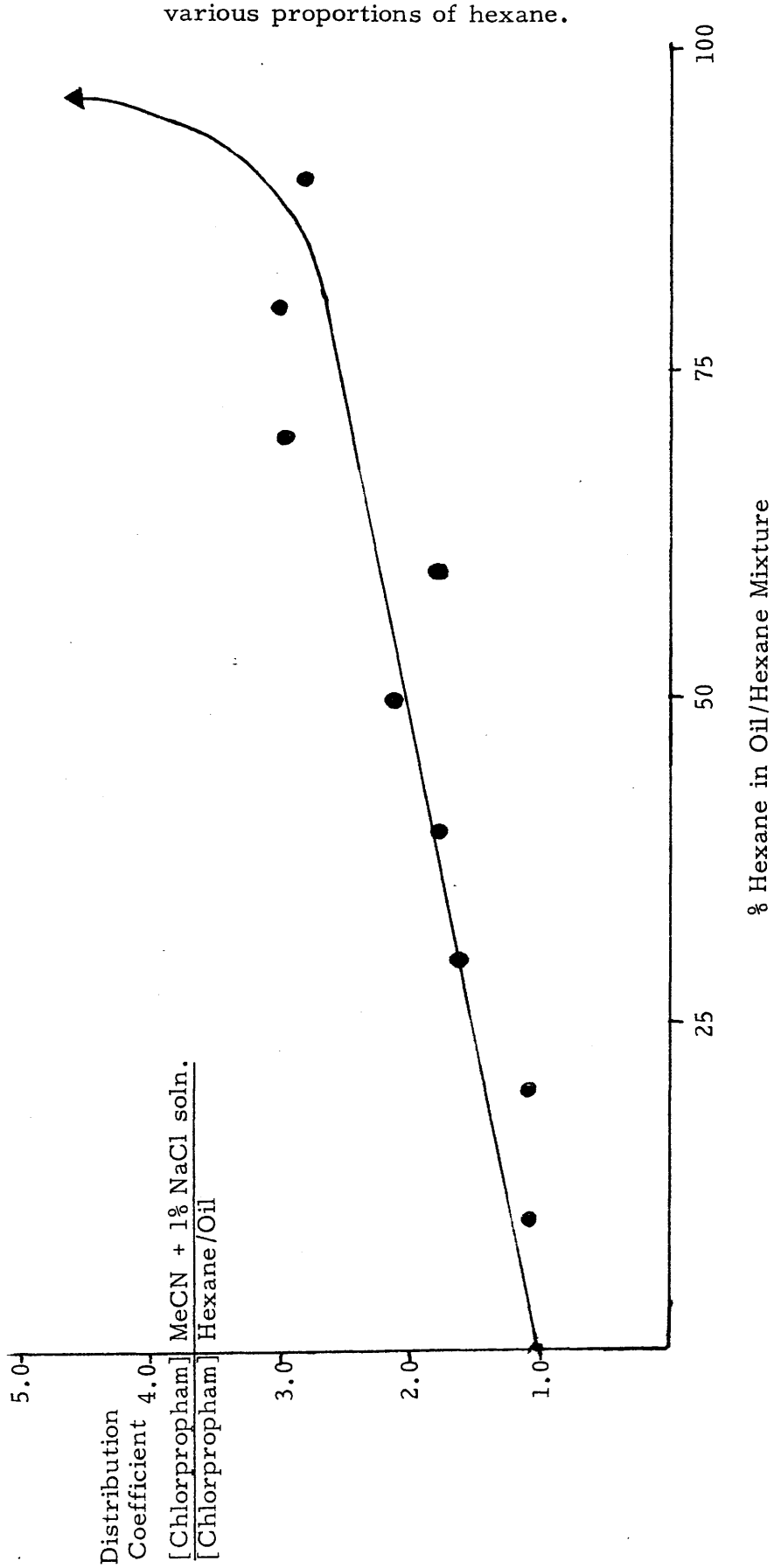


Figure 3.IV. Linear response of the flame ionisation detector to chlorpropham.

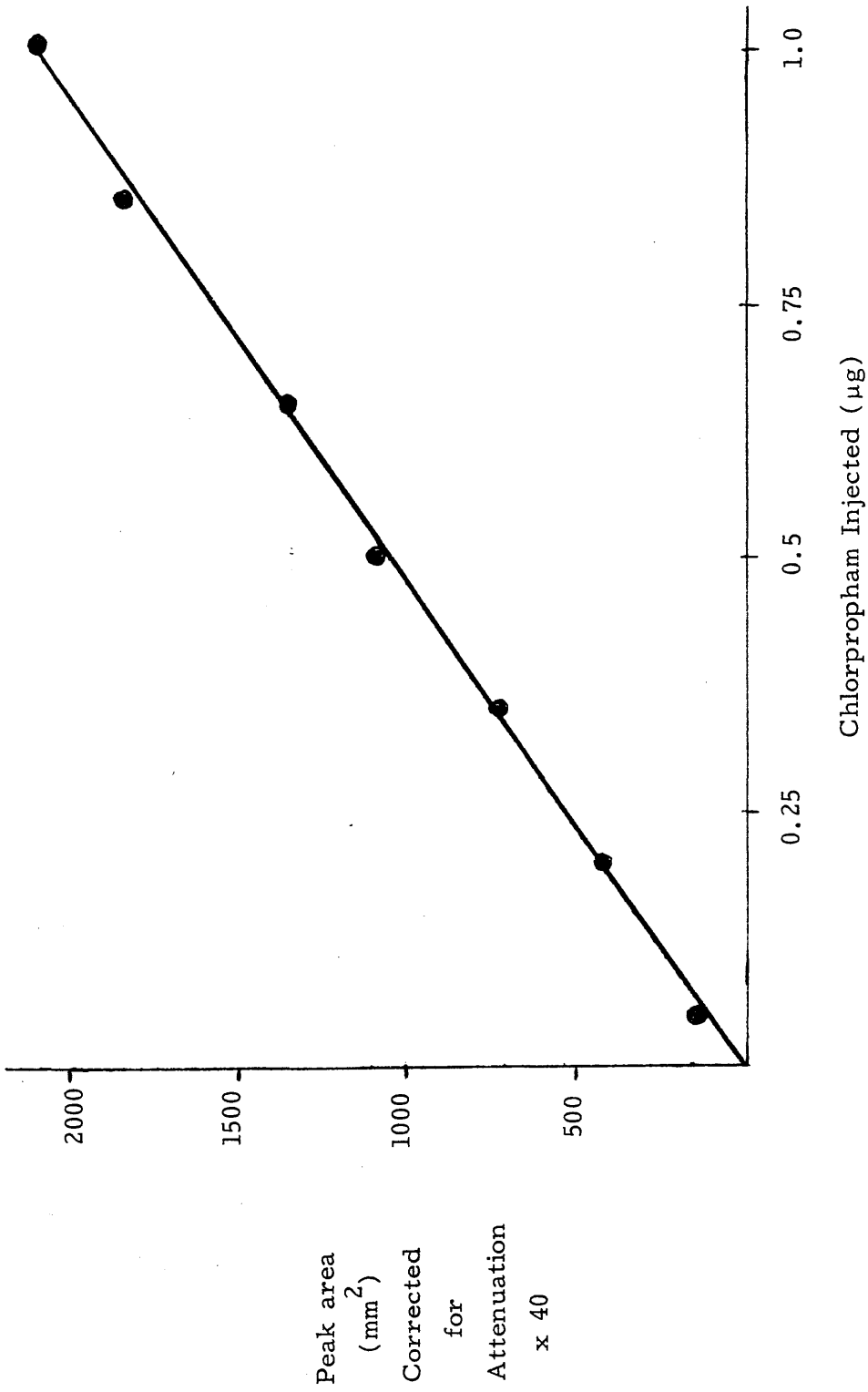


Figure 3.V. Chromatogram of hexane extract of potato slices using FID equipped GC fitted with an OV101 column (see section 3.3.3). Injection volume 5 µl, attenuation x 40. Detector response is equivalent to a concentration of 0.22 µg chlorpropham/g slices.

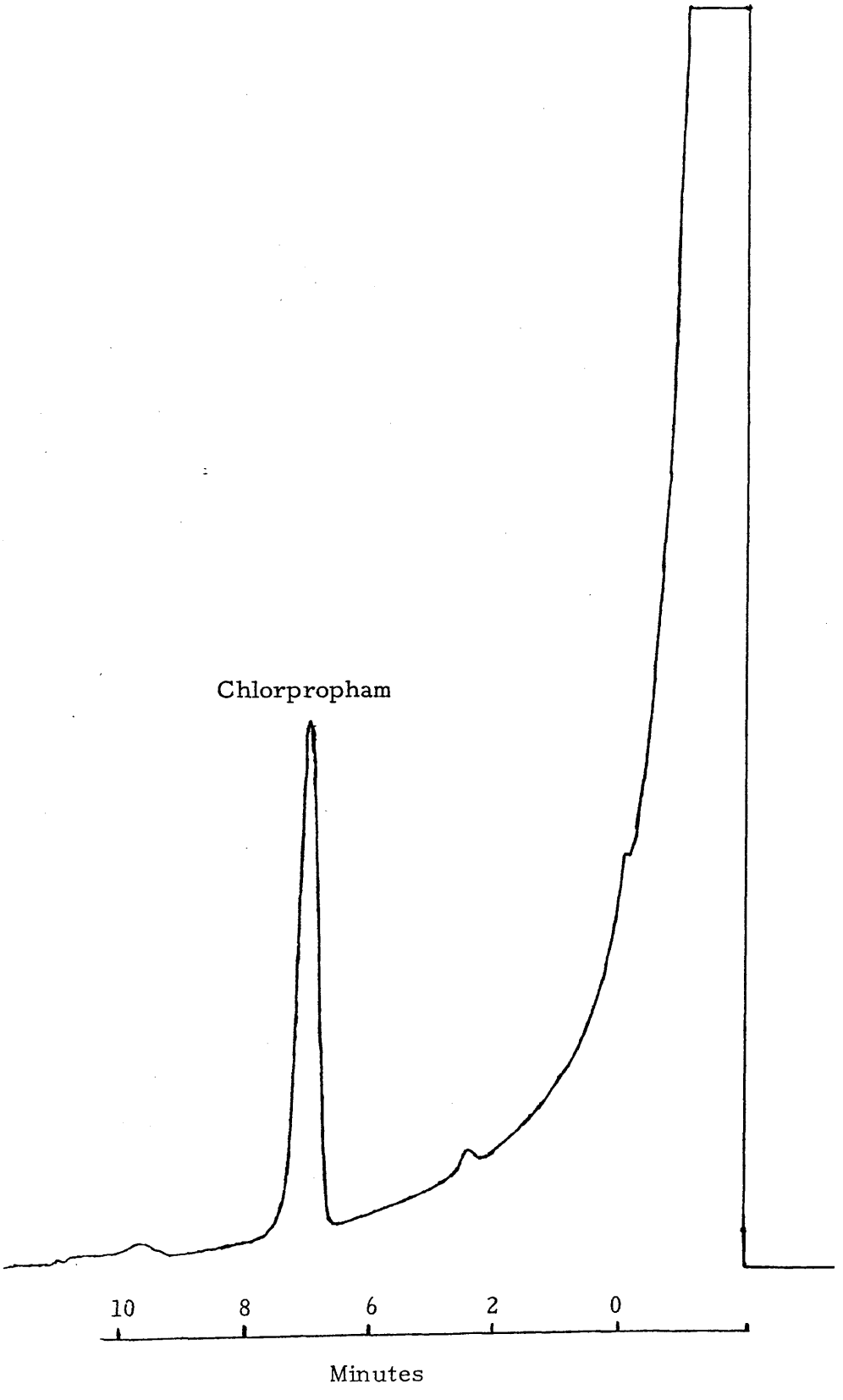


Figure 3.VI. Chromatogram of an alumina column cleaned-up crisp extract on a Carbowax 20M-TPA column. Injection volume 7 µl. Attenuation x 160. Detector response equivalent to 0.48 µg chlorpropham/g crisp. For full GC conditions see text (Section 3.4.3).

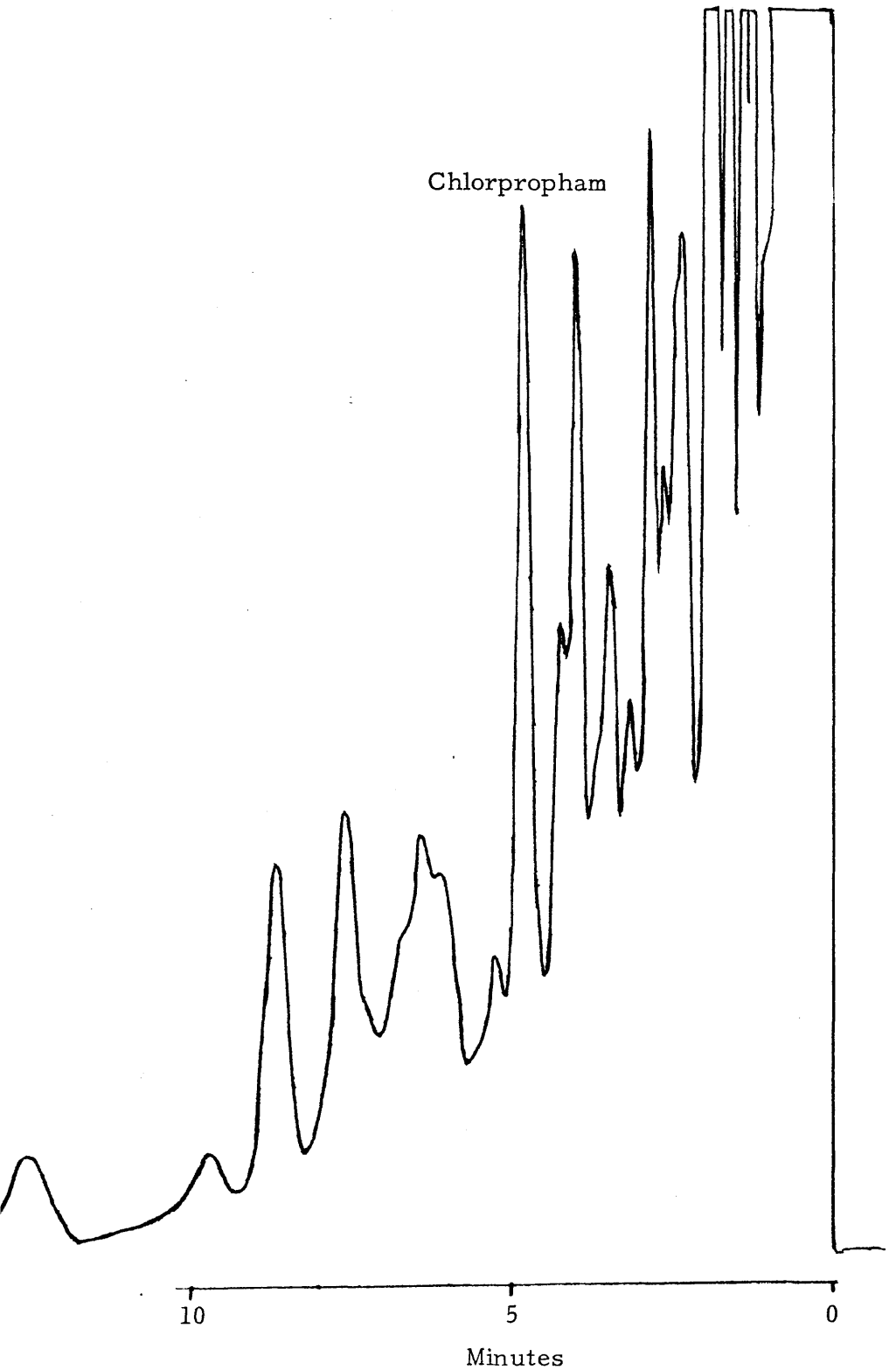


Figure 3.VII. Chromatogram of crisp extract ona GC fitted with an OV101 column after alumina column clean-up. Injection volume 7 µl. Attenuation x 160. Detector response equivalent to 0.57 µg chlorpropham/g crisp. For full GC conditions see Section 3.3.3.

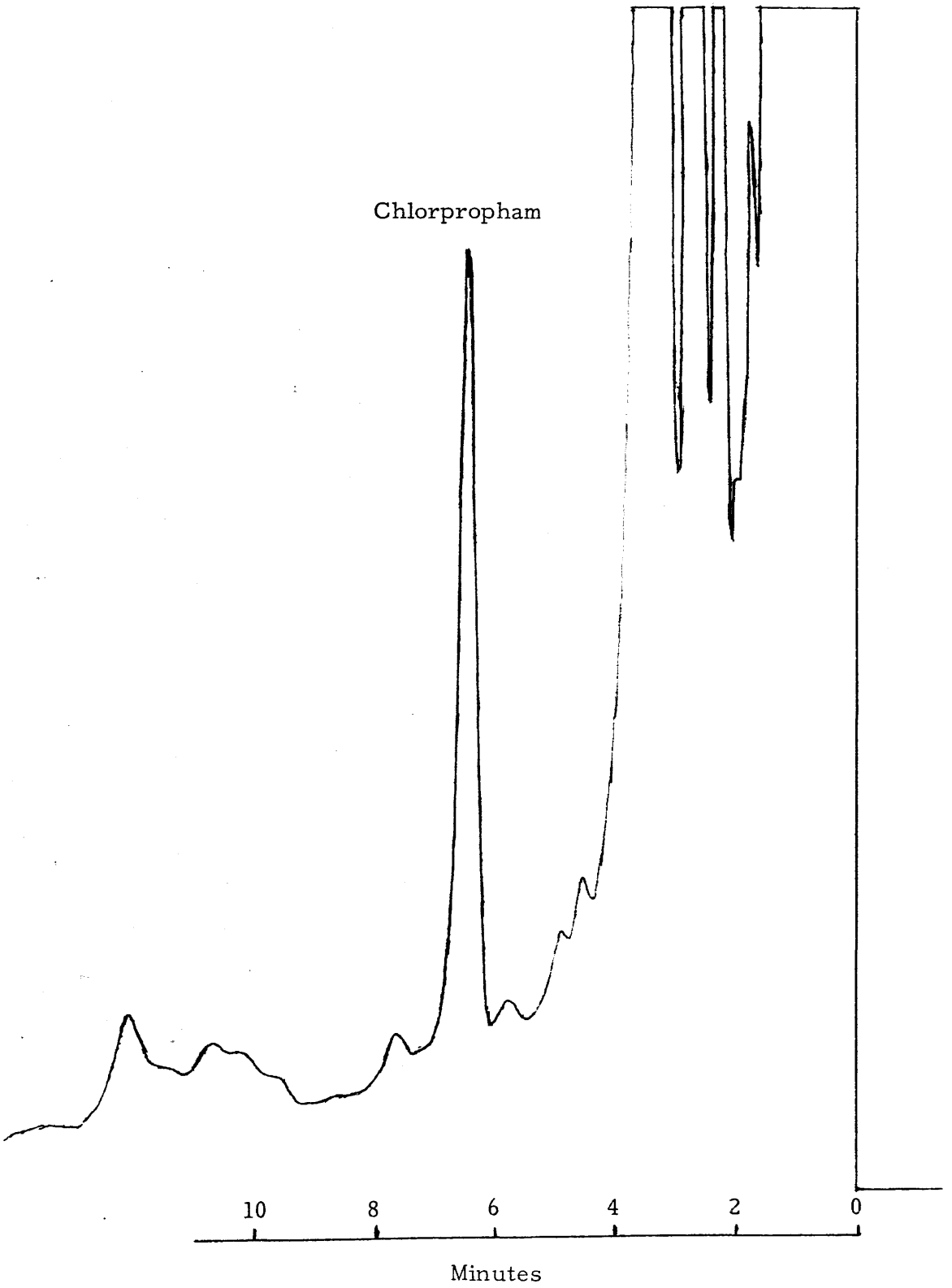
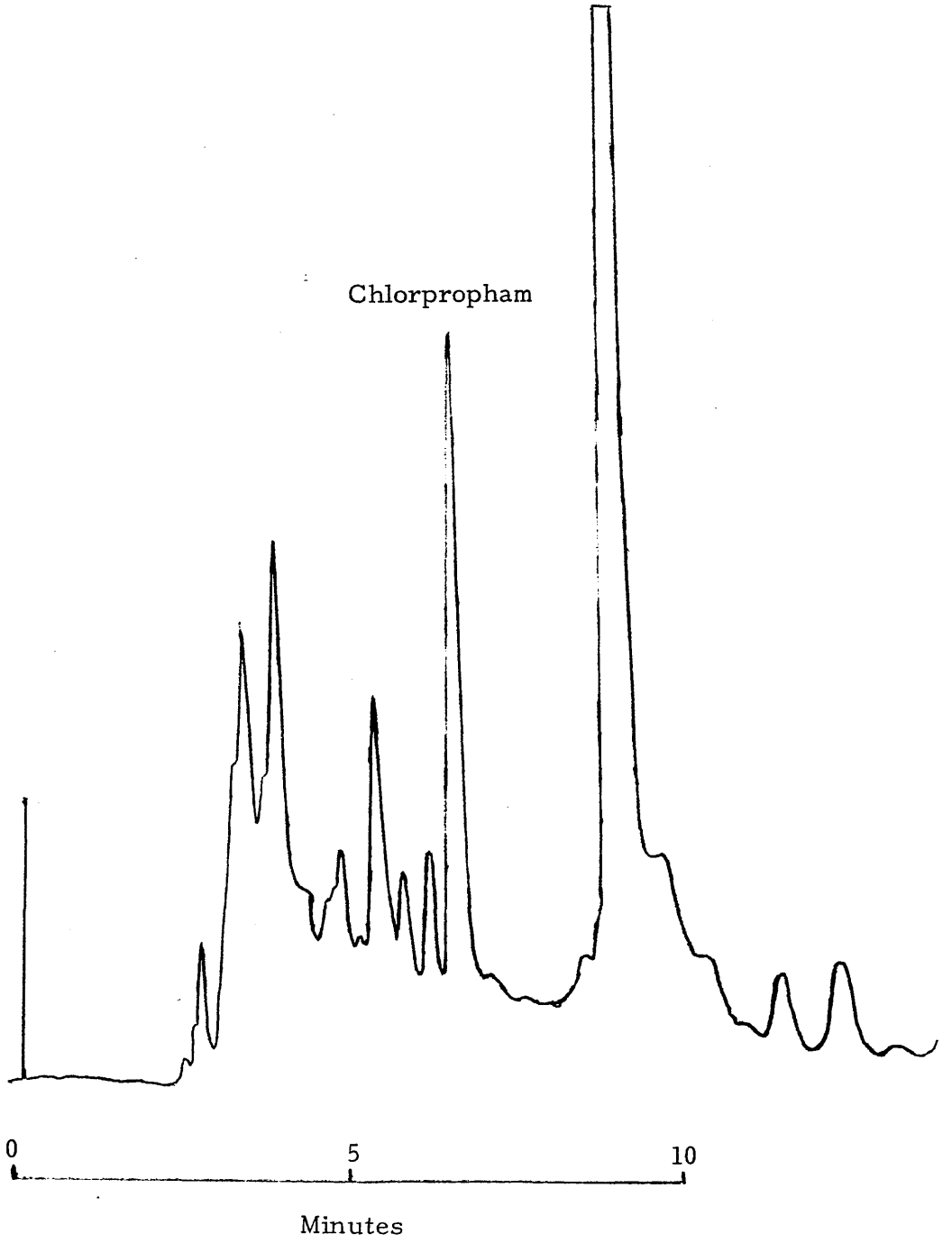


Figure 3.VIII. HPLC chromatogram of the crude acetonitrile extract of crisp prior to clean-up on an alumina column.
For full chromatographic condition of this reverse phase ODS-Hypersil system see Section 3.8. Injection volume 10 μℓ. Detector response equivalent to 0.55 μg/g crisp.



CHAPTER 4

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.1 Operation of the HPLC

Much of the analytical work described in this thesis involved the use of high performance liquid chromatography (HPLC). The chromatograph was manufactured by Waters Associates. While the products of this manufacturer are essentially standard, the way the basic chromatograph was operated is, perhaps, worthy of some explanation.

The chromatograph consisted of two M6000A dual piston reciprocating pumps with the flow rates being controlled by a 660 solvent programmer. The samples were introduced to the column via a U6K external loop injection valve that was fitted with a 2 ml capacity loop of capillary tubing. The column eluent was monitored by a Model 450 variable wavelength UV spectrophotometer. All of the above items were manufactured by Waters Associates. The output of the spectrophotometer was recorded with a Honeywell Electronic 19 potentiometric chart recorder. A home-made event marker was also fitted, which produced a signal of about 0.5 mV whenever the injection valve lever was switched to bring the injection loop onstream to the column. 25 cm x 0.5 cm i.d. stainless steel columns (Shandon Southern Products Ltd.) were used throughout this work although the shorter 10 cm and 16 cm columns were evaluated but the lower efficiencies of these columns compared to those obtained with the 25 cm columns resulted in the exclusive use of the longer column.

No thermostatting of the columns was undertaken. A 7 cm length of fine bore (0.15 mm i.d.) stainless steel tubing was used to connect the injection valve to the column head. Zero dead volume couplings were used for all capillary tubing connections downstream of the injection valve. The column outlet was connected directly to the spectrophotometer, using a PTFE ferrule without the need for an additional length of capillary tubing.

The mobile phase solvents used were supplied by Rathburn Chemicals (unless stated otherwise) and were of HPLC grade. An exception to this was water. Distilled water that had been filtered through a 0.5 μm Fluoropore PFFE filter (Millipore) was used since this produced a similar UV spectrum to HPLC grade water (Fisons Scientific Apparatus). Furthermore, when water was pumped through an ODS-Hypersil (Shandon) column at 1 ml/min for 20 min, prior to the start of a gradient elution run of 0-100% methanol in water, little difference was noted between a chromatogram produced using distilled water and one produced using HPLC grade water, indicating that both had a low amount of UV absorbing contaminants.

The M6000A pumps had three solvent lines supplying the pumps and this allowed the changeover of solvents without the need to replace solvents contained in the reservoirs. One solvent line to each pump supplied propan-2-ol. This was used as an intermediate solvent when the chromatograph was changed from operating in reverse phase to normal phase mode, owing to its miscibility with the common HPLC solvents. Sintered metal filters were fitted to the solvent supply lines to prevent particulate material reaching the pumps.

Degassing of the solvent or at least partial removal of the dissolved oxygen and nitrogen is necessary to prevent bubble formation in the detector cell. Oxygen degassing has been quoted as being the main reason for bubble formation in the detector flow cell (Kirkland, 1971) but it is difficult to envisage one set of gas laws operating for oxygen and another for nitrogen. The quantity of gas dissolved in a liquid is proportional to the partial pressure of that gas above the liquid (Henry's law). The solubility of a gas varies from solvent to solvent. For a mixture of water and methanol, the concentration of oxygen which can be held in solution (saturation concentration) is not linearly related to the mole fraction of a component of the mixture, i.e. there is negative deviation from a straight line relationship. Thus when oxygen saturated water and methanol are mixed, bubbles form and oxygen is lost from the admixture until the saturation level is attained (Bakalyar et al., 1978). When two solvents are mixed by the HPLC pumps under high pressure, the gases remain dissolved. It is only when the mobile phase pressure returns to a value close to atmospheric pressure does the concentration of dissolved gases exceed the saturation level and therefore bubbles form. These lower pressures are encountered towards the very bottom of the column, the capillary tubing leading from the column, and the flow cell of the spectrophotometer itself. The presence of bubbles in the flow cell has a marked effect on the apparent absorption of light in the cell by scattering the light and preventing it being detected by the photomultiplier.

An approach which has been adopted to prevent the formation of these bubbles is to restrict the free flow of eluent out of the spectrophotometer cell. This maintains the mobile phase under pressure and

the saturation concentration value of the gas remains above the actual concentration. While the flow cell of the Waters 450 spectrophotometer can withstand pressures up to 500 psi, this approach was seldom used due to the difficulty in ensuring the pressure did not exceed this value.

Solvent degassing before use is a more common approach. Air locking in a one pump low pressure mixing gradient elution system can be commonplace when the solvents are not thoroughly degassed, since any heat evolved on mixing would result in a lowering of the saturation concentration level of the admixture. In the Waters chromatograph described above, gradient elution chromatography was seldom performed, but the two pumps were used to mix the solvents rather than using one pump and a premixed mobile phase, the composition of which could not be easily altered. The degassing of the solvents prior to use in this two pump system when used to provide a two component isocratic mobile phase, had the advantage of eliminating the problem of air-locking of the pumps due to degassing when the solvent being drawn into the piston chamber was subjected to a reduced pressure.

Of the options available for degassing of the mobile phase, refluxing, vacuum filtration, and the use of ultrasonics, were rejected as they could not be carried out with the solvent reservoirs in place. The risk of contamination of the high grade solvents was also considered as was the time involved in carrying out these degassing procedures.

For the effective control of bubble formation in a binary mobile phase that is mixed immediately before entering the column, the concentration of the gas only has to be lowered somewhat and need not be completely removed. However, in the cases where high concentrations

of dissolved oxygen are present, high absorbances can be detected at wavelengths less than 260 nm (Bakalyar et al., 1978). This is particularly noticeable for methanol and acetonitrile.

Oxidation of the solute has also been reported where oxygen was not removed from the mobile phase (Sternson and De Witte, 1977; Snyder, 1971). Oxidation of liquid stationary phase in liquid-liquid column chromatography has also been a problem where oxygen was not excluded from the column (Leitch, 1971).

The method chosen to degas water, methanol and isopropanol was helium purging as this could quickly remove nearly all the dissolved oxygen (Williams and Miller, 1962). It also reduced the risk of impurities contaminating the solvent since it was carried out in the solvent reservoir of the chromatograph. The use of this method merely involved purging say 300 ml of solvent for about 5 minutes with helium (British Oxygen Company) at a flow rate of approx. 150 ml/minute and thereafter maintaining a trickle of helium through the solvent to prevent air re-dissolving. The purging gas was introduced into the solvent through a sintered metal filter to reduce the size of the helium bubbles, thereby reducing the time taken for effective degassing and also minimised consumption of this expensive gas.

Although helium purging results in a solvent saturated with helium, bubble formation in the detector cell never appeared to be a problem. While the solubility of helium in aqueous alcohol mixtures is non-linear with respect to alcohol concentration, i.e. displaying the same negative deviation from a linear relationship as oxygen in an alcohol/water admixture, it has been proposed that the lower solubility of helium

would result in only very small bubbles being formed if the saturation concentration of the solvent mixture is exceeded and this has little effect on the output from the photometer (Cargill, 1978).

The solvents used for normal phase chromatography are seldom degassed. It was often thought by chromatographers that air was more soluble in polar solvents than organic solvents (Kirkland, 1971; Chamberlain and Marlow, 1977). In actual fact the solubility of oxygen in hexane is about one hundred times that in water (Bakalyar et al., 1978). Despite this, when hexane is used in normal phase chromatography few problems with the solvent delivery pumps air-locking or bubble formation in the photometer flow cell are encountered. It may be that the HPLC grade hexane has not been allowed to equilibrate with the atmospheric gases and it may be removed from the solvent reservoir before equilibration has been achieved. However when dichloromethane was present in the mobile phase, solvent degassing in the flow cell was observed. Unfortunately helium purging could not be used in this case and degassing, or at least a reduction in the concentration of the dissolved gases, was achieved by the use of an ultrasonic bath, Model L674 (M.E.L. Equipment Co. Ltd.). Treatment of dichloromethane in this way prevented bubble formation in the flow cell for several hours, after which a repeat ultrasonic treatment was carried out or a small restriction was placed at the outlet of the detector, or a freshly ultrasonicated batch of solvent was gently added to the reservoir. Acetonitrile was also degassed using ultrasonics and, despite reported failure of this technique to degas solvents (Williams and Miller, 1962), it proved satisfactory.

Only two packing materials were used throughout this work. They were Hypersil and ODS-Hypersil (Shandon). The normal phase adsorbant material, Hypersil, consists of spherical porous particles of silica gel. The reverse phase material, ODS-Hypersil, uses Hypersil as a starting material and has octadecylsilyl (ODS) groups bonded to the particle surface. This is achieved by reacting octadecyltrichlorosilane with anhydrous silica and results in Si-O-Si (siloxane) bonds. When water is added the chlorine is lost and silanol groups result. These are then "capped" with a small non-polar group e.g. trimethylsilyl and the resultant material is hydrolytically stable and can be operated with mobile phases of between pH3 and pH8 (Shandon Technical Bulletin). The surface of this material behaves like a liquid and the elution order of many solutes can be predicted by use of a liquid-liquid partition data and indeed reverse phase HPLC is now used to predict octanol/water partition coefficients of candidate pesticides to determine if bioaccumulation is likely (Anon, 1975b).

The particle size of the column packings used was 3 μm for the Hypersil and 5 μm for the ODS-Hypersil. 3 μm ODS-Hypersil was available and while the effect of particle size on column efficiency and resolution is appreciated, the high column backpressures of the viscous solvents used in reverse phase chromatography resulted in the decision to use the 5 μm packing material. At this point it may be worthwhile to explain some of the terms used in chromatography. This is not intended to be an expedition into the theory of chromatography, but merely an explanation of terms which were found to be useful in practical applications of HPLC. For further reading on this subject the reader is

referred to Knox (1978), Pryde and Gilbert (1979) for general topics on liquid chromatography and to Done et al. (1972a & 1972b), Majors (1973) and Gruska et al. (1975) for aspects of column efficiency and band spreading.

The main advantages of high performance liquid chromatography over conventional column chromatography (where only gravity drives the solvent through the column) that can be easily observed are increased resolution, increased column efficiency and decreased analysis time. The resolution (R_s) of two chromatographic peaks is the degree to which the solutes are separated by the column and is defined by the peak separation divided by the mean peak width, i.e.

$$R_s = \frac{t_2 - t_1}{\frac{1}{2}(W_1 + W_2)}$$

W_1 and W_2 represent the width at the base of peaks 1 and 2

t_1 and t_2 represent the retention time of solutes 1 and 2

The terms used can be in either volume or time units, resulting in R_s being unitless. For two adjacent isosceles triangles baseline resolution corresponds to $R_s = 1$. For two Gaussian peaks effective baseline resolution is obtained at $R_s = 1.5$. The column efficiency is measured in terms of the number of theoretical plates N, and is calculated from

$$N = 16\left(\frac{rt}{W}\right)^2$$

where rt is the retention time and W the width of the peak at the baseline, again the units of rt and W must be the same. The width of peak at the baseline is determined by extending the tangents to the points of

inflection of the peak to the baseline. The efficiency of the column N, is a measure of the amount of spreading and dilution of the solute band as it travels down the column. It, of course, includes all band spreading/dilution that occurs between the injection point and the detector. The higher the value of N, the more efficient the column. Often the column efficiency is quoted in terms of height equivalent to a theoretical plate (HETP) or H. This is simply the length divided by the efficiency of the column, i.e.

$$H = \frac{L}{N}$$

While these parameters can be used to give a numerical value to the resolution and efficiency of a column, a chromatographer can easily tell merely by looking at the chromatogram when peaks are resolved and when a column is operating efficiently. Sharp peaks at long retention times are the result of minimised dispersion of the solute and minimised dilution by the solvent. The obsession of many manufacturers with column efficiency is probably a consequence of rapidly developing column technology. This has led to a wide range in the efficiencies of columns offered for sale at any one time.

Van Deemter is credited with studies into factors influencing the HETP of gas chromatographic (GC) columns. He explained the need for an optimum linear gas velocity in order to maximise column efficiency (i.e. a minimum value of HETP).

Three band dispersing effects which are related to gas flow rates are:

1. longitudinal diffusion of the sample in the mobile phase
2. dispersion due to the "tortuous" nature of the flow through the column
3. dispersion due to slow equilibration between mobile and stationary phase.

When each of these three factors are considered independently it can be seen that dispersion due to diffusion (1) is dependent on the residence time of the sample in the column and therefore is inversely related to the flow rate of the mobile phase. Dispersion due to the "tortuosity" of flow (2) is a result of varying pathlengths of different mobile phase streams and eddy currents within the column and shows only a weak positive dependence on flow rate. In the case of slow equilibration (3) dispersion increases and therefore the HETP values increase when flow rate is increased. The cumulative effect of these three factors gives a linear gas velocity - v - HETP plot which is hyperbolic, i.e. it falls rapidly then levels off to a minimum and then increases again as the linear gas velocity is increased. Knox (1978) explains band dispersion in liquid chromatography by the same three band dispersing processes but stresses the point that equilibrium between the mobile and stationary phases is much slower than in gas chromatography, due to the diffusion rate of the solute in the liquid mobile phase being slow. However if the particle size is small then only a thin film of mobile phase covers the stationary phase, therefore diffusion distances are short and equilibration fast.

The main drawback with this is the high pressures that are required to obtain flow rates which allow the column to operate at its

optimum efficiency. In the analogous situation with GC optimum flow rates are obtained at much lower pressure due to the low viscosity of the carrier gas, but GC suffers from the disadvantage of the mobile phase being compressible therefore the linear flow can only be optimum at one point along the length of the column. With HPLC high pressures are required to chromatograph solutes on small particle columns if high column performance is to be obtained. For this reason, HPLC is referred to as both high pressure liquid chromatography and high performance liquid chromatography.

Manufacturers pre-packed columns were not purchased for the work described in this thesis. The columns were packed using a Shandon column packing instrument. This instrument was housed in the Botany Department of the University and while the packing procedure was essentially that of the instrument manufacturer, the procedure used is given below.

The columns were all packed in the upward configuration and involved the packing of dilute slurries of packing material into the column at high pressure. For a 25 cm x 0.5 cm i.d. column, 3.8g of packing material was added with stirring to 33 ml of slurry solvent (methanol in the case of Hypersil, propan-2-ol in the case of ODS-Hypersil). The slurry was ultrasonicated in a low power ultrasonic bath (Semat Technical UK Ltd.) for 1-2 min before being placed in the solvent reservoir. After the column had been fitted to the top of the reservoir, the pump was switched on to give an instantaneous packing pressure of 9-10,000 psi, which powered the slurry up into the column at speed to give a compact, stable bed.

For ODS-Hypersil columns, three different solvents were pumped through the column, namely, a packing solvent, an intermediate solvent and a conditioning solvent, and consisted of 100 ml of hexane, 80 ml of propan-2-ol and 50 ml of 50% methanol in water. After half of the propan-2-ol had been eluted the column was inverted to the downward configuration. It is a feature of this packing instrument that all of the above solvents can be eluted without interruption.

With the Hypersil columns, 100 ml of methanol was used to pack the column, after which the column was inverted and eluted with a further 40 ml of methanol, followed by 50 ml of ethyl acetate as conditioning solvent.

Once all the solvent had been pumped through the columns the pump was switched off and the pressure allowed to drop to zero. The pressure control valve was then set to give an output pressure of about 12,000 psi. The pump was then switched on and allowed to continue to pump at this higher pressure for 30 seconds before being switched off again. This was repeated twice more. After the pressure had fallen to zero, the column was removed and the excess packing material removed from the top of the column and the surface made level with a spatula. A top filter mesh, spreading plate and sealing ring were inserted into the top of the column and a storage fitting was clamped on.

A problem which was encountered when the columns were first fitted to the HPLC was one of very high back pressures even at flow rates of less than 0.5 ml/min. It was found that the spreader plate was the cause of the restriction. This plate, as its name suggests, spreads the incoming mobile phase as it emerges from the capillary tubing at high speed. With the spreader plate pressed hard against the end of the

column inlet tubing, high backpressures resulted. These were overcome by scratching the surface of the metal spreader plate, thus removing the restriction to the flow of the eluent.

The efficiency of the columns were determined by the use of test mixtures. For ODS-Hypersil columns the test mixture consisted of 370 μg/ml benzamide, 60 μg/ml acetophenone, 70 μg/ml benzophenone and 100 μg/ml of biphenyl in a 70% methanol in water solution. A chromatogram of the separation of these compounds is shown in Figure 4.I. The chromatographic conditions were as follows:

column: 25 cm x 0.5 cm

packing: 5 μm ODS-Hypersil

eluent: 70% methanol in water

flow rate: 1.0 ml/min

pressure drop: 2,500 psi

detector (UV spectrophotometer): 0.1 AUFS, 254 nm

injection volume: 2 μl

The number of theoretical plates which the column is equivalent to for the biphenyl solute is about 13,700, i.e. 54,800/metre and a HETP of 4.56 μm.

The testing of ODS-Hypersil columns could be performed with ease due to the fact that the solvents making up the mobile phase used in this test procedure, recommended by Shandon, were routinely used for practical separations. This was not the case for Hypersil columns and, once a solvent system had been developed for quantification of chlorpropham then standards of this were used to assess column efficiencies.

The HPLC was found to be capable of detecting low levels of chlorpropham. Figure 4.II shows the chromatograms produced by the injections of 10 and 100 ng of chlorpropham into the chromatograph. The column conditions were as follows:

column: 25 cm x 0.5 cm

packing: 3 μm Hypersil

eluent: 40% dichloromethane, 60% hexane

flow rate: 1.2 ml/min

pressure: 1500 psi

detector: 0.1 AUFS and 0.02 AUFS 237 nm.

The optimisation of the detector to allow the quantification of small amounts of chlorpropham, and to reduce the error involved in quantifying higher concentrations, the best operating conditions had to be found. This involved merely determining the UV spectrum of chlorpropham in hexane and a maxima was found at 237 nm. At 254 nm, the wavelength used in many fixed wavelength photometers, the absorbance was some 93% less than at 237 nm. The molar extinction coefficient of chlorpropham at 237 nm is 1.98×10^4 l/mol/cm. Unless there was some need to reduce the sensitivity, the detector was operated at 237 nm for chlorpropham determinations. The variable filter of the detector was found to affect sensitivity. The variable filter is designed to reduce noise levels by averaging the signal (in the case of the 450 spectrophotometer) over anything from half a second to five seconds. At the longer time settings the response of the detector to sharp, rapidly eluting peaks was reduced and although the baseline noise was increased at the higher sensitivity settings of the detector, the variable filter remained at the half second setting throughout this work.

4.2 Linear Response of the Detector

The linear response of the detector to chlorpropham was verified over a range of 40 ng to over 7 μg of solute in a range of injection volumes between 5 and 10 μl. This was performed on a normal phase Hypersil column under the chromatographic conditions specified above (Section 4.1). The linearity of the detector response to chlorpropham in terms of peak height is shown in Figure 4.III (correlation coefficient > 0.999) and in terms of peak area using the method of triangulation in Figure 4.IV (correlation coefficient > 0.999). The use of peak height alone to determine the quantity of solute eluted from the column can involve lower errors as well as being more convenient, but peak area was often used when the width of the peaks produced by standards and samples were dissimilar.

4.3 Fraction Collection from the HPLC

Often fractions were collected from the column for further study and in order to ensure that all of the material could be recovered without being contaminated by other solutes and in a minimum of mobile phase, the elution of a solute with regard to the detector signal was determined.

An injection of a concentrated chlorpropham sample was made with the detector operated at a low sensitivity setting, and at a wavelength of 254 nm, (as mentioned above chlorpropham has a low extinction coefficient at this wavelength). Fractions were collected, as detailed on the chromatogram, Figure 4.V and the histogram, Figure 4.VI, shows the proportion of the total quantity of solute injected that appears in each sample. The chromatographic conditions used in this reverse phase

system are those given above for the ODS-Hypersil equipped chromatograph (Section 4.1).

In this unreplicated study only 94.7% of the injected chlorpropham was recovered. However once the points at which collection had to start and finish were established, which are also detailed on the chromatogram (Figure 4.V), one fraction containing all of the eluted solute was collected and the recovery from the columns was calculated. Using five replicates, the average recovery from the ODS-Hypersil column was 97.4% ± S.D. 2.5. The recovery from a 3 μM Hypersil column was 99.1% ± S.D. 1.5. The HPLC proved extremely useful for the separation of components from reaction mixtures prior to being subjected to mass spectrometry and for fractionation of radioactive biological extracts for activity determination by liquid scintillation counting.

Figure 4.I. Chromatogram of test mixture for reverse phase HPLC. For chromatographic conditions see text (Section 4.1)

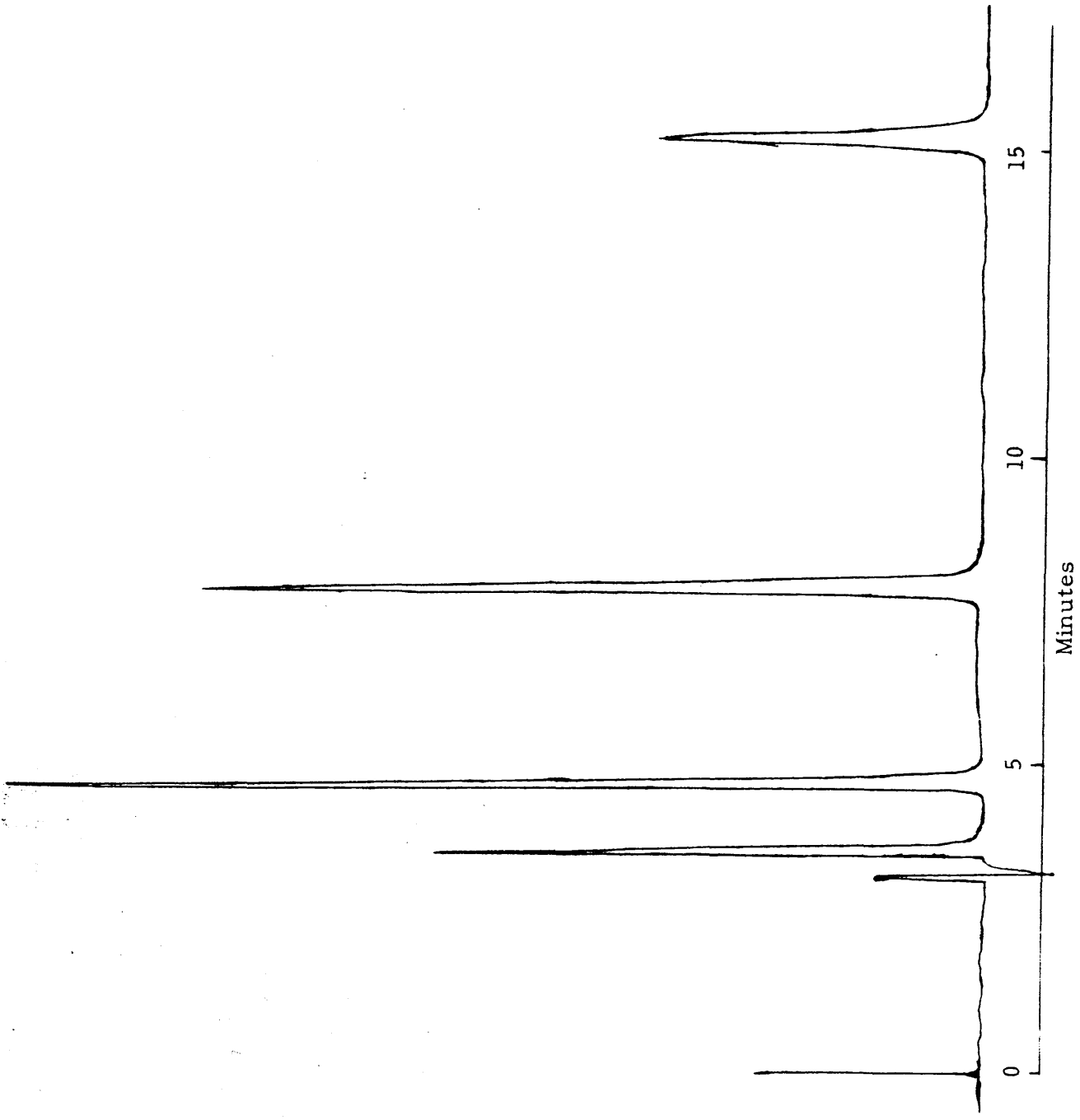


Figure 4.II. Chromatogram of 10 ng and 100 ng injections of chlorpropham. HPLC equipped with normal phase Hypersil column. For chromatographic conditions see text (Section 4.1)

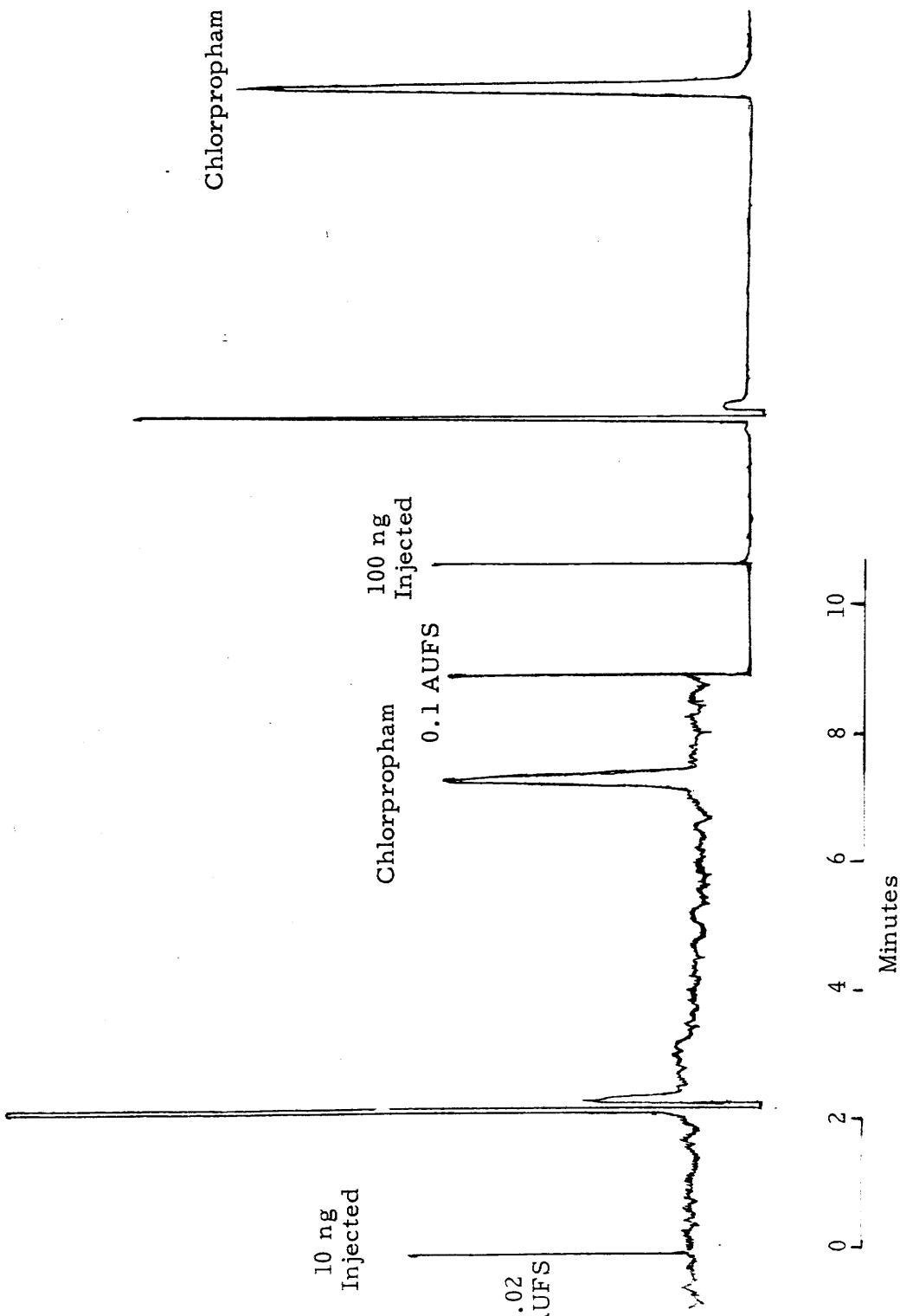


Figure 4.III. Linear response of HPLC Spectrophotometric detector to chlorpropham (in terms of peak height).

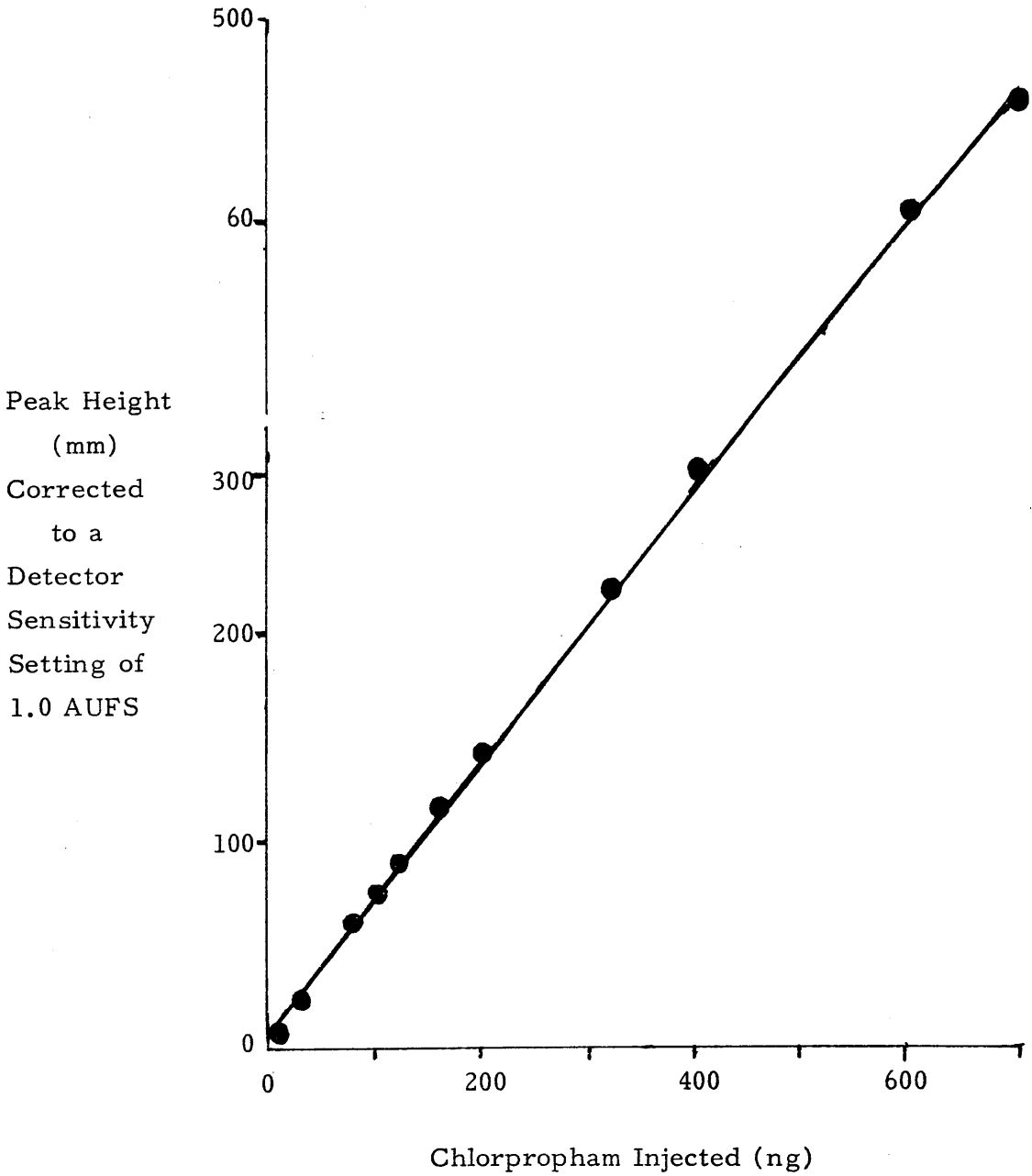


Figure 4.IV. Linear response of HPLC spectrophotometric detector to chlorpropham (in terms of peak area).

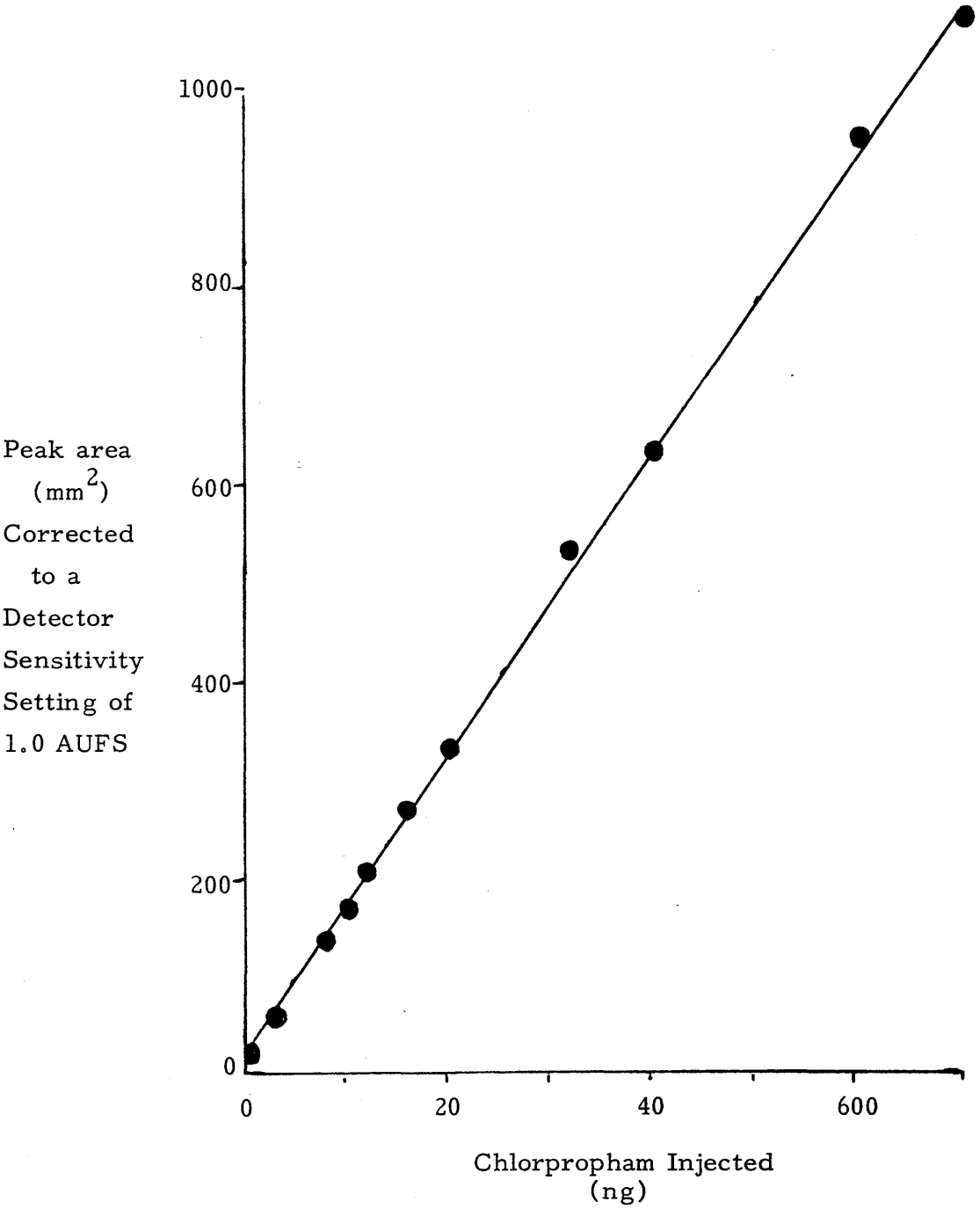


Figure 4.V. Recovery of chlorpropham from a reverse phase ODS-Hypersil equipped HPLC. For chromatographic conditions see text (Section 4.1).

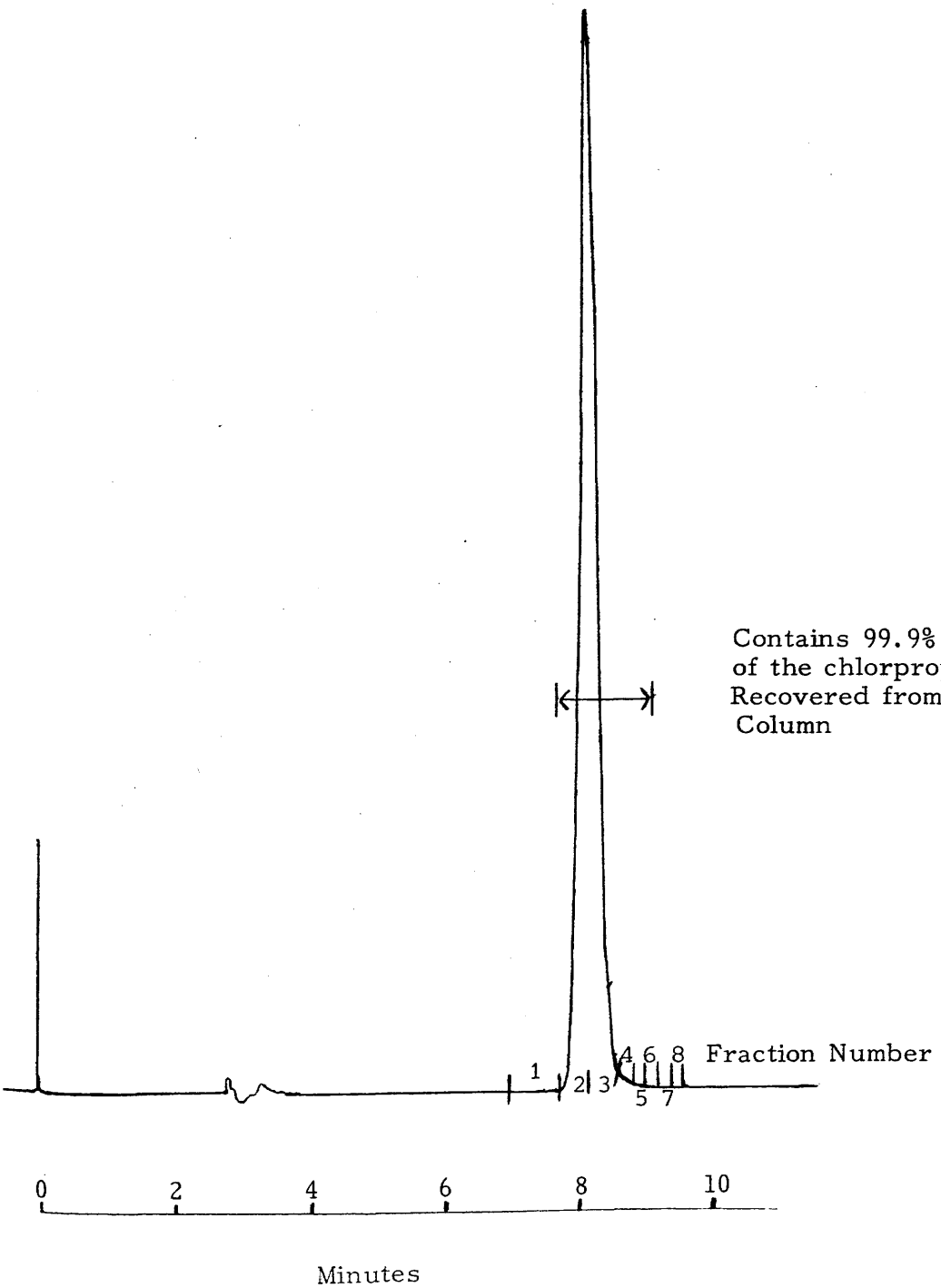
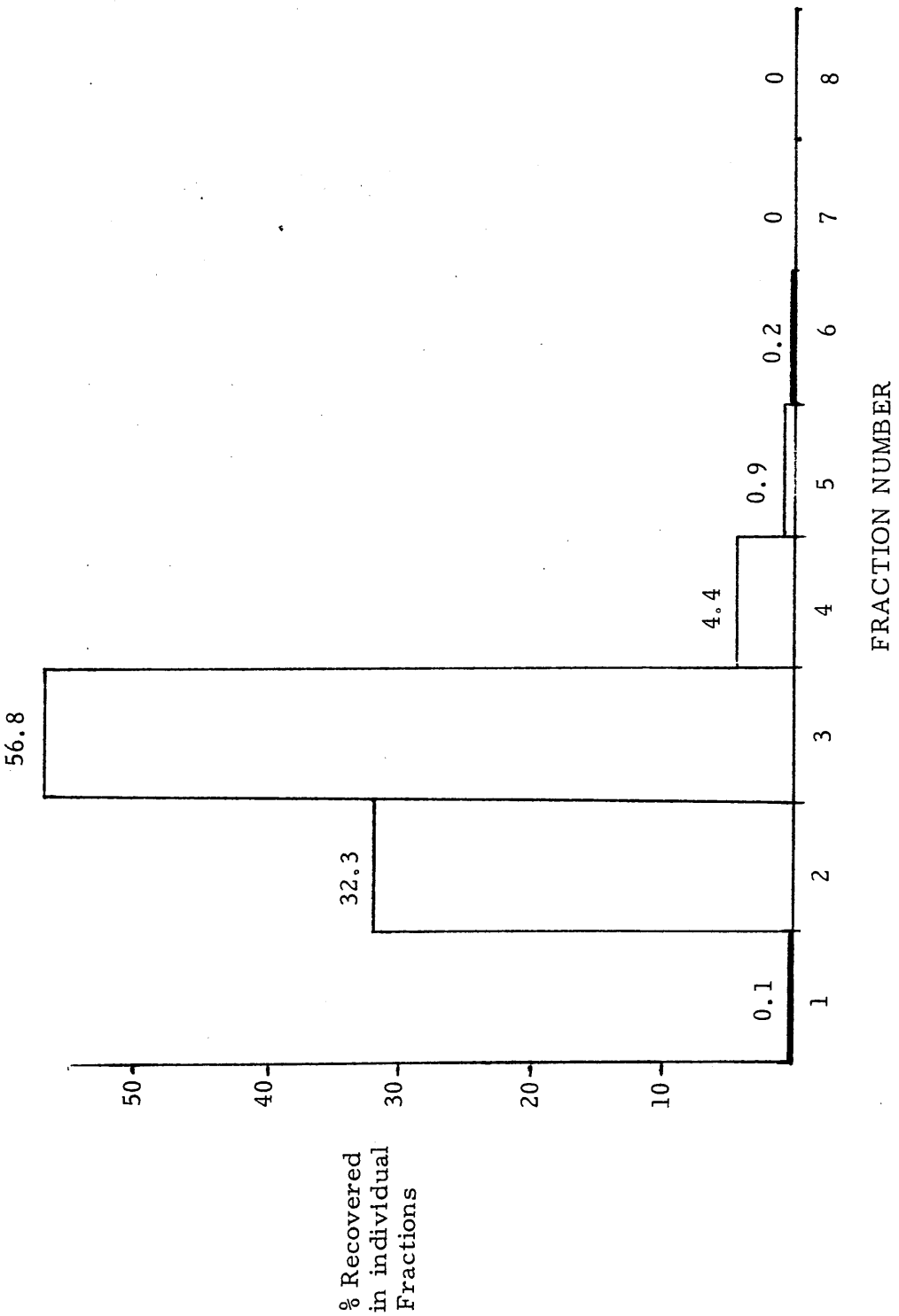


Figure 4.VI. Recovery of chlorpropham from ODS-Hypersil column.



CHAPTER 5

PREPARATION OF RADIOLABELLED CHLORPROPHAM

5.1 INTRODUCTION

At the present time, before an agrochemical is released onto the market, the Pesticide Safety Precautions Scheme (PSPS) requires extensive data covering toxicology, metabolism and residue chemistry to be submitted prior to clearance for widespread use being granted. Evidence of chemical's efficacy need only be submitted to the Ministry of Agriculture, Food and Fisheries (MAFF) if the manufacturer seeks approval under the Agricultural Chemicals Approval Scheme (ACAS). Both of these schemes are voluntary and of course cover only the United Kingdom. The inevitable consequence of an extensive research effort prior to a chemical gaining clearance is that the development of an agrochemical for minor uses is considered uneconomic. This would be the case whether the regulation of agrochemical use was voluntary or statutory. Much of the data required by statute prior to approval in other countries can be of use in the gaining of PSPS clearance in the United Kingdom and of course the converse is true. It can also be argued that if the allowed uses of a chemical already include a food crop, then the widening of the range of uses requires only a fraction of the expense that has already been incurred. The economic benefit to the manufacturer from developing a new use for one of its agrochemicals may not be great to justify additional studies. However where the chemical has been on the market for some time and is no longer protected by patent then

even relatively small amounts of expenditure becomes more difficult to justify.

Chlorpropham has been used as an agrochemical for some time now, initially as a herbicide with its sprout suppressant properties first reported by Marth and Schultz (1952). Since then there has been a considerable amount of work published relating to the analytical methods for the determination of chlorpropham residues, the residue values themselves, toxicology, metabolism (microbial, plant and animal) as well as its effect at cellular level in many systems, including those not likely to be affected by agrochemical applications. For a discussion of these aspects, see Chapter 2.

While residue studies of chlorpropham treated potatoes have been conducted, little information of the metabolic fate of chlorpropham in potato stores has been published. In order to conduct metabolic fate and indeed environmental fate studies where the fate of the agrochemical is unknown, some form of labelling of the compound being studied is required. Radiolabelling is the most commonly used method. This chapter describes the synthesis, purification, and activity determination of the radiolabelled chlorpropham used during the work of this thesis.

5.2 Experimental

The use of a radiolabel allows the degradative pathway of an agrochemical to be followed by providing a unique (unique, that is, to the system under study) indication of where the label is situated and the compounds in which it subsequently appears. In the case of chlorpropham where ^{14}C is to be the label, three distinct label positions

are possible. These positions are, the aromatic ring, the carbonyl carbon, and the ester moiety.

For obvious reasons the fate of the labelled compound need only be followed to the point where it appears in some inoccuous or naturally occurring compound. It follows then that the label should be present in the most recalcitrant part of the molecule. In the case of chlorpropham this position would be the aromatic ring since, if the label was part of the alkyl moiety or in the carbonyl carbon position and ester or amide hydrolysis occurred, labelled carbon dioxide or propan-2-ol would be produced, depending on the initial position of the label (Herrett, 1969). If such reactions were to proceed at significant rates then labelling of the aromatic ring would be essential in metabolic studies. However, in many non-soil systems the amide and ester bonds appear stable (Fletcher and Kirkwood, 1982). Labelled anilinium chloride and propan-2-ol had been purchased by the Agricultural Chemistry section to allow the synthesis of both ester and ring labelled chlorpropham. At the time the work described in this thesis was being carried out, ester labelled chlorpropham had been synthesised in the Department by Isabel Boyd.

The ester labelled chlorpropham was synthesised from ^{14}C-labelled propan-2-ol and 3-chlorophenylisocyanate. The reaction was carried out in a 3 ml "Reacti-vial" (Pierce Chemical Co.). The 500 μCi of [1,3-^{14}C]-propan-2-ol with a specific activity of 9.1 mCi/mmol (152 μCi/mg) (Radiochemical Centre, Amersham) was initially contained in an acetone/dry ice bath prior to the propan-2-ol being transferred to the Reacti-vial with the aid of 0.3 ml of hexane. 0.1 ml of unlabelled propan-2-ol (Rathburn Chemical Co.) was then added to the Reacti-vial

followed by 0.2 ml of 3-chlorophenylisocyanate. A PTFE coated magnetic stirrer was placed in the Reacti-vial which was then sealed. After applying a gentle heat to the Reacti-vial for two hours it was allowed to cool. 0.5 ml of methanol was then added to the reaction mixture, followed by water which was added dropwise until a precipitate formed. Despite attempts to crystalise the chlorpropham by plunging the Reacti-vial into an ice-bath, scratching the wall of the container and, seeding with a small crystal of chlorpropham, little crystallization resulted. Following these attempts to crystallize the chlorpropham, the reaction mixture was dried with anhydrous sodium sulphate, and filtered into a pear-shaped flask which was then sealed. It was this material which Isabel Boyd had anticipated purifying for use in her studies of sprout suppressant properties of chlorpropham but, since then, she has concentrated on aspects of potato quality which did not require labelled chlorpropham.

Before using this labelled material, its activity, purity, and the total quantity of chlorpropham present had to be established. The reaction mixture in the pear-shaped flask was transferred quantitatively with the aid of hexane washings to a 10 ml volumetric flask. 30 µl of this was taken and diluted to 5 ml with hexane. The concentration of chlorpropham in the 5 ml volumetric flask was determined using the high performance liquid chromatography described in Chapter 4. The chromatographic conditions were as follows:

column: 25 cm x 0.5 cm i.d.

packing: 3 μm Hypersil

eluent: 40% dichloromethane, 60% hexane (by volume)

flow rate: 1.0 ml/min

pressure drop: 1000 p.s.i.

detector: (UV spectrophotometer): 237 nm

injection volume: 10 μl

Figure 5.I is a chromatogram of the crude reaction mixture and shows a late eluting component contaminating the sample. The concentration of this impurity amounts to some 18% of the chlorpropham present, if the molar absorptivities of the chlorpropham and the impurity are assumed to be similar. Some "stop-flow scanning" of the impurity was carried out but since the UV cut-off of dichloromethane is around 235 nm then it was only possible to tentatively suggest that the impurity had a λ_{max} of about 237 nm.

The eluant from the column was collected manually in vials, into which had been placed 10 ml of Dimilume liquid scintillation fluid (Packard Instruments Inc.). The activity of each fraction was determined using a Phillips Model PW4510 liquid scintillation counter (LSC). The mean of the three replicates showed that the chlorpropham contained 97.4% of the radiolabel after correction for a column recovery factor of 99.1% (see Section 4.3). No other fraction produced counts significantly different from the background. The total amount of chlorpropham product was 204 mg and the total activity was 1.28×10^{10} disintegrations per minute (dpm). Using the SI unit the Becquerel (Bq), which is a disintegration per second (dps), the activity was 2.13×10^{6} Bq

(2.13M Bq). In terms of Curies (Ci) this corresponds to 57.8 μCi total activity and a specific activity of 60.6 μCi/mmol chlorpropham. The yield of the reaction with respect of propan-2-ol was 71.7% but the yield with respect to the radiolabel starting material was only 12.1%. Clearly considerable losses must have occurred when the labelled propan-2-ol was transferred from the sealed delivery ampoules to the reaction vessel. The losses would have been lower had all the labelled alcohol been delivered in just one ampoule.

The activity was less than what had been hoped for but the high concentrations of chlorpropham that were to be used in subsequent studies allowed the use of radiolabelled material of low specific activity.

The impurity, it was thought would most likely be the methyl ester of N-3-chlorophenylcarbamic acid which was formed when methanol was reacted with the excess isocyanate agent and was used to dissolve the reaction products. Its presence would of course confirm the fact that an excess of isocyanate had been added to ensure that all of the propan-2-ol reacted to produce chlorpropham. Before any metabolism studies could be undertaken this impurity would have to be removed. This was accomplished by using an alumina column. The alumina column conditions are given below (Section 5.3). The chromatographic behaviour of the impurity on the high pressure liquid chromatograph when equipped with a normal phase (Hypersil) column was used as a starting point. The large separation between the chlorpropham and the impurity on the high pressure liquid chromatograph it was hoped should allow complete separation even on a conventional clean-up column system. To minimise the risk of losing a large proportion of the radiolabel a small

quantity of the crude reaction mixture was purified on an alumina column prior to large aliquots of this mixture being applied to the column. To obtain a dilute mixture of the crude reaction products 0.2 ml was removed from the 10 ml flask and diluted to 25 ml with hexane.

Using 2 ml aliquots of this diluted material, it was possible to maximise recovery of the chlorpropham from the column while minimising the concentration of the impurity. 10 ml fractions were collected from the alumina column and analysed by high pressure liquid chromatography using the conditions given above. This showed that fractions covering the 40-80 ml band contained 98% of the applied chlorpropham with a trace of the impurity only becoming evident in the 70-80 ml fraction. If the 70-80 ml fraction was not included in the main bulked fraction then the 40-70 ml fractions contained only 93.8% of the chlorpropham applied to the column, but prevented the inclusion of the impurity which amounted to some 0.3% of the total chlorpropham which was present in the 40-80 ml fraction. It was therefore decided not to bulk the 70-80 fractions with the 40-70 ml fractions.

5.3 Final Purification Method

The method used to pack the columns was that described in Section 3.32. A 25 cm x 0.9 cm i.d. column fitted with a glass sinter was used. The adsorbant bed depth was 25 cm and a 1 cm plug of anhydrous sodium sulphate was placed on the top of the column. The adsorbant material used was neutral alumina (Woelm) and was deactivated by addition of 20g of distilled water to 100g of alumina. The water was added dropwise and shaken overnight on a reciprocating shaker

(Griffin and George). Hexane was used as the eluent and 20 ml was run through the column prior to application of the sample. Samples were drawn from the 10 ml volumetric flask containing the crude reaction mixture and the volumes depended on the amount of material required for the particular study and included about a 20% excess of material. The 40-70 ml fractions from the alumina column were collected and retained. The 0-40 ml and 70-120 ml fractions were assayed for chlorpropham prior to being discarded. The 40-70 ml fraction was run down on a rotary evaporator at 35°C under reduced pressure prior to being transferred to a preweighed graduated flask. Acetone, which was the solvent used to introduce the radiolabelled chlorpropham to growth media, was added to the graduated flask on a weight basis.

A smaller than anticipated, but known volume of acetone, was added and the total amount of chlorpropham in the flask was determined by high pressure liquid chromatography. Once this was known a further addition, again by weight, of acetone was made to give the concentration that was required. Figure 5.II shows a chromatogram of purified ^{14}C-labelled chlorpropham of the 40-70 ml fraction.

The exact chemical purity of the chlorpropham could not be determined due to the difficulties in obtaining a solvent free weight for the total chlorpropham present, in order that the HPLC response could be compared to that of a standard of known purity. However the chromatogram (Figure 5.II) shows no UV absorbing impurities to be present. In the absence of any of the likely reaction product contaminating the alumina column purified chlorpropham, it was concluded that it was of high purity.

Figure 5.I. HPLC chromatogram of the crude reaction mixture. For chromatographic conditions of this normal phase Hypersil column see Section 5.2.

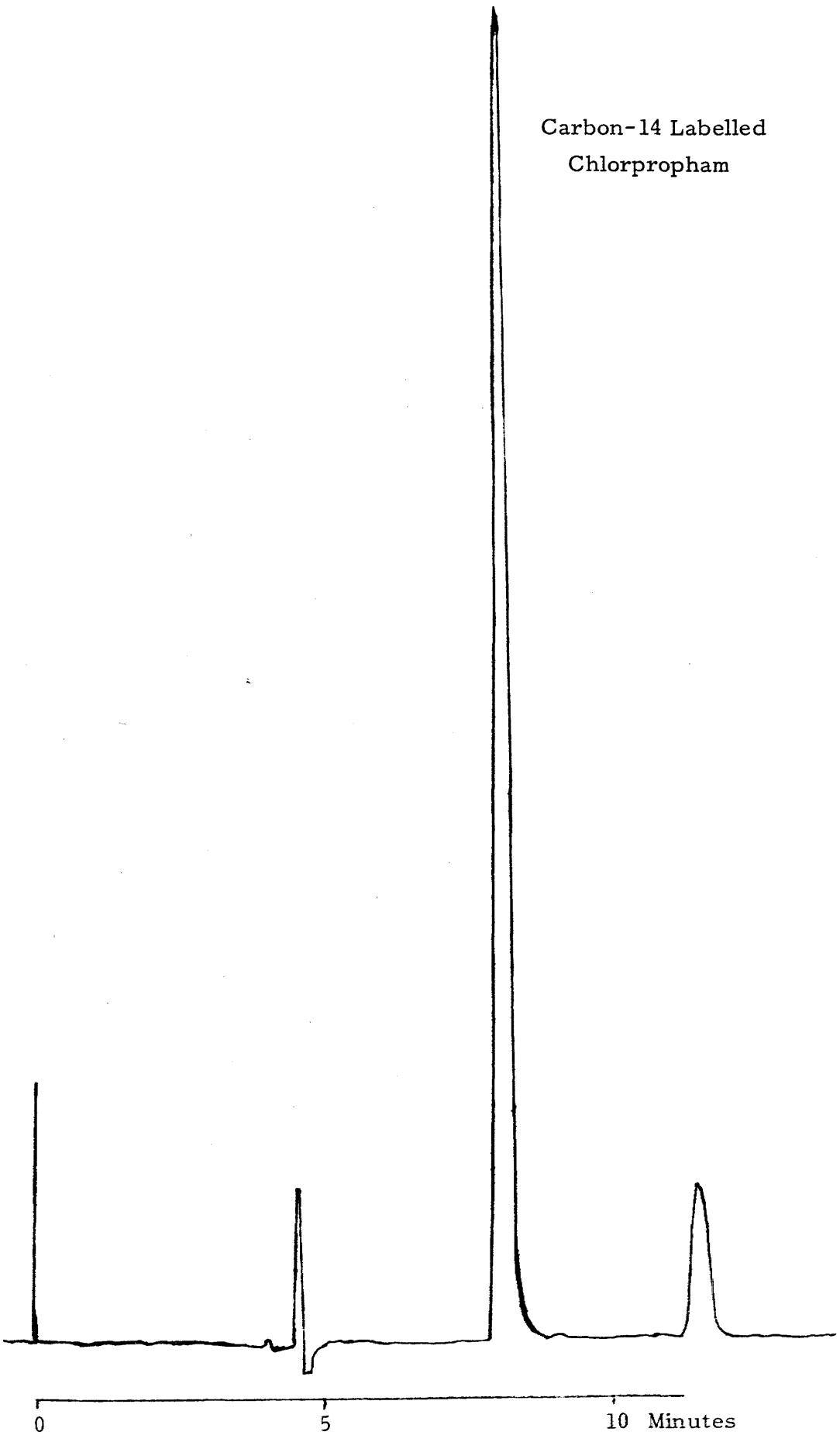
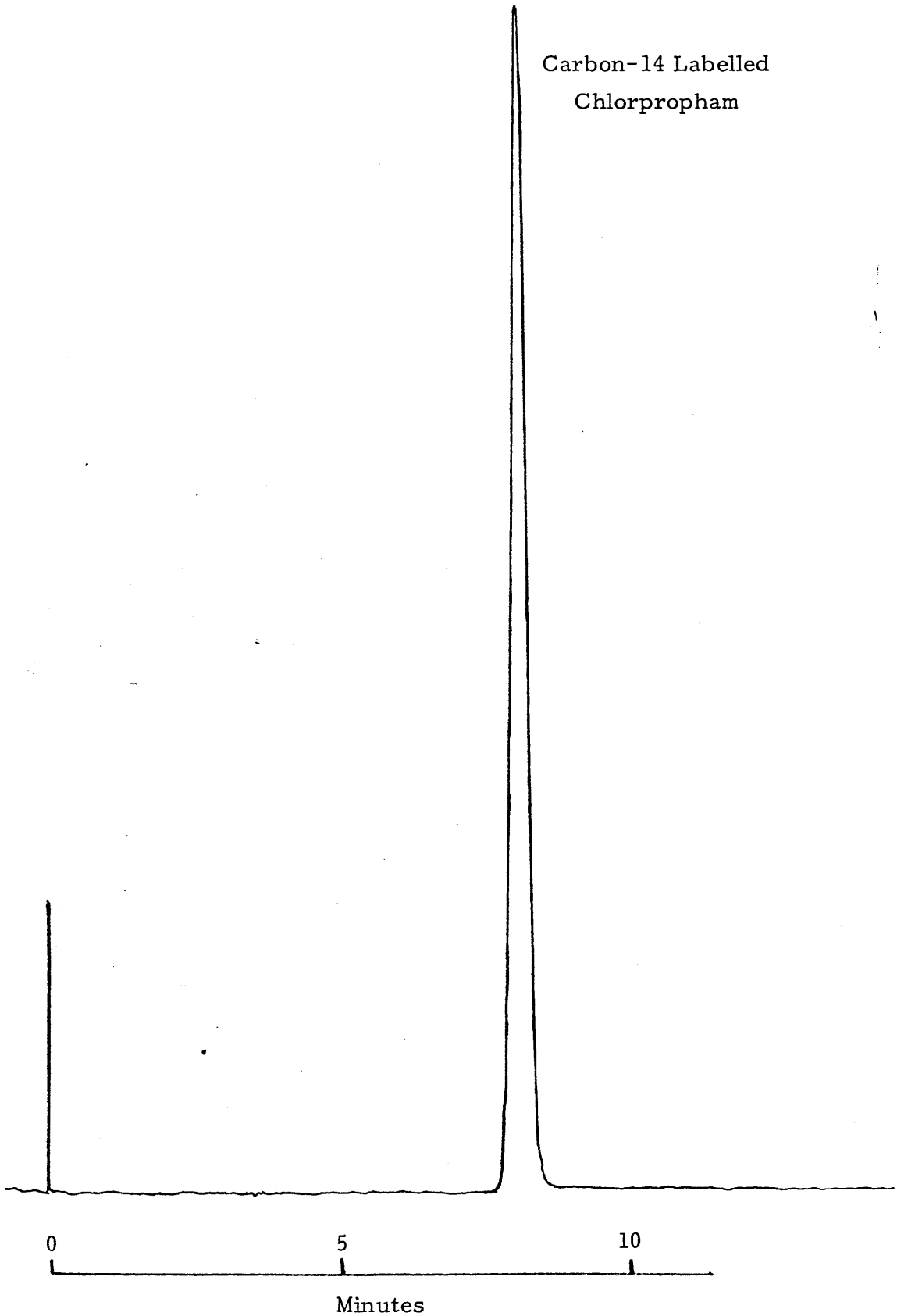


Figure 5.II. HPLC chromatogram of carbon-14 labelled chlorpropham after purification of reaction mixture on an alumina column. For chromatographic conditions on this normal phase Hypersil column see Section 5.2



CHAPTER 6

METABOLISM OF CHLORPROPHAM BY ERWINIA CAROTOVORA VAR. ATROSEPTICA (BACTERIAL SOFT ROT OF POTATOES)

6.1 INTRODUCTION

It could be considered that the scope for biotransformation of chlorpropham in a potato store is limited since metabolic activity is low for most of the time. Respiration of the tubers however continues throughout the storage season with the highest rate being just after harvest when wound healing takes place. During the wound healing period the store temperature is allowed to rise in order to speed up the process. These elevated temperatures are maintained for around 14 days before the cooling system in large modern stores brings the temperature down to 8-10°C for long term storage. If, however, the potatoes are wet, then drying and cooling takes priority over wound healing since the wet conditions when coupled with high temperatures encourages the development of bacterial soft rots.

After wound healing is complete chlorpropham is applied to the tubers, although the point should be made that commercial stores are never free of chlorpropham, as considerable quantities of this volatile chemical have built up in the fabric of the store (Boyd, 1984). Therefore, the bulk of the chlorpropham is applied to the potatoes after this initial high respiration rate of the tubers has declined and at a time when the small amount of soil adhering to the tubers has dried out somewhat.

Although the overall respiration rate in the store is low, the long contact period of the sprout suppressant with biological and non-biological systems in the store may result in significant amounts of transformation. Also there is present an array of pathogenic and saprophytic organisms in and on potato tubers which may metabolise the chlorpropham. The respiring potatoes may also metabolise the chlorpropham though only small amounts penetrate the tuber to greater than 1 or 2 mm (Coxon and Filmer, 1985; Peisker et al., 1972). Actively growing micro-organisms are not a pre-requisite to pesticide transformation since UV photolysis, extra-cellular enzymes, non-enzymic catalysed reactions and purely chemical reactions can all have a role.

One of the most active microbes in a potato store is the causal agent of bacterial soft rot - Erwinia carotovora var. atroseptica. Soft rots are not evenly distributed throughout the store, but often pockets of soft rotting develop and unless this is noticed and dealt with by removal of the tubers in the affected area, large tonnages of potatoes can be destroyed. This chapter describes the work carried out to identify metabolites of radiolabelled chlorpropham when it is incorporated into the growth medium of this bacterium.

6.2 Preliminary Work Involved in the Setting up of E. carotovora Suspension Cultures

All the solvent used in the extraction of cultures was of AR grade, supplied by Hopkins and Williams.

Before carbon-14 labelled chlorpropham could be used, several aspects of the growth of E. carotovora in culture had to be investigated.

It was anticipated that the chlorpropham would be added to the growth medium at a concentration which would not adversely affect growth rates while, at the same time, it should reflect the situation that exists in the store. Ideally the solvent used to apply the chlorpropham to the medium should also have little effect on growth or be the same solvent applied at about the same rate as that used to apply chlorpropham in the stores. The short duration of this study would however require minimal effects of the solvent on the growth rate of the bacterium.

Preliminary experiments using the usual growth medium of E. carotovora, Nutrient Agar (Oxoid, Basingstoke, England) showed that extraction of solidified medium was difficult. Extraction of liquid cultures of E. carotovora was more successful. Increased growth rates, ease of inoculation, an ability to add chlorpropham to cool medium immediately prior to inoculation, as well as even distribution of the chemical throughout the medium, are all advantages over the gel medium.

The initial growth experiments to determine the effect of chlorpropham concentration, and the solvent it was applied in, on the growth of E. carotovora posed two problems. The first of these was that of ensuring the same amount of inoculum was added to each culture flask. The second problem was that of assessment of growth rates. In the case of fungal cultures, such problems do not arise, due to the ease of obtaining uniform inoculum from concentric rings around a radially growing fungus, and growth assessment is merely the measurement of the radial extension of the mycelium.

In order to achieve similar inoculum size, E. carotovora cells were harvested from agar slants which were maintained by the Mycology

Department in the University and distributed throughout a medicine bottle of fresh medium. Often the inoculum was divided between two medicine bottles, with one being sealed and only used in the latter stages of a prolonged inoculation session. The removal of a fixed volume from the medicine bottle ensured similar inoculum size. This was attained by marking batches of Pasteur pipettes at the same level (ca. 0.7 ml) prior to autoclaving in brown paper. Cotton wool plugs pushed into the ends of the pipette eliminated the need to autoclave the rubber teats.

The assessment of growth rate was initially considered more of a problem. A Haemocytometer can be used to count the number of cells in a drop of culture medium but this was considered tedious and of questionable accuracy even for these studies. Nephelometry, which is a measure of the degree of light scattering by cells in suspension, is an alternative and commonly used by microbiologists (Koch, 1970). Standard spectrophotometers (UV/visible) have also been used to assess growth rates of suspension cultures. The use of such an instrument seemed logical and was adopted here since differences in growth rates between flasks could be detected by eye (where essentially the degree of turbidity of light transmittance was being assessed.

In order to calibrate the spectrophotometer (Pye Unicam, Model SP1800), uninoculated medium was scanned and a low optical density (OD) value was found at 570 nm. A series of dilutions of a suspension culture of E. carotovora, which contained a high cell "concentration", from 0% to 100% in fresh uninoculated medium was prepared and the optical density of these determined at 570 nm. When cell "concentration" was plotted against optical density, a straight line relationship was observed

(Figure 6.I) which proved more than adequate for the purposes of assessing growth rates in this work.

The growth of E. carotovora in medium containing chlorpropham across the range of 0 to 100 μg/g was assessed. A concentration of 30 μg/g chlorpropham in the medium resulted in growth rates being reduced by about 25% in the case of aerobic cultures and 35% in the case of anaerobic cultures when compared to acetone controls. This concentration is also one which is likely to be encountered in a potato store where 20 g/tonne is a common application rate. The use of acetone as the solvent was convenient since, like methanol, it can be used for maintaining aseptic conditions and therefore special sterilisation procedures were not required for the stock solutions used to add chlorpropham to the growth medium.

Erwinia carotovora var. atroseptica is a facultative anaerobe and it is likely that in a rapidly decaying pocket of potatoes, anaerobic conditions would prevail. Anaerobic growth conditions were also used in a set of E. carotovora cultures. These produced similar growth rates to the aerobic cultures when measured by the spectrophotometric method. The anaerobic flasks consisted of 500 ml conical flasks with B24 ground glass stoppers fitted instead of non-absorbent cotton wool in the case of the aerobic flasks. All the flasks containing medium were autoclaved with non-absorbent cotton wool stoppers to allow expansion of the flask contents. The stoppers were autoclaved separately and a little silicon grease on the stoppers ensured a good seal after inoculation. The glass stoppers had to be sealed and held down with cellotape after inoculation to prevent them blowing off as a result of gas pressure building up in the

flask during the growth of the anerobic cultures. Anaerobic conditions would only build up in the flask as the oxygen was consumed and may, it was thought, result in a different degradative pathway for the chlorpropham.

Later in the course of this work a further set of flasks, which had been flushed with nitrogen to ensure anaerobic conditions from the start of growth, were also used. To facilitate nitrogen flushing, modified Dreschnel bottle heads were used to replace the glass stoppers on the flasks. These Dreschnel bottle heads had ground glass taps and B19 cones fitted to both the inlet and outlet of the head. Sterilised air condensers packed with non-absorbent cotton wool were then fitted to both the inlets and outlets and nitrogen was passed through the flask for 2 minutes at a flow rate of approximately 5 ℓ/minute. The growth rates of the nitrogen flushed cultures were extremely slow, taking about 4 times as long to produce a similar amount of growth as the aerobic cultures or the anaerobic cultures which merely had glass stoppers fitted to the flask.

Since chlorpropham has an appreciable vapour pressure 10^{-5} - 10^{-6} mm Hg (Alexandrova and Klisenko, 1982), losses from the aerobic culture flask are possible. While loss of unlabelled chlorpropham is of little concern, contamination of the surrounding areas with radio-labelled chlorpropham had to be minimised. With this in mind, the loss of chlorpropham from water solutions was investigated. 500 ml cotton wool stoppered flasks, to which had been added by weight 90 ml of a 30 μg/ml chlorpropham solution, were placed on an orbital shaker at a temperature of 30°C and speed of 90 revolutions per minute (RPM). At various time intervals from 0 to 19 days, duplicate flasks were reweighed and the chlorpropham concentration in the water determined by reverse

phase high pressure liquid chromatography (for conditions see Section 4.1). The results showed that after an initial drop in the chlorpropham concentration at Day 4, the rate of chlorpropham loss and water loss were similar so that after 19 days there had been a water loss of 8% and a chlorpropham loss of 9%. Clearly, losses of this amount of a radiolabelled compound could not be tolerated.

Polyurethane plugs have been used to trap organic vapours and since chlorpropham is structurally related to urethane, efficient capture and retention could be expected (Kearney and Kontson, 1976).

In order to assess the ability of a polyurethane trap to retain any volatilised chlorpropham, use was made of the modified Dreschnel bottle heads described above. The inlet and outlet of the bottle heads were fitted with polyurethane packed B19 air condenser. A piece of low density polyurethane "foam" (40 mm x 100 mm x 25 mm) was packed between two plugs of non-absorbent cotton wool. 90 ml of water containing 30 μg/ml chlorpropham was placed in flasks fitted with the polyurethane traps and air was pumped through at a flow rate of about 400 ml/minute for a period of 72 hours while the flasks were maintained at a temperature of 20°C. The airstream was directed through the inlet air condenser and down the glass tube inside the flask which terminated about 1 cm above the water level. The air, together with the chlorpropham vapour, was exhausted through the second condenser. The object of the first condenser was to remove impurities in the air which, if not removed by the first polyurethane trap, might have contaminated the chlorpropham trap on the exhaust.

To remove the material retained by the trap, a B19 Quickfit conical flask with suction side arm, was used to apply a slight negative pressure to the polyurethane plug and 100 ml of methanol was drawn through the condenser and reduced in volume to 10 ml by rotary evaporation. The concentration of chlorpropham in the methanol extract was determined by high pressure liquid chromatography, using the same conditions as above (see Section 4.1).

Again, the water loss and the chlorpropham loss from the flask was comparable. The average water loss for the two replicates was 21% while the average chlorpropham loss was 18%. The duplicate polyurethane traps recovered 80% and 85% of the chlorpropham lost from their respective flasks. These traps, therefore, while capable of preventing most of the loss of chlorpropham vapour from the flask, could not recover all of the chlorpropham vapour in this force draught system.

Vapour traps similar to those above were used on aerobic culture flasks containing carbon-14 labelled chlorpropham, although the condenser had to be shortened to fit into the orbital shaker. The short growth period of the E. carotovora cultures combined with the ability of the vapour trap to retain most of the chlorpropham should result in low losses of radiolabelled chlorpropham.

The rate of metabolism of chlorpropham by E. carotovora cultures was determined using unlabelled chlorpropham with the flasks being extracted after a two day growth period. The method of extraction used was the modified Bligh/Dyer procedure of Still and Mansager (1973a). This involved the addition of methanol and chloroform to give a methanol: chloroform:water ratio of 2:1:0.8 with the culture medium supplying the

water. This one-phase system was initially used to extract the cultures and break open the cells before the solvent ratio was altered to 2:2:1.8. The resultant two-phase system has about 99% of the unaltered chlorpropham in the chloroform phase. The methanol/water phase contains very little chlorpropham but hydroxylated metabolites of chlorpropham have been shown to partition into the aqueous phase (Still and Mansager, 1973a).

Due to the difficulty involved in removing water from the aqeuous phase, no additional water was added to the E. carotovora cultures. The required methanol was added to the culture media after the growth period had elapsed and the chloroform was added in lots with shaking, so as to retain the advantages of a one-phase extraction system until a point was reached when the required volume of chloroform had been added and the phases separated. Occasionally, a few extra ml of chloroform were added as this facilitated the separation of the phases. Complete cell disintegration was assumed, due to the complete solvation of the culture in the one-phase extract.

When preliminary studies were carried out using unlabelled chlorpropham, it was impossible to say if the metabolism of this agrochemical took place in the culture flask due to the difficulty of adding an exact volume of chlorpropham solution under aseptic conditions, thus resulting in a large variation in the apparent recovery of the chemical. The extraction procedure itself was not at fault since extraction of spiked mature E. carotovora cultures proved thorough ($> 99\%$). The accuracy of such extractions however could not be repeated under aseptic conditions since these recovery studies involved a larger addition of

chlorpropham solution (2 ml of acetone) to a correspondingly larger amount of growth medium, and were carried out where accuracy was the main criterion of the operation, rather than a combination of accuracy and good sterile technique. The use of the same stock solution of chlorpropham in the quantification of the chlorpropham concentration in the extracts increased the accuracy of the recovery determination.

With the use of radiolabelled material, however, the calculated recoveries themselves are only of small importance, provided of course that no actual loss of the radiolabel to the surroundings is allowed. The distribution of the label within the system is the interesting aspect. A simplified detection system (radioactivity monitoring) allows quantification of the amount of radioisotope present as a first step followed by a second step of the identification of the compounds in which it appears. This is invaluable, not only in determining the origin of a particular compound, but it can greatly speed up the more routine traditional steps of an analysis.

6.3.1 Metabolism of Carbon-14 Labelled Chlorpropham by Erwinia carotovora var. atroseptica

The first study using growth medium incorporating radiolabelled chlorpropham was conducted under aerobic and anaerobic conditions. 500 ml conical flasks with B24 glass stoppers were used to allow anaerobic conditions to build up in the flasks. In the case of the aerobic flask, polyurethane packed air condensers were used to stopper the flasks.

The growth medium was a standard bacterial nutrient medium and each litre of this contained 1g beef extract (Oxoid, Basingstoke,

England), 2g yeast extract (Oxoid), 5g peptone (Difco Laboratories, Detroit) and 5g sodium chloride (AR grade, Hopkins and Williams), and the pH was adjusted with dilute sodium hydroxide to at least 7.4 (ideally between 7.5-7.6). 90 ml of this solution was added to each flask and cotton wool stoppers were fitted prior to sterilisation by autoclaving for the standard time of 20 minutes at 121°C.

The carbon-14 labelled chlorpropham was prepared as described in Section 5.3. 2 ml of the crude reaction mixture was purified to give 5.15 mg/ml chlorpropham in 6.4 ml acetone. 0.5 ml of this was pipetted into the culture flasks under aseptic conditions. The method of inoculation used was that described in Section 6.2. For the same reasons as cotton wool filters were placed in the inoculating Pasteur pipettes, graduated pipettes were fitted with these also since the pumpettes were not sterilised. Care had to be taken not to pack the filter too tightly as condensation on the filter had to be avoided so as not to create difficulties in drawing up exact quantities of material into the pipette. Condensation can be minimised by using brown paper in preference to aluminium foil to wrap utensils for autoclaving.

0.5 ml of the radiolabelled chlorpropham solution was incorporated into duplicate flasks of aerobic and anaerobic cultures. 0.5 ml of acetone was added to a further four flasks (2 aerobic, 2 anaerobic) and 2.5 mg of unlabelled chlorpropham in 0.5 ml of acetone was added to duplicate flasks of aerobic and anaerobic cultures. The twelve flasks were placed on an orbital shaker (Gallenkamp) for 40 hours. The shaker was run at 90 RPM and a temperature of 22°C.

6.3.2 Extraction of the Culture Medium

After a growth period of 40 hours, glass stoppers were used to replace the polyurethane packed air condensers, which were then sealed with "Parafilm" at the top and a 50 ml round bottom (RB) flask at the base. These traps were extracted some time later by placing on a B24 Quickfit conical flask with suction side arm and pulling through 100 ml of methanol which was run down to less than 5 ml and quantitatively transferred with the aid of methanol washings to a 5 ml volumetric flask. The activity of three lots of 1 ml was determined by placing in 15 ml of Dimilume (Packard Instruments Inc.) and counting on a Phillips Model PW 4510 liquid scintillation counter (LSC) for the usual period of 20 minutes. Occasionally very short counting times were used to obtain some idea of the activity in samples prior to the batch being counted for the full 20 minutes. The quantity of chlorpropham retained by the polyurethane trap is shown in Table 6A.

Three 0.5g aliquots of culture medium were removed and placed in a LSC vial containing 1 ml of methanol then 13 ml of Dimilume scintillation fluid and 1 ml of Soluene 350 (Packard Instruments Inc.) solubiliser was added. Soluene is a solution of a 0.5N quaternary ammonium hydroxide in toluene which is added to the xylene based Dimilume in order to maintain aqueous samples in solution. The activity was then determined on the LSC and this figure appears in Table 6A and represents the total activity present in the flask.

The aniline concentration in the acetone controls and the flasks containing unlabelled chlorpropham was determined using the Bratton-Marshall procedure, as described by Brocklesby and Moggleton (1973).

This involved taking 2g aliquots directly from the medium of the flasks and adding to a boiling tube containing 0.5 ml of 6N HCℓ. 0.5 ml of a 0.5% sodium nitrite solution was added and left to stand for 10 minutes with occasional swirling. 0.5 ml of a 5% ammonium sulphamate solution was then added to remove the excess nitrite reagent. 10 minutes later 0.5 ml of a 0.2% solution of N-(1-naphthyl)ethylene diamine dihydrochloride was added to couple with any diazonium salts that were produced by the reaction of the nitrite with any aromatic amines which were present in the culture medium. Using this method, concentrations as low as the equivalent of 0.1 μg 3-chloroaniline in 1 ml of culture medium could be determined. No aniline was found in any of the culture media analysed.

60 ml of methanol was added on a fresh weight basis to all the flasks, which had been previously weighed. This effectively killed off the cultures and prevented other micro-organisms from colonising the flask. The culture media were then extracted using the same modified Bligh/Dyer solvent system of Still and Mansager (1973a), outlined in Section 6.2. This involved transferring the contents of the culture flask to a 1L separating funnel and the balance of the methanol required for the extraction of the known volume of culture material was calculated and added to the separating funnel via the culture flask to wash out the remainder of the culture medium. The required weight of chloroform was added in stages to the separating funnel, again via the culture flasks. Towards the end of the series of chloroform additions, the two phases could be seen to separate. Experience determined whether it was best to add all the chloroform. Sometimes the last few ml were not added to the separating funnel since rapid separation of the phases occurred without this, but

occasionally a further 2-3 ml of chloroform over and above the allotted quantity had to be added to speed the separation.

The chloroform layer was drawn off into an RB flask and reduced to 2-3 ml on a rotary evaporator at reduced pressure and at a temperature not exceeding 35°C. No drying of the chloroform was necessary. This 2-3 ml was then quantitatively transferred with the aid of chloroform washings to a 10 ml volumetric flask. The aqueous phase was drawn off and 10-20 ml of methanol was washed down through the separating funnel to remove traces of remaining extract and bulked with the aqueous phase. Again, rotary evaporation was used to reduce the volume to 100 ml. The radioactivity present in both phases from radiolabelled and unlabelled cultures was then assessed. All activity determinations were done in triplicate. 0.5 ml (by weight) from the 10 ml chloroform flasks was added to 15 ml of Dimilume. 0.5 ml (by weight) of the 100 ml aqueous phase was added to LSC vials containing 15 ml of Dililume and 1 ml Soluene. The activity in these vials was then determined. Unfortunately, the quenching of the chloroform extract of inoculated flasks was severe and could not be accounted for solely by the quenching action of the chloroform. Due to the high activity of these samples, it was possible to reduce the volume of sample added to the LSC vials. When 25 µl of chloroform extract was used, the quenching was well within the correction range of the LSC. The results of the activity determinations are given in Table 6A.

From these results and, in particular, the Bligh/Dyer distribution ratio of the radiolabel between the phases, it was initially thought that some dechlorination of the chlorpropham had occurred to produce

propham. To investigate this possibility but also to determine the distribution of the radiolabel in the chloroform extract, 25 µl aliquots of the chloroform extract were chromatographed by high pressure liquid chromatography under conditions given in Section 4.1, with the eluant being collected in LSC vials. Collection of the eluant at the retention time of propham was also made along with fractions making up the entire eluant from the chromatographic run. Figure 6.II shows a chromatogram of a sample from the chloroform of the first Bligh/Dyer separation of the medium. However, none of the fractions, other than the chlorpropham peak, showed activity greater than that of the background and therefore propham was not a metabolite of chlorpropham under these culture conditions. Although there was some reduction in sample activity (see Table 6A) after chromatographing the chloroform extract on the high pressure liquid chromatograph, this is not likely to be due to the label being present in a polar compound retained by the column since this would have partitioned into the polar phase of the Bligh/Dyer separation.

A second chloroform partition of the 100 ml of aqueous phase was carried out and, as can be seen from Table 6A, nearly all the activity was extracted into the chloroform phase. This would substantiate the conclusion that only chlorpropham contained the radiolabel.

To complete the E. carotovora study, two flasks, fitted with the modified Dreschnel bottle heads, were used to culture E. carotovora on a medium that incorporated radiolabelled chlorpropham. No unlabelled chlorpropham or acetone controls were considered necessary. These cultures were set up and extracted in the same way as described above

for the aerobic and anaerobic label incorporated cultures, the only difference being that the flasks were flushed with nitrogen, as described in Section 6.2 above, and the period allowed for growth was extended from 40 hours to 136 hours on account of the fact that nitrogen flushing of the culture flasks reduces the growth rate.

The flasks were placed in a Gallenkamp orbital shaker but the overall height of the flasks did not allow the lid to be closed. The shaker settings were the same but fine control of the temperature could not be expected. Although the room temperature was thermostatically controlled, the temperature fluctuated between 20-24°C.

Aniline analysis was carried out using the same procedure as that used for the unlabelled chlorpropham incorporated medium. As was the case in the aerobic and glass stoppered anaerobic flasks, none was detected at the end of the 136 hour growth period. The cultures were extracted and the radioactivity determined in the same way as the glass stoppered flasks above and the results are given in Table 6B.

These results are essentially the same as the other two growth condition treatments and show that in pure cultures, Erwinia carotovora var. atroseptica does not metabolise chlorpropham to any perceivable extent. The significance and relevance of the results obtained from the E. carotovora metabolism studies are discussed more fully in Chapter 9.

TABLE 6A. DISTRIBUTION OF LABELLED CHLORPROPHAM IN THE AEROBIC AND ANAEROBIC (GLASS STOPPERED) ERWINIA CAROTOVORA CULTURES

	TOTAL ACTIVITY PRESENT IN MEDIUM	% OF TOTAL ACTIVITY APPEARING IN CHLOROFORM PHASE OF FIRST BLIGH/ DYER SEPARATION	% OF TOTAL ACTIVITY IN* FLASKS WHICH APPEARS IN CHLORPROPHAM PEAK	% OF TOTAL ACTIVITY IN MEDIUM APPEARING IN FIRST METHANOL/ WATER PHASE	% OF TOTAL ACTIVITY IN CHLOROFORM PHASE OF SECOND BLIGH/ DYER SEPARATION	AMOUNT (µg) EXTRACTED FROM POLYURETHANE TRAP
AEROBIC REPLICATE I	100% (1.76×10^6 dpm)	100.6	94.6	2.36	2.72	10.5 µg
REPLICATE II	100% (1.64×10^6 dpm)	99.1	94.7	1.65	2.69	18.3 µg
ANAEROBIC (GLASS STOPPERED) REPLICATE I	100% (1.78×10^6 dpm)	98.3	95.1	2.03	2.47	N.A.
REPLICATE II	100% (1.58×10^6 dpm)	99.7	95.9	1.77	2.16	N.A.
ALL OTHER FLASKS CONTAINING UNLABELLED MATERIAL		ACTIVITY NOT SIGNIFICANTLY DIFFERENT FROM BACKGROUND				

* Corrected for recovery of 99.1% from the HPLC when equipped with Hypersil column (see Section 4.3)

N.A. Not applicable

TABLE 6B. DISTRIBUTION OF LABELLED CHLORPROPHAM IN NITROGEN FLUSHED ANAEROBIC CULTURES OF ERWINIA CAROTOVORA

	TOTAL ACTIVITY PRESENT IN MEDIUM	% OF TOTAL ACTIVITY APPEARING IN CHLOROFORM PHASE OF FIRST BLIGH/ DYER SEPARATION	% OF TOTAL* ACTIVITY IN FLASKS WHICH APPEARS IN CHLORPROPHAM PEAK	% OF TOTAL ACTIVITY IN MEDIUM APPEARING IN FIRST METHANOL/ WATER PHASE	% OF TOTAL ACTIVITY IN CHLOROFORM PHASE OF SECOND BLIGH/ DYER SEPARATION
NITROGEN FLUSHED FLASK REPLICATE I	100% (1.82×10^6 dpm)	97.1	96.1	1.87	1.89
REPLICATE II	100% (1.74×10^6 dpm)	102.2	96.8	2.28	2.52

* Corrected for recovery of 99.1% from the HPLC when equipped with Hypersil column (see Section 4.3)

Figure 6.I. Relative cell "concentration" of Erwinia carotovara var. atroseptica cells versus optical density.

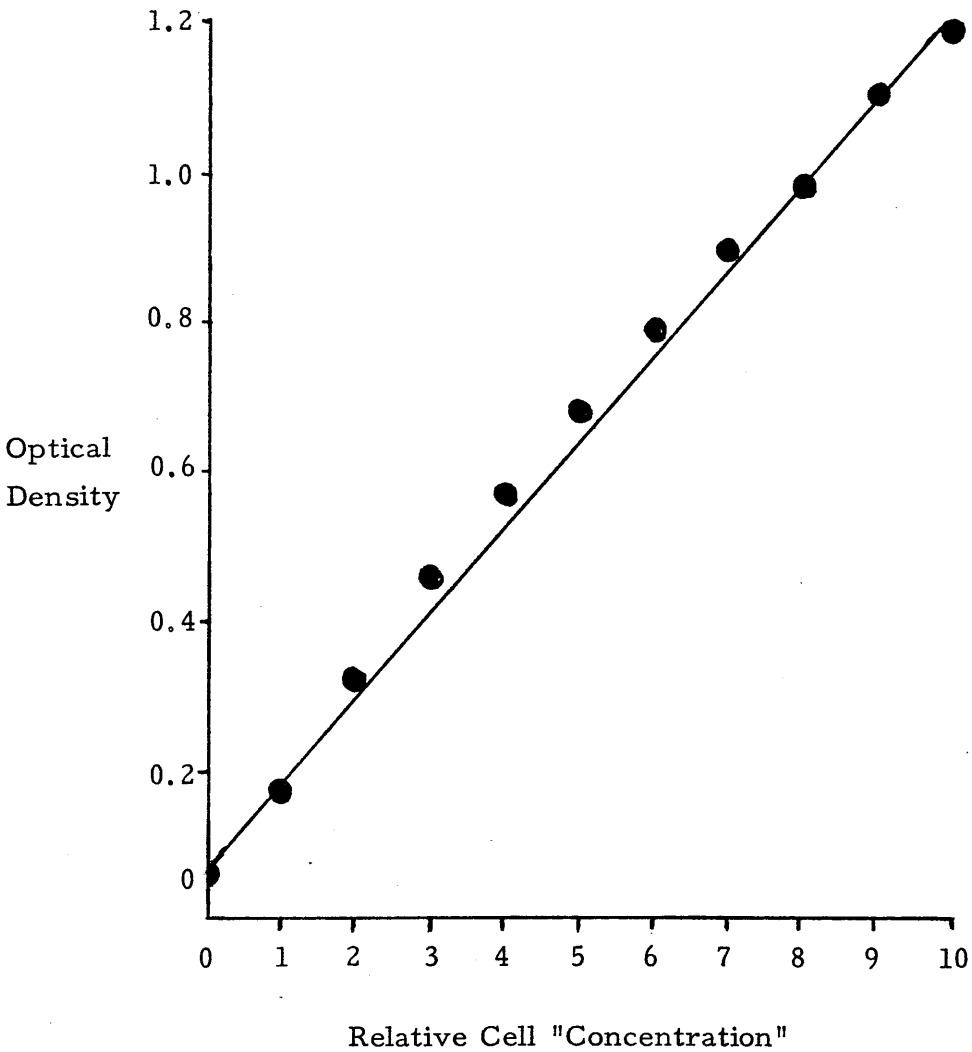
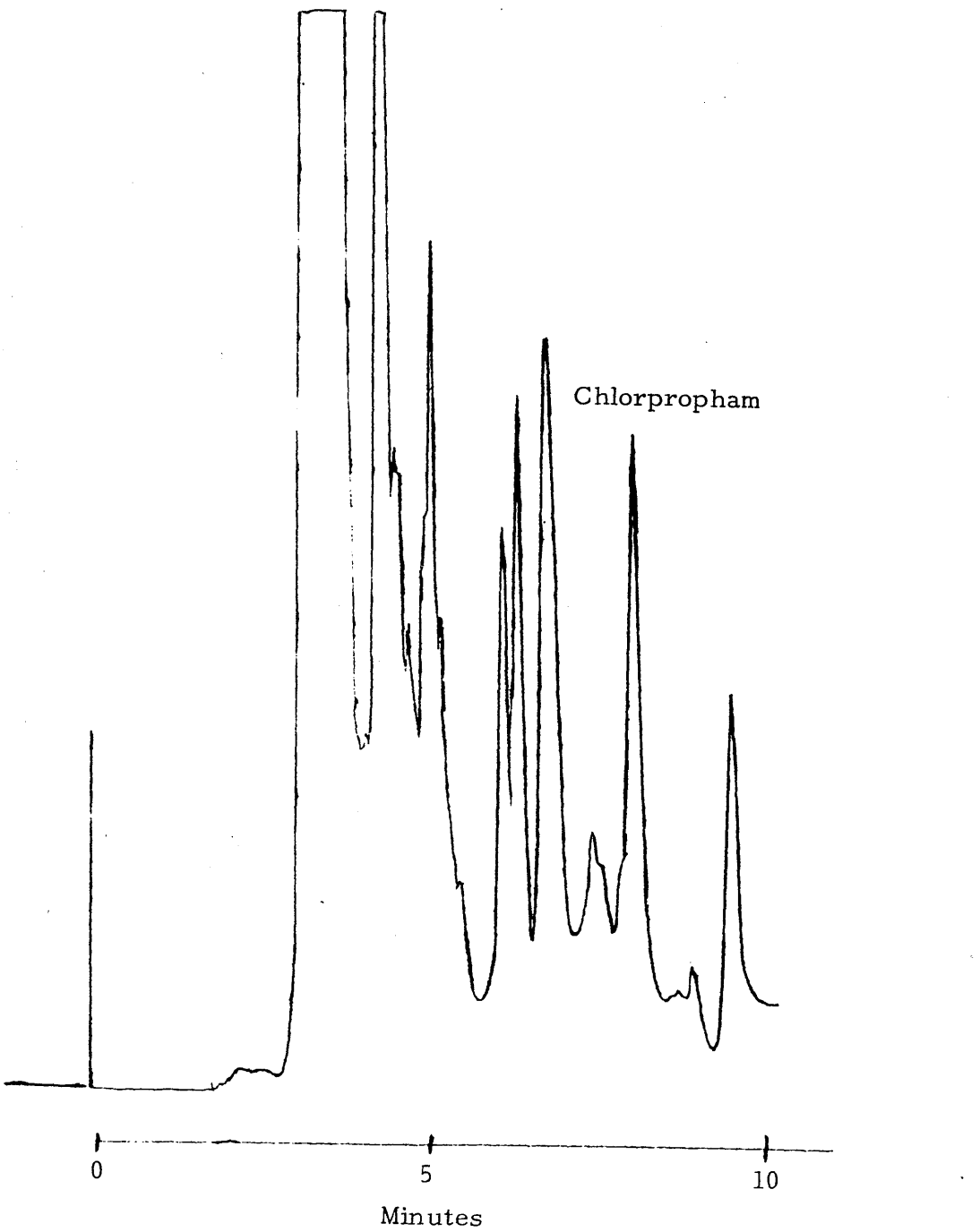


Figure 6.II. HPLC chromatogram of chloroform phase of the Bligh/Dyer separation of Erwinia carotovora var. atroseptica culture medium. Normal phase column (Hypersil).
For full details of chromatographic conditions see Section 4.1.



CHAPTER 7

METABOLISM OF CHLORPROPHAM BY POLYSCYTALUM PUSTULANS (SKIN SPOT)

7.1 INTRODUCTION

During the long term storage of potatoes disease levels have to be carefully monitored. The spread of some diseases is rapid and can lead to total destruction of the stored crop.

During the storage of potatoes deterioration of the tubers can take many forms. Bacterial soft rots can cause rapid destruction of potatoes. The speed at which rots develop and spread requires store managers to be alert when conditions that are favoured by the causal agent are maintained in the store for significant periods of time. The presence of blight (Phytophthora infestans) in and on the tubers can result in a complete loss of the stored material despite a slow development and spread rate although modern field applied fungicides have all but eliminated this disease in tubers.

Deterioration of a stored crop is not confined to complete loss of individual or even pockets of tubers. In the case of potatoes used by the processing industry for high temperature frying, high concentrations of reducing sugars in the tuber would be considered as deterioration in the quality of stored stocks since this results in a loss of brightness of the product through browning reactions which occur during the frying process. Surface blemishes that develop as a result of mechanical damage

or insect or fungal attack often cause economic loss to the processor through greater peeling losses and increased hand trimming costs.

Skin spot is a disease which develops only slowly on the surface layers of stored potatoes and usually is of little significance when the necrotic region remains within 1 mm of the surface of the tubers. However deep forms of the disease often result in damage to the flesh of the tuber to depths of up to 5 mm after a prolonged storage season. The development of this deep form has been associated with chlorpropham applications and some mechanism of impaired wound healing as a result of chlorpropham application would appear feasible (French, 1976). Skin spot is caused by the fungus Polyscytalum pustulans. This chapter describes work done to determine if chlorpropham is metabolised by this potato pathogen.

7.2.1 Preliminary Work on the Culture of Polyscytalum pustulans

In order for a study on the metabolism of chlorpropham by P. pustulans to proceed the culture conditions should satisfy the same criteria as those in the case of E. carotovora. The medium should contain chlorpropham at a high concentration. The addition of the radiolabel to the medium must be straightforward and without loss and the extraction of the chlorpropham as well as any metabolites should be complete.

Initially the growth of P. pustulans on plated medium containing various chlorpropham concentrations was observed. The medium consisted of 2% malt extract (Oxoid, Basingstoke, England) and 0.8% agar (No. 3, Oxoid). Chlorpropham in acetone was added to the hot medium prior to the pouring of the plates. Discs of inoculum were taken from a concentric ring of a P. pustulans culture grown on plated medium.

The assessment of growth was only complicated by the fact that P. pustulans does not always grow perfectly radially and that sporulation of the fungus results in the development of other colonies in the Petri dish. In large cultures sporulation is often advantageous as it allows more rapid mycelial development than growth which only emanates from the point of inoculation.

To obtain liquid growth medium merely involved the omission of the agar constituent of the plate medium and this allowed chlorpropham to be added to cool medium thereby lessening the concern over having to add radiolabelled chlorpropham to warm medium. Liquid medium was poured into Petri dishes to a depth of 3-4 mm, and the inoculum used for these dishes was drawn from a deep plate culture so that the inoculum would stand above the liquid medium surface, at least initially. The submerging of the inoculum however had little effect on subsequent growth. When the inoculum disc did not remain in the centre of the Petri dish problems in estimating growth resulted.

The growth of P. pustulans in both liquid and gel medium was similar for equivalent treatments. In the case of chlorpropham incorporated medium at the concentration of 25 μg/ml both sets of cultures showed a decrease in growth rate of just under 30% in comparison to acetone controls. The cultures grew slowly with control treatment taking 20 days at a temperature of 15°C to achieve a 75 mm diameter mycelium. The mycelium in the liquid cultures was of variable depth with some areas growing only on the surface of the medium and other areas where growth extended to a depth of 3-4 mm as well as a layer above the surface of the medium with the greater amount of growth seeming to have occurred as

a result of the medium flooding over the mycelium when the Petri dishes were disturbed in the incubator. This "reinoculation" is a widely used technique and was used with large scale fungal cultures to obtain greater amounts of mycelial growth per flask. Recolonisation of the surface layers was more rapid after reinoculation than the initial inoculation, presumably due to the larger inoculum present.

For large scale cultures two litre Roux bottles were used. These are similar to very large medicine bottles with the neck displaced to one side so that they could be stored flat without the medium spilling out. The necks were stoppered with a cheesecloth and cotton wool wrapped piece of glass tubing and therefore did not allow the polyurethane traps from the E. carotovora experiment to be used. Some loss of radiolabelled chlorpropham was expected but these cultures had to be grown in the Mycology Department "walk-in" growth room which had a ventilation system that exhausted directly to the outside of the building. Experience had shown however that the chlorpropham losses would not be great and the low specific activity of the available carbon-14 labelled chlorpropham would result in only relatively low total amounts of radioactivity in the culture flasks.

7.3.1 Large Scale Cultures of P. pustulans using Unlabelled Chlorpropham

Four 150 ml lots of growth medium containing 2% malt extract were placed in two litre Roux bottles and autoclaved for the standard time of 20 minutes at 121°C. 0.5 ml of 7 mg/ml chlorpropham in acetone was added to each flask. Two of the four Roux bottles were not inoculated

and these served as controls to indicate the likely loss of chlorpropham over the duration of the experiment. Each of the remaining two Roux bottles were inoculated. A 5 mm cork borer that had been dipped in methanol and flame sterilised was used to cut discs from a concentric ring mid-way between the centre and the edge of the P. pustulans mycelium growing on deep plated medium. This area of the culture was used as it was thought to be more likely to be at a growth stage involving sporulation. By cutting the discs out while the cork borer was still hot meant that the discs were completely freed from the surrounding agar and this allowed the discs to be lifted out by placing a scalpel into the side of the column of agar and lifting free. Four discs were then placed upright in the Roux bottle and two of these were pushed to the far end of the culture flask with a glass rod. This was an awkward and lengthy inoculation procedure and had to be undertaken with great care to avoid contamination of the culture flask.

After completion of the inoculation and incorporation of the chlorpropham into the medium, the flasks were placed in the growth room at 15°C. After three weeks the fungal mycelium covered the surface of the growth medium having spread out from the inoculum discs and from other colonies arising either from spores or from fragments of discs that had dislodged during the inoculation procedure.

The flasks were reinoculated twice during the growth period by allowing fresh medium to run over the surface of the mycelium. This resulted in the submerging of around 90% of the mycelium. Recolonisation of the newly exposed surface was relatively rapid. After a total of 41 days the culture media were extracted.

7.3.2.1 Extraction Procedure

The cotton wool stoppers were removed and placed in an eight ounce screw capped jar to be extracted at a later date (see Section 7.3.2.3 below). The culture medium together with the mycelium which had to be pulled through the neck of the Roux bottle with forceps was filtered through a Whatman No. 1 filter paper in a Büchner assembly under slight suction.

The filtrate in the Büchner was weighed and duplicate two gram samples of filtrate were placed in boiling tubes prior to the carrying out of the Bratton-Marshall determination for anilines as described in Section 6.3.2. of the E. carotovora study.

In the case of the extraction of the unlabelled material the entire mycelium and culture medium weight was determined and after deduction of an arbitrary one gram (for the dry weight of the mycelium) from the total, the volume of water could be obtained. The mycelium and filter paper were washed with a volume of methanol corresponding to 80% of that required for the Bligh/Dyer separation with the methanol being first used to wash the culture flask. The filter paper and mycelium were then transferred to a Soxhlet thimble for extraction (see Section 7.3.2.2 below). The filtrate was then transferred to a 1L separating funnel and the remainder of the methanol added. The required volume of chloroform was added in stages with shaking until some signs of separation of the phases was observed. Additional chloroform had to be added to separate the phases and even with this, separation was slow. By not accurately determining the amount of water washed out of the mycelium by the methanol washings and that which was transferred to the Soxhlet thimble along with

the mycelium, the exact quantities of chloroform and methanol required could not be accurately predicted.

The chloroform was run off and since it did not require drying was then reduced to 10 ml by rotary evaporation under reduced pressure and at a temperature not exceeding 35°C. The aqueous phase was run off and a few ml of methanol was used to wash the sides of the separating funnel and this was evaporated until condensate ceased to appear on the condenser of the rotary evaporator. The total weight was noted. Three lots of 0.5 g of this residue was then transferred to liquid scintillation counter (LSC) vials containing 12 ml of Dimilume and 1 ml Soluene which were then counted. Both unlabelled chlorpropham incorporated medium and carbon-14 labelled medium extracts were counted. The inclusion of extracts from radiolabel-free medium assessed the background level of radiation picked up by the LSC and checked that phosphorescence or chemiluminescence was not contributing to the activity detected by LSC. After the removal of the 1.5g for radioactivity determination the remainder was returned to the separating funnel and the methanol and chloroform replenished. Again the chloroform was drawn off and reduced to less than 10 ml by rotary evaporation and transferred quantitatively to a 10 ml volumetric flask. The aqueous phase was also run down until the point where no condensate formed on the rotary evaporator condenser and was then transferred to a preweighed volumetric flask to determine the total amount present.

7.3.2.2 Soxhlet Extraction

The filter paper and mycelium or alternatively the filter paper and any particulate material (in the case of the uninoculated flasks) was placed in a cellulose 30 mm x 80 mm Soxhlet thimble which was plugged with cotton wool. 100 ml of methanol was then added and the extraction was carried out during two 12 hour periods. Following this period the methanol that would drain from the thimble together with that in the lower reflux flask was combined and concentrated to 10 ml on a rotary evaporator. 70 ml of water was then added and a further 12 hour extraction was made. This was then drained and its weight noted. The Soxhlet thimble was placed in an eight ounce screw capped jar and retained.

7.3.2.3 Extraction of Cotton Wool and Cheesecloth Stopper

The Roux bottle stopper was extracted with 100 ml of methanol using a conical flask with suction side arm and a filter funnel. The glass tubing insert was held against the base of the funnel and about 50 ml of methanol was pulled through the core. The outside of the tube, which was wrapped in cheesecloth and a little cotton wool, had methanol poured around it and this was allowed to drain into the conical flask. The methanol was evaporated on a rotary evaporator and transferred quantitatively to a 5 ml volumetric flask.

7.3.2.4 Quantification of Chlorpropham in each Fraction

The main purpose of the extraction of unlabelled chlorproham from the growth media was to check out the extraction method and to provide some background information which would be of use when carbon-14

labelled chlorpropham was used. The determination of the concentration of chlorpropham in extracts was performed using high pressure liquid chromatography. In the case of the chloroform extracts a normal phase Hypersil column operated under the conditions given in Section 4.2 was used. Reverse phase chromatography using an ODS Hypersil column (for conditions see Section 4.2) was carried out on samples of the methanol and methanol/water extracts.

7.3.2.5 Results

The aniline determination showed that no aniline could be detected in the filtrate of the culture media.

The quantities of chlorpropham appearing in each fraction after correction for the quantities previously removed for analyses are shown in Table 7A (i.e. the figures represent the total amount that would have been present had no subsamples been removed for analyses). The figures give the percentage of the total which was added into the flask which appears in a particular fraction.

Recoveries from the uninoculated flasks show that little could have been lost from the flasks. In the case of the inoculated flasks the amount of chlorpropham lost and/or metabolised and/or unextracted is significant. The methanol Soxhlet extraction removed significant quantities of chlorpropham from the mycelium but when water/methanol was used as the extracting solvent very little additional chlorpropham was extracted.

The distribution of chlorpropham between the organic and aqueous phases in the Bligh/Dyer separation in the inoculated treatments

TABLE 7A. % OF UNLABELLED CHLORPROPHAM INITIALLY ADDED (3500 µg) TO CULTURE APPEARING IN EACH FRACTION (CORRECTED FOR SUBSAMPLES REMOVED FOR ANALYSES)

	METHANOL EXTRACT OF FLASK STOPPER	CHLOROFORM PHASE OF FIRST BLIGH/ DYER PARTITION OF MEDIUM FILTRATE AFTER METHANOL WASHING OF FILTER PAD	METHANOL/ WATER PHASE AFTER FIRST BLIGH/DYER SEPARATION	CHLOROFORM PHASE OF SECOND PARTITION OF MEDIUM FILTRATE	METHANOL SOXHLET EXTRACT	METHANOL/ WATER SOXHLET EXTRACT	% RECOVERY
INOCULATED & CHLORPROPHAM INCORPORATED MEDIUM							
REPLICATE I	0.4	33.1	6.0	4.0	21.7	< 2%	59.2
REPLICATE II	1.9	34.4	6.0	4.2	14.4	< 2%	54.9
UNINOCULATED CHLORPROPHAM INCORPORATED MEDIUM							
REPLICATE I	1.2	86.8	N.D.	1.2	4.9	< 2%	94.1
REPLICATE II	0.76	90.5	N.D.	1.4	3.7	< 2%	96.3

N.D. NOT DETERMINED

was not as expected. This was probably due to the difficulty in determining the correct amounts of water present in the mycelium. These results also highlight the problems which arise in the absence of a label when it is difficult to know whether the low recovery of chlorpropham is due to metabolism, loss from the flask, or low extraction efficiency.

7.4 Metabolism of Carbon-14 Labelled Chlorpropham by Polyscytalum pustulans

The radiolabelled chlorpropham was prepared as described in Section 5.3. 2 ml of the crude reaction mixture was purified to give 7.0 µg/ml chlorpropham in 4.9 ml of acetone. 0.5 ml aliquots of the acetone solution was added to four Roux flasks containing medium prepared and sterilised as described in Section 7.3.1. Two of the four flasks remained uninoculated while the remaining two were inoculated using the procedure described in Section 7.3.1.

All the culture flasks were placed in a walk-in growth room in the Mycology Department which was maintained at a temperature of 15°C. Reinoculation was carried out after 23 days by which time the fungus had completely colonised the entire surface of the medium. After submerging the mycelium by tipping the culture flasks to bring the medium to the surface, growth was allowed to continue for a further 9 days when the reinoculation procedure was repeated. After a total of 44 days the culture was extracted using the methods given in Section 7.3.2. Deviations from this method were that no samples were removed for aniline analysis but liquid scintillation counter (LSC) samples were taken (again on a weight

basis) from the medium filtrate before and after washing of the filter pad with methanol. In the case of radiolabelled samples the first analyses were those of activity determination while identification of the compound in which the label appeared was carried out later.

LSC samples were prepared in the normal manner with 0.1 ml to 0.5 ml aliquots of chloroform extracts being counted in 12 ml of Dimilume. 0.5 ml - 2 ml aliquots of methanol or methanol/water extracts were counted in 12 ml of Dimilume and 1 ml of Soluene. Quenching in the chloroform containing samples was not as severe as with the chloroform extracts of E. carotovora cultures and consequently larger sample sizes could be used.

The activities of the extracts were determined at the following points of the extraction procedure:

1. The methanol extract of the flask stopper
2. Liquid filtrate prior to washing filter pad with methanol
3. Liquid filtrate after washing filter pad with methanol
4. Chloroform phase of first Bligh/Dyer separation
5. Methanol/water phase of first Bligh/Dyer separation
6. Chloroform phase of second Bligh/Dyer separation
7. Methanol/water phase of second Bligh/Dyer separation
8. Methanol Soxhlet extract
9. Methanol/water Soxhlet extract.

The results shown in Table 7B are the activities of the complete extracts (again being corrected for any subsample which had previously been removed). They are expressed as a percentage of the total amount of chlorpropham added based on the assumption that exactly 0.5 ml of

TABLE 7B. % OF TOTAL RADIOACTIVITY INITIALLY ADDED (3500 µg x 630 dpm/µg) APPEARING IN EACH FRACTION

(CORRECTED FOR SUBSAMPLES REMOVED FOR ANALYSES)

	METHANOL EXTRACT OF FLASK STOPPER	MEDIUM FILTRATE PRIOR TO METHANOL WASHING OF FILTER PAD	MEDIUM FILTRATE AFTER METHANOL WASHING OF FILTER PAD	CHLOROFORM PHASE OF FIRST BLIGH/ DYER SEPARATION	METHANOL/ WATER PHASE OF FIRST BLIGH/ DYER SEPARATION	CHLOROFORM PHASE OF SECOND BLIGH/ DYER SEPARATION	METHANOL/ WATER PHASE OF SECOND BLIGH/ DYER SEPARATION	METHANOL SOXHLET EXTRACT	METHANOL/ WATER SOXHLET EXTRACT	ASSESS - MENT OF RESIDUAL ACTIVITY IN MYCELIUM	% RECOVERY
INOCULATED & CHLORPROPHAM INCORPORATED MEDIUM											
REPLICATE I	0.7	8.2	39.6	34.0	6.2	4.6	3.3	32.5	1.0	0.1	73.9
REPLICATE II	0.4	14.9	47.0	31.0	10.3	8.8	3.7	21.1	0.3	0.1	68.9
UNINOCULATED CHLORPROPHAM INCORPORATED MEDIUM											
REPLICATE I	1.4	83.2	90.7	94.5	2.1	2.7	0.8	2.2	0.1	N.A.	94.4
REPLICATE II	1.0	86.6	91.5	93.2	2.8	3.2	0.5	2.6	0.1	N.A.	95.2

N.A. NOT APPLICABLE

7 mg/ml acetone solution was added to each flask.

The results show only a similar recovery rate of the radiolabel from these cultures as the chlorpropham recovery rate obtained when unlabelled chlorpropham was incorporated into the medium. The only part of the culture which had not been assessed in the case of the unlabelled cultures was the unextracted portion that may have remained in the mycelium. In order to obtain some idea of the radioactivity remaining in the mycelium of the carbon-14 labelled cultures, pieces of the mycelium visually approximating to 5% of the total mycelium were placed in an LSC vial containing 14 ml Dimilume and 1 ml Soluene and counted. While it was appreciated that the results obtained from such a count would be of little quantitative value due to the poor physical distribution of these samples in the LSC vial, the opportunity was nevertheless taken to assess the residual activity in the mycelium. The results show only small quantities of activity were displayed by mycelium samples.

The chloroform extracts were chromatographed on the high pressure liquid chromatograph equipped with a normal phase Hypersil column (for conditions see Section 4.1) with the column eluant being fractionated prior to activity determination by LSC. Figure 7I is a chromatograph of the chloroform phase extract of a first Bligh/Dyer extraction of medium filtrate from an inoculated flask.

No radioactivity above background levels were detected in any eluant fraction other than the chlorpropham peak. There was however a 4-5% loss in activity from the sample after chromatography and collection of the chlorpropham peak. This was the loss over and above the 0.9% loss associated with columns packed with Hypersil (see Section 4.3). It

is unlikely that a compound which would be retained by the column or have a very long retention time (i.e. a polar compound) would appear in the chloroform phase since it is more likely to partition into the aqueous phase.

The methanol Soxhlet extracts were also chromatographed and the eluent fractionated by the high pressure liquid chromatography using a reverse phase system employing an ODS Hypersil column operated under conditions given in Section 4.1. Figure 7II is a chromatograph of a methanol Soxhlet extraction of a P. pustulans culture. Again the only fraction displaying activity was that of the chlorpropham peak and some loss in activity of the sample was observed. This average loss in activity was 2.7% above the 2.5% of applied chlorpropham which is not usually recoverable from an ODS column (see Section 4.3). This unrecovered radiolabel was not thought to represent an unidentified metabolite since the order of elution on a reverse phase column is that of decreasing polarity and therefore metabolites with short retention times would be more likely rather than fully retained metabolites.

The low recovery of the radiolabel from the culture flasks is lower than would have been expected. Loss of intact chlorpropham through volatilisation is unlikely in view of the low losses recorded by the uninoculated flasks. Losses of the carbon-14 isotope as radiolabelled carbon dioxide is possible and trapping of the carbon dioxide from the culture flask would have been useful in confirming or eliminating this possibility. Loss of the label during Soxhlet extractions when water was used as the solvent could have been possible at the elevated temperatures used. Cleavage of the carbamate linkage of the chlorpropham molecule followed by loss of the labelled propan-2-ol through volatilisation or metabolism to carbon dioxide may have occurred. Such a route of loss would however

have to exist alongside rapid degradation or occlusion of the aniline since no aniline was detected in the culture medium when unlabelled chlorpropham was used. The low recovery of the radiolabel is more likely to be attributable to the label remaining in the mycelium either as an integral part or located in an area inaccessible to extracting solvents. The P. pustulans mycelium concentrates the chlorpropham from the medium as shown by the low concentration of chlorpropham left in the medium. Washing with methanol however removes much of this and further extraction with methanol in Soxhlet removes approximately the same amount again but whether the extraction was complete it is impossible to say with any degree of certainty. The activity determinations of pieces of mycelium produced very low counts but this method of counting can be very inaccurate (Horrocks, 1974). With the benefit of hindsight it would have been more useful if an accurate total activity determination had been carried out. If the mycelium had been combusted the carbon-14 labelled carbon dioxide activity could have been assayed when in intimate contact with scintillation fluid. Alternatively, drying and grinding of the mycelium into a fine powder prior to dispersing in a gel suspension scintillation cocktail could have provided more meaningful results. It was however felt, at the time, that the provision of the necessary equipment and the gaining of the expertise to accurately determine the total activity in the mycelium would prove too great a hurdle to overcome merely to complete the "balance" aspect of this study. Perhaps more exhaustive extraction of the mycelium would have removed more radiolabel and mechanical fragmentation of some sort prior to the Soxhlet extraction may have facilitated penetration and removal of occluded chlorpropham by the solvent. It is feasible that the

Soxhlet extraction only removed the radiolabel from areas of mycelium close to the surface and this would result in the activity of the samples being grossly underestimated since only carbon-14 on the surface of the mycelium would have been detected by the LSC.

In conclusion then it has to be said that no metabolites of chlorpropham were identified which was also the situation with the E. carotovora study. However in the E. carotovora study where recoveries were high it was possible to state with some certainty that no metabolites were produced. In the case of the pure culture of Polyscytalum pustulans with recoveries not being as high it was not possible to make such a statement.

The results of the metabolism investigations are more fully discussed in Chapter 9.

Figure 7.I. HPLC chromatogram of chloroform phase of the Bligh/Dyer separation of Polyscytalum pustulans culture medium
column: normal phase (Hypersil).
For chromatographic conditions see Section 4.I.

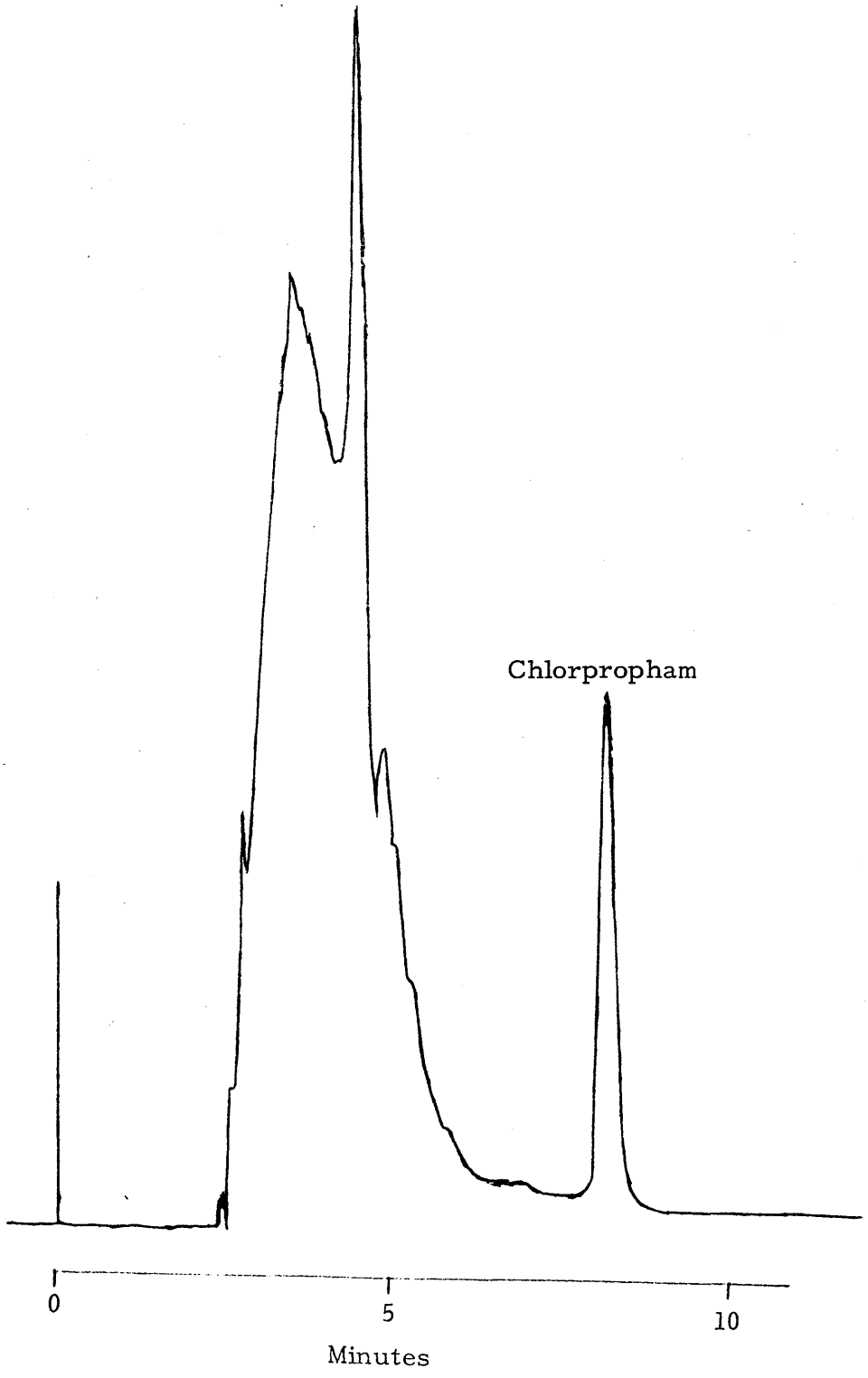
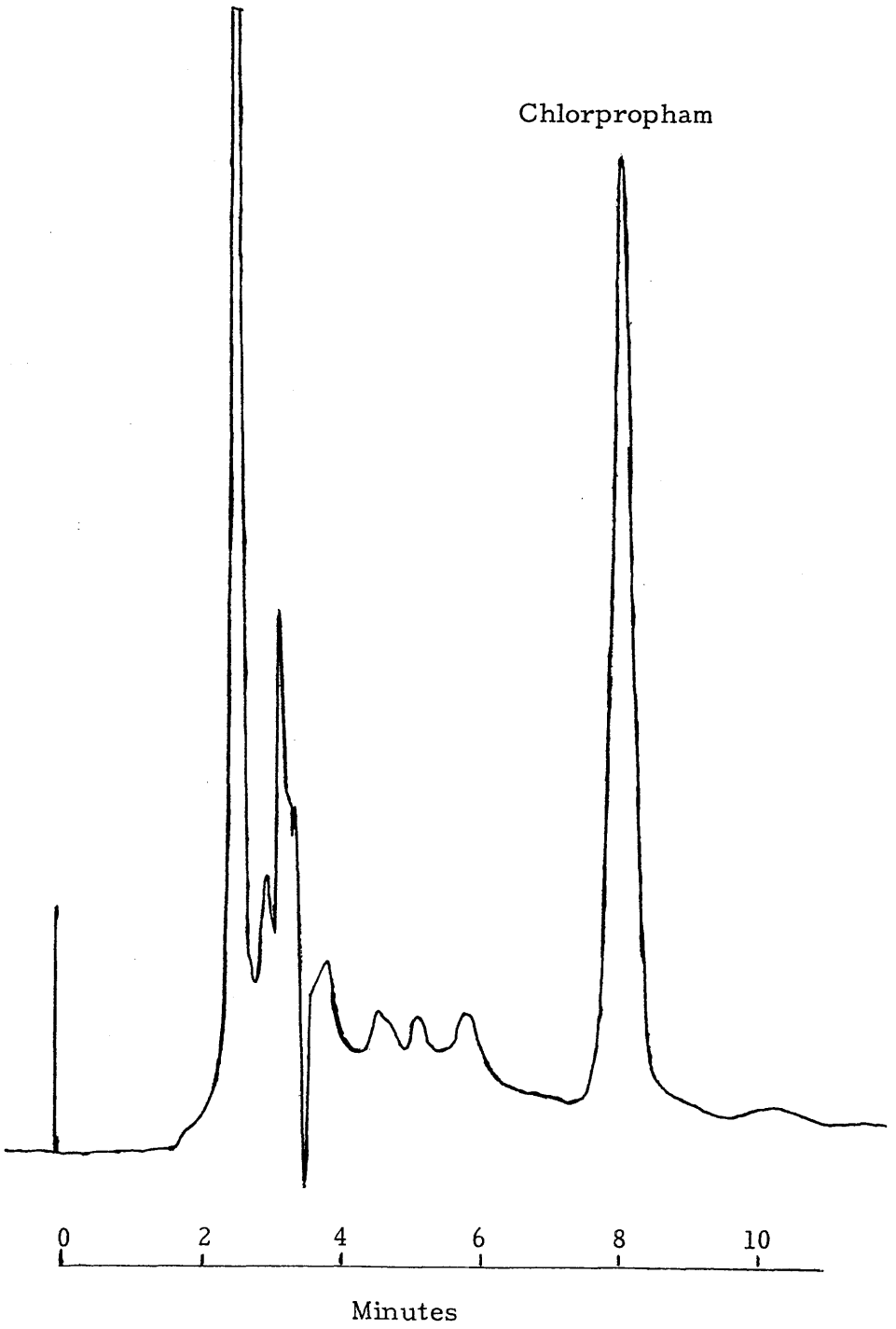


Figure 7.II. HPLC chromatogram of methanol Soxhlet extract of Polyscytalum pustulans mycelium. Column reverse phase ODS-Hypersil. For chromatographic conditions see text (Section 4.I).



CHAPTER 8

METABOLISM OF CHLORPROPHAM BY *PHOMA EXIGUA* VAR. *FOVEATA*

8.1 INTRODUCTION

Phoma exigua var. *foveata* is the causal agent of gangrene, a disease of stored potatoes. This disease tends to become evident when potatoes are stored at low temperatures. Where reducing sugar concentration in the tubers is of importance, the storage conditions include temperatures which are well above those that are associated with the development of this disease. It therefore could be argued that chlorpropham would not always be applied to potatoes suffering from this disease or indeed likely to develop symptoms of the disease since the majority of the chlorpropham is used on high temperature stored material. This is unlike the cases of *E. carotovora* and *P. pustulans* which are frequently present in stores of potatoes be they high temperature or low temperature stores.

It was nevertheless felt that although *P. exigua* was not a common disease in the stores which use the majority of the sprout suppressant formulations of chlorpropham in this country it was worthy of study since it is a significant potato storage disease.

8.2 Preliminary Work on the Culturing of Phoma exigua var. foveata

The same approach as that used in the P. pustulans study to determine the concentration of chlorpropham that the cultures could tolerate without reducing the growth rate too much was followed. Using Petri dishes and a 2% malt extract medium it was found that a 25 µg/ml chlorpropham concentration in the medium reduced the average growth rate by 24% compared to acetone controls. The incorporation of acetone into the medium had only a marginal effect on growth rates.

8.3 Large Scale Cultures of P. exigua on Medium Incorporating Radiolabelled Chlorpropham

No large scale cultures that utilised medium incorporating unlabelled chlorpropham were used.

The radiolabelled material used in the P. exigua metabolism study was from the same batch as that used in the P. pustulans study which was a 7 µg/ml chlorpropham in acetone solution. The preparation of media, incorporation of the radiolabel, preparation of the inoculum and inoculation of the flasks were as described in Section 7.4 of the P. pustulans study. Again 0.5 ml of the radiolabelled chlorpropham in acetone was added to four Roux flasks each containing 150 ml of autoclaved media (2% malt extract). Two of the culture flasks were inoculated with 4 discs of inoculum from cultures on plated malt extract medium and the remaining two Roux flasks remained uninoculated. The four flasks were placed in a walk-in growth room which was maintained at a temperature of 15°C.

The cultures were grown for a total of 34 days with reinoculation of the flasks being carried out after 20 days growth and after a further 8 days growth in order to increase the yield of mycelium. After a total of 34 days the cultures were extracted.

8.4.1 Extraction of Cultures

The cotton wool/cheesecloth stopper was extracted with methanol as described in Section 7.3.2.3 and evaporated to 5 ml. The medium was filtered and duplicate 2g aliquots were removed for aniline analysis using the procedure described in Section 6.3.2. The filtration and extraction of the mycelium was essentially the same as the method given in Section 7.3.2.1 except that an allowance of 10 ml was made for the water retained by the mycelium and not added to the filtrate by methanol washing of the filter pad. Rapid separations were not achieved in these P. exigua extracts although they were somewhat quicker than those of P. pustulans extracts. The other deviation from the method used in the extraction of the P. pustulans medium filtrate was that the methanol/water phases of the Bligh/Dyer separation were not evaporated but a proportion of these was removed and the radioactivity present determined by liquid scintillation counting (LSC) with the remainder of the phase being re-extracted with chloroform. Aliquots of the resultant aqueous phase were used for activity determination by LSC and the remainder of the phase retained without being evaporated.

8.4.2 Soxhlet Extraction

The Soxhlet extraction with methanol was carried out using the method described in Section 7.3.2.2 but only where methanol was used as the extracting solvent. Unlike the Soxhlet extraction of the P. pustulans culture when water was used as the second extracting solvent, 70 ml of chloroform was used in the case of these P. exigua cultures. The chloroform extraction time, like that of the water was one 12 hour period after which it was allowed to cool and reduced in volume to 10 ml by rotary evaporation under reduced pressure and at a temperature not exceeding 35°C.

8.5 Activity Determinations of the Fractions

The activity of the fractions was determined by LSC. The points in the extraction procedure and the volume of the samples used are given below.

1. The extraction of the cotton wool/cheesecloth stopper - 1 ml
2. Liquid filtrate prior to methanol washing of the filter pad - 0.5 ml
3. Liquid filtrate after methanol washing of filter pad - 0.5 ml
4. Chloroform phase of first Bligh/Dyer separation - 0.1 ml
5. Methanol/water phase of first Bligh/Dyer separation - 0.5 ml
6. Chloroform phase of second Bligh/Dyer separation - 0.1 ml
7. Methanol/water phase of second Bligh/Dyer separation - 0.5 ml
8. Methanol Soxhlet extract - 0.5 ml
9. Chloroform Soxhlet extract - 0.2 ml
10. Mycelium after methanol and chloroform Soxhlet extractions - About 5% of total mycelium

The samples were placed in 12 ml of Dimilume. 1 ml of Soluene 350 was added to methanol/water or methanol extracts.

8.6 Results and Discussion

The aniline determinations showed that no anilines were detected with the optical density of the reagent blanks and those of the culture filtrate recording a similar low value.

The results given in Table 8A are the activities displayed by the above extracts (Section 8.5). They are expressed as a percentage of the total activity added that appears in each extract. They have been corrected to show the amount of radiolabel that would have been present had no subsamples been removed for analysis. In calculating the percentages in Table 8A the assumption has been made that exactly 0.5 ml of 7 mg/ml chlorpropham in acetone was added to each flask. Clearly this could not have been the case. More than this amount must have been added with the likely cause of this being the evaporation of some of the solvent resulting in the solution being more concentrated than 7 mg/ml.

The recoveries of the radiolabel from these cultures are higher than those achieved with the P. pustulans cultures and this could be due, at least in part, to the more open and filamentous nature of the P. exigua mycelium.

High pressure liquid chromatography was carried out on both of the chloroform extracts of each replicate using a normal phase Hypersil column under conditions given in Section 4.1. Collection of the column eluant fractions was made and the activity determined by LSC. The only fraction returning above background counts originated from the chlorpropham peak.

TABLE 8A. % OF TOTAL RADIOACTIVITY INITIALLY ADDED (3500 µg x 630 dpm/µg) APPEARING IN EACH FRACTION

(CORRECTED FOR SUBSAMPLES REMOVED FOR ANALYSES)

	METHANOL EXTRACT OF FLASK STOPPER	MEDIUM FILTRATE PRIOR TO METHANOL WASHING OF FILTER PAD	MEDIUM FILTRATE AFTER METHANOL WASHING OF FILTER PAD	CHLOROFORM PHASE OF FIRST BLIGH/ DYER SEPARATION	METHANOL/ WATER PHASE OF FIRST BLIGH/ DYER SEPARATION	CHLOROFORM PHASE OF SECOND BLIGH/ DYER SEPARATION	METHANOL/ WATER PHASE OF SECOND BLIGH/ DYER SEPARATION	METHANOL SOXHLET EXTRACT	CHLOROFORM SOXHLET EXTRACT	ASSESS-MENT OF RESIDUAL ACTIVITY IN MYCELIUM	% RECOVERY
INOCULATED & CHLORPROPHAM INCORPORATED MEDIUM											
REPLICATE I	1.95	32.0	65.2	53.4	14.2	3.1	7.2	31.6	0.3	0.2	99.2
REPLICATE II	1.41	34.9	47.2	36.1	14.5	2.0	7.2	46.9	0.9	0.2	96.6
UNINOCULATED CHLORPROPHAM INCORPORATED MEDIUM											
REPLICATE I	4.82	82.4	100.6	99.3	3.0	N.D.	N.D.	3.3	0.05	N.A.	108.8
REPLICATE II	1.77	86.2	104.6	103.3	2.6	N.D.	N.D.	3.1	0.07	N.A.	109.5

N.A. NOT APPLICABLE; N.D. NOT DETERMINED

Prior to the aniline determinations being carried out, 25 µl aliquots of the filtrate medium of the inoculated flasks were chromatographed on an ODS-Hypersil equipped high pressure liquid chromatograph (for conditions see Section 4.1) with the chlorpropham peak being collected and placed in a LSC vial containing 12 ml Dimilume and 1 ml Soluene 350. Figure 8I is a chromatogram produced from such an injection. The results from the LSC showed that only 55% in the case of replicate I and 59% in the case of replicate II of the radioactivity was present in the chlorpropham fraction of the eluent. Unfortunately it was only the chlorpropham peak which was collected. It was impossible to determine at which point the remainder of the label was eluted since the high pressure liquid chromatographic separation was carried out on the aniline analysis fraction and by the time the LSC results were to hand no more liquid filtrate sample remained.

The chloroform Soxhlet extracts were not subjected to high pressure liquid chromatography since the activity was too low. The methanol Soxhlet extracts however were chromatographed on the high pressure liquid chromatograph, see Figure 8II (for chromatographic conditions see Section 4.1). For this chromatographic run a 30 µl injection was made and eluant fractions I to X as detailed on the chromatogram were collected. The average activity displayed by 30 µl aliquots placed directly into a LSC vial was 2375 dpm. Table 8B shows the activity of each of the fractions after the background count had been subtracted.

TABLE 8B

FRACTION NUMBER	ACTIVITY (dpm)
I	16
II	40
III	29
IV	18
V	18
VI	26
VII	12
VIII	34
IX (Chlorpropham)	2188
X	23

It would seem that all of the radiolabel was still present in the chlorpropham and would appear to be inconsistent with the HPLC/LSC results obtained when medium filtrate was injected into the HPLC.

The high activity of the methanol/water fraction even after two chloroform extractions suggested that the label was not present in the chlorpropham and that a metabolite more polar than chlorpropham had been produced. The activity was however low and since it was present in a large volume of the methanol/water phase some form of clean-up and concentration was necessary in order to proceed further with this study.

Still and Mansager (1973a) used an Amberlite XAD-2 column purification procedure to clean-up metabolites of chlorpropham produced by soybean plants. Use was made of a similar column to purify and concentrate the radiolabelled compound present in the aqueous phase of the Bligh/Dyer extract of the inoculated flask. Only Replicate I was used. The total quantity present was not large, being equivalent to 250 μg (assuming the same molecular weight as chlorpropham).

The first step in the purification was the reduction in volume of the aqueous phase of Replicate I on a rotary evaporator under partial vacuum and at a temperature not exceeding 35°C until condensate ceased to appear on the evaporator condenser. The residual aqueous phase was then ready for application to the column and its activity was determined by LSC (0.5 ml in 12 ml of Dimilume and 1 ml of Soluene 350).

The Amberlite XAD-2 (BDH Chemicals Ltd.) was added slowly and continuously to a 0.9 mm i.d. x 400 mm glass column fitted with a sintered glass base which had been previously filled with diethylether while maintaining a flow rate of 2 ml/minute. 200 ml of diethylether was used altogether and was followed by 200 ml of methanol and then 200 ml of distilled water.

After these solvents had conditioned the column the flow rate was reduced to 1.2 ml/minute and maintained at this rate throughout the purification. The evaporated aqueous phase was then added to the column. After the last of the large volume had passed below the top of the column bed, 50 ml of distilled water followed by 150 ml of methanol was used to elute the column. The eluant during the addition of the sample was collected. The batches containing a large proportion of methanol were reduced in volume by rotary evaporation under reduced pressure and at

a temperature not exceeding 35°C until no further condensate appeared on the evaporator condenser. The activity of the various fractions were determined by LSC. 0.5 ml aliquots of sample were then used and 12 ml Dimilume and 1 ml of Soluene 350 made up the scintillant mixture. Table 8C gives the activities of the various fractions.

TABLE 8C

FRACTION	ACTIVITY dpm ($x10^3$)
Methanol/water phase prior to evaporation	158
Methanol/water phase after evaporation	121
Fraction collected during application of sample to column (120 ml)	41
120 ml - 170 ml column eluant collected during the period when distilled water was used as eluant	15.1
170 ml - 220 ml (methanol eluant)	48.3
220 ml - 270 ml (methanol eluant)	9.5
270 ml - 320 ml (methanol eluant)	2.7

The 170 - 220 ml fraction was transferred to a flask and evaporated until condensate ceased to appear on the condenser to give a 10 ml concentrate. 50 µl aliquots were chromatographed on the HPLC equipped with an ODS-Hypersil column (for chromatographic conditions see Section 4.1) and fractionation of the HPLC eluent for activity determination by LSC was carried out. Figure 8.III is a resultant chromatograph with the fraction collection points indicated on the chromatogram. The

activities of the individual fractions, after correction for background counts, are given in Table 8D.

TABLE 8D

FRACTION NUMBER	ACTIVITY (dpm)
I	14
II	22
III	194
IV	42
V	19
VI	15
VII	14
(Chlorpropham) VIII	42
IX	21
X	16

A radiolabelled compound was eluted close to the "solvent front" (void volume). Without the use of reference compounds it is difficult to establish what this compound might be. Certain possibilities can however be discounted. Dechlorinated chlorpropham i.e. propham, has a retention time shorter than chlorpropham on an ODS Hypersil column but under the chromatographic conditions used on this run the retention time of propham is 7.7 minutes i.e. it would appear in fraction VI.

Propan-2-ol is a possible candidate. It is however unlikely that losses of radiolabel would have been as low as they were during the extraction and in particular during the evaporation stages if radiolabelled propan-2-ol had been present. The production of aniline, which is the likely by-product should radiolabelled propan-2-ol have been liberated from ester labelled chlorpropham, was not detected and co-metabolism or occlusion would have to accompany any production of labelled propan-2-ol. It could therefore be concluded that while labelled propan-2-ol is a possible metabolite it is an unlikely candidate.

No early eluting radiolabelled compound was detected in the methanol Soxhlet extracts. It is difficult to envisage this being possible when a short retention time radiolabelled metabolite in aqueous extracts was observed. Although methanol washing of the fungal mycelium in the filter pad was undertaken, it could not have been expected to remove all of a water soluble metabolite which would have been widely distributed throughout the culture flask. It may be that the mycelium retains or occludes little water soluble material and the small HPLC injection volume used and the relatively high activity of the methanol Soxhlet extract (due to presence of radiolabelled chlorpropham) prevented detection of this metabolite.

The polar metabolite observed would be present at a concentration of greater than the 7% that was still present in the methanol/water phase after two chloroform extractions. The 43% of the total radioactivity in the medium filtrate which was not collected along with the chlorpropham peak may be a reasonable means of estimating the amount of metabolite produced. Bearing in mind that the medium filtrate contained an average

of 56% of the radioactivity originally incorporated into the medium then a figure of around 25% metabolism can be calculated using the activity figures obtained from the unreplicated HPLC fractions of the culture medium filtrate.

Since the existence of a metabolite has been established it is immeasurably easier to characterise this metabolite and design new extraction procedures specifically aimed at extracting this metabolite. This is discussed in greater detail in Chapter 9. The Phoma exigua var. foveata metabolism study was in fact one of the most interesting aspects of the work undertaken during the course of this thesis and had time allowed then this would undoubtedly have been an area worthy of further investigation.

Figure 8.I. HPLC chromatogram of medium filtrate of Phoma exigua var. foveata cultures. Column: ODS-Hypersilreverse phase. For chromatographic conditions see Section 4.I.

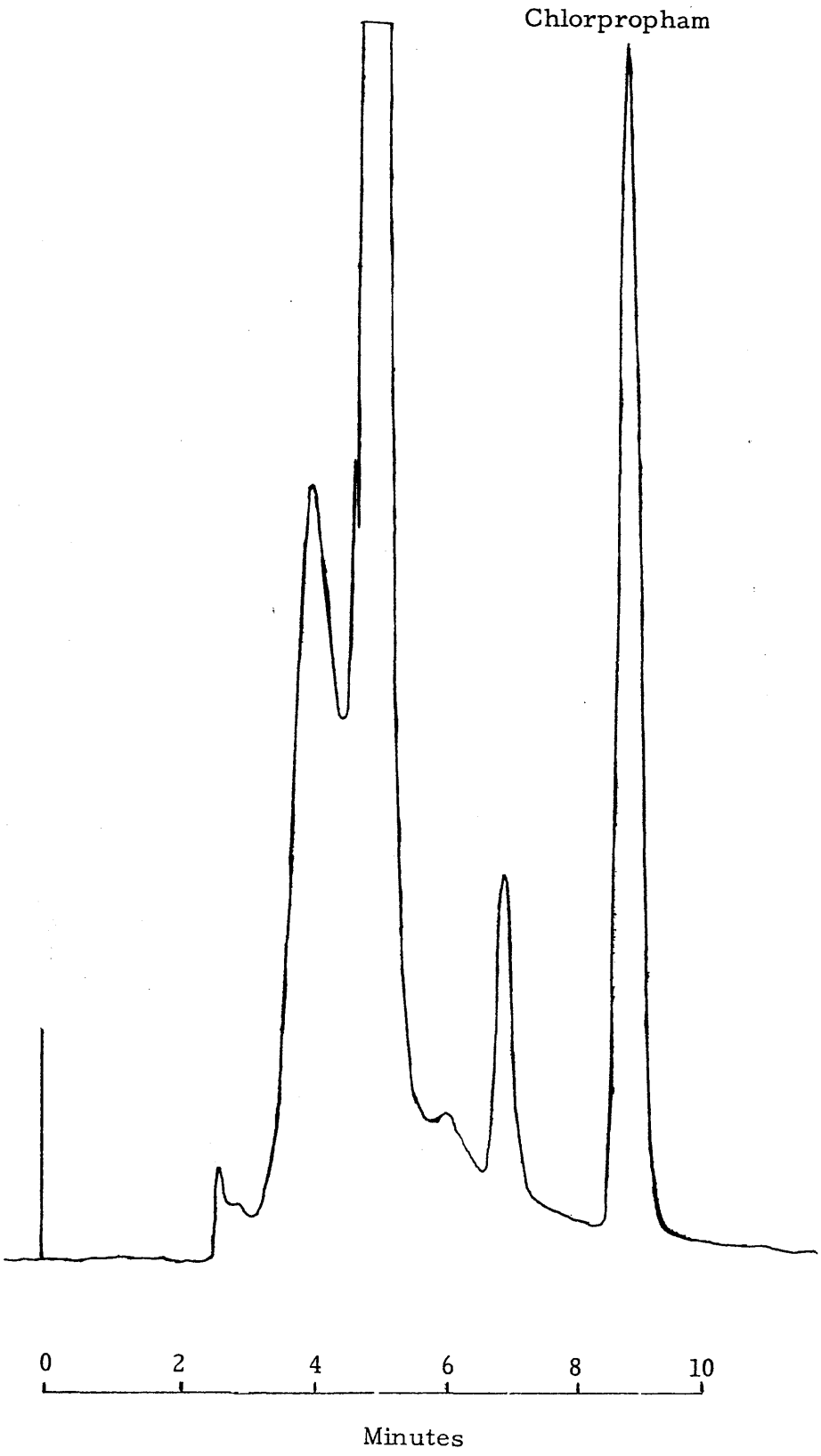


Figure 8.II. HPLC chromatogram of methanol Soxhlet extract of Phoma exigua var. foveata mycelium column: reverse phase ODS-Hypersil. For chromatographic conditions, see text (Section 4.I).

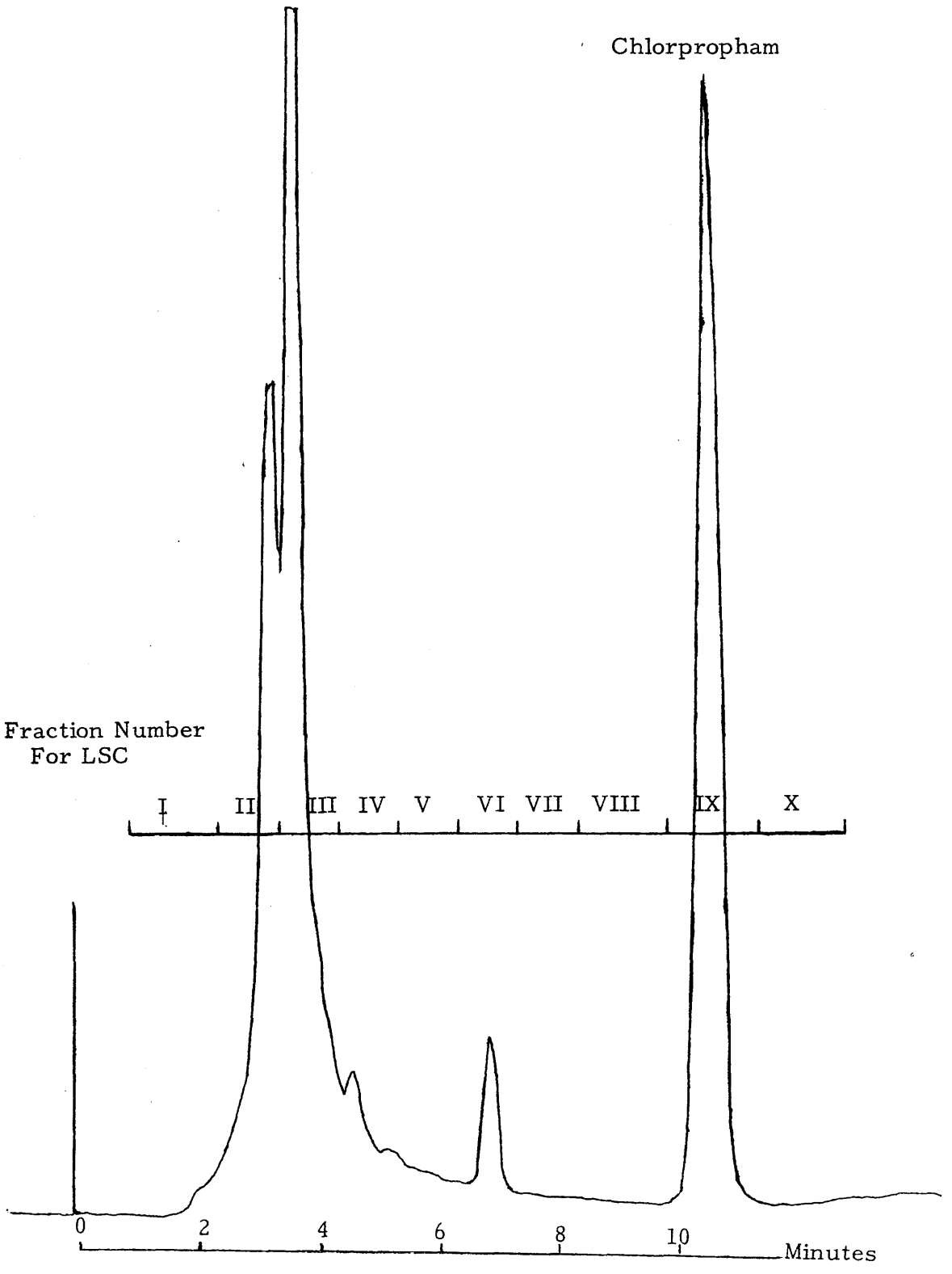
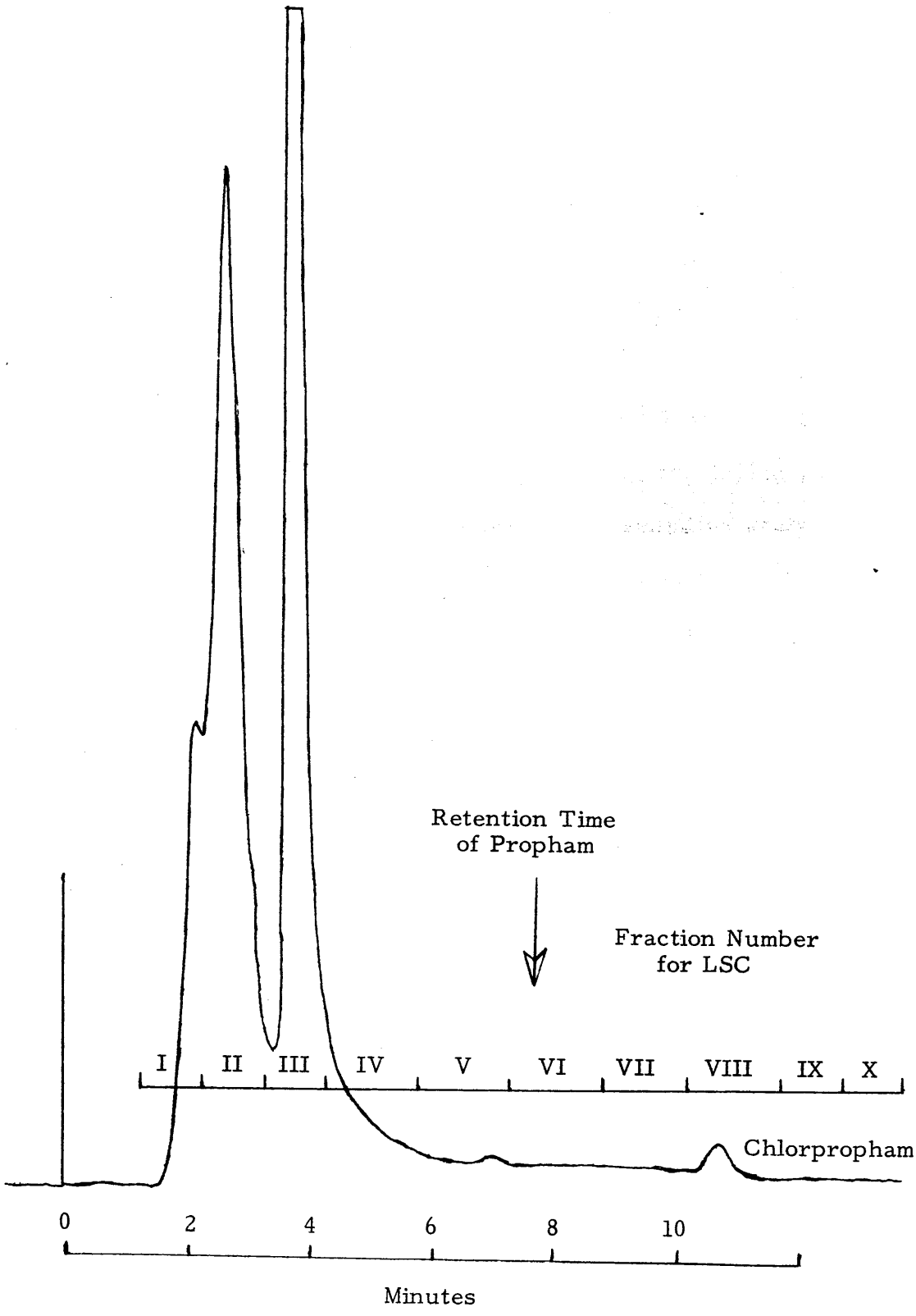


Figure 8.III. HPLC chromatogram of aqueous phase of Bligh/Dyer separation of liquid medium filtrate from Phoma exigua var. foveata cultures after chromatography on XAD-2 column - see text (Section 8.6). For chromatographic conditions of this reverse phase system which utilised an ODS-Hypersil column, see Section 4.I.



CHAPTER 9

CONCLUSIONS

The object of this thesis was to provide some additional information on the fate of chlorpropham that is applied to stored potatoes as a sprout suppressant. Investigation of the residues of chlorpropham was restricted to the effect of the manufacturing process on the residue levels and as mentioned earlier was not concerned with volatilisation losses from the store. The original direction of the investigation was dictated by the available literature on the subject, which suggested that the chlorpropham residue which remained in the tubers after washing and peeling was lost from the potato slices during the frying process used to produce crisps. The development of a sensitive analytical technique was carried out in order to determine what parameters of operation of the processing line have an effect on the loss and rate of loss of the chlorpropham from the friers.

Previous work in the Agricultural Chemistry Section, prior to the analysis of crisps for chlorpropham residues, had been concerned with ensuring efficient use of the chlorpropham as a sprout suppressant and as far as the processing industry was aware, very little of the chlorpropham that was present in the potatoes at the beginning of the processing line remained by the time the crisps had been fully fried.

The development of the analytical method described in Chapter 3 to determine chlorpropham residue levels in crisps and oil below those attainable by the method of Gard and Rudd (1953) was only partially successful. The "limit of sensitivity" of the colorimetric method of Gard

and Rudd was reported as 0.05 μg/g of product. The minimum amount which can be detected and quantified using the procedures developed in the course of this thesis is equivalent to 0.035 μg/g crisp when the GC is used to quantify the chlorpropham extracted from crisps. The use of high pressure liquid chromatography (HPLC) lowers the detection limit and residues of as little as 0.02 μg/g can be detected.

The use of this method to determine very low residues of chlorpropham was not in fact required, due to the residue values found in crisps and crisp frying oils being much greater than the limit of detection. This finding therefore changed the direction of the study to one where actual residue levels in the final product were determined. The effects of some processing procedures on the final residue were examined. It was shown that potatoes which had not been treated with sprout suppressant became contaminated after being processed on the same production line and, in addition, it can be tentatively suggested that the infra red heaters used to reduce the oil content of the crisps do not reduce the chlorpropham residues.

The most interesting aspect is, of course, the fact that a measurable chlorpropham residue is present in the crisp. It can be argued that the loss of the chlorpropham through vaporisation would be expected. As explained more fully in Chapter 3, the GC temperature used to determine chlorpropham on an OV 101 column was 170°C and relatively short retention times result. It could also be suggested that a significant vapour concentration of chlorpropham exists over the oil in the frier and if no means of returning the chlorpropham to the frier exists then irreversible loss of the chlorpropham from the oil would result.

The chlorpropham residue in crisps reported by Martens et al. (1971) and Gard (1959) was below the limit of sensitivity of their analytical method and might have been as a result of fresh frier oil being used. This could have resulted in the chlorpropham that was present in the slices being dissipated throughout the oil and such a dilution may have lowered the chlorpropham residue to a value of less than that detectable by their analytical procedures.

A copy of the analytical method described in this thesis was given to staff at the IBVL (the Potato Research Centre) in the Netherlands. Members of the Potato Crisp Industry's Research Group (PCIRG), which represents processors in Europe (including the United Kingdom) participated in an extensive residue study carried out by the IBVL and residues of chlorpropham in crisps were found to range from 0.09-1.38 μg/g crisp with an average residue of all submitted samples of 0.69 μg/g chlorpropham/g crisp (Wolsink,1984).

Continental processors also participated in the IBVL study to determine the residues of chlorpropham and propham that are present in crisps. The method was apparently used without modification and resulted in low recoveries of propham. This was probably as a result of the alumina column clean-up procedure not being modified to take account of the different polarity and therefore different adsorption characteristics of propham in comparison to chlorpropham. Recently in the United States, residue figures have been reported for baked potatoes, potato chips (American term for crisps) and French fried potatoes (Heikes, 1985). These were determined as part of the Total Diet Program of the Food and Drug Administration (FDA) and based on market baskets which reflect the dietary preferences of eight age-sex adult

groups and infant and toddler diets in the United States. The summarised results of these are published periodically (see Johnston et al., 1984a; Manske and Johnston, 1977; Johnston et al., 1981a for adult diets and Johnston et al., 1984b; Johnston et al., 1981b for infant and toddler diets) with the most recent covering the period of October 1977-September 1978 (Podrebarac, 1984a - adult diet; Podrebarac, 1984b - infant and toddler diet).

Heikes is a member of the Total Diet Research Centre team and reports (Heikes, 1985) the individual chlorpropham residues detected in baked potatoes (0.25 μg/g), crisps (2.4 μg/g) and French fried potatoes (1.6 μg/g). The residue levels in the crisps appear very high but it should be remembered that the collection of the market baskets is from retail outlets in order to authentically reflect the pesticide residues, industrial chemicals and toxic metals to which the United States population is exposed. The result of this is that the history of the foodstuffs is unknown and details such as the time of year and whether or not the potato skin was removed prior to crisp frying would be lacking.

Even the higher residue detected by the FDA would not be cause for concern. The Environmetal Protection Agency (EPA) allow a residue value in or on potatoes of 50 μg/g, but this seems to reflect the highest possible residue, rather than being based on some safety parameters since the residue on soybean is restricted to 0.2 μg/g of chlorpropham and the metabolite of chlorpropham [1-hydroxy-2-propyl N-(3-chlorophenyl)carbamate]. This is a likely reflection of the lower residues found on or in soybeans and is in contrast to the Dutch limit of 0.5 μg/g of chlorpropham and propham in washed peeled tubers. In

most European countries a residue of up to 5 μg/g in whole washed, unpeeled tubers is permissible which is, in effect, almost identical to the Dutch limit (Hesen, 1984).

Although the reported levels of chlorpropham residue in the United States food basket samples are higher than those generally accepted in Europe for washed and peeled tubers, it should be remembered that only a relatively small proportion of crisps is present in the diet. Nevertheless, the processors are rightfully concerned about the fact that much of their crisp and potato snack products are consumed by young children. By using the EPA one hundredfold safety factor applied to the 2,000 mg/kg no observable effect level (NOEL) reported for rats (Anon, 1978) then a residue of 20 mg/kg of food intake could be "supported". Based on a 1.5 kg daily dietary intake of a 60 kg human the acceptable daily intake (ADI) would be 0.5 mg/kg of body weight, i.e. 30 mg total for a 60 kg human. To exceed this intake level then 60 kg of crisps with a chlorpropham residue similar to those reported in Chapter 3 would have to be consumed. This is equivalent to consuming 2,400 standard 25g packets in one day. However, if the same 60 kg human happened to consume potatoes that contained a chlorpropham residue which was equal to, but did not exceed the EPA maximum permissible residue, then only 0.6 kg of this material would be required to exceed the ADI. It must, however, be stressed that the 50 μg/g residue is uncommon even in or on whole unwashed tubers. Work carried out in this laboratory showed that washing and peeling of individual tubers which had chlorpropham residues of greater than 100 μg/g of unwashed tuber resulted in the reduction of the residue to less than 1 μg/g (Boyd, 1980).

Suggestions of areas worthy of further investigations arising from the residue study would include determination of the chlorpropham residue in the starch recovered from the washings of the slices. Any further studies of an extensive nature may be more profitably undertaken if the high pressure liquid chromatograph (HPLC) was the instrument used to determine the chlorpropham content in the sample extracts. As mentioned in Chapter 3, the traditional colorimetric method of analysis of Gard and Rudd (1953) could be successfully combined with the solvent extraction steps used in the residue study described in Chapter 3 as a means of analysis. The hydrolysis of the chlorpropham extracted, together with the saponification of the co-extracted oil prior to synthesis of the azo dye, which itself involves a degree of selectivity, in conjunction with open column chromatography or high pressure liquid chromatography, may provide a method capable of detecting very low levels of chlorpropham. Of course, direct analysis of the crude acetonitrile extract by high pressure liquid chromatography may provide an acceptable method for routine analysis of residue at the levels which are present in the crisps.

The objective of Chapter 3 was essentially fulfilled in that the fate of at least some of the chlorpropham added to the friers as a slice residue was identified. A more complete study involving the monitoring of the chlorpropham entering the frier and that removed from the frier in crisps would be required to determine if significant quantities of chlorpropham are lost from the friers through volatilisation.

The work of Chapter 4 was mainly associated with the operation of the high pressure liquid chromatograph and the discussion of some

background theory. In general, this Waters Associates chromatograph and the operator packed columns performed well. The only suggestion for further work would be concerned with the packing of GC columns. As mentioned in the text of Chapter 4, there is an optimum linear mobile phase velocity which results in a maximum separation efficiency of the column. This is the combined effect of three sample band dispersion processes. Lateral diffusion of the sample and dispersion of the sample due to the tortuous nature of the flow have little effect at higher mobile phase velocities. Dispersion due to slow equilibrium between the mobile and stationary phase, however, is dependent on mobile phase velocity, such that, at high mobile phase velocities, increased sample dispersion is observed. At high mobile phase velocities dispersion due to slow equilibration greatly reduces the chromatographic efficiency of the column. An important factor here is the thickness of the film of mobile phase which surrounds the stationary phase particles since the equilibration time required for the sample to partition between the two phases is dependent on the diffusion rate of the sample in the mobile phase. In liquid chromatography the use of small particle sized packings results in only a thin film of solvent flowing over the particles and since diffusion distances are short, rapid equilibration occurs and therefore rapid separations and high chromatographic efficiency result. In gas-liquid chromatography, equilibration rates are dependent on the thickness of the film of liquid coating and on the diffusion distances of the sample in the gaseous mobile phase. Due to the faster diffusion rates of the sample vapour in a gaseous mobile phase compared to solute molecules in a liquid mobile phase then the particle size of gas chromatographic packings does

not have to be as small as liquid chromatographic packings. In the case of liquid chromatography the optimum mobile phase velocity observed for a column remains constant along the length of the column. In gas chromatography the observed optimum mobile phase velocity is an average of the linear velocities along the column and since, unlike the liquid chromatography situation where the mobile phase is essentially uncompressable the mobile gas phase is compressed and therefore the linear velocity will increase towards the end of the gas chromatographic column. Different GC carrier gases produce different linear carrier gas velocity -V- HETP (column length/column efficiency) plots (Van Deemter plots). In the case of helium the use of high flow rates (i.e. high linear velocities of the carrier gas) do not lead to as great a reduction in the efficiency of the column as when the more common nitrogen carrier gas is used at high flow rates. This is probably due to the lower pressure drops (for the same average linear velocity) observed when helium is used and thus a narrower range of linear carrier gas velocities within the column results.

The use of the expensive gas, helium, for GC work can often not be justified but if a range of column packing particle sizes was to be used with the large particles at the beginning of the column and reducing to the smaller particle size at the end of the column, then this may allow the use of nitrogen as the carrier gas and result in higher column efficiencies since the linear gas velocity would be nearer the optimum for a greater proportion of the column length.

The use of different thicknesses of liquid coating has been used with a thicker coating being used at the start of the column then a thinly

coated packing material being used at the detector end of the column. Higher efficiencies resulted since rapid equilibration took place under conditions of high linear velocities of the carrier gas towards the end of the column (Poole and Schuette, 1984). Use could also be made of a thinner coating of liquid phase towards the end of the column in conjunction with reducing particle sizes.

This may be an area worthy of appraisal since columns of higher efficiency may be able to be packed and used on a low financial budget.

The objective set out in Chapter 5 to purify the crude reaction mixture was fulfilled. The most pertinent comment that can be made regarding the synthesis and purification of the radiolabelled chlorpropham is that the reaction yield with respect to the radiolabelled propan-2-ol starting material was low.

The greatest inprovement which may be brought about in the reaction yield would probably be a result of improved handling of the labelled propan-2-ol since the reaction conditions and apparatus required are relatively simple. The fact that the 500 μCi of labelled material was delivered in two break-seal ampoules would contribute to handling losses. Nowadays Amersham International offer delivery of radioisotopes in a variety of delivery containers. If the synthesis of radiolabelled chlorpropham was to be undertaken in the delivery vial without the need to transfer the labelled starting material, then higher reaction yields would be anticipated. The addition of the reactants without having to break the seal of the delivery vial, i.e. by syringe through a Teflon coated rubber inner seal would also lead to an increased yield of radio-

labelled product. The Amersham Microvial or one of the Duoseal range would therefore be considered appropriate delivery and reaction vials for use in the synthesis of radiolabelled chlorpropham.

The metabolism studies of Chapters 6, 7 and 8 are worth discussing together and in relation to each other.

The aim of these studies was to provide some additional information on the fate of chlorpropham when in contact with potato pathogens. No metabolites of chlorpropham were observed in the extracts of the Erwinia carotovora var. atroseptica cultures. Reductive dechlorination of chlorpropham to produce propham was thought to have been a possible metabolic route of this facultative anaerobe. However, no propham was detected under growth conditions that were anaerobic from the time of inoculation. The E. carotovora growth conditions and extraction procedures of the cultures were the simplest of the three pathogenic micro-organisms studied and this was reflected in the highest recoveries of the radiolabel that was originally incorporated into the medium.

Closed systems were used in two of the three culture conditions of the E. carotovora and no radiolabel was observed in any compound other than chlorpropham. In the case of the fungal cultures, longer growth periods and a more complex extraction procedure had to be utilised. In the Polyscytalum pustulans study, no metabolites were detected and a low recovery or a high level of bound or occluded radio-isotope was noted. Under the constraints of limited time and resources it was decided not to attempt combustion of the mycelium and an activity determination of the liberated radioisotope and with the benefit of

hindsight this may have been a wrong decision, since it would have completed the balance aspect of this study. It should, however, be borne in mind that results from these total activity determinations are only of value in defining the total amount of activity present and do not provide additional information that is of use in the characterisation of the compounds in which the radioisotope is present. Total activity determinations of a destructive nature should be a last resort in a sequential extraction procedure, although destruction of only part of the sample can be carried out to provide information as to whether it would be worthwhile to use a more exhaustive extraction method. An observation which was made, although not directly related to the metabolism of chlorpropham was the mycelium of the P. pustulans fungus is capable of concentrating the chlorpropham from the nutrient medium. This may be an important factor in the development of P. pustulans (skin spot) infection of potato tubers. Chlorpropham applications have been associated with the development of the disease to a point where at the end of the season, deep lesions have developed. It may be that the fungus can concentrate the chlorpropham, perhaps from the vapour phase or surrounding deposits of chlorpropham, and this provides a route, through the fungal hyphae to the subsurface layers of the tuber. Once at this location it may exert an effect on the cork layer which allows penetration by the fungus (French, 1976), or it may affect development of a new cork barrier (Reeve et al., 1963).

Phoma exigua var. foveata did metabolise chlorpropham to a water soluble metabolite. The recovery of the radiolabel was more complete in the P. exigua study but in a case where a metabolite is

produced, high recovery values are not so important. It is where no metabolism has been detected, that 100% recovery of the radiolabel as unaltered chlorpropham is required in order to show that no metabolism has occurred.

The failure to fully characterise and identify this metabolite was a disappointment, but some information was gained from the study. First and foremost was that biotransformation of the chlorpropham did occur. The amount present was not insignificant and some retention characteristics on reverse phase high pressure liquid chromatography have been determined.

Metabolism chemists face a situation which has been described as 'like looking for the proverbial "needle in the haystack" when one does not know the size, shape and composition of the needle, or even how many needles there are in the stack' (Harvey, 1980).

Even with the small amounts of information gained from the P. exigua study it should be possible to design an extraction and purification scheme to cater for this one metabolite.

Since this polar metabolite appears essentially in the liquid medium, extraction of the mycelium would be of little value. A Bligh/ Dyer partition would remove most of the unaltered chlorpropham from the liquid medium filtrate to leave most of the metabolite in the aqueous phase. Concentrating procedures performed by chromatographing the whole of the medium filtrate on a medium polarity high pressure liquid chromatograph packing may retain the metabolite and a change in solvent to try and obtain it as a short elution band may prove of use. Mass spectrometry would provide a means of elucidating the structure.

Without more information on the structure of this metabolite, it is impossible to say if the metabolic pathway of the chlorpropham biotransformation resembles any of those previously reported, a review of which is given in Chapter 2.

Early in the course of this work, potato suspension cultures were considered for use in determining the metabolism of chlorpropham by potato tissue. The original experimental protocol was to obtain rapidly growing suspension cultures and add chlorpropham to the culture flask and allow growth and multiplication to proceed in the presence of chlorpropham. While the potency of the chlorpropham as a mitotic poison was appreciated, it was considered that this would be the best approach. Initiation of callus growth was relatively easy and callus was used for the initiation of suspension cultures. The main problems with the suspensions were that sometimes the callus remained intact and continued to grow in large aggregates while sometimes the callus broke up well and a finely divided suspension culture, with a high cell load was produced in 5-6 weeks. However predictable, consistent growth rates were seldom observed and because of this, potato suspension cultures were not used in any metabolism studies. It is now considered that the requirements of the culture to produce consistent and predictable growth rates when using low concentrations of chlorpropham to allow reasonable growth rates were unrealistic constraints. Addition of radiolabelled chlorpropham to flasks which had present a large volume of potato cells could have been carried out. To have the potato suspension culture growing and dividing in the presence of the chlorpropham may not be essential. While high chlorpropham concentrations in the growth medium

certainly would inhibit cell division, the cells would still retain many biochemical functions and the elucidation of pathways of biotransformation is more likely when larger amounts of biological material are present as well as larger quantities of chlorpropham.

The concern over chlorpropham in potatoes and potato based products has been lessened by the low acute toxicity of chlorpropham, and the absence of any data showing deleterious effects on animals when fed sub-lethal chlorpropham concentrations (Anon, 1978). It should, however, not be forgotten that metabolites have to be considered also, and that the effect of processing can result in unforeseen problems arising. For instance, caustic peeling of potatoes is often used to remove the skin from the potato rather than the use of abrasive peeling. The 3-chloroaniline hydrolysis product of chlorpropham was considered to have been a likely product, but it would appear that the conditions used in caustic peeling and the resistance of the chlorpropham molecule to alkaline hydrolysis would result in aniline production being unlikely (Bergon and Calmon, 1983).

Low residue values of chlorpropham are usually detected after prolonged storage with this being attributed to volatilisation losses of the chlorpropham (Corsini et al., 1979; Peisker et al., 1972; Steinbeiss et al., 1972) and this has also reduced the concern over the use of this agrochemical. Recently, however, it has been shown that chlorpropham applied to the surface of the potato is not readily desorbed from the potato surface (Coxon and Filmer, 1985). These workers applied radiolabelled chlorpropham in methanol by brush to the surface of unwashed tubers at the rate of 100 µg/g of tuber. After storage for 34 weeks in a vessel which was force ventilated at rates

greater than that used in commercial stores, less than 1% of the applied radioactivity was removed in the airstream exhausting from the storage container and an average of 28% of the radioisotope initially applied remained unextracted in the peel layer following a methanolic extraction procedure. This residual 28% was determined following combustion of the peel layer in which it was present. Furthermore if the maximum possible loss through volatilisation of chlorpropham is calculated using saturated vapour pressures, the maximum concentration of chlorpropham in air, and the ventilating pattern of modern commercial stores, then it can be shown that maximum losses over the whole season are small in comparison to the total that is applied over the season within the store.

Coxon and Filmer (1985) also reported that the vapour pressure displayed by chlorpropham when absorbed on sheets of filter paper was 15 times greater than when it was applied to the surface of potato tubers. It would therefore seem that the ventilation losses of chlorpropham do not account for all of the shortfall between that which was applied throughout the storage season and the extractable residues of chlorpropham from potatoes.

Boyd (1984) has shown that the fabric of the store retains large amounts of chlorpropham. The floor, wall and potato storage boxes in a store which he studied had an average chlorpropham content of 5,700 μg/g, 200 μg/g and 270 μg/g, respectively. The time of sampling was in mid-October, some 4 months after the last application of chlorpropham and some 3 months after the store had been cleared of the previous season's potatoes. Clearly the fabric of the building is a reservoir of chlorpropham and will account for some of the shortfall.

When Coxon and Filmer (1985) used washed tubers, the non-extractable radiolabel in the peel fraction amounted to less than 2% of the applied radiolabel after 21 weeks of storage but the total radiolabel recoveries were low. In this study the activity of trapped volatile radiolabelled material was less than 2% of the total applied to the tubers. When the unwashed tubers had been used, the soil fraction did not present any extraction problems since Soxhlet extraction with diethyl-ether followed by methanol resulted in near complete extraction of the radioactivity in the soil. Despite the large quantities of chlorpropham retained in the fabric of the store, it seems likely that much of the chlorpropham originally applied to the potatoes remains as bound residues and/or chlorpropham metabolites. The only metabolite of chlorpropham which has been isolated from chlorpropham treated potatoes is isopropyl N-(3-chloro-4-methoxyphenyl)carbamate, reported by Heikes (1985), but only 1% of the chlorpropham used to treat the potatoes was metabolised to this product after 6 weeks storage of the treated potato tubers.

While chlorpropham is an agrochemical with low mammalian toxicity (Naylor, 1976), high levels of unidentified bound residues which have been reported by Coxon and Filmer (1985) are in need of further study. Identification of the metabolite produced by P. exigua var. foveata and the study of the remaining range of pathogens normally found in potato stores would contribute to a more complete knowledge of the fate of chlorpropham in potato stores.

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