ENVIRONMENTAL FATE AND TRANSFORMATIONS OF ANILINE-BASED HERBICIDES WITH SPECIAL REFERENCE TO CHLORPROPHAM

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B.Sc., M.Sc. (Chemistry)

Thesis Submitted for the Degree of

Doctor of Philosophy (Ph.D.)

August 1990

Agricultural, Food and Environmental Chemistry

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Acknowledgements

I am extremely grateful to Dr. Harry Duncan for his kind assistance, interest and supervision during the course of this study.

I also wish to thank Dr. Isabella Boyd for her kind help and fruitful discussions.

In addition I wish to express my sincere thanks to all staff and colleagues for their help in various ways.

I am extremely grateful to Jordan University of Science and Technology (JUST), and the British Council for financial support during the period of this study.

Finally my sincere and heartfelt thanks go to all my family, particularly my wife for her patience and encouragement during my study.

Contents

Summary

The work described in this thesis is principally an investigation into the fate and possible transformations of the aniline-based herbicides or phenylamides with particular reference to chlorpropham in potatoes. This xenobiotic chemical also known as isopropyl N-3-chlorophenylcarbamate or CIPC, has been widely used since the early 50's as a selective herbicide. It is also used on staple food-potatoes, which are often eaten without peeling, to suppress sprouting during storage.

Special attention has been focussed on three aspects including: (1) Analysis of the peel of laboratory and commercially treated and stored potatoes for residual chlorpropham and three of its potential metabolites, (2) Photolysis of chlorpropham in aqueous and organic solvents, and (3) The effect of horseradish peroxidase, HRP, system on chlorpropham and related anilines.

A comprehensive review of the literature pertaining to the environmental fate of phenylamides revealed that chemicals belonging to this group all have in common an amide linkage which is susceptible to hydrolysis at least enzymatically especially where no organophosphorus or methylcarbamate insecticides exist. Hydrolysis of the aniline-based herbicides produces aniline or substituted aniline(s), which are very unstable and may undergo various competing reactions with the possibility of forming ultimately more or less toxic

derivatives than their precursors.

In chapter three analytical methods with high precision and good overall recoveries were developed which allowed simultaneous determination of chlorpropham and three suspected metabolites viz. 3,3^{-}-dichloro-azobenzene, 3,3^{-}-dichloroazoxybenzene and 4-methoxy-chlorpropham. Some effort was given to synthesising and identifying these metabolites. Quantification of the chlorpropham was made possible by GC-ECD or GC-FID, while for the azo and azoxy derivatives quantification was best made only by GC-ECD and for the methoxy metabolite by GC-FID. The minimum detection limits were 4, 8, 8 and 20 ng g^{-1} for the chlorpropham, the azo, azoxy and methoxychlorpropham respectively.

Analysis results revealed on the one hand the existence of significant amounts of chlorpropham in potato peel, on the other hand, that none of the analysed peel samples from laboratory and commercially treated and stored potatoes contain any of the questionable metabolites within the detection limits already stated even after long periods of storage.

The photolysis investigation in chapter four demonstrated that chlorpropham is a photolabile chemical especially under the uv-lamp. The rate of its photo-transformation followed first order kinetics with the highest rate at the lowest concentration, and was affected by the nature of the solvent in the order, water>hexane>methanol.

The principal pathways of chlorpropham photolysis

in the various media were reductive dechlorination to the more stable propham, solvolysis and coupling or dimerization with the formation of chlorpropham-propham or propham-propham biphenyl dimers.

Finally, the enzymatic work in this study clarified that chlorpropham did not respond to the peroxidase even after a long period of incubation, similar to the behaviour of nitroaniline and sulfanilamide, probably because chlorpropham hydrolyses too slowly if at all in the buffer media of the enzyme system providing not sufficient chloroaniline to react with the enzyme. The susceptibility of other tested anilines to react with HRP especially where no efficient reducing agent such as Na_2SO_4 or ascorbic acid was present, correlated in a positive manner with the electron densities on the nitrogen atoms. Susceptibilities were in the order: P-anisidine> 2-amino-p-chlorophenol≥3-chloro-p-anisidine>> 3-chloroaniline.

A complex mixture of products and oligomers such as substituted acylanilide, azo, azoxy, anilino azo, diphenylamine, benzoquinone and phenazine type compounds were successfully isolated from the reaction media by thin layer chromatography and eventually identified by GC-MS and/or mass spectroscopy. The mass spectra of forty compounds are reported.

In the light of these findings, together with the controversial and/or undiscovered toxicity of chlor-propham and many of the identified products, it may be

adviseable to stay alert to the possibility that such metabolites may contaminate the environment and to suggest that chlorpropham treated potatoes should as a first precaution be peeled before consumption or processing.

CHAPTER ONE

Introduction and Thesis Objectives

1.1 Introduction

As the population of the world is ever increasing, with an estimated figure of 6.35 billion forecast for the year 2000 (Moore, 1986), adequate food for the exploding population becomes more critical. Otherwise starvation and malnutrition are inevitable.

Food production can be enhanced by using improved plant varieties together with crop rotation, or else by using agrochemicals viZ. fertilizers and pesticides. Fertilizers are usually used in the proper amounts to supply or replace the removal of soil nutrients. On the other hand, pesticides are used in recommended doses to save crops from competing weeds and grasses, and to protect plants from the attacking fungi and pests.

Despite the undoubted benefits accruing to the use of agrochemicals, there are various drawbacks, e.g. their inevitable residues, their persistence and degradation into more hazardous compounds, together with acute and chronic toxicity, especially to consumers and non-target organisms. All of these potential consequences have created public concern and promoted governments' awareness leading to better regulation, monitoring and control of pesticide residues especially in food commodities to insure that their use does not affect public health.

Aniline-based herbicides are the most common herbicides in current use. Two main problems are often associated with their widespread use and application. These include the residual contamination which they leave and their degradation in the environment with the ultimate release of the active aniline or substituted anilines. Some of the aromatic amines and/or their condensation products are known to have undesirable consequences, and may constitute serious health hazards, including the possibility of cancer and mutations.

Recognition of the impacts, and the existence of these chemicals as environmental pollutants, consititutes an obligation for further investigation appropriate to their ultimate fate and behaviour.

As chlorpropham, a member of the aniline-based herbicide group with plant regulating property, is regularly and directly used on a staple food item such as potatoes, to suppress sprouting during storage, it is advisable if not essential to reinvestigate its terminal residues, and upgrade data related to its use. The need for these is unquestionable for the safety and risk assessment. However, instead of re-evaluating and updating data for chlorpropham and its related propham, for the Codex Commission, these chemicals are to be considered as new, and full data are due to be submitted by 1992 (Anon, 1988). Hence, the work undertaken here has immediate relevance.

1.2 Thesis Objectives

This thesis is principally an investigation in the environmental fate and possible transformations of aniline-based herbicides with particular reference to chlorpropham. Toward achieving this objective, several steps were set up and divided as follows:

Chapter one set out to introduce the subject and clarify the objectives of the work to be undertaken.

Chapter two comprised a review of the available literature pertaining to the environmental fate of aniline-based herbicides. The aim behind this was to build up a solid background on the subject and to provide sound information crucial to an understanding of the environmental behaviour of the aniline-based herbicides and to evaluate their potential hazards. Special attention was given to the various factors which affect the fate and dissipation of these chemicals and their potential intermediates.

Chapter three dealt with the analysis of potato peel for chlorpropham and three of its suspected metabolites viz. 3,3^{-}-dichloroazobenzene, 3,3^{-}-dichloroazoxybenzene and 4-methoxychlorpropham. The objective behind this was to build up a picture of chlorpropham concentration regularly found in commercial treated potatoes and to ascertain whether the mentioned metabolites have been formed and if so to what extent. Considerable effort was put into the synthesis and identification of the three mentioned metabolites.

Chapter four was geared at investigating the photolability of chlorpropham in aqueous and organic solvents, and characterizing the various photoproducts formed. As field photochemical transformation can be difficult to repeat or to interpret, due to the interaction of various environmental factors, this has resulted in a preference for a laboratory photolysis investigation.

The aim of chapter five was to study the effect of a model peroxidase system on chlorpropham and related substituted anilines. This study was set up on the basis that environmental fate studies need not consider only the degradation of the parent molecules but also the ultimate fate of the intermediate products as well. Added to this were the reports of Bartha et al. (1968) and Bordeleau and coworkers (1972) which stated that the metabolites of substituted anilines and/or their parent herbicides in the soil and in peroxidase systems are similar if not the same. An attempt was made to clarify this point.

Finally with regard to implications of the findings and the general outcome of the study, conclusions were drawn and made clear at the end of each individual chapter rather than grouped together at the end.

CHAPTER TWO

Environmental Fate of the Aniline-based Herbicides

The work in this chapter comprised the literature pertaining to the environmental fate of the aniline-based herbicides. The objectives were to build up a firm background appropriate to the subject and to provide sound information necessary to evaluate the potential hazards of these herbicides. Emphasis was put on the various factors affecting the dissipation and fate of these chemicals. The formation and transformation of substituted anilines also has been thoroughly reviewed.

2.1 Introduction

Aniline-based herbicides or conventional phenylamides comprise a large number of chemicals including the acylanilides, phenylcarbamates (carbanilates) and substituted phenylureas (ureides) (Kaufman, 1973; Cripps and Roberts, 1978). Altogether they constitute a substantial portion of the currently used herbicides. Several additional chemicals might be added, if one wishes to consider toluidines and nitro compounds which degrade ultimately to aniline or substituted anilines.

Aniline-based herbicides all have in common an aniline or substituted aniline moiety and a carbonyl bond with imino hydrogen similar to the peptide linkage in protein and penicillin. They are structurally related

with the formula of, Ar-NHCOR, where Ar represents an aromatic or phenyl moiety with or without additional substituents; R stands for alkyl or phenyl moiety in the acylanilide family, aloxyl group in the carbanilate family or dialkylamine in the ureide family.

The importance of these chemicals as successful herbicides came as a result of their effectiveness and selectivity, particularly at low level concentration (Eshel and Warren, 1977). Added to this they are relatively stable under normal conditions of storage and application, and all have low mammalian toxicity (Hance, 1965). In the environment, phenylamides are not persistent as compared to the organochlorine compounds, because they are bio- and photochemically degradable, especially in the absence of organophosphorus or methylcarbamate insecticides (Kaufman et al., 1970; Hassall, 1982).

Examples of the phenylamides, accompanied by their trivial and chemical names, and some of their physical properties are presented in table 2.1 (Hartley and Kidd, 1983; Suntio, 1988).

Table 2.1 Examples of the common phenylamides and their physical properties

Names: Trivial; chemical	mp,°C	S	VP	$\log K_{ow}$	$LD_{50} \times 10^3$
Propanil, stam; N-(3,4-dichlorophenyl)-propanamide	91-93	300	0.0005	2	1.4
Solan, pentanochlor; N-(3-chloro-4-methylphenyl)-2-methyl pentanamide	85-86	8-9	very low	-	>10
Propham, IPC; isopropyl N-phenylcarbamate	87-88	250	sublime	2.38	>4.4
chlorpropham, CIPC; isopropyl N-(3-chlorophenyl) carbamate	40.7-41.1	100	0.001	3.1	5-7.5
monuron, CMU; N-(4-chlorophenyl)-N^-, N^--dimethylurea	176-177	200	0.003	1.8	3.7
Diuron, DMU; N-(3,4-dichlorophenyl)-N^-, N^--dimethylurea	158-159	40	0.0002	2.6	3.4-3.6

mp = melting point

K_{ow} = octanol/water partition coefficient

VP = vapor pressure (pascal at 20°C)

LP_{50} = rat acute oral toxicity (mg/kg)

S = solubility (g/m^3 at 20°C)

2.2 Synthesis

Aniline-based herbicides may be synthesised by reacting aromatic amines or their respective isocyanates with the appropriate reagent (Sweig, 1964; Maier-Bode and Hartel., 1981) according to the following equations:

$$Ar\text{-}NH_2 + ClCOR/(RCO)_2O \xrightarrow{OH^-} Ar\text{-}NHCOR + HCl \quad \text{---- (2.1)}$$

$$Ar\text{-}NH_2 + ClCOOR \xrightarrow{OH^-} Ar\text{-}NHCOOR + HCl \quad \text{--- (2.2)}$$

$$Ar\text{-}NCO + ROH \longrightarrow Ar\text{-}NHCOOR \quad \text{-------- (2.3)}$$

$$Ar\text{-}NCO + HNRR^- \longrightarrow Ar\text{-}NHCONRR^- \quad \text{------ (2.4)}$$

2.3 Mode of Action

The mode of action of the phenylamide herbicides in susceptible or sensitive plants, after being absorbed and translocated, is principally based on their ability to interfere with or inhibit biosynthetic systems, such as photosynthesis, oxidative phosphorylation, cell division, RNA and protein synthesis (Ashton and Crafts, 1973; Geissbuhler et al., 1976; Mitsunake et al., 1986). In doing so, phenylamides cause cell destruction and hence prevent susceptible plants from growth and/or starve them to death. However, in tolerant or resistent species, the parent herbicides are readily hydrolyzed or biodegraded into water soluble products, followed by conjugation with the endogenous plant constituents such as carbohydrates, proteins and/or glutathione (Smith and

Sheets, 1967; Ashton and Crafts, 1973).

In this context, it is worth mentioning that methylcarbamates and/or organophosphorus insecticides compete with the phenylamides for the hydrolysing enzyme, thereby increasing the persistence and the phototoxicity of these herbicides (Kufman et al., 1970; Hassall, 1982). However in animals the insecticides deactivate choline esterase, resulting in the accumulation of acetylcholine and hence block the transmission of the nerve impulses (Hassall, 1982).

2.4 Factors Affecting the Fate of Phenylamides

A multiplicity of physical, chemical and biological processes in the environment govern the fate and behaviour of xenobiotics in general, resulting finally in a residue consisting of the original parent chemical and/or various metabolites. Apart from the biological factors which play the key role in the mineralization and transformation of xenobiotics, other influencing factors, which are interrelated, can be classified into two categories (Lichtenstein, 1972; MacKay, 1980). The first includes physico-chemical, formulation and synergists; while the second represents the environmental factors and the agricultural techniques.

2.4.1 Physico-chemical Factors

The availability, movement and rate of degradation of the herbicides are generally specific to

their physiochemical nature (Hartley and Graham-Bryce, 1980). Formulation type and adjuvants are also of value. Three of the major influencing physico-chemical properties which are often utilized as predictive tools for environmental behaviour of the xenobiotics are highlighted below.

2.4.1.1 Water and Lipid Solubility

Water solubility of a chemical is an intrinsic physical property, and is basically determined by saturating distilled water with the chemical, and after equilibration at a specific temperature, the analyte is measured by a suitable technique and expressed as mg DM^{-3} or part per million (ppm).

Solubility values may serve as useful indicators for leaching and partitioning out of water into sediments and/or biota (Haque et al., 1980; Mill, 1980a).

The water solubility of the phenylamides is relatively low and decreases as the number of halogen substituents in their structure increases (table 2.1). Consequently these compounds are liable to partition out from water and accumulate in biota. This attitude is best described as octanol/water partitioning and expressed as K_{ow}.

K_{ow} is defined as the relative solubility of the chemical in pure octanol, C_o, to that in water, C_w, i.e.

$$K_{ow} = C_o/C_w \quad \text{----------} \quad (2.5)$$

Experimentally, this property can be determined as described by Fujita *et al.* (1964) by mixing known volumes of

dilute octanol solution of the chemical with distilled water, and after shaking gently, to avoid emulsion, and equilibration at a certain temperature, the concentration of the analyte in water is measured and that in octanol is deduced by difference. More recently Weith and coworkers (1979) described a rapid method for estimating K_{ow} by using an HPLC technique.

Various correlations have been observed between the solubility (S), K_{ow}, bioconcentration factor (K_{BCF}), absorption coefficient onto organic carbon (K_{oc}), melting point (MP) and ecological magnification (EM). Several authors (Neely et al., 1974; Lu and Matcalf, 1975; Mill, 1980a; Briggs, 1981; Yalkowsky, 1983) reported the following regression correlations respectively.

$$\log BCF = 0.524 \log K_{ow} + 0.124 \quad \text{---------} \quad (2.6)$$

$$\log EM = 0.6335 \log K_{ow} + 0.7285 \quad \text{--------} \quad (2.7)$$

$$\log K_{oc} = -0.782 \log [S] - 0.27 \quad \text{---------} \quad (2.8)$$

$$\log K_{oc} = 0.52 \log K_{ow} + 0.62 \quad \text{---------} \quad (2.9)$$

$$\log K_{ow} = -\log [S] - 0.01MP + 0.7 \quad \text{-------} \quad (2.10)$$

These correlations facilitate the estimation of the value from the others (Hague et al., 1980).

2.4.1.2 Volatilization

The mass transfer of a substance from open surfaces to the vapour phase into the atmosphere is a well-known phenomenon, especially for volatile substances with relatively high vapour pressures such as alcohol or

kerosine. However, many chemicals such as DDT and phenylamides, despite their low vapour pressures, low water solubilities and low polarities, were lost from open surfaces via volatilization at rapid rates, owing to their high activity coefficients in solution, which in turn enhance their effective concentrations (Spencer et al., 1973; Mill, 1980a).

The magnitude of vaporization is affected positively by the temperature, water solubility and the air flow rate. MacKay and Wolkoff (1973) showed that an estimation of the air/water partitioning coefficient (H) can be obtained from the equation:

$$H = P/[S] \quad \text{---------- (2.11)}$$

where P, represents the vapour pressure of the chemical in mm Hg and [S] stands for molar solubility.

Adsorption onto the soil and sediments influence the evaporation negatively because it lowers the partial pressure of a chemical (Spencer and Farmer, 1980). However, Parochetti and Warren (1966) reported that moisture in the soil competes with the xenobiotics for the available sorption sites and hence increases their dissipation through volatization. Spencer et al. (1973), Spencer and Claith (1975) demonstrated that soil incorporation, granulation, plant cover and high relative humidity, all exert negative effects on volatilization.

2.4.1.3 Adsorption

The attachment of chemicals onto soil constituents is governed by the sum of physical and

chemical factors (Stevenson, 1976; Huang, 1980). The strength of the adsorption is affected by various factors including the nature of the adsorbate and the adsorbent, temperature, pH, moisture content and cation exchange capacity (Hsu and Bartha, 1976; Huang, 1980). Hance (1965) and Hague et al. (1980) revealed that the adsorption increases with the increase of the hydrophobicity of the adsorbate and/or with the increase of the organic content of the adsorbent. However, Parachotti and Warren (1966) demonstrated that the water solubility, the temperature and the soil moisture content especially above the sorption limit all inhibit the sorption of chemicals in a negative manner.

It is noteworthy that adsorption of chemicals is an equilibrium process (Osgerby, 1973; Khan, 1980), where the solute partitions itself between soil and water. The process can be described by the Freundlich isotherm equation, which states that, at equilibrium, the amount of the adsorbate adsorbed per unit mass of the adsorbent (C_s) in a unit of $\mu g\ g^{-1}$, is proportional to the concentration of the adsorbate in solution (C_w) in $\mu g\ cm^{-3}$.

Mathematically, the Freundlich isotherm is represented (Hague et al., 1980) as:

$$C_s = K(C_w)^n \quad \text{----------} \quad (2.12)$$

where K and n are empirical constants, the first describes the capacity of the adsorption and hence provides indications for leaching and volatilization

potentials and n describes the nature of sorption.

The isotherm constants K and n can be deduced from the fitted linear relationship of log C_s v log C_w where n and log K are represented by the slope and the intercept of the fitted line respectively.

For dilute solutions, it has been found by Mill (1980a) that the distribution of a hydrophobic substrate between the soil and water was constant and obeys the relation:

$$K_d = C_s/C_w \quad \text{----------} \quad (2.13)$$

where K_d stands for adsorption coefficient, and can be determined by mixing one gram of the soil with multiple known concentrations of the adsorbate in 100 cm^3 aqueous solution (0.01M $CaCl_2$). The suspension is then allowed to attain equilibrium by shaking for some time (8-16h). Finally, the equilibriate mixture is centrifuged and the remaining analyte in the various solutions is estimated by a suitable technique against a soil blank (Briggs, 1981).

The amount of K_d for a single substance may vary from one soil to another. To account for this and to place soils and sediments on a nearly equal basis, Mill (1980a) and Briggs (1981) used the formula:

$$K_d = K_{oc}.A \quad \text{----------} \quad (2.14)$$

where A represents the fraction of organic content in the soil or sediment (mg adsorbate/mg sorbent) and K_{oc} stands for the sorption constant corrected for the organic

content.

The general adsorption trend of the phenylamides on suspended bentonite clay as revealed by El-Dib and Aly (1976) was found to be in the order:

phenylureas>phenylcarbamates>acylanilides

El-Dib and coworker also found that the adsorption of the phenylamide herbicides conform with the Freundlich isotherm. Rajagolal (1984) and references therein showed that activated carbon was the most effective adsorbent in deactivation of pesticides including chlorpropham and 2,4-D in particular. Several workers (Hsu and Bartha, 1974; Bartha and Hsu, 1976) demonstrated that chloroanilines and their condensation products were adsorbed firmly, especially at low concentrations, onto soil constituents forming non-exchangeable residues in the forms of hydrolysable and nonhydrolysable residues.

Finally it is worth mentioning that adsorption of chemicals onto the clay or humic matter may catalyze their degradation (Saltzman et al., 1976), or it may slow down their dissipation and/or transformation over a long period of time (Stevenson, 1976; Bartha, 1980).

2.4.2 Environmental Factors and Agricultural Techniques

The persistence and the fate of xenobiotics in the environment are usually influenced by their direct interaction with their surroundings, although indirect influences may be equally important.

Lichenstein (1972) and Hill and Wright (1978) reported the following influencing factors:

1 - Climatic factors of temperature, precipitation, humidity and sunshine.

2 - Soil type and properties, including oxygen status (aerobic or anaerobic), the pH, clay, organic matter and moisture content.

3 - Count and species of microorganisms and biota.

4 - Methods, rates and frequency of application.

5 - Cultivation techniques and irrigation.

Kaufman (1967) and Anderson and Lichenstein (1971) revealed that environmental factors of warmth, moisture and nutrients which are in favour of microbial growth also are in favour of xenobiotic degradation. This conclusion came from the observed extended activity of herbicides in sterile soils as compared to that in nonsterile counterparts.

2.5 Biological and Non-biological Transformations

Although pesticides, of which phenylamides constitute a major portion, are usually dissipated and transported from one environmental compartment to another, via a sum of interchangeable processes such as leaching, run off, washing, volatilization, adsorption and biota uptake, the remaining portion of these chemicals in the soil, air, water and/or biota are often subject to biological, chemical and photochemical effects capable of causing various transformations in their structures (Hill and Wright, 1978). The term transformation, however, encompasses any change or alteration in the chemical structure, whether degradative

or additive for the purpose of activation or detoxification (Hill, 1978). Bollag (1974) and Hill and Wright (1978) pointed out that transformation products of a chemical in the environment are often the same or similar, and it is not easy to distinguish which action has caused a specific transformation, since different factors may act upon chemicals separately or simultaneously.

Before discussing the environmental metabolism of phenylamides in particular, it may be of value to consider the general potential pathways of biological and non-biological transformations of the pesticides.

2.5.1 Non-biological Transformations

2.5.1.1. Chemical Transformations

Once a xenobiotic e.g. a phenylamide herbicide finds its way into the environment deliberately or accidently, some may volatilize in the atmosphere, whereas the bulk may come in contact with water, soil and/or sediment which may eventually act as a sink for it (Hague et al., 1980). The most important non-biological processes that may act upon a chemical include hydrolysis, pyrolysis, oxidation-reduction and photolysis (Mill, 1980; Draper and Wolfe, 1987)

2.5.1.2 Abiotic Hydrolysis

Hydrolysis of organic compounds such as esters, amides or nitrites results in the introduction of a hydroxyl group into the chemical with the loss of a

leaving group (Mabey and Mill, 1978). In the environment non-biological hydrolysis is slow and negligible as compared with the enzymatic one. However, this can be enhanced by the involvement of OH^- or H_3O^+. In soil, although sorption to humic matter and clay may be regarded as a kind of sequestering to a certain extent, metal ions $[M]^{x+}$, such as Cu^{2+}, Ca^{2+} and Zn^{2+} and/or their ligand aqua complexes $[M(H_2O)(OH)L]^{x+}$, act as carriers for H_2O or OH^-, thereby catalyzing the hydrolysis process (Saltzman, 1976; Mill and Maybe, 1988).

As all members of the phenylamides contain principally an amide group together with or without an ester linkage, the hydrolysis of the carbamoyl bond or the ester linkage preferentially results in the formation of the aniline precursor as follows:

$$Ar\text{-}NHCOR + HOH \xrightarrow{H_3O^+/OH^-} Ar\text{-}NH_2 + RCOOH \quad \text{--- (2.15)}$$

Several workers (Wolfe et al., 1978; Hartley and Graham-Bryce, 1980; Wolfe, 1980) pointed out various factors such as steric, inductive, pH, temperature, water solubility and catalytic activity of the media which have a direct influence on the rate of hydrolysis. El-Dib and Aly (1976) indicated that phenylamides are expected to maintain their stability in natural water, as they hydrolyse very slowly in the following order:

phenylcarbamate>anilides>phenylureas

2.5.1.3 Pyrolysis

Pyrolysis or cleavage by heat is a result of thermal instability, expecially at high temperatures. Although phenylamides are stable at normal conditions of application and storage (Hartley, 1983), at temperatures around 200°C as in gas chromatography or mass spectrometry, these compounds show thermal instability and resort to pyrolysis (Mukaiyama and Hashino, 1956; Rogmagnoli and Bailey, 1966).

Pyrolysis of the phenylamides proceeds in a reverse manner to their synthesis giving signals that correspond to the respective arylamine or arylisocyanate. The dissociation may occur at any of the linkages attached to the carbonyl group (Onuska, 1985). It is noteworthy that the arylisocyanate which may be formed is not stable and may undergo secondary reactions with alcohol, water or amines giving urethane, arylamine or phenylurea respectively (Dyer and Wright, 1959).

Arylisocyanates and/or their parent phenylamides hydroxylated ortho to the nitrogen atom, may undergo thermal intramolecular cyclization giving the corresponding benzoxazolinone or benzoxazoline respectively (Still and Mansager, 1972; Fletcher and Kaufman, 1979).

Thermal degradation of salts of chlorinated phenols by comparison resulted in the formation of high yields of dioxins (Rappe, 1980).

2.5.1.4 Oxidation Reactions

Oxidation of chemicals in the environment usually requires oxygen, but almost never involves direct reaction with it, because oxygen and other reacting species are in different states (Mill, 1980).

In the aquatic media and the atmosphere, oxidation of pollutants and chemicals requires active oxidants such as singlet oxygen ($^1O_2$) or O (^1D), ozone (O_3) or free radiacls e.g. $OH^\cdot$, $RO^\cdot$, $RO_2^\cdot$. The generation of these oxidants in the atmosphere can be visualized as:

$$NO_2 \xrightarrow{h\nu} NO + O(3p) \quad \text{---------- (2.16)}$$

$$O(3p) + O_2 \xrightarrow{M} O_3 \quad \text{---------- (2.17)}$$

$$O_3 \xrightarrow{h\nu} O_2 + O(^1D) \quad \text{---------- (2.18)}$$

$$O(^1D) + H_2O \longrightarrow 2OH^\cdot \quad \text{---------- (2.19)}$$

$$O(^1D) + O_2 + RH \longrightarrow OH^\cdot + RO_2^\cdot \quad \text{---------- (2.20)}$$

$$RO_2^\cdot + NO \longrightarrow RO^\cdot + NO_2 \quad \text{---------- (2.21)}$$

In natural water, sensitized photolysis of dissolved and suspended matter with production of either hydrated electrons (e_{aq}) or excited sensitizers leads to the formation of singlet oxygen, $OH^\cdot$ and $RO_2^\cdot$ (Zepp et al., 1977; Drapper and Wolfe, 1987).

The involvement of the various mentioned oxidants depends on their concentration, relative stability and selectivity. In the atmosphere, though the concentration

of OH· is very limited, ca. 3.4×10^{-15}M, it represents the principal oxidant for many organic chemicals but not for haloalkanes, alcohols and esters (Mill, 1980). In comparison, O_3, which averages at about 1.7×10^{-9}M in the lower atmosphere (Mill, 1980) is only important in the oxidation of olifens and some sulphur or phosphorus compounds. On the other hand singlet oxygen is a selective oxidant. It initiates the formation of other free radicals from arylamines or phenols which then undergo oxidative coupling reactions, or else $^1O_2$ and similarly OH· may add to olefins and/or aryl moities (Mill, 1980; Parris, 1980; Fishbein, 1984). Free radical attack to alkyl moieties, produces aldehydes and alcohols.

In chlorine water, anilines and phenols yield principally chloroanilines and chlorophenols together with quinones, quinoneimines or indoanilines ($HO\text{-}C_6H_4\text{-}N{=}C_6H_4{=}NH$) but no chloramines as expected (Fishbein, 1984).

2.5.1.5 Photochemical Reactions

Photochemical reactions of organic compounds in the environment are brought about directly or indirectly by absorption of solar radiation, with maximum effect at the shortest wavelength of the spectrum. The cut-off for the solar spectrum by ozone in the upper atmosphere is about 290 nm (Crosby and Li, 1969). Consequently, only absorption of photons at this or longer wavelenghth in the environment can result in direct photolysis, especially over an extended period of exposure. However

many compounds which do not absorb or absorb very little in the uv-visible region violate this trend and undergo photodecomposition as in the case of ureides and 2,4-D, presumably due to changes in their absorption spectrum in the field, or due to the presence of natural photosensitizers (Rosen, 1972). In this context Parlar (1980) revealed that the absorption behaviour of certain compounds changed markedly in the adsorbed state. One such change may be a red shift and an increase in the intensity of specific absorption bands, e.g. the absorption peak for photodieldrin recorded at 193 nm in hexane was displaced to 264 nm on silica gel.

The absorption of light radiation with subsequent excitation may eventually result in various chemical changes including reductive dehalogenation, oxidation, nucleophilic substitution, isomerization, dimerization and/or polymerization (Menzie, 1988; Mill, 1980a).

Clarifying examples and more discussion on the phototransformation of the phenylamide herbicides are detailed in chapter four.

2.5.2 Biological Transformations

2.5.2.1 General Consideration

Although significant alteration in structure and behaviour of xenobiotics in the environment result from non-biological processes, the principle and often the most important reactions by which the majority of organic compounds are transformed and/or mineralized is biological. This is justified from the fast degradation

in non-sterile soils, microbial and enzymatic cultures as compared to that in autoclaved or sterilized media (Freed, 1951; Parochetti and Warren, 1966; Bartha and Pramer, 1970).

Microbial and biochemical transformations comprise a group of enzymatically mediated reactions carried out by living organisms or biota. They occur to a more or less degree via similar routes, although significant differences in terms of rates and extents exist, depending on the physico-chemical structure of the chemical and its concentration. Also it depends on the microbial population and other environmental factors (Kaufman, 1967).

Generally, biotransformations occur through two phases. In phase one xenobiotics undergo such alteration reactions as oxidation, reduction, hydrolysis and hydroxylation, resulting in the formation of polar metabolites which may be less or more toxic than their parents. This process involves mixed function oxidases (MFO), which catalyse incorporation of oxygen in the presence of a hydrogen donor such as reduced nicotinamide adenine dinucleotide (NADH) or its phosphate counterpart.

In phase two, products of phase one undergo secondary reactions or conjugations with endogenous matter such as carbohydrates, amino acids, sulphuric acid or glutathione (GSH), forming eventually water soluble conjugates, thus facilitating its incorporation or storage as glycosides or lignin complexes. However, conjugates in animals are usually excreted as glucuronide

or sulphate esters.

Conjugation in this way blocks the nucleophilic sites of xenobiotics and prevents them from binding with the electrophilic centres on DNA, RNA and proteins (Morrison and Cohen, 1980; Menzi, 1978).

In this field, it is noteworthy that certain microorganisms have the potential to mineralize or cometabolise some organics such as phenylamides as nutrients and/or energy sources (Clark and Wright, 1970; Bollag, 1974; Marty et al., 1986).

What follows is a summary of the potential routes of biodegradation which are relevant to phenylamides and similar compounds.

2.5.2.2. Reaction Types

2.5.2.2.1 Hydrolysis

Enzyamtic incorporation of water molecules into organic structures is well known for numerous pesticides (Kearney, 1965; Engelhardt et al., 1972; Menzi, 1988). The incorporation results in the cleavage of a suitable bond such as ester, amide, alkylhalide or nitrile bond. Chlorpropham, hydrolysis enzymatically to m-chloroaniline via carbamic acid or arylisocyanate intermediates (Still and Herrett, 1976). Ureides and acylanilides hydrolyse similarly to the respective arylamines (Geissbuhler, 1969; Bartha et al., 1971; Hill, 1978).

Practical studies on this aspect revealed that the type, number, size and position of the substituents on the ring and/or the side chain of phenylamides affect

the stability of the hydrolysable carbonyl bond and influence the rate of their degradation. Kearney (1967) and Sharabi and Bordeleau (1969) pointed out that the more electrophilic the substituents, the more susceptible was the hydrolysable bond to nucleophilic attack by water. The authors also revealed that bulky groups may sterically hinder the nucleophilic approach. Kaufman et al. (1970) demonstrated that the organophosphate and methycarbamate insecticides compete with phenylamides for the hydrolysing enzymes, thereby increasing the persistence and residual activity of these herbicides.

2.5.2.2.2 Oxidative Dealkylation

The stepwise removal of alkyl groups attached to nitrogen or oxygen atoms in organic structures has been reported as a first step for detoxification of numerous chemicals such as ureides, methylcarbamates and triazenes (Dalton et al., 1966; Geissbuhler and Voss, 1971).

Dealkylation with eventual yield of phenols or arylamines proceeds via N-methylol or hydroxymethyl intermediates. The process entails incorporation of an oxygen atom in the presence of MFO and NADH as hydrogen donors (Kearney and Helling, 1969).

2.5.2.2.3 Hydroxylation

The insertion of a hydroxyl group into aromatic or alkyl moieties is of practical importance and is necessary for conjugation and/or ring opening (Dagley, 1972; Hill, 1978). The rate and position of the hydroxylation depends upon the type, number, size and

position of the substituents on the ring. The most suitable positions for hydroxylation are para- and/or ortho- to a lesser extent. Aromatic moities with halogen substituents undergo hydroxylation by displacement of the hydrogen or the halide atom (hydrolytic dehalogenation) or by substitution with concomitant p-halide migration to an adjacent free position. Such intramolecular halide migration induced by hydroxylation is often termed an NIH-shift (Guroff et al., 1967; Still and Mansager, 1972).

In comparison to carbon hydroxylation, the N-hydroxylation of the amides or haloanilines which has been reported in soils and animals (Irving, 1964; Kaufman et al., 1973), seems to be of only minor significance in plants (Still and Herrett, 1976).

2.5.2.2.4 Nitro- and Azo- Reduction

Aromatic nitrocompounds in oxygen poor media, such as soil sediments, flooded water and intestine are susceptible to reduction to the corresponding amine probably via nitroso and hydroxylamine labile intermediates, in the reverse manner to amine oxidation under aerobic conditions (Kaufman et al., 1973; Bollag, 1974; Parris, 1980).

Similarly the azo and azoxy compounds under anaerobic conditions or *in vivo* may be reduced to the respective amines or aminophenols. Prontosil, for instance, the first antibacterial azodye has been reduced to sulfanilamides (Miyadera, 1975).

Ryan and coworkers (1958) and Weber (1988) reported that azo and azoxy reduction proceeds via the respective hydrazo intermediates. Also Dobin and Wright (1975) revealed that the reduction exhibited zero order kinetics and suggested that the reaction was extracellular and non-enzymatic.

2.5.2.2.5 Dehalogenation

The enzymatic removal of halides from aliphatic and aromatic moieties is an important step in the biodegradation. The process involves reductive dehalogenation and/or hydroxy replacement with or without halogen migration (NIH).

In this context, Hill (1978) and references therein revealed that m-chloroanilines metabolize readily in contrast to 3-chlorophenols and 3-chloroanisoles, which reflects the ease with which hydroxylation of the first may proceed as a prerequisite for catechol formation and ring opening. Also, bromo-substituted analogues in soils are more persistent than chlorosubstituted analogues, probably because they are strongly adsorbed which in turn inhibits their availability to microorganisms.

2.5.2.2.6 Conjugation and Complex Formation

Conjugation of xenobiotics and/or their metabolites with the endogenous constituents of the biosystem or soil organic matter constitute a major route for their detoxification (Tweedy et al., 1970; Morrison and Cohen, 1980). The conjugation of a chemical usually

proceeds after an acquisition of a polar group such as -OH, $-NH_2$ or -COOH.

Hsu and Bartha (1974; 1976) and Bartha (1980) demonstrated that substituted aromatic amines and phenols have the capability to bind firmly with humic matter in a reversible manner forming unextractable terminal residues in the form of hydrolysable and nonhydrolysable matter. Examples of the first are anils (Shiff's base) and anilinoquinones, and for the others are phenazines and phenoxazines.

Bartha (1971 and 1980) reported that the remobilization and degradation of xenobiotic-humic matter complexes proceeds very slowly and may extend up to 10 years as compared with culture metabolism and, thus may actually mediate crop contamination.

Finally it is of interest to know that acylation and methylation may be regarded as a kind of conjugation and detoxification particularly to arylamines and phenols (Loose and associates, 1967; Kearney and Plimmer, 1972).

2.6 Phenylamides Metabolism

2.6.1 Acylanilides

The metabolism of acylanilides has been reviewed by several workers (Bartha and Pramer, 1970; Matsunaka, 1971; Cripps and Roberts, 1978). Propanil, for instance, the best and most important acylanilide herbicide, metabolizes in soils, plants, animals and pure cultures similarly. It hydrolyses enzymatically to the

corresponding aniline and propionic or lactic acid (Yih et al., 1968; McClure, 1974). Hughes and Corke (1974), however, pointed out that the N-trisubstituted acylanilides were not readily metabolized giving rise only to a small and negligible amount of anilines.

The hydrolysis process is dependent upon the selectivity of the plant or microbial species, and on the specificity of their enzymes which are not equally distributed among different plant species or even in different parts of the plant (Ischizuka and coworker, 1966). Still and Kuziran (1967) for example reported that rice seedlings were at least twenty times more effective in deactivating propanil than barnyard grasses. Also Freer and Still (1968) demonstrated that the degradation of propanil in the leaves proceeded much faster than in the roots.

Bouling and Hodgins (1966) and Freer and Still (1968) reported that the steric and inductive effects caused by the side chain length and the substituents on the aryl moiety influence the activity of the hydrolysing enzymes and the rate of the hydrolysis as well. Also Matsunak (1971) revealed that the activity and the resistence of the acylanilides were enhanced by the organophosphorus and methylcarbamate insecticides.

Propionic and/or lactic acids, the primary metabolites of propanil are often used as growth substrates and further metabolize via the B-oxidation route into CO_2 and H_2O (Still, 1968; Yih et al., 1968). However, the aromatic amine produced undergoes either

incorporation and/or conjugation to organic matter (Bartha, 1971; Kearney and Plimmer, 1972), or else, it may undergo a multitude of competing reactions such as acylation, mineralization, oxidative coupling and polymerization (Parris, 1980; Freitag and associates, 1984).

In an attempt to determine the residual level of substituted azobenzene which might be formed from 2,4-dichloroaniline in rice producing soil treated with high rates of propanil and/or 2,4-dichloroaniline, Bartha et al. (1968), Kearney et al. (1970) and Hughes and Corke (1974) demonstrated that only small amounts of the azo were formed probably due to the strong adsorption of the aniline onto the soil, which in turn restricts its dissipation and its reactions.

Bartha et al. (1968); Bartha (1969); Lieb and coworker (1969) and Still (1969) also revealed that rice plants and barnyard grasses treated with propanil or chloroanilines for two weeks were free from any amounts of chloroaniline condensation products, although rice plants have highly active peroxidase, which were thought to catalyze the condensation of chloroanilines into azobenzene analogues. The reason for that may be attributed to the high specificity of the enzyme for its primary substrate (Lieb and Still, 1969).

In another attempt to determine the tendency of azobenzenes to be absorbed and translocated by the plants, Still (1969) treated 20-day old rice seedlings for twelve days with a saturated solution of

3,3¯,4,4¯-tetrachloroazobenzene in liquid culture, and found that only 0.18% of the total applied chlorobenzene was absorbed and translocated, probably due to the low water solubility of this azo which was only 0.313 μg cm^{-3}.

2.6.2 Phenylcarbamates

As the biodegration of the carbanilate herbicides is concerned Hodgson (1967), Ashton and Crafts (1973), and Still and coworker (1976) reviewed the subject and showed that hydrolysis of these chemicals to the respective aniline and alcohol moieties constitute the major metabolic route in the tolerant plants. Sensitized plants, however, incorporate and/or conjugate the intact herbicides or their modified forms (Still and Mansager, 1971, 1972).

Molecular modification of these chemicals proceeds through hydroxylation of the alkyl side chain and/or the aryl moiety without hydrolysis. The eventual polar conjugates are rarely translocated and remain at the sites in which they were produced as methylol glycoside or lignin complexes (James and Prendivelle, 1969; Still and Mansager, 1973; Cole, 1983).

In soils and microbial cultures, the predominant scheme of degradation occurs through an enzymatic cleavage of the carbamoyl bond with production of arylamine, alcohol and CO_2. The organic products may be co-metabolized or mineralized as sources of carbon or they may undergo secondary reaction and conjugation (Bartha and Pramer, 1969; Clark and Wright, 1970; You

and Bartha, 1982; Marty and associates, 1986).

When ingested by animals phenylcarbamates were readily absorbed from the gut, followed by metabolism and elimination via urine and faeces. Holder and Ryan (1968), Grunow et al. (1970), Bobike and associates (1972) and Paulson (1975) pointed out that the major mode of carbanilate detoxification proceded through hydroxylation of the alkyl and/or aryl moieties followed by conjugation with sulphuric and/or glucuronic acids. A minor route for carbanilate metabolism in animals included hydrolysis with subsequent acylation, hydroxylation and conjugation (Paulson et al., 1972; 1973).

Finally it is worth noting that the formation of N-hydroxy derivatives, although revealed in animals (Irving, 1964) and suggested to be involved in the metabolism of propham and chlorpropham as a route of activation of these chemicals, James and Prendivelle (1969) and Still and Mansager (1973) were not able to isolate these metabolites from plant tissues which suggests that N-hydroxylation is of minor significance in plants.

2.6.3 Phenylureas

A literature survey on the metabolism of substituted phenylurea herbicides (Geisbuhler, 1969; Geissbuhler and Voss, 1971; Ashton and Crafts, 1973; Cripps and Roberts, 1978; Maier-Bode and Hartel, 1981) revealed that ureide transformations in plants, animals and soils have sufficient common features to eliminate

separate discussion in these media, although differences as to the rates and quantities produced have been met. The general and principal route of their metabolism proved to proceed through successive N- or O-dealkylations giving monodemethyl or didemethylated phenylurea via the corresponding methylol or formyl intermediates. The demethylated phenylureas are often subsequently subjected to either hydroxylation and conjugation or to stepwise losses of ammonia and carbon dioxide to produce the respective aniline (Tweedy et al., 1970; Geissbuhler and Voss, 1971; Maier-Bode and Hartle, 1981).

The hydrolysis of the ureides, although reported in soils (Wallnofer and Bender, 1970; Engelhardet et al., 1972), seemed to be of minor significance or very limited in animals (Paulson, 1975).

Several workers (Geissbuhler and Voss, 1971; Paulson, 1975) reported ring hydroxylation to the intact ureides or their demethylated derivatives. The position of the hydroxyl insertion occurred at the ortho position preferentially and was influenced by the steric and inductive effects of the original substituents.

The formation of aniline, although not always been detected, has been generally assumed to be a secondary metabolite. Some workers have noted its presence to a small extent (Dalton et al., 1966; Nashed and Ilinikis, 1970; Wallnofer and Bender, 1970) while others could not trace its formation but felt obliged to suggest its formation, at least in soils (Onley et al., 1968;

Geissbuhler and Voss, 1971).

In light of this evidence the formation of the azobenzene derivatives from phenylurea herbicides must be doubtful. Belasco and Pease (1969) and Geissbuhler (1976) could not trace the formation of azobenzenes in soil specimens from numerous soils previously treated once or more with ureide herbicides in doses common in weed control. This was possibly due to the low level of anilines formed which would not allow any synthesis of azobenzenes.

Analysis of potatoes and carrots from soils treated with linuron for the azo derivatives revealed their absence despite the low level of detection ca. 0.1 mg kg^{-1} (Maier-Bode and Hartle, 1981). In a previous study, Maier-Bode could not trace the absorption of dichloro and tetrachloroazobenzene in dwarf bean plants after pre-emergence application of wettable powder of these chemicals in a greenhouse. Consequently it was pointed out that there was no reason to suspect that azobenzene metabolites might get into food or plant origin from field application of ureide herbicides.

2.7 Formation and Environmental Fate of Aromatic Amines

With the widespread use and distribution of numerous aniline based chemicals and their environmental degradation into aniline or substituted anilines, much interest has arisen as to the formation and fate of these intermediates (Kaufman et al., 1973; Pillai and coworkers, 1982; Fishbein, 1984).

In plants and animals, several researchers (Onley et al., 1968; Still and Manasager, 1969; Rouchaud et al., 1988) reported or assumed the presence of arylamines there at low levels resulting from the hydrolysis of their parent compounds. Others such as Smith and Sheets (1967) and Geissbuhler (1969) could not trace the formation of these metabolites to any extent, probably due to their high reactivity which renders them prone to further rapid conversions and/or conjugations with the endogenous bioconstituents.

In soils, natural water and microbial cultures, aniline-based herbicides are being degraded with the ultimate liberation of free aniline or its substituted analogues. As to the fate and transformations of these intermediates under laboratory and outdoor conditions, a literature survey revealed the following.

1 - In higher plants and animals, aromatic amines are always subjected to various reactions such as N-acylation, N-oxidation or aryl hydroxylation (Bray et al., 1956; Onley and coworker, 1968; Kaufman et al., 1973; Bollag and associates, 1978; Parris, 1980; Fishbein, 1984)

2 - In soils, some of the anilines may be dissipated through volatilization, leaching or mineralization (Still, 1969; Kearney et al., 1970). Viswanathan and coworkers (1978) and Pillai et al. (1982) reported that about one third of the applied chloroanilines has been volatilized into the atmosphere as converted or unconverted products, less than 1% has been

leached, whereas more than two thirds of the applied chemicals have been recognized as unextractable soil-bound residues. Several investigations were made to clarify the nature of these bound-residues. Bartha (1971, 1980); Hsu and Bartha (1974, 1976) and Bollag et al., (1978) demonstrated that arylamines were adsorbed onto soil organic matter and were immobilized in the form of unextractable masked residues as hydrolysable anils or anilinoquinones or as non-hydrolysable phenazines or phenoxazines. As far as their bioactivity is concerned, Hsu and Bartha (1974, 1976), Bartha (1980) and Still et al. (1980) stated that though bound residues are resistent to microbial attacks, they may be remobilized and made available to microbial attacks, but very slowly over a long period of time which may extend up to ten years. Consequently humic conjugates may become sources of crop contamination even if crops were not directly treated with a particular pesticide.

In this context, many investigators (Kaufman, 1967; Clark and Wright, 1970; Briggs and Walker, 1973) showed that only aniline and 3-chloroaniline, especially at low concentrations, demonstrated complete degradation or mineralization via catechol intermediates. Other monochloro and highly halogenated analogues were relatively more stable. The trend of resistence increases as the number of halogen substituents increases as well.

3 - In water, Fishbein (1984) and references therein reported that aromatic amines as they degrade, may act as pollutants in terms of increasing the biological oxygen demand (BOD) and producing ammonia. In chlorinated water, aniline for instance may undergo ortho-chlorination or para-hydroxylation and coupling into indoaniline violet ($HO-C_6H_4-N=C_6H_4=NH$) similar to indophenol (a coupling adduct from phenol and ammonia (Scheiner, 1976).

4 - Concerning the behaviour of aromatic amines in the atmosphere, it has been stated that anilines may act as free radical scavengers and/or ozone formation inhibitors. They can react with singlet oxygen or hydroxy radicals producing the respective arylamino radicals, which further react with more hydroxy radicals or nitrogen oxides (NO_x) according to the following equations:

$$Ar-NH_2 + O(^1D) \longrightarrow Ar-NH^{\cdot} + OH^{\cdot} \quad \text{-------- (2.22)}$$

$$Ar-NH_2 + OH^{\cdot} \longrightarrow Ar-NH^{\cdot} + HOH \quad \text{-------- (2.23)}$$

$$2Ar-NH^{\cdot} + 2OH^{\cdot} \longrightarrow Ar-NHOH + HO-Ar-NH_2 \quad \text{-- (2.24)}$$

$$Ar-NH^{\cdot} + NO_2 \longrightarrow p-O_2N-Ar-NH_2 \quad \text{-------- (2.25)}$$

Also, it has been demonstrated that arylamino radicals, especially at relatively high concentration may undergo oxidative coupling via N-N, N-C or C-C bond formation producing the respective azo and azoxybenzenes, phenazine, semidine and biphenylamine type compounds (Engelhardet et al., 1977; Parris,

1980; and Pillai et al., 1982). However, Roberts and Caserio (1965) showed that the C-C coupling is not likely to occur unless the other two possibilities are inhibited by steric factors in contrast to phenol coupling.

Beside mineralization and/or conjugation of the arylamines in microbial cultures and soils, both natural and sterilized, these chemicals especially at considerable concentration levels, demonstrated several competing reactions with the formation of numerous identified and unidentified products (Chisaka and Kearney, 1970; Kaufman et al., 1973; Engelhardet and coworkers, 1977; Pillai et al., 1982; Feshbein, 1984; Rajogolal and associates, 1984). Of the various modes of transformation which have been described by the previous authors are the following:

a - Coupling of two arylamines with the formation of phenazenes or symmetrical and asymmetrical azo and azoxybenzenes, probably via arylhydroxylamine, nitroso and hydrazo labile intermediates.

b - Condensation of two molecules of a primarily ortho hydroxylated chloraniline with the formation of chlorophenoxazinone.

c - Diazotization and/or coupling of two molecules into§ bis-triazene, azobenzene or biphenylamine. The kind of products produced are dictated by the pH of the media, the presence or the absence of nitrate or nitrite ions and the nature of other substituents on the ring.

d - Condensation of three molecules with the formation of substituted anilinoazobenzene (Plimmer et al., 1970; Bordeleau and Bartha, 1972c).

e - Acylation with the production of formanilide, acetanilide or malonic acid conjugates.

f - Nitrogen or aryl hydroxylation resulting in the formation of arylhydroxylamine or aminophenol (ortho and/or para), iminoquinone, benzoquenone and/or catechol (Briggs and Walker, 1973; Fletcher and Kaufman, 1979).

g - Oxidation of the amino group to the respective nitro, probably via hydroxylamine and nitroso intermediates.

Finally it is of interest to note that the oxidation products of arylamines, though they were primarily identified from soils, microbial and/or enzymatic cultures, the formation of these products were also demonstrated to be possible photochemically (Plimmer and Kearney, 1969; Rosen et al., 1970; Rosen and Seiwerski, 1971; Miller et al., 1980) or via catalysed induced reactions in autoclaved and azide treated soils under aerobic conditions (Hughes and Corke, 1974; Pillai et al., 1982).

It is of equal interest to know also that aniline condensation reactions are concentration dependent and are limited to the availability of the arylamines (Tweedy et al., 1970; Kearney and Plimmer, 1972; Worobey, 1984) probably due to the acylation and binding competing processes (Kaufman et al., 1970).

2.8 Impact of Arylamines and Azoarenes

The concern over arylamine condensation products began some time ago when Bartha and Bramer (1967); Bartha (1968) and Bartha et al. (1968) reported the formation of minor quantities of substituted azobenzene, particularly in soils which received repeated high doses of acylanilides or chloroanilines. This concern was promoted as the formation of these metabolites was being substantiated by Chisaka and Kearney (1970), Bartha (1971) and Viswanathan and coworkers (1978).

In mammals, the biotransformation of arylamines into azo analogues is minor but rare (Miyadera, 1975). However, Katz and associates (1969) and Call et al. (1983) could not trace the formation of azoarenes in rumen fluid and fish respectively after being fed or incubated with chloroanilines or phenylamides. In treated plants, particularly tolerant species, aniline-based herbicides are expected to be hydrolysed into their respective anilines (Still, 1968; Still and Mansager, 1969). The formation of azobenzenes could not be detected in these tissues (Still, 1969; Coxon and Filmer, 1985). However, Viswanathan et al. (1978) and Worobey and Sun (1987) pointed out the presence of very small amounts of azo derivatives in barley and potato peels.

The controversy over the undesirable formation of the azoarene type compounds centres around the following:

1 - The widespread production, use and distribution of the aniline-based chemicals, with the possibility of their being contaminated with azobenzenes (Bunce et al., 1979; Worobey and Sun, 1987) or being degraded with the ultimate release of aniline(s), the precursors for the azo and azoxy derivatives (Worobey, 1984).

2 - Azo compounds are either very rare e.g. cyacasin ($CH_3N=N(O)CH_2O$-glycoside) or do not occur in nature (Miyadera, 1975).

3 - Despite the relative stability of the azo and azoxy derivatives they are degradable to a certain extent to the respective arylamines, some of which are known or suspected to be mutagenic and/or carcinogenic agents (Katz et al., 1969; Fishbein, 1984; Kimmel et al., 1986).

4 - Azoarenes in general are potentially toxic, some have been identified as mutagenes or carcinogens (Weisburger and Weisburger, 1966; Parasad, 1970), others were reported isosteric and structurally related to tetrachlorodibenzodioxin (TCDD) and tetrachlorodibensofuran, all of which are known as potent toxins, teratogenic and/or acnegenic agents and inducers of arylhydrocarbon hydroxylase activity in chick embryoes (Poland et al., 1976; Worobey, 1984).

5 - Azobenzenes and related compounds are biologically active. They can activate selectively the oxidative coupling of glutathione, the intracellular free

radical scavenger and the cofactor of several biosynthesis enzymes, thereby inhibiting protein synthesis and suppressing the bioimmunity (Miyadera, 1975).

Finally, it is worth mentioning that the carcinogenicity potential of some azoarenes such as 4-dimethylaminoazobenzene (butter yellow) and arylamines such as aniline and 2-naphthylamine is correlated to the electron density on the nitrogen atom. These compounds are believed to be bioactivated first into hydroxylamines and/or nitroso intermediates directly or after N-demethylation. The formed labile intermediates are capable of producing nitrinium ions which bind covalently with the nucleophilic sites in proteins, DNA and/or RNA, leading eventually to mutation and tumor formation (Weisburger and Wiesburger, 1966; Hill et al., 1978; Parris, 1980; Fishbein, 1984). This type of mechanism, however, is different from the one that aliphatic azo compounds might follow. Methylazoxymethane for instance acts as a methylating agent via a diazonium ion intermediate (Miydera, 1975).

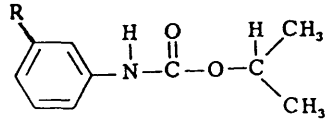
In the light of reported results and concerns, coupled with the fact that no concentration of a carcinogen is completely safe, it seems reasonable that condensation of arylamines and the formation of azobenzene derivatives constitute potential risks to man's health and his environment, and should be always monitored especially in staple food items.

CHAPTER THREE

Residue Analysis of Chlorpropham and its Potential Metabolites in Potato Peels

3.1 Introduction

Chlorpropham, chloro-IPC, chlor-IFC (USSR) or IPC are the approved names for a synthetic amide ester known as isopropyl m-chlorocarbanilate or isopropyl N-3-chlorophenylcarbamate. It has the formula $C_{12}H_{12}ClNO_2$. Propham or IPC by anology is the trivial name for a closely related chemical without chlorine as described below.



R = H (propham); R = Cl (chlorpropham)

Chlorpropham and propham, as well, are members of the phenylcarbamate family of pesticides. They have been in use since the early 50's as pre-emergence and/or early postemergence herbicides to control weeds in various crops (Witman and Newton, 1951; Marth and Schultz, 1952; Eshel and Warren, 1967). They are also commonly used to suppress sprouting in potatoes during storage and in marketing channels (Yoshida et al., 1983; Prendeville and associates, 1968). In this respect chlorpropham and/or propham are often applied as an aerosol, a dust, a soak (sprout nip) or a spray, at a rate of 10-20 mg kg^{-1}

potatoes, depending upon conditions and intended storage periods (Hartley and Kidd. 1983; Koivistomen and Karimpaa, 1965). While effectively inhibiting potato sprouting, chlorpropham influences many aspects of tuber metabolism. On the one hand it enhances some enzymatic discoloration and phenol content, on the other hand it reduces phospho-lipids and ascorbic acid contents, which concentrate at the end buds of the tuber (Ponnampalam and Mondey, 1986). As chlorpropham is regularly and directly used on a staple food item, such as potatoes which often are eaten without peeling, a concern has arisen as to the inevitable residue which it leaves and to the transformations it may undergo (Kearney and Kaufman, 1976; Corke and coworkers, 1979; Coxon and Filmer, 1985; Worobey et al., 1987).

The behaviour and fate of chlorpropham in potato tubers has been the subject of various studies. The early work in this context (Jumer and Sieber, 1964; Coxon and Filmer, 1985) revealed the absence of any detectable metabolite, although there was evidence of a non-polar, unextractable conjugate associated with the peel already treated with chlorpropham. Recently Heikes (1985), Worobey et al. (1987) and Worobey and Sun (1987) reported traces of some metabolites.

In the light of these controversial results, coupled with the concern being expressed about the toxicity of chlorpropham and its metabolites, instead of just a re-evaluation by the Codex Commission, chlorpropham and propham are to be treated as new

chemicals and full data are to be submitted in 1992 (Anon., 1988). Based on these considerations, it was felt that residual amounts of chlorpropham and its potential metabolites in potatoes should be thoroughly investigated aiming to fulfill the following:

1. To build up a picture on the concentrations of chlorpropham regularly found on commercial potatoes.
2. To ascertain whether suspected metabolites such as 3,3^{-}-dichloroazobenzene, 3,3^{-}-dichloroazoxybenzene and 4-methoxychlorpropham have been found in potato peel and to what extent.

Finally as it is understood that analysed metabolites should be available, which was not the case, some effort has been givev to preparing and characterizing those metabolites. Also a short review in relation to chlorpropham residual analysis, toxicology and environmental fate has been introduced so that an overall view of chlorpropham metabolism is conceived.

3.2 Analysis of Chlorpropham Residues

The detection and/or determination of chlorpropham microamounts in different environmental matrices can be achieved by various analytical methods and techniques, namely spectroscopic, chromatographic, radiometric, polarographic and bioassay (Mitchell, 1961; Zweig, 1964; Clark and Wright, 1970; Onley and Yip, 1971; Boyd et al., 1982; Coxon and Filmer, 1985; Hajslova and Davidek, 1986).

In the following a brief summary of the most important methods which have been used in the analysis of chlorpropham terminal residues is included to serve as a guide rather than a full review.

1. Colorimetric methods

These entail hydrolysis of chlorpropham directly or after extraction with an appropriate solvent, followed by distillation of the respective chloroaniline and eventually formation of a coloured complex with either N-1-naphthylethylenediamine or phenol/ammonia-hypochlorite mixture. The colour produced from the previous treatment has maximum absorption at wavelength 540 and 650 nm respectively (Gard and Rudd, 1953; Montgomery and Freed, 1959; Gard et al., 1959; Gard and Ferguson, 1963; Ferguson and Gard, 1969;). Though colorimetric methods gave high recoveries and were able to detect levels down to 0.025 mg dm^{-1}, they were not without disadvantages, since they lack specificity to chlorpropham.

2. Spectrometric methods

These methods were based on the measurement of the absorbance of chlorpropham analyte relative to a standard and finally estimation of the concentration from Beer-Lambert's Law.

The absorption measurements in this respect were made at 236 nm in methanol by uv and at 1210 and 1110 cm^{-1} in CS_2 solution by IR (Ferguson et al., 1963).

3. Bioassay methods

This kind of assay was based on the measurement of the inhibition of the primary root elongation growth of pre-germinating seeds caused by chlorpropham in solution or soil suspension relative to a standard in distilled water (Yoshida et al., 1983; Clark and Wright, 1970). The bioassay, as in spectrometrical methods, lacks specificity and sensitivity as well.

4. Chromatographic methods

HPLC and GLC combine good resolution of a multi-component residue with detection techniques, which are both sensitive (quantitative) and selective for most pesticides. The principal in these techniques is based on partitioning of the analyte between two phases, a mobile phase (liquid or gas), and a stationary liquid phase of high boiling point, on a solid support. The quantification in these methods is usually made through various kinds of detector(s), which respond in a direct proportion to the concentration of the analyte(s).

Several workers have adapted HPLC (Lores et al., 1978; Wilson et al., 1981; Heras and Rasero, 1982; Ritchie, 1986) or GC (Romognali and Bailey, 1966; van Vliet and Hertag, 1966; Onley and Yip, 1971; Corsini et al., 1978; Bradway and Shafik, 1977; Heikes, 1985; Worobey and Sun, 1987) to quantify chlorpropham directly or after chemical derivitization.

3.3 Toxicology

Although the toxicity of chlorpropham is a matter of controversy, the impression gained from literature was that chlorpropham, and similarly propham, seemed to have relatively low mammalian toxicity, probably due to their ready absorption and excretion, shortly after their administration (van Esch and Kroes, 1972).

The acute oral toxicity, LD_{50}, for rats and rabbits has been reported between 5 and 7.5 g kg^{-1} of the body weight (Zweig, 1964; Hartley and Kidd, 1983). Other values for LD_{50}, also have been reported with the lowest value 1.2 g kg^{-1} in rats previously fed no dietary protein (Boyd and Carsky, 1969; Sittig, 1984). The variation in LD_{50} could be attributed to the nature of the amount of protein in the diet previously fed to the animals from the time of weaning (Boyd and Carsky, 1969).

As chlorpropham and propham are derivatives of mutagenic and carcinogenic urethanes (Barnes, 1976; Benignet al., 1989), which are considered as natural constituents of most fermented beverages in concentration as high as 192 mg dm^{-1} in a commercial sale (Ough , 1976). Also the inhibition of mitosis by chlorpropham in plants urged some workers to find out whether chlorpropham inhibits mitosis in animal cells or not. Esch and associates (1958) reported that chlorpropham and propham have weak tumour initiation action, of the same nature as urethane. However, van Esch and Kroes (1972) found that long-term exposure to either chlorpropham or

propham in the diet or by subcataneous injection produced no signs of carcinogenisis. Concerning chlorpropham mutagenicity action, Woo (1983) and Benigni et al. (1989) reported various experiments with positive and negative results depending on the type of mutagenicity test used. Recently, the International Agency for research on cancer has re-evaluated the carcinogenic risks of chlorpropham to humans and experimental animals. From the re-evaluated data, the working group concluded that chlorpropham could not be classified as to its carcinogenicity to humans (Anon, 1987).

In view of these findings, and the scientific thinking which considers no level of carcinogen as safe, a point of risk concomitant with the use of this chemical should be always borne in mind, particularly if some attention is paid to the toxicity of its potential metabolites as well.

3.4 Metabolism of chlorpropham

Although the overall condition of the environmental fate of xenobiotics including aniline-based herbicides was discussed in the previous chapter, it is perhaps worth investigating the environmental fate of chlorpropham in some detail, particularly as this chapter is primarily concerned with the residue analysis of chlorpropham in potato tubers onto which soil particles are often adhered.

3.4.1 Metabolism in soil

As various processes and factors were proved (Freed, 1951; Hance, 1967; Kaufman, 1967; Herrett (1969) to influence the fate and persistence of xenobiotics in soil, chlorpropham was no exception in this respect. Volatilization from soil and adsorption onto its constituents played major roles in chlorpropham immobilization, dissipation and/or fate, especially at high temperature and moisture soil content (Parachetti and Warren, 1968; Rajagolal and associates, 1984).

Several workers revealed the involvement of soil microflora and blue-green algae in the degradation of chlorpropham (Kaufman and Kearney, 1965, 1967; Kaufman, 1967; Still and Herrett, 1976; Vega et al., 1985). Many of the previous authors were able to isolate soil fungi and bacteria which degraded chlorpropham or propham and utilized them as a sole source of carbon. Viga et al., (1985) demonstrated that 3-chloroaniline degraded through catechol as a source of carbon and energy in a similar rate to chlorpropham itself. This is in contrast to the utilization of dichloroanilines, which mineralize very slowly, probably due to their binding and/or polymerization with soil constituents (You and Bartha, 1982).

Wolfe et al. (1976) and Rouchaud et al. (1988) reported 10 and 3 days as the half life period of chlorpropham in the soil and in microbial culture respectively. However, Bollag (1974) and Rajagolal et al. (1984) found out that the persistence of chlorpropham

in the soil may extend up to eight weeks. This is in contrast to 10^4 days and six months, the calculated half life periods of chlorpropham in water and in acid/base media at 70°C respectively (Koivistonen and Karinpaa, 1965; Wolfe et al., 1978).

In general the predominant route for chlorpropham metabolism in soil as reported by Kaufman (1967), Still and Herrett (1976) and Cripps and Roberts (1978) proceeds through hydrolysis, yielding isopropanol, carbon dioxide and 3-chloroaniline. The latter may principally be incorporated in soil organic matter (Kaufman, 1967) or be further metabolized via mineralization (Viga et al., 1985), acylation (Tweedy et al., 1970), N-oxidation (Kaufman et al., 1973), hydroxylation (Fletcher and Kaufman, 1979) and/or condensation into products similar to its transformation in the peroxidase model system (Bartha et al., 1968; Kearney et al., 1969; Bordeleau et al., 1972; Marty et al., 1986).

3.4.2 Metabolism in Animals

Animals may be exposed to chlorpropham directly or indirectly through forage and feedstuffs. In vivo experiments revealed that orally administered chlorpropham to various animals (rats, goats and chickens) was readily absorbed, translocated and excreted as conjugated metabolites in their urine and faeces over a period of a few days after administration (Grunow and associates, 1970; Bobike et al., 1972; Fang et al., 1974; Paulson and coworkers, 1972, 1973, 1975). However, chlorpropham insoluble residues in plant sources were

not available to animals, as they passed through their guts with little or no modification (Paulson et al., 1975). In fish and crustacea, chlorpropham has been demonstrated to concentrate in their bodies (Erb et al., 1980).

The major route for chlorpropham metabolism in various animals as reviewed by Still and Herrett (1976) seemed to occur via hydroxylation at para and/or ortho positions to a smaller extent. Similarly hydroxylation to the 3-chloroaniline or its acylated form and to the isopropyl side chain of the intact chlorpropham also were reported (Grunow et al., 1970; Fang et al., 1974; Still and Herrett, 1976). The hydroxylated metabolites once formed, were subject to conjugation and excretion as glucuronide or sulphate esters (Bobike et al., 1972; Paulson et al., 1972, 1973, 1975). Finally, it seems interesting to note that the reported sulphate conjugates of m-hydroxy-, 3,4-dihydroxypropham and 2-aminophenol were specific metabolites for propham in chickens, since these metabolites were not detected either in animals or in plants when chlorpropham was used instead of propham (Bend et al., 1971; Paulson et al., 1972, 1973).

3.4.3 Metabolism in Plants

By its nature as a herbicide and sprout suppressant, chlorpropham should eventually come into contact with plants. Several workers (Prendeville et al., 1968; James and Prendeville, 1969; Still and Mansager, 1971, 1972, 1973; Russness and Still, 1977; Still, 1968b, 1969) demonstrated that plants absorb,

translocate and metabolize chlorpropham principally through hydroxylation, with subsequent conjugation forming glycosides and/or lignin-cellulose complexes. The hydroxylation occurred at the ortho- and para- sites to the amide bond, and/or at the isopropyl side chain to a smaller extent.

The work of the forementioned authors revealed that tolerant plants metabolized chlorpropham extensively and rapidly as compared to the susceptible species, thereby providing an explanation for chlorpropham differential toxicity.

In this respect Koivistoinen and Karinpaa (1965) in their study, on the stability of chlorpropham residues on fruits, failed to degrade chlorpropham by tomato and spinach homogenate at pH's 4.2 and 5.8 respectively. This indicates that the enzymatic degradation of chlorpropham was not significant. However, Rouchaud and associates (1988), in their research on the metabolism of chlorpropham, pointed out the existence of 3-chloro-aniline as a metabolite in plants. As far as the fate of chloroaniline in plant systems is concerned, Yih et al. (1968) and Still (1968b) revealed the formation of glucoside and saccharideamine polar metabolites together with lignin and cellulose incorporated residues. The work of the previous authors showed no evidence for the formation of the corresponding azobenzene which was in accordance to the findings of Lieb and Still (1969) in rice plants and barnyard grasses, probably due to the specificity of peroxidases in the plant system or to the

high level of cysteine and ascorbate that are available in the plant which could potentially make the contribution of plant peroxidase less relevant.

3.4.4 Metabolism in Potatoes

As chlorpropham is regularly and widely used as a sprout suppressant on ware potato, it is possible that tuber surfaces could be degraded to some extent, producing the same kind of metabolites in the soil-plant system.

The metabolism of chlorpropham in relation to its use as a sprout suppressant on stored potatoes has been investigated by many workers (Jumer and Sieber, 1964; van-Vilet and Hertog, 1966; Coxon and Filmer, 1985; Ritchie, 1986; Worobey and Sun, 1987; Worobey et al., 1987). The early work in this field demonstrated the absence of any detectable metabolite in chlorpropham treated and stored tubers. It also demonstrated that intact chlorpropham and/or its probable metabolites were concentrated in the outer layer of potato tubers in the form of unextractable residues similar to what has been reported for swep, a phenylcarbamate herbicide, in plants (Chin et al., 1964). Jumer and Sieber (1964) reported that potatoes did not metabolize chlorpropham to any extent, which may be unlikely, although the process may be very slow or not significant.

In their investigation on the fate and distribution of chlorpropham, when applied to stored potatoes, Coxon and Filmer (1985) pointed out the absence of any extractable degradation product, probably due to

a little or no metabolism of chlorpropham. However, there was evidence for bound, unextractable residues in the peel fraction, in accordance to the finding of Koivistoinen and Karinpaa (1965) who stated that chlorpropham and propham residues were firmly bound to plants and fruit surfaces, probably dissolved in the lipid of the cuticle. Coxon and his associates also reported that little of the applied chlorpropham penetrated beyond the peel layer, even after six months of storage.

Heikes (1985) reported for the first time the formation of a biologically inactive metabolite in chlorpropham treated commercial potatoes at a level of 4 - 63 ng kg^{-1}. The author identified the metabolite by GC-MS as 4-methoxychlorpropham. The formation of such a metabolite is not without precedent, especially in animals and microbial cultures. Paulson et al. (1972, 1973), Iwan et al. (1976) and Heikes et al. (1979) reported similar methoxylated products for propham, carbaryl, 2,4-D, pentachlorophenol and nitrobenzene. However a recent study by Griffith (1989) revealed the absence of 4-methoxychlorpropham in chlorpropham treated potatoes, despite the low levels of detection ca. 2 ng kg^{-1} the author achieved.

In a study on the fate of chlorpropham and the possibility of its biotransformation by common potato pathogens, Ritchie (1986) pointed out the absence of any identifiable metabolite, although there was evidence for a polar, unidentified metabolite associated with a

gangrene pathogen. Currently, Worobey et al. (1987) and Worobey and Sun (1987), using GC-MS fitted with a selective ion monitor, found traces of both 3-chloraniline and 3,3^{-}-dichloroazobenzene in the peel of commercial tubers at levels of 0.18 - 0.36 ng kg^{-1} and 2.1 - 39 ng kg^{-1} respectively, despite the very low level of chlorpropham which Worobey and associates reported, 21 - 166 ng kg^{-1}. In view of these findings, it is essential to screen potatoes for chlorpropham and its suspected metabolites so that chlorpropham terminal residues in potatoes may be fully assessed.

3.5 Experimental

3.5.1 The Determination of Chlorpropham, 3,3^{-}-dichloro-azobenzene and its Azoxy Analogue in Potato Peel

3.5.1.1 Materials and Equipment

Chlorpropham (CIPC), 99% pure, was supplied by Sigma Chem. Co.; hexane, glass distilled from Rathburn Chem. Ltd., Scotland; alumina, neutral active, Brockmann grade 1, BDH Chem. Ltd.; 3,3^{-}-dichloroazoxybenzene was obtained from Aldrich ABC and purified as described later. All other chemicals and solvents were analytical grade and were used as such. Analyses were carried out by GC-ECD and/or GC-FID as described later (section 3.5.1.6)

3.5.1.2 Potato Treatment and Storage

Washed and unwashed tubers, four weeks after harvest, were separately packed in twelve cardboard

boxes, each containing approximately 10 kg of tubers. Triplicates of the washed and unwashed tuber boxes were equally dusted with a single application of chlorpropham on alumina carrier, at a level of 8 mg g^{-1}, to give a recommended treatment rate of 20 mg kg^{-1} potato. Similarly the rest of the boxes were treated with alumina alone, 25 g per box, to serve as controls. The treated replicas and their corresponding controls were closed with loosely fitted lids. Eventually, the boxes were labelled and stored in a cold room at a temperature below 10°C for up to 9-10 months, until required for analysis. Prior to chlorpropham application, two representative samples, 1 kg each, of the washed and unwashed tubers were taken and stored in a deep-freeze until needed for analysis as blanks.

3.5.1.3 Synthesis of 3,3^{-}-dichloroazobenzene

As environmental fate studies entail the availability of reference metabolite(s), and because the examined analytes of chemicals were not available commercially, some effort has been given to their preparation. The title compound, also known as azo bis(3-chlorophenyl) or diazene bis(3-chlorophenyl), can be synthesized by reduction of 3-chloronitrobenzene or by oxidation of the corresponding 3-chloroaniline chemically or biochemically with the appropriate reagent(s) (Linke et al., 1969, and references therein; Kulshrestha and Mukerjee, 1985). In this laboratory the reduction method with lithium aluminium hydride, $LiAlH_4$, was adopted as the method of choice for the azobenzene

synthesis (Nystrom and Brown, 1948; Corbett and Holt, 1963) as follows:

Two grams of 3-chloronitrobenzene in 50 cm^3 of anhydrous ether was treated with $LiAlH_4$ (1 g), which was added portionwise, as the reaction was vigorous. The mixture was stirred for one hour and refluxed for 15 minutes. After being cooled, water was added very carefully. The mixture was then transferred to a separating funnel and twice extracted with ether. Extracts were bulked, dried over magnesium sulphate and evaporated to give 1.4 g crude product containing small amounts of an azoxy analogue as detected by mass spectrometer and gas chromatography. Finally the solid was recrystallized three times from ethanol to give orange red needles with melting point, 101-102°C, the same as in literature (Vogel, 1978). Analysis calculated for $C_{12}H_8Cl_2N_2$:C 57.4, H 3.21, N 11.16, found: C 57.38, H 3.19, N 11.11. Mass spectral analysis gave the right molecular ion and chlorine isotopic abundances at 250, 252, 254 with the ratios of 18:12:2. The electron impact fragmentations were identical to that of substituted azobenzenes.

3.5.1.4 Preparation of 3,3'-dichloroazoxybenzene

This compound was purchased from Aldrich Chem. Co. and tested by GC-ECD. The detector revealed multi-peaks response, which suggested that the purchased chemical was impure and required purification. The crude solid, however, was purified through multi-recrystallization from ethanol-water, using a little charcoal in the first trial to give faint yellow needles

with m.p. 97-98°C, identical to the literature value (Vogel, 1978). Analysis calculated for the formula $C_{12}H_8Cl_2N_2O$:C 53.96, H 3.02, N 10.5, found: C53.88, H 2.97, N 10.48. Mass spectral analysis gave the right even molecular ion and chlorine isotopic abundances at 266, 268, 270 with the ratio of 18:9:2. The electron impact fragmentations were identical to that of the azoxyphenyl compounds.

Small quantities of this compound and the previous one were synthesized biochemically by the action of peroxidase on 3-chloroaniline in acetate buffer solution at pH 4.7 (chapter 5).

3.5.1.5 Development of the Analytical Method

A considerable body of literature exists describing the wide range of methods and techniques available for determining low levels of chlorpropham in various matrices but with no account of its metabolism (section 3.2). However, a limited number of articles describe residue analysis of chlorpropham together with its metabolites. Coxon and Filmer (1985) used radio-chromatographic T.L.C. to investigate the fate of chlorpropham when applied to stored potatoes and the authors could not identify any metabolite and hence reported the existence of chlorpropham only, with the bulk, ca. 95%, concentrated in the peel. Recently Worobey et al. (1987) and Worobey and Sun (1987) analysed potato peel for chlorpropham terminal residues, using capillary GC-ECD and combined GC-MS fitted with a low resolution selective monitor, and despite the low level

of chlorpropham they detected 21-166 ng g^{-1}. They reported traces of 3-chloroaniline and 3,3^{-}-dichloro-azobenzene at levels of ng g^{-1} as metabolites of chlorpropham in the seeds of chlorpopham treated potatoes.

Since chlorpropham was found mainly in the outer layers of treated potatoes and can penetrate very little even after a long period of storage (Jumer and Sieber, 1964; van Vliet and Hertog, 1966; Dalziel and Duncan, 1980; Boyd et al., 1982; Coxon and Rilmer, 1985), only peel was considered in this study. Preliminary attempts to transfer the method of Worobey and coworker(s) were not satisfactory, as partitioning of the methanol extracts of the peel with methylene chloride produced a stable emulsion. Also, the technique which they used was not available at the time. Consequently it was felt that a method that was available should be adopted or developed to allow residue analysis of chlorpropham and its potential metabolites. Bearing this in mind and recognizing the semi-polar nature of chlorpropham and its metabolites, methanol was chosen as a suitable solvent for extraction of the various analytes from peel matrices. However, it was understood that the polar character of methanol will enable it to extract some extraneous matter and hence a further step(s) of clean-up prior to the eventual analysis was also considered. Several experiments were conducted to determine the best conditions under which low concentrations of the spiked residue could be recovered. Consequently, the most suitable method was

developed as follows:

3.5.1.5.1 Extraction

Prior to extraction, tubers were washed thoroughly under running cold water to remove all adhering particles of soil and alumina carrier. Peel was removed by a household knife to a depth of about 1 mm., equivalent to ca. 5% of the whole weight of tubers. Triplicate samples of 50 gram each were homogenized with 100 cm^3 methanol in a stainless steel jar of an electric blender for 3 minutes at medium speed. The homogenates were spiked at this step and/or filtered with suction through a Buchner funnel fitted with Whatman No. 1 filter paper, into a 500 cm^3 suction flask. The blender jar, and the filtrate cake were repeatedly washed and rinsed with extra methanol to ensure high extraction recovery.

Finally the methanol extract was transferred quantitatively to a 250 cm^3 round bottomed flask and concentrated to about 30 cm^3 through a rotary evaporator under reduced pressure at about 40°C.

3.5.1.5.2 Partitioning

The concentrated methanol filtrate from the previous step containing chlorpropham and its potential, or the spiked metabolites, was transferred quantitatively to a 250 cm^3 separating funnel containing 50 cm^3 saturated sodium chloride solution. The mixture was then extracted three times with 50 cm^3 portions of 5% diethyl ether in hexane. In this context, sodium chloride was added to speed up the separation of the layers and to

make doubly sure that chlorpropham and its metabolite are partitioned in favour of the organic phase. After this, the ether-hexane extracts were combined, washed with 2x50 cm^3 portions of water, dried over anhydrous sodium sulphate and filtered quantitatively into a 250 cm^3 round bottomed flask. Finally the filtrate was evaporated under reduced pressure, at about 40°C to almost dryness. The residue concentrate eventually collected was redissolved in hexane, transferred into a volumetric flask and adjusted by hexane to a final volume of 10 cm^3. It is noteworthy here that replacing the diethyl ether in the partitioning solvent gave similar results. However, using methylene chloride as a partitioning solvent gave a stable emulsion, and hence it was excluded in the analytical method as a suitable partitioning solvent.

3.5.1.5.3 Clean-up

Preliminary investigation on the untreated tubers which were reserved as controls, showed that they contained co-extracted materials, some of which had the same retention time as that of the analytes, thereby interfering with their quantification. Accordingly, it was felt that a further step of clean-up should be undertaken. For this purpose different chromatographic materials, including activated alumina, florisil and different types of bond-elut cartridges were tested to gauge their suitability for eliminating or at least reducing the degree of the interferences in the GC-analysis.

Following the conditioning of the bond-elut cartridges, sample application and hexane elution, it was revealed that the non-polar octadecyl silyl or C-18 cartridge retained a yellow pigment and allowed chlorpropham, azo and azoxy compounds to pass through in the first 2 cm^3 of hexane. On the other hand, it was shown that cyanopropyl and diol polar cartridges retarded chlorpropham relatively with a yellow pigment and allowed the azo and the azoxybenzene to pass through in the first few cm^3 of hexane. Eluates from all tested cartridges were accompanied by some co-extractives and hence were not further considered. Activated alumina and florisil were also tested and both gave similar results. However, taking into consideration the availability and cost of these materials, alumina was chosen for the clean-up process. Unfortunately, working with more than one batch demonstrated that different batches behaved differently in their adsorption behaviour and hence it was necessary to make separate elution profiles for each batch used.

Eventually the clean-up step was performed in a glass chromatograpic column, 10 mm (i.d.) x 40 cm (long), equipped with a glass stopcock as follows.

A glass wool plug was placed in the bottom of the column and filled with hexane. 15 gram activated neutral alumina, grade 1 was then added slowly and steadily with hexane running through the stopcock at a rate of 5 cm^3 per minute. It was necessary not to add alumina too quickly as the air bubbles which were carried into the hexane had to be given enough time and space to come to

the surface, otherwise, it would be entrapped and lower the performance of the column. Gentle tapping on the side of the column produced a more dense bed. Any alumina adhering to the surface of the column was washed down with hexane, and finally topped with 3 grams anhydrous sodium sulphate. 30 cm^3 of hexane were added and allowed to reach the top of the sodium sulphate, after which the analyte mixture was transferred quantitatively to the top of the column using a Pasteur pipette with minimum amounts of washing and allowing the level of the solvent to drop to the column surface after each addition. The flow rate was adjusted to 2 cm^3 per minute. The column was then eluted with hexane first of all 60 cm^3 followed by diethyl ether in hexane (5% v/v) to quickly remove the strongly adsorbed chlorpropham. Finally the eluents coming out were collected portionwise, immediately after the application of the sample, in centrifuge tubes of 10 cm^3 capacity, till 400 cm^3 were collected. Solvents in the centrifuge tubes were then concentrated to 5 cm^3 and subsequently anlysed by GC-ECD to determine which portion should be reserved for the specific analyte(s). Three columns for blank, spiked potato peel and a standard mixture of chlorpropham, 3,3^{-}-dichloroazobenzene and its azoxy analogue were set up and processed as described in figure 3.1.

3.5.1.6 Residue Analysis

The instruments used for the determination of chlorpropham terminal residues in this experiment were a

Pye-Unicam GCD chromatograph equipped with nickel-63 electron capture detector, ECD and a Pye-Unicam Pu 4500 chromatograph fitted with a flame ionization detector, FID. The chromatographic column in the GC-FID was a glass column, 2 m x 4 mm (i.d.), packed with a semi-polar silicon oil, 3% ov-17 + 1.95% ov-202 on 100/120 mesh chromsorp WHP, and in GC-ECD was a 5% ov-17 on 100/120 mesh chromsop Q. Both columns were conditioned for overnight at 250°C with nitrogen carrier gas at a flow rate of 55 cm^3 per minute. The temperature of the injection port, column and detector for GC-FID and GC-ECD were 200°C, 185°C, 250°C and 230°C, 220°C, 275°C respectively. Peak integration was made by Spectra Physics SP 4290 integrator. Hamilton Syringes were used for all injections. Aliquots of injected samples were 1 mm^3 and 5 mm^3 for the GC-ECD and GC-FID respectively.

The linearity of detector(s) response was checked over ranges of 0.2-50 or 0.2-100 $\mu g\ cm^{-3}$ of standards prepared by appropriate dilutions of 1000 mg dm^{-3} stock solutions of the analytes in hexane. A standard curve of the integrated peak areas against concentrations were constructed and linear responses over the range tested were observed (figure 3.2, 3.3). The minimum detection limit for chlorpropham was 0.1 $\mu g\ cm^{-3}$ potato peel which is equivalent to 4 ng g^{-1} potato peel, and for 3,3^{-}-dichloroazobenzene and its azoxyanalogue was 0.2 $\mu g\ cm^{-3}$ which is equivalent to 8 ng g^{-1} of potato peel, based on processing 50 g peel as previously described and eventually adjusted to a final volume of 2 cm^3 in

hexane.

Trials were made to find the optimum condition for performance. Consequently it was found that the sensitivity of the instruments was enhanced with an increase of detector(s) temperature and/or with a decrease of column(s) temperature. The latter also affected the shape of the peak and made it look comparatively wide. As a result of these trials, the prementioned isotherm conditions were arbitrarily chosen. The GC-chromatograms of the blank methanol extract following its partition with hexane showed that it contained co-graphs. However, the alumina clean-up process eliminated the co-extract interferences with chlorpropham and 3,3¯-dichloroazoxybenzene derivative, while for the 3,3¯-dichloroazobenzene, the co-extract interference remained. Attempts to improve the chromatogram resolution by using different columns such as ov-17 and carbo · wax in addition to the pesticide mixture column, or by temperature programming were not successful. The average amount detected for blank co-chromatograph impurities/or contaminants was nearly twice the minimum detection value and thus, it was substracted in the recovery studies. As to what properties or nature this small concentration was attributed, it would be impossible to judge with certainty at this stage.

3.5.1.7 Recovery Determinations

In the overall recovery determination to test the efficiency of the analytical method used, and to correct

the residual amounts analysed. Clorpropham untreated tubers which were assigned as control, were thoroughly washed and peeled. Triplicates of the peel, 50 gram each, were homogenized separately with 100 cm^3 methanol, and then spiked at this stage with two separate levels of 2, 0.06, 0.02 $\mu g\ g^{-1}$ and 6, 0.2, 0.06 $\mu g\ g^{-1}$ of chlorpropham, 3,3$^-$-dichloroazobenzene and 3,3$^-$-dichloroazoxybenzene respectively in three cm^3 hexane. The extraction and clean-up processes were repeated as described earlier. Another set of triplicates of the peel from the untreated and unspiked tubers were similarly processed to serve as blanks. Eventually aliquots from the blank and the spiked peels were analysed as previously described to give results shown in tables 3.1-3.4.

All subsequent values particularly for chlorpropham in laboratory treated and commercial potatoes were corrected for the respective recovery factor.

Figure 3.1 The elution profile of a mixture of chlorpropham, 3,3^{-}-dichloroazobenzene and 3,3^{-}-dichloroazoxybenzene spiked on potato peel at 2, 0.06 and 0.02 $\mu g\ g^{-1}$ respectively, and eluted through activated alumina with hexane, 50 cm^3, followed by 350 cm^3 of diethyl ether in hexane (5% v/v).

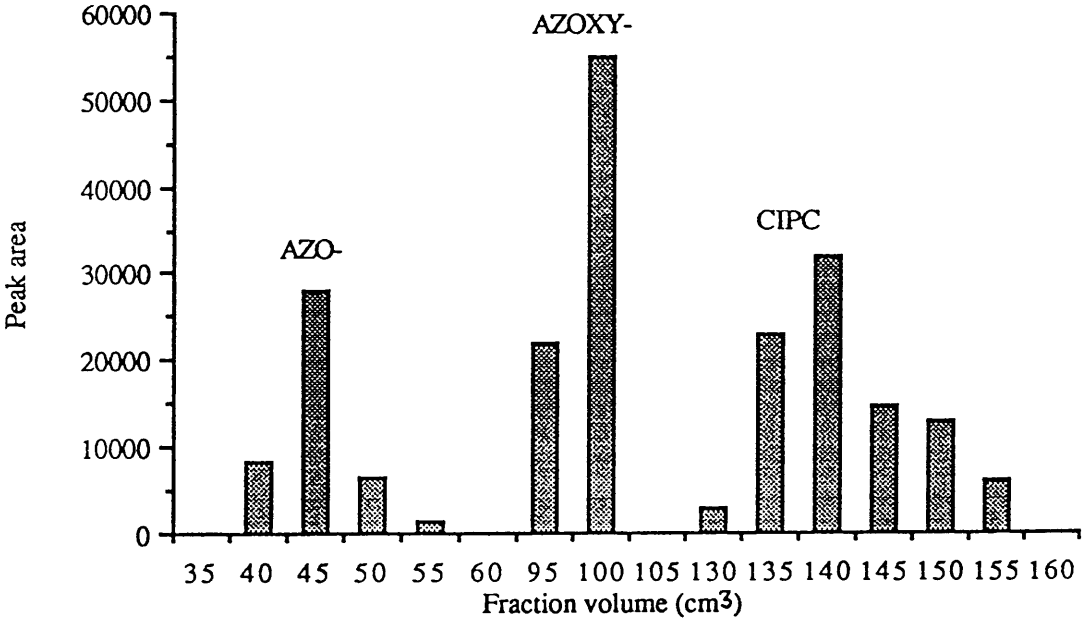


Figure 3.2 Chlorpropham standard curves as determined by GC-FID and GC-ECD.

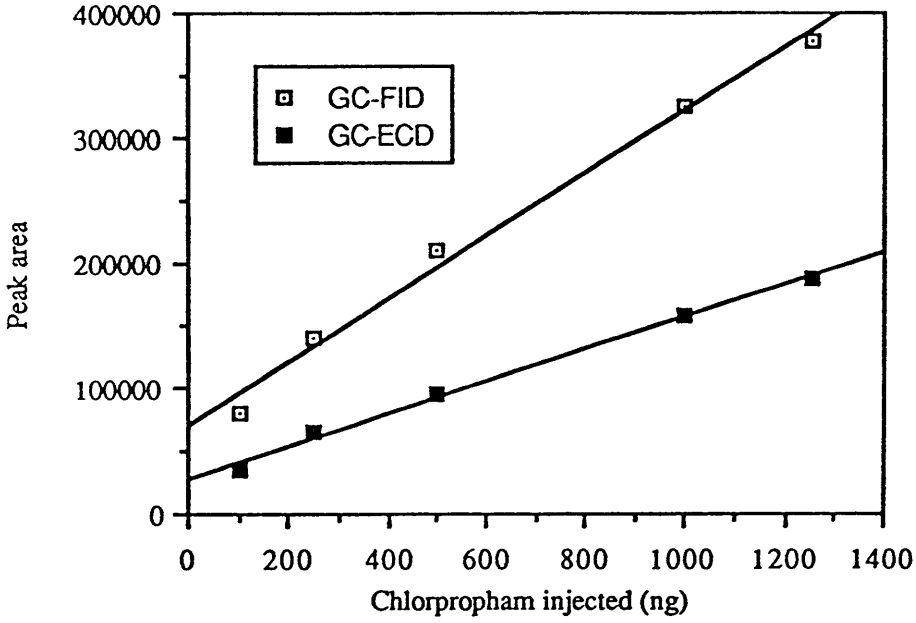


Figure 3.3 3,3^{-}-dichloroazobenzene and 3,3^{-}-dichloro-azoxybenzene standard curves as determined by GC-ECD.

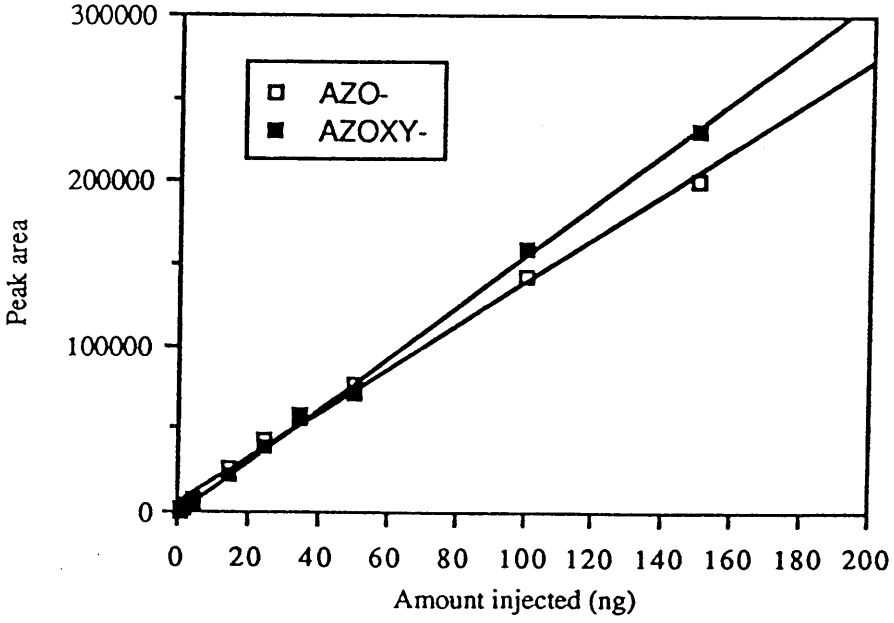


Table 3.1 Recoveries of chlorpropham as based on the fortified amounts and determined by GC-FID

Replicate	Amount added μg	Amount found μg	Recovery %
R1	100	82.44	82.44
R2	100	80.91	80.91
R3	100	86.14	86.14
Mean ± SD	100	83.16±2.69	83.16±2.69
R11	300	231.13	77.04
R12	300	244.94	81.65
R13	300	251.69	83.90
Mean ± SD	300	242.59±10.48	80.86±3.50

Table 3.2 Recoveries of chlorpropham as based on the fortified amounts and determined by GC-ECD

Replicate	Amount added μg	Amount found μg	Recovery %
R1	100	86.74	86.74
R2	100	80.33	80.33
R3	100	89.33	89.33
Mean ± SD	100	85.47±4.63	85.47±4.63
R11	300	244.76	83.25
R12	300	241.37	80.46
R13	300	256.31	85.44
Mean ± SD	300	247.84±7.83	83.05±2.5

Table 3.3 Recoveries of 3,3¯-dichloroazobenzene based on the fortified amounts and corrected for the blank as determined by GC-ECD

Replicate	Amount added µg	Amount found µg	Recovery %
R1	3	1.88	62.67
R2	3	1.92	64.00
R3	3	1.91	63.62
Mean ± SD	3	1.90±0.02	63.45±0.69
R11	10	7.59	75.9
R12	10	7.54	75.4
R13	10	7.45	74.5
Mean ± SD	10	7.53±0.07	75.27±0.71

Table 3.4 Recoveries of 3,3¯-dichloroazoxybenzene based on the fortified amounts and corrected for the blank as determined by GC-ECD

Replicate	Amount added µg	Amount found µg	Recovery %
R1	1	0.80	80
R2	1	0.92	92
R3	1	0.88	88
Mean ± SD	1	0.87±0.06	86.67±6.11
R11	3	2.70	90.0
R12	3	2.42	80.7
R13	3	2.59	86.3
Mean ± SD	3	7.53±0.07	85.67±4.68

3.5.1.8 Final Determination

1. Laboratory Treated Tubers

Laboratory treated samples of washed and unwashed potato tubers, after 8-9 months of chlorpropham treatment and storage, were eventually peeled and analysed as prescribed for chlorpropham, 3,3¯-dichloroazobenzene and its azoxy analogue. The results as determined by both GC-FID and GC-ECD were in good agreement as shown in table 3.5.

2. Commercial Treated Tubers

To check that commercial samples in large scale potato stores, which often receive multi-treatments of chlorpropham, were producing comparable results to those from laboratory experiments, and to check whether they contain traces of the azo and azoxy metabolites, commercial samples were obtained and similarly peeled and analysed. Analysis results as determined by GC-FID and GC-ECD were in good agreement for chlorpropham. However, no indication of the presence of the azo and azoxy metabolites was observed within the detection limits used. In table 3.6 is shown the analysis results of chlorpropham residues in commercial samples.

3.5.1.9 Soil Analysis

The examination of soil was not intended to be a full residue study, but it was merely intended to give an indication as to the presence or absence of 3,3¯-dichloroazobenzene and its azoxy counterpart. No adequate control samples were available, so the recovery

data were not available either. Soil analysis was determined as follows. 50 grams soil from tuber surfaces or that which had fallen off were collected, sieved, weighed and divided equally in two cellulose parchment thimbles. The thimbles were extracted with 100 cm^3 of methanol in a soxhlet for 10 hours. The methanol of the extract, after cooling was then transferred quantitatively through filtration to a 250 cm^3 round bottom flask, where it was concentrated to about 30 cm^3 under suction at a temperature around 40°C. The methanol concentrate was then partitioned three times with diethyl ether in hexane (6% v/v) particularly after an addition of 50 cm^3 aqueous solution saturated with sodium chloride. The combined organic layers were washed three times with distilled water, dried over sodium sulphate and filtered into a 250 cm^3 round bottom flask, and finally evaporated under suction to almost dryness. A faint yellowish residue obtained was redissolved in hexane and adjusted to a total volume of 2 cm^3. Aliquots were then analysed by GC-ECD, and the rest were subjected to alumina column clean-up as previously described.

In a similar way, 25 grams soil from chlorpropham untreated tubers and solvent methanol were separately processed as mentioned above to serve as blanks. Eventually aliquots were analysed by GC-ECD for the azo and azoxy derivatives. The results indicated that none of the metabolites were detected in the analyzed soils within the detection limits used.

Table 3.5 Residual amounts of chlorpropham and its potential metabolites in the peels of laboratory treated potato as determined by GC-FID for chlorpropham and by GC-ECD for the azo and azoxy derivatives

Replicate	chlorpropham ($\mu g\ g^{-1}$)		3,3'-dichloroazobenzene	3,3'-dichloroazoxybenzene
	found	corrected	$\mu g\ g^{-1}$	$\mu g\ g^{-1}$
WR1	83.12	102.79	ND	ND
WR2	76.81	94.99	ND	ND
WR3	76.54	94.66	ND	ND
Mean ± SD	78.85±3.78	97.48±4.6		
UWR1	79.73	98.60	ND	ND
UWR2	83.04	102.20	ND	ND
UWR3	70.28	86.92	ND	ND
Mean ± SD	77.68±6.62	96.07±8.19		

WR = washed tuber replicate

UWR = unwashed tuber replicate

ND = not detected

SD = standard deviation

Residual amounts of chlorpropham were corrected for the recovery factor (80.86)

N.B. The mean of chlorpropham in the peel of commercial samples as determined by GC-ECD and corrected for the respective recovery factor was found to be 91.39±6.41 (SD).

Table 3.6 Residual amounts of chlorpropham and its potential metabolites in the peels of commercial samples as determined by GC-FID for chlorpropham and by GC-ECD for the azo and azoxy derivatives

Replicate	chlorpropham ($\mu g\ g^{-1}$)		3,3'-dichloroazobenzene	3,3'-dichloroazoxybenzene
	found	corrected		
R1	65.56	81.08	ND	ND
R2	59.29	73.29	ND	ND
R3	56.48	69.85	ND	ND
Mean ± SD	60.43±4.65	74.74±5.75		

R = commercial replicate

ND = not detected

SD = standard deviation

Residual amounts of chlorpropham were corrected for the recovery factor (80.86)

N.B. The mean of chlorpropham in the peel of commercial samples as determined by GC-ECD and corrected for the respective recovery factor was found to be 70.18±2.43 (SD).

3.5.1.10 Results and Discussion

The mean values for chlorpropham, 3,3^{-}-dichloroazobenzene and 3,3^{-}-dichloroazoxybenzene in the peel of laboratory and commercial treated and stored potatoes are given in tables 3.5 and 3.6. The analyzed data were determined by GC-FID and corrected for the respective recovery figure. The values shown in tables 3.5 and 3.6 indicate that the concentration of chlorpropham in the quantified samples were significant as compared to the value of 5 mg per kg for the whole potatoes, the maximum permissible limit of chlorpropham in the Netherlands (Coxon and Filmer, 1985). The residual amounts of chlorpropham in washed, unwashed and commercial samples were found to be 97.48±4.6, 96.07±8.14 and 74.79±5.79 (SD) $\mu g\ g^{-1}$ respectively. These values demonstrate that the concentration of chlorpropham in washed potato is higher than that in the unwashed tubers. However, the difference is not significant, probably due to the small amount of soil particles adhered to the tuber surfaces which may inhibit penetration of the chlorpropham. The standard deviation of chlorpropham in unwashed potatoes is greater than that in the washed ones, probably due to uneven distribution and penetration of chlorpropham into potato tubers.

The residual content of chlorpropham in the commercial samples from a large scale potato store, which often receive multitreatment of chlorpropham shows comparable results to that of laboratory experiments, though the concentration is slightly less on this

occasion.

The variation in the residual amounts of chlorpropham in the treated tubers is not unexpected, as it is normally influenced by various factors such as level and repeatedness of treatment, storage period and storage conditions, method of application, potato cultivar and the status of the treated tubers (Jumer and Sieber, 1964; Koivistoinen and Karinpaa, 1965; Coxon and Filmer, 1985 and Hajslova and Davidek, 1986).

Applying the Q-test to all experimental data showed that none of the measurements would be regarded as gross error and all results were retained. Q-test is defined as the deviation of any susceptible value from its nearest neighbour, divided by the range of results.

Analysis of potato peel and the adhered soil samples for 3,3^{-}-dichloroazobenzene and its azoxy counterpart, revealed that in no case did the analysed samples reveal the presence of these metabolites within the detection limit, 8 ng g^{-1}, for both compounds.

Recovery calculations over several fortified samples, as compared and/or corrected to untreated blanks, were found to have high values and high precision as follows: 83.16±2.69% and 80.86±3.50% for 2 and 6 μg g^{-1} CIPC, 63.45±0.69% and 75.27±0.71% for 0.06 and 0.2 μg g^{-1} 3,3^{-}-dichloroazobenzene and 85.67±4.68% for 0.2 μg g^{-1} azoxy derivative.

Though the recovery figure obtained for 3,3^{-}-dichloroazobenzene is greater than the reported one 49.1±4.9% (Worobey and Sun, 1987), it is relatively small

as compared to that of chlorpropham or the azoxy analogue. The reason for this was the presence of an interfering co-extracted fraction with the same chromatographic retention time as that of the azo standard. The amount corresponding to the interference was equal to twice the minimum detection limit. Several trials were carried out to get rid of the extraneous matter but these were unsuccessful. Thus, the respective interfering value was subtracted from the recovery data which in turn affected the overall recovery percentage especially at the low spiked level.

As to what properties or nature the small interference fraction had, it would be impossible to judge with certainty at this stage.

The formation of 3,3^{-}-dichloroazobenzene and/or 3,3^{-}-dichloroazoxybenzene may be brought about microbially, photochemically or via soil-catalysed synthesis (Worobey, 1984). Other sources of such metabolites could be irrigated water and/or cross contamination from the analysis or formulations.

The absence of the azo and azoxy metabolites in all of the analysed samples including the peel and soil samples indicates that chlorpropham on or in the outer layers of potatoes where it usually concentrates, resists degradation or else metabolizes very little if any. This is not without justification. Wolfe et al. (1978) reported 10^4 days as the hydrolysis half life period of chlorpropham in natural water. Similarly Koivistoinen and Karinpaa (1965) calculated the hydrolysis half life

period of chlorpropham at pH 1 and 9 at 70°C as six months. The previous authors also reported the failure of spinach and tomato macerates in buffer media at pH's 4.2 and 5.8 respectively at 37°C to hydrolyse chlorpropham. Moreover the low temperature and low moisture content in potato stores would inhibit the microbial and/or enzymatic activity (Kaufman, 1967). It is worth mentioning also, that 3-chloroaniline the precursor for the azo and azoxy metabolites, if it is formed may bind to the peel constituents or it may undergo acylation which will compete and/or inhibit azo and azoxy formation. The presence of ascorbic acid in potato, may inhibit the action of peroxidase enzyme, which is known to catalyze the oxidative coupling of arylamines. Lieb and Still (1969) and references therein reported that although azo compounds were synthesized from arylamines by peroxidase, they could not detect their presence in rice plants which were permeated with chloroaniline, probably due to the specificity of the enzyme in the plant system to its primary substrate. Kaufman et al. (1970) was able to detect tetrachloro-azobenzene only in two soil samples out of 47 previously treated with propanil, an acylanilide, at a rate of 3.4 kg ha^{-1}. More recently Viswanathan et al. (1978) revealed the presence of tetrachloroazobenzene at a level below 5 ng g^{-1} together with acylated products in barley grains and peeled potato collected from soil previously treated with dichloroaniline.

As far as the azoxybenzene derivation is concerned, Kaufman et al. (1972) stated its absence in soil which received an exceptionally high dose treatment of dichloroaniline. However, Freitag et al. (1984) reported its detection in an outdoor soil and in plant systems.

Jumer and Seiber (1964) reported that chlorpropham did not metabolize in potatoes to any extent, which may be unlikely, although the degradation is not significant. Later on Koivistoinen and Karinpaa (1965), Coxon and Filmer (1985), and Ritchie (1986) all reported the absence of any detectable metabolites in potatoes (section 3.4.4). Similarly Geissbuhler et al. (1971) in his investigation on the acaricide, chlorophenamidine (N^{-}-(4-chloro-o-tolyl)-N,N-dimethylformamine) in plants that received high doses of this chemical, mentioned the absence of any indication of any significant amount of the respective azo, probably because the binding sites in the plant system compete successfully for the chloroaniline especially at low concentration levels. Recently Rouchaud et al. (1988) revealed that chlorpropham and 3-chloroaniline were quickly mineralized in chlorpropham treated soil at a level of 1.6 kg ha^{-1}. The authors also reported the absence of 3-chloroaniline in the harvested roots of the witloof (chicory) plant, which was grown in the treated soil for more than nine months.

In this context it was only Worobey and Sun (1987) who reported very small concentrations of

3,3^{-}-dichloroazobenzene at a level of 2.1-39 ng g^{-1} (section 3.4.4). This is despite the low recovery of the azo derivative and the low level of chlorpropham which Worobey and coworker reported.

Finally the results from this study indicate the presence of high levels of chlorpropham in potato peel and the absence of the questionable azo and azoxy metabolites. In view of these findings combined with the general attitude in the previous discussion, it seems reasonable to suggest that, if chlorpropham is to be continued in use as a potato sprout suppressant, potato tubers should always be monitored for the presence of excess chlorpropham with the residual limit in mind. Potatoes should also be peeled before consumption or processing if they have been suspected of being treated with chemicals.

3.5.2 The Determination of Chlorpropham and 4-methoxychlorpropham in potato peel

3.5.2.1 Materials and Equipment

Chlorpropham, 99% pure, and Florisil(R) 60/100 mesh were supplied by Sigma Chem. Co. and used as such. Hexane, glass distilled or HPLC grade, were purchased from Ruthburn Chem. Ltd. All other solvents and chemicals were analytical grade and were used as received. Isopropyl chloroformate was purchased from Aldrich, U.S.A.. 4-methoxychlorpropahm was synthesized as described later (section 3.5.2.3).

Analyses were determined by a Pye-Unicam PU 4500 chromatograph equipped with a flame ionization detector and glass column 4 mm (i.d.) x 180 long and filled with a semipolar silicon oil of a mixed phase made of 3% ov-17 together with 1.95% ov-202 on a 100/120 mesh WHP. Chromatograms were reproduced on a spectraphysics SP 4290 integrator and/or Shimadzo C-R 113 chromatopac integrator.

3.5.2.2 Potato Treatment and Storage

Washed and unwashed healthy tubers that remained from the setting up of the previous experiment were placed in new cardboard boxes and dusted for the second time with chlorpropham on alumina carrier at a rate of 10 ppm. Boxes were closed with loosely fitted lids and stored at a temperature below 10°C for 2-3 months. Finally replicates from the treated tubers were separately peeled and subsamples of the peel, 50 gram

each, were reserved in polyethylene bags in a deep-freeze until required for analysis.

3.5.2.3 Synthesis of 4-methoxychlorpropham

The title compound, also known as isopropyl N-(3-chloro-4-methoxylphenyl)carbamate, was synthesized by adoption of the general procedure for the preparation of phenylcarbamates (Heikes, 1985) as follows.

3-chloro para-anisidine, (Aldrich Chem. Co.) was purified by vacuum distillation. Equimolar amounts (0.1 mol) of the purified chemical in 50 cm^3 toluene and sodium hydrogen carbonate in 25 cm^3 H_2O, were mixed vigorously by a stirring magnet and cooled to about 5°C. To the stirring mixture was then added dropwise over a period of two hours 0.1 mole of isopropyl chloroformate in 50 cm^3 toluene while the temperature was kept at about 5°C. At the end of the reaction, dilute hydrochloric acid was added to the reaction mixture till slightly acidic. The organic layer was separated, washed repeatedly with dilute hydrochloric acid and water and dried over anhydrous sodium sulphate. Finally the toluene solution was treated with charcoal on a steam bath, filtered and left in the hood overnight to give a white solid residue, which was crystallized twice from ethyl alcohol to yield white needles with m.p. 97-98°C and in 90% yield. Analysis calculated for $C_{11}H_{14}ClNO_3$:C 54.4, H 5.76, N 5.75, found: C 54.19, H 5.83, N 5.68. Mass spectrum revealed the right molecular ion. Isotopic abundances at 243/245, and fragmentation pattern were identical to that in the literature (Heikes, 1985).

Attempts to synthesise 4-methoxychlorpropham from 3-chloroaniline, carbon monoxide and isopropanol in presence of HCl, O_2, $CuCl_2$ and $PdCl_2$ as a catalyst, as an alternative route for carbamate synthesis (Alper and Hartstock, 1985) were unsuccessful.

3.5.2.4 The Analytical Method

3.5.2.4.1 Extraction and Partitioning

The method adopted for the extraction was made with regard to the Official Methods of Analysis, AOAC, which were developed for the analysis of herbicide multiple residues in non-fatty foodstuffs (Anon., 1975) and with reference to the work of Yip (1975) and Heikes (1985).

In this method, triplicate samples of the peel, 50 grams each, were homogenized separately for 3 minutes in an electric blender with 100 cm^3 acetonitrite and 5 grams celite as a filter aid. The homogenates were filtered under suction through Buchner funnels fitted with filter paper into a 500 cm^3 suction flask. The blender jar and the filter pad were washed and rinsed with more acetonitrile to ensure quantitative extraction. The combined acetonitrile filtrate was then transferred to a 1000 cm^3 separating funnel containing 100 cm^3 hexane. The mixture was shaken vigorously for 2 minutes and 20 cm^3 of a saturated sodium chloride solution was then added followed by 500 cm^3 of distilled water. The mixture was shaken again for 2 minutes, and the layers were allowed to separate. Finally the organic layer was

washed with 2 x 100 cm^3 portions of distilled water, dried over anhydrous sodium sulphate and filtered into a 250 cm^3 round bottom flask. The hexane was evaporated with suction at about 40°C. A greenish yellow residue obtained was eventually redissolved in a total volume of 5 cm^3 ethyl acetate prior to the following clean-up and analysis steps.

From preliminary investigation on purification and crystallization of the synthesized methoxy-chlorpropham it was revealed that its solubility in hexane is limited, and hence attempts were made to improve its recovery from potato sample matrices. The result of the various attempts demonstrated that an addition of a small percentage of diethyl ether in hexane (6% v/v) and repartitioning the acetonitrile-aqueous extract in hexane improved the recovery of both chlorpropham and its methoxy derivative. Eventually all subsequent extractions were made according to this modification.

3.5.2.4.2 Florisil Column Clean-up

The clean-up studies were made according to the method of food and drug administration (FDA, 1980) as follows.

To a glass chromatographic column, 22 mm (i.d.) x 25 cm, fitted with stopcock, 19 grams of activated Florisil was added and topped with 3 grams anhydrous sodium sulphate. The dry filled column was then pre-wetted and washed with 50 cm^3 hexane. The sample concentrate was transferred quantitatively and eluted at

a rate of 2 cm^3 per minute with the following stepwise gradient of solvents, 50 cm^3 of hexane, 100 cm^3 of 6% diethyl ether in hexane, 100 cm^3 of 15% diethyl ether and 200 cm^3 of 50% diethyl ether in hexane (v/v).

Eluates were collected portionwise immediately after the application of the sample in centrifuge tubes of 10 cm^3 capacity and concentrated by nitrogen gas to 2 or 5 cm^3 prior to analysis. To determine the elution cuts which should be reserved for analysis, three columns of the blank, spiked potato peel and a standard mixture of chlorpropham and methoxychlorpropham were set up and processed as above. Figure 3.4 shows the elution of a spiked potato peel sample with a mixture of chlorpropham and its methoxy derivative.

3.5.2.5 Residue Analysis

Determination of chlorpropham and its methoxy counterpart was made by GC-FID (section 3.5.2.1). The operating temperature of the injection port, column and detector were 200, 180 and 250°C respectively. Injected samples were 5 mm^3 transferred by a Hamilton syringe. The linearity of the detector response was checked over a range of 0.5 - 100 $\mu g\ cm^{-3}$ of standards prepared by appropriate dilution of 1000 mg dm^{-3} stock solution of the analytes in ethyl acetate. Standard curves of the integrated peak areas versus concentrations were constructed and linear responses over the range tested were observed (figure 3.5). The minimum detection limit for 4-methoxychlorpropham was 0.5 $\mu g\ cm^{-3}$ which is equivalent to 20 ng g^{-1} of potato peel.

3.5.2.6 Recovery Determination

In the overall recovery determination of chlorpropham and its methoxy derivative, triplicate samples of the chlorpropham untreated peel, 50 grams each, were separately homogenized with acetonitrile in a ratio of (1:2 w/v), then spiked at this point with chlorpropham and 4-methoxychlorpropham at 2 and 1 $\mu g\ g^{-1}$ respectively in two cm^3 ethyl acetate. Extraction, clean-up and residue analysis were made with regard to the prescribed steps and with reference to a blank and standard mixture. Trials were made to improve the efficiency of extraction and clean-up. In table 3.7 is shown the various recoveries of the analytes as determined from several trials.

Figure 3.4 The elution profile of a mixture of chlorpropham and 4-methoxypropham spiked with potato at 2 and 1 $\mu g\ g^{-1}$ respectively, and eluted through activated florisil.

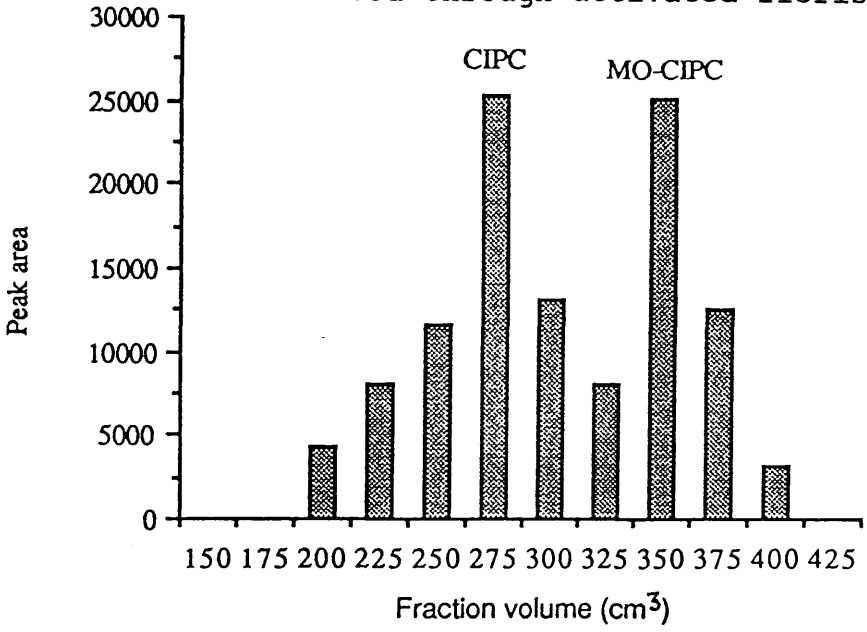


Figure 3.5 Standard curves of chlorpropham and 4-methoxychlorpropham as determined by GC-FID

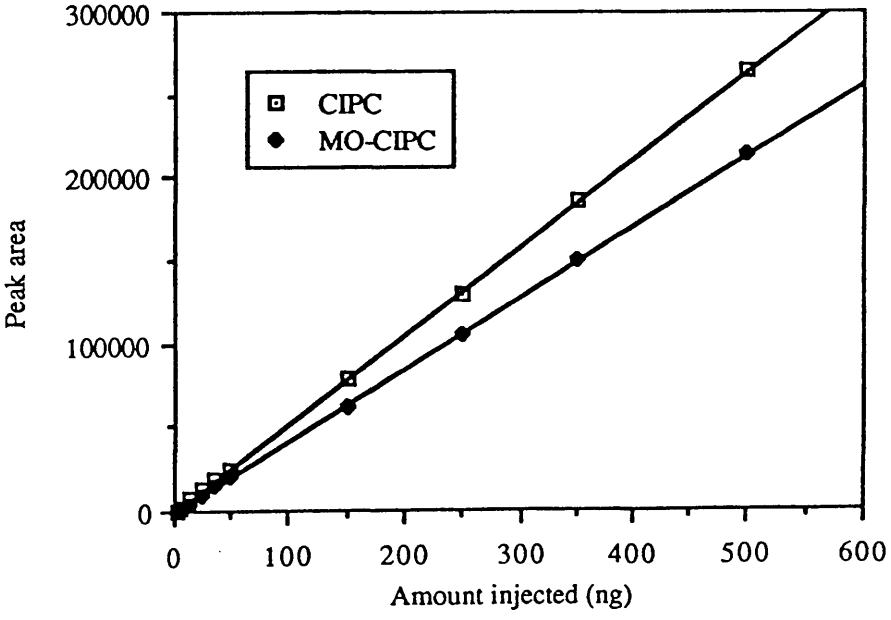


Table 3.7 Recoveries of chlorpropham and methoxychlorpropham from spiked potato peel as determined by GC-FID after florisil clean-up.

Replicate	chlorpropham ($\mu g\ g^{-1}$)			4-methoxychlorpropham ($\mu g\ g^{-1}$)		
	applied	found	% recovery	applied	found	% recovery
R1a	2	1.434	71.70	1	0.391	39.1
R2a	2	1.559	77.95	1	0.347	34.7
R3a	2	1.352	67.60	1	0.361	36.1
Mean ± SD		1.448±0.104	72.42±5.21		0.365±0.022	36.63±2.25
R1b	2	1.613	80.65	1	0.417	41.7
R2b	2	1.443	72.15	1	0.362	36.2
R3b	2	1.576	78.80	1	0.361	36.1
Mean ± SD		1.54±0.04	77.2±4.77		0.38±0.03	38.0±3.2
R1c	2	1.60	80.0	1	0.485	48.5
R2c	2	1.53	76.5	1	0.483	48.5
R3c	2	1.54	77.0	1	0.555	55.5
Mean ± SD		1.56±0.04	77.83±1.39		0.508±0.04	50.77±4.10

R = replicate

a = recovery from one partitioning with hexane alone

b = recovery from one partitioning with 6% diethyl ether in hexane (v/v)

c = recovery from double partitioning with 6% diethyl ether in hexane (v/v)

SD = standard deviation

3.5.2.7 Final Determination

1. Laboratory Treated Tubers

Peel samples from chlorpropham treated potatoes stored in the laboratory for up to 12 months, were eventually analysed for chlorpropham and its 4-methoxy derivative. In table 3.8 is demonstrated the result of this analysis as determined by GC-FID and based on the prescribed method.

2. Commercial Treated Tubers

To check that commercial samples from large scale potato stores which often receive multitreatments of chlorpropham were producing comparable results to those from the laboratory experiment which received a double application of chlorpropham and to see whether they contain any residual amounts of the potential 4-methoxychlorpropham, commercial samples were obtained from top, medium and bottom of a layered potato store and similarly analysed. Analysis results are shown in table 3.9.

Table 3.8 Residual amount of chlorpropham and 4-methoxychlorpropham in the peel of laboratory treated potatoes as determined by GC-FID after florisil clean-up.

Replicate	clorpropham ($\mu g\ g^{-1}$) found	corrected	4-methoxy-chlorpropham
WR1	125.05	160.67	ND
WR2	121.28	155.83	ND
WR3	117.12	150.48	ND
Mean ± SD	121.15±3.97	155.66±5.10	
UWR1	103.01	132.35	ND
UWR2	120.62	154.98	ND
UWR3	118.78	152.61	ND
Mean ± SD	114.14±9.68	146.65±12.44	

WR = washed replicate

UWR = unwashed replicate

ND = not detected

SD = standard deviation

Residual correction as based on the recovery factor (77.83)

Table 3.9 Residual amounts of chlorpropham and 4-methoxychlorpropham in the peel of commercial potatoes as determined by GC-FID after florisil column clean-up.

Replicate	clorpropham ($\mu g\ g^{-1}$) found	corrected	4-methoxy-chlorpropham
CT1	70.88	91.07	ND
CT2	80.34	103.22	ND
Mean	75.61	97.15	
CM1	124.53	160.00	ND
CM2	132.82	170.65	ND
Mean	128.68	165.33	
CB_1	128.98	165.72	ND
CB_2	115.77	148.75	ND
Mean	122.38	157.23	

CT = Top stored replicate

CN = Medium stored replicate

CB = Bottom stored replicate

ND = not detected

Residual correction as based on recovery factor (77.83)

3.5.2.8 Results and Discussion

The GC-FID determination of chlorpropham and 4-methoxychlorpropham revealed significant and high residual amounts of chlorpropham in the peel of both commercial and laboratory treated tubers. The residual concentrations found were 155.66±5.1, 146.65±12.44 $\mu g\ g^{-1}$ of the peel of washed and unwashed laboratory treated tubers respectively. Chlorpropham residues in samples from top, middle and bottom of a commercial store were 97.15, 165.33 and 157.22 $\mu g\ g^{-1}$ respectively.

In no case with the analysed samples were there any indications of the presence of the suspected methoxychlorpropham metabolite within the detection limit 20 $ng\ g^{-1}$ (table 3.8 and 3.9).

The overall recovery from triplicate determinations were 77.83±1.89% from 2 $\mu g\ g^{-1}$ chlorpropham, and 50.77±4.1% from 1 $\mu g\ g^{-1}$ 4-methoxychlorpropham.

Heikes (1985) reported for the first time the presence of the methoxy metabolite in market basket samples at levels of 0.25±1.6 $\mu g\ g^{-1}$. Heikes also reported that homegrown potatoes dipped in a 5% aqueous solution of a sprout nip formulation showed concentrations of the methoxy metabolite at a level of the 170 $\mu g\ g^{-1}$ after six months of treatment. Recently Griffith (1989) reported the absence of the 4-methoxy-chlorpropham in potato peel despite the very low level of detection, ca. 2 $ng\ g^{-1}$, the author achieved. Previously Coxon and Filmer (1985), Ritchie (1986) and others (section 3.4.4) reported the absence of any detectable

metabolites in treated potatoes.

As far as the formation of such metabolites is concerned, Heikes (1985) stated that the formation of the methoxy metabolite occurs possibly through the attachment of a methyl group to the hydroxylated structure. Recently we have identified this metabolite as a photoproduct of chlorpropham in methanol at 254 nm.

The formation of 4-methoxychlorpropham is not without precedent, particularly in animals and microbial cultures. Biological methylation of inorganic divalent mercury to methyl and/or dimethyl mercury is a noteworthy example. The methylation in such a case is brought about by methyl cobalamine, a vitamin B12 analogue in methanogenic bacteria under anaerobic conditions. Similarly, Iwan (1976) and references therein reported microbial and/or animal methylation for various compounds such as 2,4-dichlorophenoxyacetic acid (2,4-D), carbaryl p-chlorophenylmethylcarbamate, pentachloronitrobenzene and pentachlorophenol. The methylation of such compounds was thought to proceed through the hydroxylated structure by S-adenosylmethionine or glutathione. Finally, Paulson et al. (1972) demonstrated a methoxylated metabolite for propham from chickens.

In view of our findings with the previous results which state the absence of the questioned methoxy metabolite, it seems difficult to imagine the formation of such a metabolite in potatoes, particularly if we exclude other potential sources such as irrigated water and cross contamination.

3.6 Conclusions

As chlorpropham is regularly and widely used as a sprout suppressant on ware potatoes and the possibility that such a chemical in potatoes or in the soil around tuber surfaces could be metabolised to some extent, it was felt essential to screen treated potatoes for residual chlorpropham and look for potential metabolites that may be formed during storage. With reference to these objectives, this study was planned, and divided into two parts. In part one, peel from commercial and laboratory tubers were simultaneously analysed for chlorpropham, 3,3^{-}-dichloroazobenzene and 3,3^{-}-dichloro-azoxybenzene. In this part soil samples from tuber surfaces or that which fell off around the tubers also were analysed for the azo and azoxy derivatives. In part two, potato peel was screened for chlorpropham and 4-methoxychlorpropham.

As an outcome of this study the following conclusions were made.

1. An analytical method with high precision and good overall recovery was developed, which allowed simultaneous determination of chlorpropham and its metabolites in potato in the range of $\mu g\ g^{-1}$.
2. Quantification of chlorpropham was made possible with either GC-ECD or GC-FID, provided that the conditions in both GC systems were adjusted to give optimum performance. However, the analysis of the azo and azoxy derivatives were best made only by GC-ECD, while for methoxy derivative, the best

quantification was achieved by GC-FID.

3. The residual amounts of chlorpropham in the peel of commercial and laboratory treated samples were significant, even after long periods of storage. This reflects the readiness of the intact clorpropham to penetrate the peel and concentrate there, probably dissolved in the lipid of the cuticle.
4. Successive treatment of potatoes with chlorpropham enhanced the residual level of this chemical in the tubers probably due to its accumulation.
5. The residual amounts of chlorpropham in the various screened samples were comparable despite little differences amongst washed, unwashed and commercial samples.
6. The level of chlorpropham in the peel of commercial samples collected from the top of a bulk store was relatively less than that in samples from the middle or the bottom of the store.
7. In no case of the analysed potato or soil samples was there any indication of the presence of any of the searched for metabolites within the detection limits achieved which indicates that degradation of chlorpropham in potatoes or in the soil around the potatoes during storage was very little if any to allow sufficient accumulation or build-up of any metabolite. This is probably due to the low temperature and low moisture content which inhibit the microbial and/or the enzymatic activities. Also it may be due to the presence of endogenous ascorbic

acid which could potentially hinder peroxidase activity.

In the light of these findings together with the questionable and controversial toxicity of chlorpropham coupled with the trend of eating more fibre and more vitamins in potato skin, it seems reasonable to suggest and recommend that treated potatoes should be peeled before consumption or processing, simply because eating more whole tubers implies that more chemical residue would be consumed and a potential health risk would be consequently created.

CHAPTER FOUR

Photolysis of Isopropyl 3-chlorocarbanilate (Chlorpropham)

4.1 Introduction

Xenobiotics and pesticides in the environment are subject to various kinds of transformations. Such transformations are brought about by different processes including biological, chemical and photochemical (Crosby, 1969; Chisaka and Kearney, 1970; Hill and Wright, 1978). In situ, the transformation products from the various processes are often the same or at least similar, and it is not easy to distinguish which process has caused a specific change or to establish their relative involvement. Evidence based on rapid losses and conversion of pesticides in sterilized soils (Fletcher and Kaufman, 1980) and the enhancement of their efficiency by shading (Crosby, 1972) suggests that photodecomposition of xenobiotics does occur under field conditions and that considerable portions of pesticides may be transformed by solar radiation, especially those compounds which absorb radiation in the uv-visible portion of the solar spectrum (Slade, 1966; Crosby, 1969). Of the numerous examples which demonstrate the power of sunlight in bringing positive and negative chemical changes are the development of the present life from its primitive reducing atmosphere, photosynthesis, photography, destruction of refractory chemicals and

hazardous substances such as dioxin and methoxychlor; the phototherapy of rickets and jaundice in newly born infants, drying of paints, water purification, photochemical smog, ozone depletion, sunburn (erythema), fading of dyes and clothes, synthesis of vitamin-D, nylon 66 and ether peroxide (Crosby, 1972; Lightner and Park, 1979; Pfoertner, 1984).

Because of the widespread use of chlorpropham as a herbicide and/or as a sprout suppressant on ware potatoes, coupled with the possibility of exposure to sunlight especially in hot regions where sunshine dominates for long periods, it was felt relevant from the public health point of view and environmental fate interest to pay attention not only to chlorpropham residual analysis, but also to its photochemical response.

Principally this chapter was set out to fulfill the following:

1. To introduce the subject and illustrate through several known examples on the photolysis of aniline-based herbicides, the diversity of feasible phototransformations. Such an introduction will be of great value in providing a better understanding and explaining the consequences involved.
2. To study the photodecomposition pathways of chlorpropham in aqueous and organic media.
3. To identify the major photoproducts of chlorpropham, and make predictions about their environmental fate.

4.2 Principles of Photochemistry

Photochemistry can be defined as the study of chemical changes and related physical properties that are produced by the interaction of electromagnetic waves, such as the UV and the visible portions of sunlight, with matter. As light is principally involved, a brief introduction to its basics would be relevant, particularly to appreciate the amount of energy which is needed to break a chemical bond.

Light is an electromagnetic radiation with dual nature; it behaves as waves, with specific wavelength, λ, and frequency, ν, on one hand, and acts as particles known as photons or quanta on the other hand. The energy, E, of a photon or radiation is directly proportional to its frequecy according to the following equation.

$$E = h\nu = hc/\lambda \quad \text{---------- (4.1)}$$

where h is Plank's constant (6.63×10^{-34} J. sec) and c, the velocity of light (3×10^{8} m sec^{-1}).

For one mole or quanta,

$$E = Nh\nu = Nhc/\lambda \quad \text{---------- (4.2)}$$

Here, N represents Avogadro's numbers (6.02×10^{23} mol^{-1}).

Substituting the values of the constants, N, h and c in equation 4.2 results in,

$$E = \frac{11.963 \times 10^{4}}{\lambda(nm)} \text{ Kj } mol^{-1} \text{ nm} \quad \text{----- (4.3)}$$

$$= \frac{2.86 \times 10^4}{\lambda(nm)} \text{ Kcal mol}^{-1} \text{ nm}$$

nm, stands for nanometer (nm = 10^{-9}m)

Accordingly, the energy associated with the radiation of λ = 300 nm corresponds to 399 Kj mol^{-1} or 95 Kcal mol^{-1} and that of λ = 253.7 nm corresponds to 472 Kj mol^{-1} or 113 Kcal mol^{-1}, which is sufficient to disrupt most covalent bonds.

4.3 Radiation - Matter Interaction

Solar energy extends over a wide range of wavelengths; it stretches from long radiation waves of low frequency to very short gamma and ultra violet radiations of high energy content. The cut-off of solar radiation by ozone in the upper atmosphere is about 290 nm (413 Kj mol^{-1}). Accordingly, only radiation at this wavelength or longer will be responsible for the majority of environmental photochemical reactions.

The absorption of the UV-visible radiation by organic molecules is normally associated with the excitation of the non-bonded (n) or pi (π) electrons from its singlet ground state to the respective non-bonded or anti-bonded empty orbitals of π^* or σ^*. If unquenched, the excited singlet electrons may undergo intersystem crossing to a long-lived triplet state. The absorption of the infra-red, IR, or microwaves in comparison is not enough to excite electrons, but is sufficient to increase the vibrational, rotational and translational modes of

freedom which would eventually dissipate as heat. In this regard, the thermal energy by contrast is distributed about all modes of excitation. For a thermally electronic excitation, the relative number of particles at two separate levels with kinetic energy ≥ Ea is given by the Boltzmann equation (Wayne, 1988);

$$\frac{n_2}{n_1} = e^{-Ea/kt} \quad \text{----------} \quad (4.4)$$

where n_2 and n_1 stand for the number of particles in the excited and ground states respectively; Ea the activation energy which represents the minimum amount of energy that a particle should possess to be excited and start a chemical change; k, Boltzmann's constant (1.3805×10^{-23} j K^{-1}) and T, the absolute temperature.

Accordingly, for a typical energy separation of 250 Kj mol^{-1} at room temperature, the fraction, $^{n}2/n_1$, will be equal to 4×10^{-46}, and hence, to achieve a concentration of 1% excitation, for example, it would require a very high energy ~ 6800°C.

4.4 Fate of Excitation

Once a molecule is excited after being irradiated by the uv-visible radiation directly or indirectly, it may lose its short-lived excitation and return to its ground state through several pathways, including luminescence such as fluorescence and/or phosphorescence, energy transfer via sensitization or quenching, photoionization and chemical reactions, depending on the

nature of the chemical, its concentration, the neighbouring molecular species and the kind of photolysis i.e. direct or sensitized (Plimmer and Kearney, 1969; Rosen et al., 1970; Roof, 1982).

The efficiency of the various deactivation processes to occur depends on their competition for the following:

1 - Free radical quenching by oxygen.

2 - Recombination of radicals.

3 - Stability of intermediates by the solvent.

4.5 Photochemical Laws

The absorption of radiation and the photochemical behaviour of chemicals are governed by the following laws.

1. The absorption of radiation by a chromophore in a homogenous absorbing system depends upon the concentration of the absorbing matter and the pathlength that radiation crosses (Zabik and Leavitt, 1976; Wayne, 1988). Mathematically,

$$A = \epsilon CL \qquad (4.5)$$

where A is the absorbance or optical density; ϵ, the molar absorptivity or molar extraction coefficient, which is an intrinsic physical property, representing the probability that a molecule will absorb a specific wavelength, in units of $dm^{+3}\ mol^{-1}\ cm^{-1}$ and L is the light pathlength.

2. Only those molecules that absorb radiation directly or indirectly may undergo photochemical transformation.

3. Absorption of radiation is a one quantum process i.e. each photon absorbed in a primary process excites one molecule.

4. The efficiency of a photochemical change, also known as quantum yield, ϕ, may be defined as the number of photoreacted molecules per photon absorbed.

4.6 Photochemical Kinetics

The rates and routes of photochemical reactions are influenced markedly by the physical and chemical nature of the reactant and the environment in which it resides. Also, it is affected by the light intensity and by sensitization.

Mill (1980) and Zepp (1982) revealed that the rate of direct photolysis of xenobiotics especially at low concentration levels, follows first order kinetics and is affected by the average rate of light absorption and by an efficiency term.

$$\frac{-dA}{dt} = K_p[A] \quad \text{----------} \quad (4.6)$$

where $\frac{-dA}{dt}$, represents the rate of disappearance of a photoreacted matter in units of mol dm^{-3} t^{-1}, K_p, is the photoreaction constant in reciprocal time unit (t^{-1}), and

is defined as

$$K_p = I_{a\lambda}\phi \quad \text{----------} \quad (4.7)$$

In the above equation $I_{a\lambda}$ represents the rate of light absorption at a specific wavelength and is equal to $\epsilon_\lambda I_\lambda$; I_λ stands for the intensity of radiation at a specific wavelength absorbed; ϕ, the quantum yield. [A], the molar concentration of the photoreacted matter. Rearrangement and integration of the equation 4.6 produces the formula,

$$\ln Ao/A = Kt \quad \text{----------} \quad (4.8)$$

from which, $0.693 = Kt_{1/2}$, where $t_{1/2}$ represents the half life period and is defined as the time required for half of the starting material to undergo photochemical transformation.

In the case of indirect or sensitized photolysis, two routes are possible (Draper and Wolfe, 1987). In the first a donor or sensitizer, S, absorbs radiation and passes it on to a xenobiotic or an acceptor, A, which in turn undergoes chemical reaction, provided that the energy of the sensitizer in its interacting triplet state is higher than that of the acceptor.

$$S + h\nu \longrightarrow S^* \xrightarrow{ISC} {}^3A^* \quad \text{---------} \quad (4.9)$$

$$^3S^* + A \longrightarrow S + {}^3A^* \quad \text{---------} \quad (4.10)$$

$$^3A^* \longrightarrow \text{products} \quad \text{---------} \quad (4.11)$$

$$^3S^* + {}^3O_2 \longrightarrow S + {}^1O_2 \text{ (singlet) --- } (4.12)$$

It is worth mentioning in this context that photosensitizers have been identified in soils, plants

and natural water (Miller et al., 1980; Dodge and Knox, 1986).

In the second route, absorption of radiation by natural sensitizers and/or dissolved ionic chemicals may result in photoionization and generation of free radicals and/or aqueous electrons, e_{aq}, both of which in turn initiate a series of secondary reactions as described below.

$$S + h\nu \longrightarrow S^{*} \longrightarrow S^{+\cdot} + e_{aq} \quad (4.13)$$

$$ArNH_2 + h\nu \longrightarrow ArNH_2^{\cdot +} + e_{aq} \quad (4.15)$$

$$ArO^{-} + h\nu \longrightarrow ArO^{\cdot} + e_{aq} \quad (4.16)$$

$$SCN^{-} + h\nu \longrightarrow SCN^{\cdot} + e_{aq} \quad (4.17)$$

$$CO_3^{-2} + h\nu \longrightarrow CO_3^{\cdot -} + e_{aq} \quad (4.18)$$

$$Fe^{+2} + h\nu \longrightarrow Fe^{+3} + e_{aq} \quad (4.19)$$

$$e_{aq} + H_2O \longrightarrow H^{\cdot} + OH^{-} \quad (4.20)$$

$$e_{aq} + H_3O^{+} \longrightarrow H^{\cdot} + H_2O \quad (4.21)$$

$$e_{aq} + H^{\cdot} \longrightarrow H^{-} \quad (4.22)$$

$$e_{aq} + O_2 \longrightarrow O_2^{\cdot -} \quad (4.23)$$

$$2O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad (4.24)$$

$$O_2^{\cdot -} + H_2O \longrightarrow HO_2^{\cdot} + OH^{-} \quad (4.25)$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + O_2 \quad (4.26)$$

$$H_2O_2 + h\nu \longrightarrow 2OH^{\cdot} \quad (4.27)$$

$$NO_3^{-} + h\nu \longrightarrow NO_2 + OH^{\cdot} + OH^{-} \quad (4.28)$$

$$OH^{\cdot} + Cl^{-} \longrightarrow OH^{-} + Cl^{\cdot} \quad (4.29)$$

$$OH^{\cdot} + ROH \longrightarrow RO^{\cdot} + H_2O \quad (4.30)$$

The capacity of natural water and soil surfaces to generate singlet oxygen, hydrated electrons and free radicals such as $OH^{\cdot}$, $CO_3^{\cdot -}$, $Cl^{\cdot}$, $RO^{\cdot}$, $RO_2^{\cdot}$, $O_2^{\cdot -}$ is

correlated with the half life periods of the generated species and with the concentration of the dissolved organic and inorganic matter (Draper and Wolfe, 1987).

In this field, Roof (1982) reported that sunlight radiation below 325 nm could generate hydrated electrons at a rate of 10^{19} e_{aq} dm^{-3} hr^{-1} which is equivalent to 0.03 mM dm^{-3} for every daylight hour.

In natural water, $OH^{\bullet}$, $CO_3^{\bullet -}$ and $O_2^{\bullet -}$ are present in very small concentrations as compared to that of $^1O_2$ and $RO_2^{\bullet}$ (Mill, 1980). However, these radicals may play a significant role in the removal of organic pollutants such as arylamines and alkylhalides. Ebihardt and Martinez (1975); Sawyer and Gibian (1970) and Larson and Zepp (1988) demonstrated that carbonate radicals react with aromatic systems in a similar way to the reaction of $OH^{\bullet}$, but with more selectivity and in th order of o>p>m. The reaction of these radicals involves addition with subsequent oxidation or hydrogen removal.

As mentioned earlier, several factors affect the rates and routes of photoreactions, each of which should be taken into consideration, particularly in predicting the fate of xenobiotics via photolysis. The variation in light intensity for example affects the rate of light absorption, and hence directly influences photolysis rate(s). The wavelength of radiation involved also affects the rate of phototransformations with a trend of greatest change occurring at shortest wavelengths (Plimmer, 1970; Jordan et al., 1965). The physical state of the irradiated chemical as well as its absorption

spectrum both have direct influence on the photolysis rate. In this context, concentration is of particular importance, because it influences the thickness of the reacting zone that is directly related to $1/\epsilon C$ (Beer-Lambert's Law). Also, dimerization reactions are concentration dependent (Crosby and Li, 1969). Other influencing factors are related to the following:

1. The nature of solvent together with its polarity and viscosity, as it is well known that polar solvents decrease the amount of energy associated with $\pi \rightarrow \pi^*$ transitions in contrast to that of $n \rightarrow \pi^*$. Solvents may also associate with the reacting species and hence decrease its effective concentration, or it may stabilize certain intermediates, thereby enhancing its formation (Mill, 1980). Moreover, solvents may be involved directly in the photoreactions (solvolysis) or it may provide e_{aq} which initiates a series of secondary reactions (Woods and Akhtar, 1974; Wayne, 1988).

2. The absorption behaviour of chemicals is markedly altered by the adsorption state. One such change may be a red shift with an increase in the adsorption intensity of specific band(s) which are responsible for photoinduced reactions, e.g. the absorption peak for dieldrin recorded at 193 nm in hexane was displaced to 264 nm on silica gel (Parldr, 1980). Plimmer (1970) and Ruzo et al. (1973) revealed that aromatic dehalogenation was in agreement with the

case of bond dissociation, and was affected by the nature of ring substituents, e.g. electron withdrawing groups (EWG) enhance the loss of an ortho substituent in contrast to the effect of electron releasing groups (ERG) which facilitate the loss of meta halogens.

3. Finally the presence of other materials in the media may act as sensitizers or quenchers, thereby enhancing or hindering the photodecomposition e.g. acetone and surfactants act as sensitizers, the latter increases herbicide solubility and shifts its u.v. spectra to longer wavelengths, thereby increasing the total amount of energy absorbed (Harrison and Wax, 1986). Humic matter in comparison, may act as sensitizers and/or as quenchers, sensitizers even to non-absorbing radiation chemicals, because they contain ketonic and quinoid functional groups, which absorb strongly in the uv-region of the solar spectrum. However, the quenching effect in contrast may be attributed to the aromatic and polycyclic structures which absorb the excitation energy and retard the photodecomposition (Choudhry, 1982).

4.7 Photolysis of Phenylamide Herbicides

The phototransformations of the titled herbicides were mostly reviewed by Crosby and Li (1969); Crosby (1976); Zabic and coworkers (1976); and Marcheterre et

al., (1988). As photochemistry of xenobiotics became an integral part of the environmental fate study, a photolysis survey on phenylamides and cloroanilines is presented.

This survey will undoubtedly provide better understanding of the environmental fate of these herbicides, also it may allow some prediction on the photolysis of related compounds.

4.7.1 Photolysis of Acylanilides

Simple anilides, such as acetanilide and propananilide, under the influence of ultraviolet radiation, in organic solvents (alcohol), were found to undergo photo-Fries intramolecular rearrangement to the corresponding ortho- and/or para- aminoaryl ketones, similar to the photo-Fries rearrangement of aryl esters to their respective ortho- and para- hydroxy benzoates (Kosba, 1962; Anderson and Rees, 1963; Elade et al., 1965; and Crosby and Li, 1969). Elade (1963) reported the same kind of rearrangement for anilides in presence of aluminium chloride particularly at high temperature. Aniline also was found as a minor photoproduct from acylanilides.

Anilides, with chlorine on the aromatic moiety as in 3,4-dichloropropionanilide (propanil), when irradiated with ultraviolet irradiation in aqueous solution, yielded a mixture of products, involving stepwise replacement by H and/or OH, and accompanied with hydrolysis of the amide bond to the corresponding 3,4-dichloroaniline. The latter, upon prolonged irradiation, reacted further

producing a mixture of azo, azoxy and/or anilinoazo derivatives depending on the irradiation conditions (aerobic or anaerobic) and the kind of sensitizer present (Plimmer and Kearney, 1969; Rosen et al., 1970; Moilanin and Crosby, 1972; Crosby, 1972; and Miller et al., 1980), similar to the biodegradation products of phenylamide and/or chloroanilines in soil (Bartha and Bramer, 1967; Bordeleau and Bartha, 1972 and Smith and Brigs, 1978).

Two other features from the photolysis of propanil were reported by Moilanin and Crosby (1972) and Tanaka et al. (1984, 1985). The first showed the formation of an unexpected propanamide and the latter demonstrated the formation of chlorinated biphenyl.

4.7.2 Photolysis of Phenylureas

The photochemical degradation of phenylurea herbicides has been the subject of numerous studies under various conditions (Crosby, 1976; Kulshrestha and Makerjee, 1986; and Marchetrre et al., 1988). The major photoproducts isolated in most cases were the mono and didemethylated products, produced by successive losses of N-methyl and/or methoxyl groups via N-hydroxymethyl and N-formyl intermediates. The processes were then followed by further degradation of the amide group giving the respective arylamine (Tang and Crosby, 1968; Crosby and Tang, 1969 and Rosen et al., 1969).

Ring hydroxylation in the ortho position, hydrolytic dehalogenation, hydrolysis of any of the amide linkage to the corresponding arylisocyanate and/or aryl-amine, the oxidation of the produced arylamine to the

respective nitro analogue or its conjugation with the isocyanate to diarylurea or carbinilide were also reported (Jordan et al., 1965; Crosby. 1969; Crosby and Tang, 1969; Tanaka and associates, 1977 and Tanaka et al., 1982).

Photolysis of ureides under anaerobic conditions or in the presence of surfactants eliminated ring hydroxylation and enhanced reductive dehalogenation, N-dealkylation or dealkoxylation and finally isomeric coupling via radicals formed from the homolysis of the carbon halide bond. Tanaka et al. (1982) and Tanaka and associates (1984) showed that photolysis of monuron in aqueous solution gave two isomers of monochlorobiphenyl (para-para and para-meta) and three dechlorinated fenuron biphenyls (para-para, para-ortho and para-meta). However, photolysis of monuron in methanol gave fenuron and the respective methyl carbamate via aryl isocyanate intermediate (Mazzocchi and Rao, 1972). These authors also reported that fenuron photolysis under anaerobic conditions afforded aniline together with ortho- and/or para- amino-N,N-dimethyl benzamide through intramolecular rearrangement similar to the reported photo-Fries rearrangement of anilides, phenyl carbamates and aryl esters.

The formation of the azo and azoxy derivatives as a result of unsensitized photodecomposition of isoproturon, a substituted phenylurea herbicide, has been stated by Kulshrestha and Mukerjee (1986). Such a conversion usually requires sensitizers (Plimmer and

Kearney, 1969), or it involves microbial activation particularly in soils as reported for various phenylanilide herbicides (Bartha and Prammer, 1967; Chisaka and Kearney, 1970; Rosen et al., 1970).

4.7.3 Photolysis of Phenylcarbamates

Ultraviolet irradiation of N- or O-arylcarbamates in dilute solutions was observed to form their respective aminobenzoates and hydroxybenzamides as major photoproducts in agreement to photo-Fries intramolecular rearrangement of arylesters, anilides and ureides (Trecker et al., 1968). Beachell and Chang (1972) revealed that photolysis of ethyl N-phenyl carbamate afforded aniline together with the respective o- and p-aminobenzoates. The process involves photo-Fries rearrangement via homolysis of the amide bond and hydrogen abstraction. Formation of the related diethyl 4,4-azobenzene dicarboxylate also has been reported, especially in presence of oxygen and photosensitizer(s). Moreover, the previous authors revealed that polar solvents such as alcohols inhibited phenylcarbamate photo-Fries rearrangement, probably due to the intermolecular hydrogen bond between the carbamate and the protic solvent. Masilamani and Hutchins (1976) demonstrated that photo-Fries rearrangement of phenylcarbamate was concentration dependent in dilute solutions. Aniline was exclusively formed in the process via free radical hydrogen abstraction from the solvent, which in turn underwent dimerization, but in concentrated solutions, the intramolecular rearrangement was enhanced

by the cyclic or chain aggregates or clusters of the carbamate molecules.

4.8 Uv-irradiation of Propham and Chlorpropham

The effect of uv-irradiation on propham has been examined by several workers. Wolfe et al. (1977) reported that propham and chlorpropham photolyzed very slowly as compared to their microbial mediated degradation. Earlier, Mitchell (1961) did not observe any breakdown of propham when irradiated on a filter paper at 253.7 nm. However, Crosby (1976) and Crosby and Li (1969) revealed that propham photolysed in the reverse manner to its preparation yielding aniline, phenylisocyanate, diphenylurea, propylene, isopropanol and unidentified polymeric matter. Such products were observed from its pyrolysis. Wolfe and associates (1978) reported that propham underwent direct photolysis very slowly even during summer times with a half life period of 254 days and a possibility of undergoing photoreaction similar to photo-Fries rearrangement. However, the previous authors neither identified nor isolated any of the potential photoproducts, probably due to the formation of a high molecular weigh polymer. Recently, Larson and Zepp (1988) revealed that carbonate radicals generated by the photolysis of H_2O_2 at 313 nm in aqueous sodium carbonate reacted with propham significantly with a half life period of 1800 minutes, indicating the possibility of its removal environmentally, particularly in carbonate-rich water. Tanaka et al. (1981)

investigated the effect of surfactants in aqueous media on the photolysis of propham and chlorpropham and were unable to conclude that surfactants may increase their losses from water.

Chlorpropham, in contrast to propham, was somewhat affected by uv-radiation at 253.7 nm and afforded, according to Mitchell (1961) four photoproducts but with no evidence to their identity. Crosby (1976) mentioned that chlorpropham and barban, another phenylcarbamate, may be expected to photolyze in the same fashion as propham at least by the formation of aryl-isocyanate, isopropanol and diphenylurea.

Photolysis of chlorpropham at room temperature using simulated sunlight for 104 hours yielded 3-hydroxy propham as a major product. The half period for the disappearance of chlorprophanm under these conditions was estimated to be 130 hours. However a faster rate with $t_{1/2}$ 4 hours, and a second major photoproduct were obtained in presence of 2% acetone in the photolyzed medium. The additional photoproduct was isolated from a photolysed solution of 2 grams chlorpropham in 1 dm^3 of 20% aqueous solution, and identified as 2-isopropoxy-carbonylamino-1,4-benzoquinine (Guzik, 1978).

Finally Tanaka et al. (1985) reported the formation of a monohydroxylated biphenyl derivative as a result of uv-photocoupling of chlorpropham in aqueous media, similar to the formation of chlorinated biphenyls from monuron or propanil photolysis with uv-lamps or sunlight.

4.9 Experimental

4.9.1 Materials and Equipment

Chloropham (CIPC), technical grade with purity 99%, was purchased from Sigma Chemical Co., 3-chloro-aniline, boiling point 95-96°C (11 mm Hg) and 99% purity, obtained from Aldrich Chemical Co.; Hexane, glass distilled, from Rathburn Chemical Co., (Scotland). All other chemicals and solvents were Reagent grades or Analar and used as such. Thin Layer Chromatography (TLC) was carried out using self-coated or pre-coated (preparative), silica gel 60 F-254 plates of 20x20 cm and 1 or 2 mm thickness respectively.

The photochemical apparatus consisted of a three-necked pear shaped vessel of 1050 cm^3 capacity. It was equipped with a magnetic stirring bar and a water cooled internal quartz immersion well. Light source was a 125 watt Hanovia high pressure mercury lamp which produced peak radiation at 253.7 nm. The reactor was open to the atmosphere during photolysis.

Analyses were made by a GC-Pye Unicam, Pu 4500 chromatograph, fitted with a flame ionization detector (FID) and 2 m x 4 mm i.d. glass column, packed with a semipolar silicon oil (3% OV 17 + 1.95% OV 202), supported on 100/120 mesh WHP. The FID signals were recorded on a chromatopac integrator, Shimadza, C-R1B.

Mass spectra for identification, were made on a GC-MS and/or Mass Spectrometer using positive electron impact and direct inlet technique. Uv-visible spectra were made on a 550 SE uv/visible spectrophotometer

(Perkin-Elmer).

4.9.2 Photolysis of Chlorpropham in Organic Solvents

To determine the photoreactivity of chlorpropham in polar and non-polar organic media, hexane and methanol were chosen for this purpose.

1. Photolysis in Hexane

In the prescribed photoreactor vessel, a solution of chlorpropham in 1 dm^3 hexane was introduced and irradiated at 253.7 nm wavelength, with constant stirring during the photolysis at room temperature for three hours. During irradiation, aliquots were withdrawn periodically, starting from zero time, and analysed by GC-FID for the disappearance of chlorpropham and/or the build-up of any photoproducts. After the termination period, the remaining solution, which gradually acquired a pale yellow coloration, was transferred to a round bottom flask and evaporated almost to dryness by rotary evaporator under reduced pressure. Eventually the red brown residue was redissolved in a little methanol and qualitatively analysed by GC-MS, utilizing the mass spectrum departmental service.

The above experiment was conducted with the following concentrations: 100, 200, 1000 and 1500 mg chlorpropham per dm^3 hexane. A controlled experiment was also carried out under dark conditions and analysed by GC-FID.

To examine the effect of photosensitizers and inhibitors on the photolysis of chlorpropham, two

phototrials were made; the first in the presence of 2% acetone in hexane (v/v), and the second in the presence of equimolar amounts of tocopherol (vit E), both at concentrations of 100 mg dm^{-3}.

2. Photolysis in Methanol

Irradiation of chlorpropham in methanol was carried out at 253.7 nm wavelength as in hexane, but with a substitution of vitamin C for vitamin E. At the end of irradiation time, the photolysate was concentrated under vacuum to about 10 cm^3 of a brown red residue, and finally analysed by GC-MS.

4.9.3 Photolysis of Chlorpropham in Aqueous Solutions

A saturated solution of chlorpropham in distilled water (1 dm^3) was irradiated at 253.7 nm for 3 hours. Aliquots of 25 cm^3 were taken periodically, starting from the zero time of irradiation, and extracted three times with 30 cm^3 of methylene chloride in the presence of 5 g NaCl. The combined organic extract was washed with distilled water, dried over anhydrous, Na_2SO_4, filtered into a 250 cm^3 round bottom flask and evaporated to almost dryness under reduced pressure. The remaining brown-red residue was dissolved in methanol and adjusted to a total volume of 5 cm^3 prior to a final analysis for the disappearance of chlorpropham and/or any build-up of potential metabolite(s).

The photolysate bulk solution was extracted and redissolved in a little methanol for qualitative analysis by GC-MS.

Photolysis of chlorpropham in water was repeated in the presence of acetone (2% v/v) and equimolar amounts of ascorbic acid, KI and NH_4SCN. A controlled experiment was also carried out under dark conditions. All collected samples were treated as above and finally analysed by GC-FID.

4.10 Separation and Identification of the Photoproducts

The photolysate concentrates from the different solvents used, were carefully chromatographed over thin layer pre-coated, PLC, silica gel 60 F-254 plates of 2 mm thickness with fluorescent indicator. The plates were developed in a binary solvent of hexane:diethyl ether 7:3(v/v). After development, the plates were examined under uv-light. Finally the localized bands were scraped from the plates. The compounds were then eluted with methanol, concentrated and analysed for their identity by mass spectrometer.

Identification of the various photoproducts from GC-MS and/or mass spectra were made on the basis of the parent molecular ion(s) (M/e), the fragmentation pattern as compared to the mass spectra of a synthesized and/or literature analogues and on some occasions on the presence of meta stable ion(s). The amounts of the photoproducts handled were not sufficient to allow further confirmatory studies.

4.11 Results and Discussions

Attempts were made to evaluate the photolability of chlorpropham and identify its potential photoproducts. Direct photolysis studies on chlorpropham were investigated in aqueous and organic solvents at 254 nm, using a high pressure mercury lamp (125 watt), fitted with a quartz jacket. Moreover the studies were extended to involve uv-spectra, photosensitizers and photoinhibitors.

4.11.1 Uv-spectra

Preliminary investigations on the uv-spectra of chlorpropham in hexane and methanol, revealed similar absorption in both media, with the following absorption characteristics, respectively.

$\lambda_{max}(\epsilon x10^4)$:210(37.9), 238(15.8), 279(1.2), 287(0.95).

:211(22.1), 239(16.9), 278(1.1), 286(0.9).

The first two high intensity bands are associated most probably with π-π^* transitions, while the other two low intensity bands may be attributed to n-π^* transitions.

Solvent studies are often used to characterize $\pi \rightarrow \pi^*$ transitions and distinguish them from $n \rightarrow \pi^*$ transitions. The $\pi \rightarrow \pi^*$ transitions usually show hyperchromic and red shift on going from non-polar to polar solvents in contrast to $n \rightarrow \pi^*$ transitions which usually undergo blue shift ongoing from non-hydrogen bonding to hydrogen-bonding solvents. Crosby and Li

(1969) reported one absorption band at 238 nm as a characteristic uv-absorption for chlorpropham. However El-Dib (1970) reported two characteristic bands at 240 and 270 nm for chlorpropham. Sparacino and Hines (1976) revealed uv spectrum in acetonitrile, comparable to that in hexane as follows:

$\lambda_{max}(\epsilon x10^3)$:207(42.1), 238(12.1), 278(1.2) and 287(1).

In figure 4.1 the uv-spectrum of chlorpropham in hexane and methanol are demonstrated. From the spectrum it can be predicted that chlorpropham in the environment is not a good candidate for direct photolysis as it shows negligible absorbance above the solar cut-off radiation. Moreover chlorpropham absorbs very little in the uv region down to 254 nm. Wolfe et al. (1978) revealed that chlorpropham, and propham as well, underwent direct photolysis only very slowly in distilled water, with half life periods of 121 and 254 days respectively. Guzik (1978) photolyzed a dilute solution of chlorpropham directly in water under simulated noon daylight and reported 130 days as the half life period for the disappearance of chlorpropham. Preliminary investigation on the laboratory uv-radiation of chlorpropham suggested that chlorpropham is photolysable, as the solution becomes increasingly yellow and turbid with some adhering to the walls of the photoreactor over the time of irradiation.

4.11.2 Photoirradiation Studies

In order to establish the profile of chlorpropham disappearance and estimate its residual half life period, direct photolysis of chlorpropham in various media were conducted and the remaining percentage of chlorpropham was periodically monitored by GC-FID.

Figure 4.2 shows the photolysis of chlorpropham in hexane at concentrations of 100, 200, 1000 and 1500 mg dm^{-3}. From the represented hollow curve, it is clear that the disappearance of chlorpropham follows first order kinetics with the highest rate of change at lowest concentrations. The correlations of $\ln C_o/C$ v time gave straight lines with negative slopes. To avoid using mathematical calculations, the presented graphs of the different slopes were chosen which gave direct estimations of the $t_{1/2}$ values.

The slower rates noticed with high initial concentrations of chlorpropham may be attributed to interaggregation of chlorpropham molecules. Masilamani and Hutchins (1976) reported that the photoinduced transformations of N-phenylcarbamate is concentration dependent and revealed a reduction in the quantum yield at high concentrations, probably due to the association via interhydrogen bonds.

The photolysis behaviour of chlorpropham in hexane compared to that in the presence of equimolar amounts of vitamin E or acetone (Figure 4.3) demonstrated an inhibitory action of the vitamin and the acetone as well. This is probably due to the antioxidant property

and free radical scavenging ability of the vitamin, or due to an interassociation between acetone and chlorpropham, or because acetone may be preferentially photolyzed. Similarly Rosen and Siewierski (1970) reported that benzophenone can not act as a sensitizer in cyclohexane, because it preferentially abstracts a hydrogen atom from the solvent, instead of passing its energy to a photoacceptor. The major photoproduct of chlorpropham in hexane was propham. This was identified and confirmed by GC-FID and GC-MS analysis. Figure 4.4 represents the profile formation of propham from chlorpropham in various media. From the graph it is evident that vitamin E and/or acetone inhibit propham phototransformation. Also it is evident that propham, although it is more stable than chlorpropham as it accumulates with time of irradiation, still shows a similar photolysis pattern to chlorpropham especially with regard to the trend of being slowly photolysed at high concentrations. Presumably this is due to the same reasons already mentioned for chlorpropham.

The photolysis of chlorpropham in methanol demonstrated the same trend as that in hexane (figure 4.5). However, the presence of acetone in the methanol enhanced the phototransformation of chlorpropham (see figure 4.6) in contrast to that in hexane (see figure 4.3). The enhancement action of acetone could be associated with its triplet sensitizing behaviour that mimics the sensitizing effect of dissolved materials present in natural water. Guzik (1978) reported that

acetone increased the rate of degradation of chlorpropham, ethylene thiourea and heptachlor and influenced the nature of the photoproducts. In the case of chlorpropham the presence of acetone in the photolysed medium gave rise to a secondary photoproduct identified as 2-isopropoxycarbonylamino-1,4-benzoquinone, in addition to the 3-hydroxypropham (Guzik, 1978). In the case of ethylene thiourea, acetone enhanced its photodegradation into a glycinate derivative (Ross and Crosby, 1973).

Initially, the use of organic solvents was made to enhance chlorpropham solubility and to facilitate its analysis, as hexane and methanol solution can be injected directly into the GC. However aqueous solution is the one that is most available in the environment but because it is poorly detailed and highly variable in composition; distilled water was chosen as a model medium to evaluate its polar effect on the photolysis of chlorpropham.

The photoirradiation of chlorpropham in water at 254 nm gave 3-hydroxypropham as a major product in agreement to what has been previously reported by Guzik (1978). In figure 4.7 the decline curves of chlorpropham and the formation of hydroxypropham during chlorpropham photolysis in water are presented. Comparing the rates of chlorpropham phototransformation in aqueous media with that in organic solvents (see figure 4.8) revealed that the rate of transformation in water was much faster than that in organic solvents in the following order: water>hexane>methanol. This trend is probably due to

differences in the routes followed for transformation in different media. Banerjee et al. (1978), Miller et al. (1979) and Mill (1985) reported that nucleophilic and/or electrophilic substitution of the haloarenes are predominant in the aqueous media, while homolytic cleavage with subsequent hydrogen or solvent abstraction is the general and dominant mechanism in organic solvents. The slower rate of transformation in methanol, as compared to that in hexane may be attributed to the association of chlorpropham with the protic solvent via hydrogen bond. This is in agreement with what has been reported by Beachell and Chang (1972). Another reason could be based on the observation that propham is the major photoproduct in hexane and methanol. The higher rate of change may be attributed to the better hydrogen donating ability of hexane as compared to that of methanol. Figure 4.9 demonstrates the influence of the various factors which have been studied on the rate of chlorpropham disappearance during its photolysis in water. From the graphs, it is understood that chlorpropham phototransformation is concentration dependent with the highest rate at lowest concentrations. The addition of acetone to the media enhanced the rate of change in contrast to the effect of ascorbic acid, KI and NH_4SCN. The inhibitory action of these reducing chemicals may be asociated with their quenching ability.

Figure 4.1 The uv-spectra of chlorpropham.
1: In hexane. 2: In methanol.

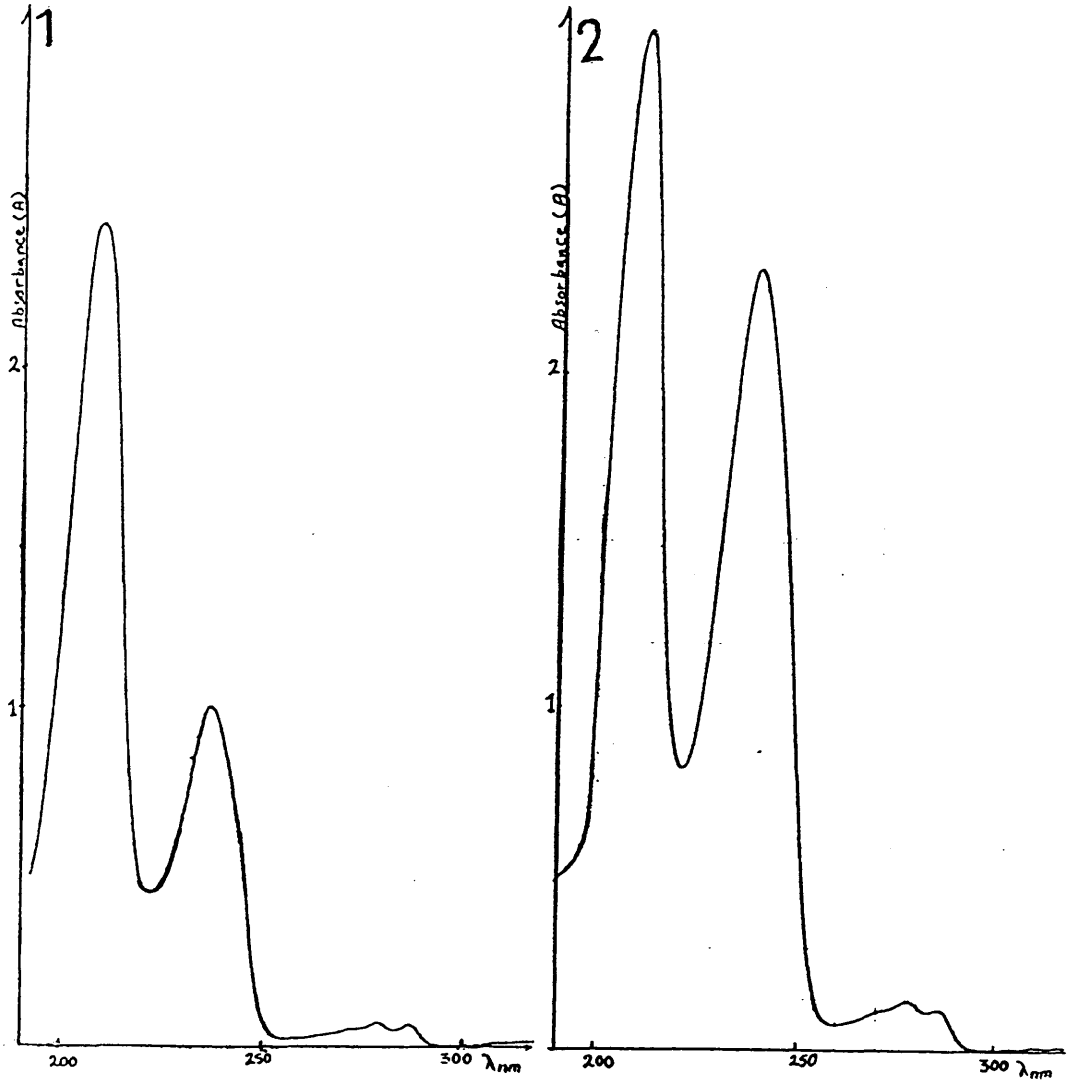


Figure 4.2 Rate of phototransformation of chlorpropham and formation of propham in hexane at different concentrations.

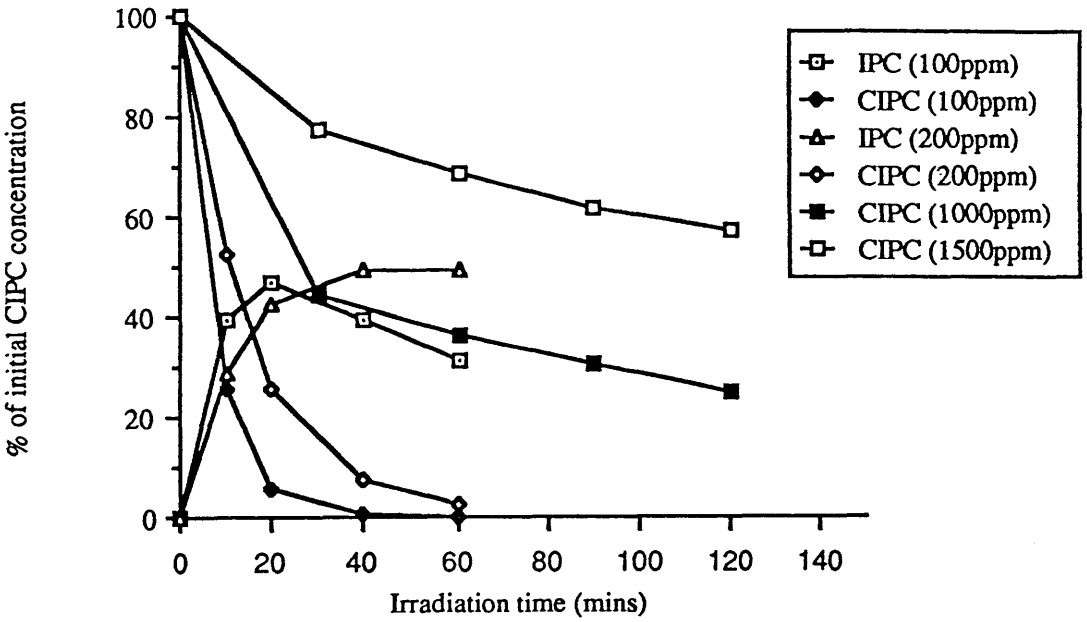


Figure 4.3 Rate of chlorpropham photochange in hexane and in the presence of acetone (2% v/v) and equimolar concentration of vitamin E.

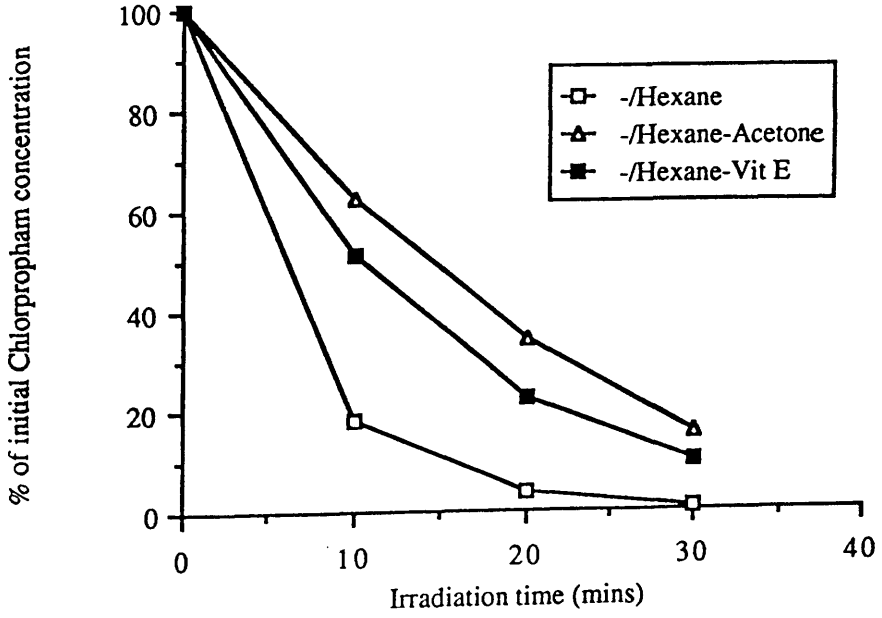


Figure 4.4 Photoprofile formation of propham from chlorpropham in hexane and in the presence of acetone and vitamin E.

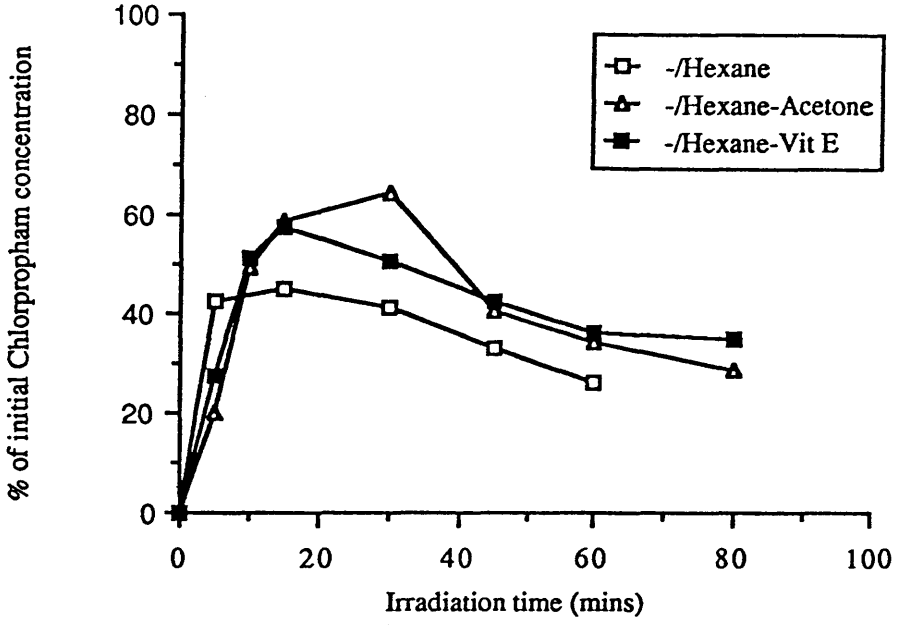


Figure 4.5 Rate of chlorpropham photochange and propham formation in methanol at different concentrations.

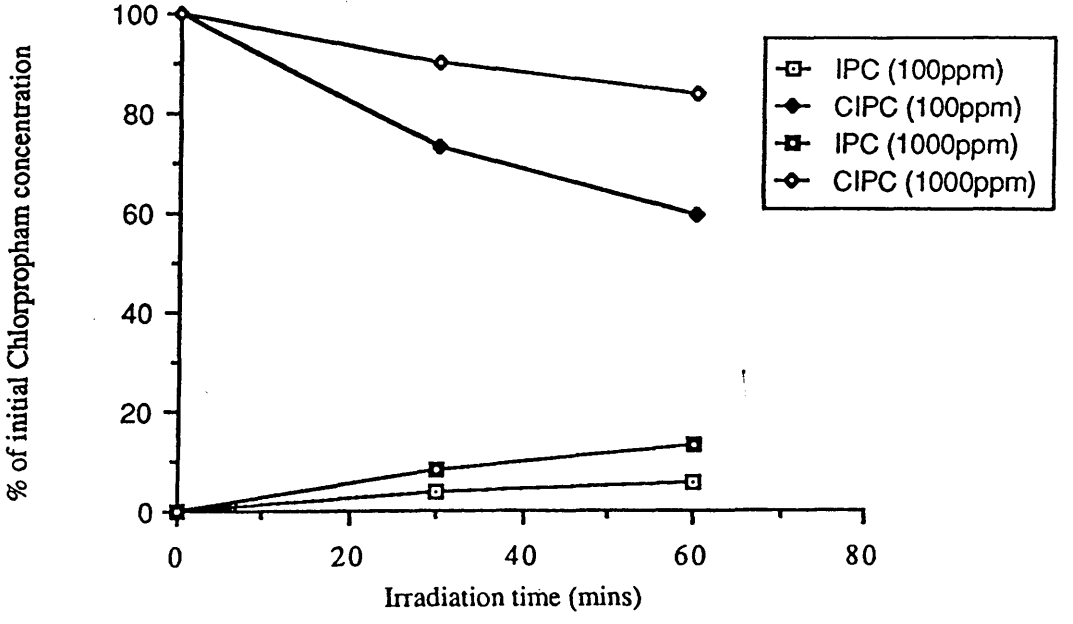


Figure 4.6 Rate of chlorpropham photochange in methanol and in presence of acetone (2% v/v) and equimolar concentration of vitamin C.

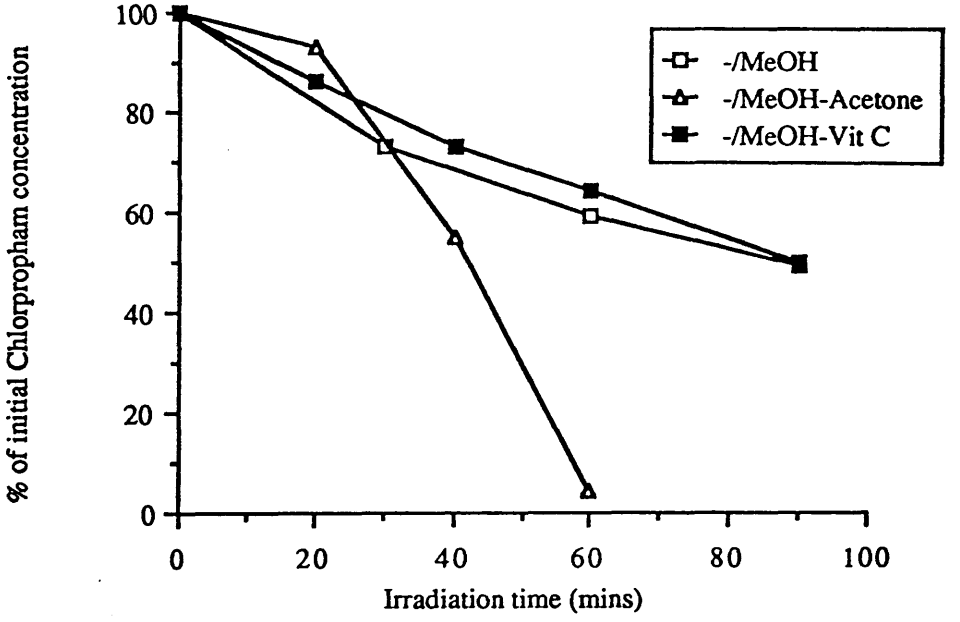


Figure 4.7 The photochange of chlorpropham and the formation of hydroxypropham in distilled water saturated with chlorpropham.

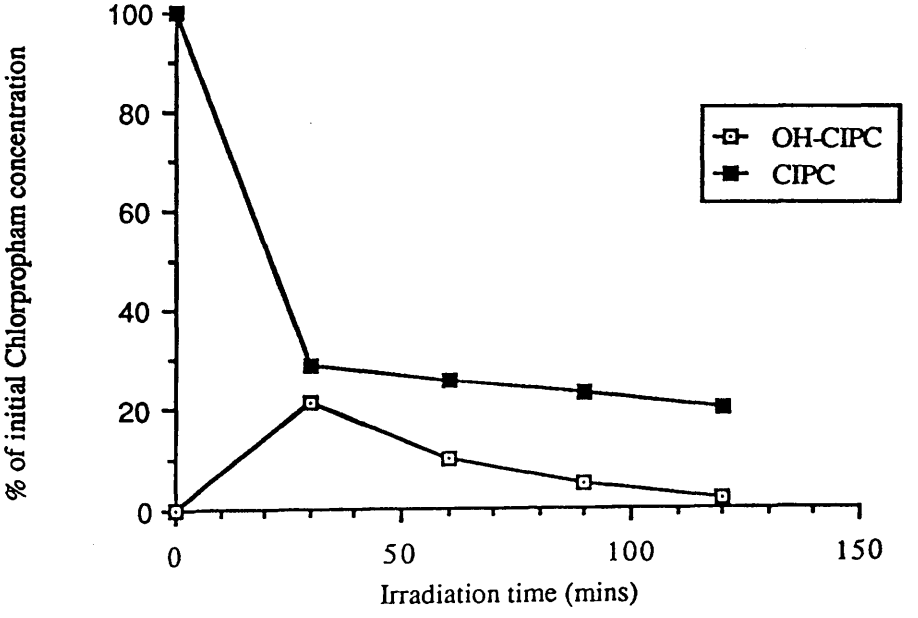


Figure 4.8 Photolysis pattern of chlorpropham disappearance and propham appearance in hexane, methanol and water.

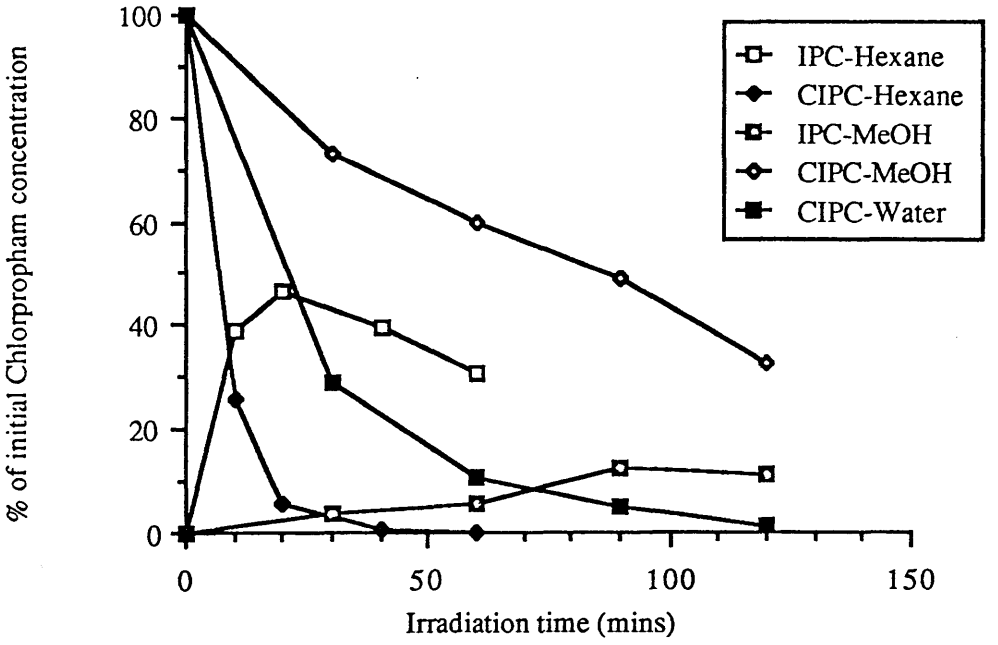
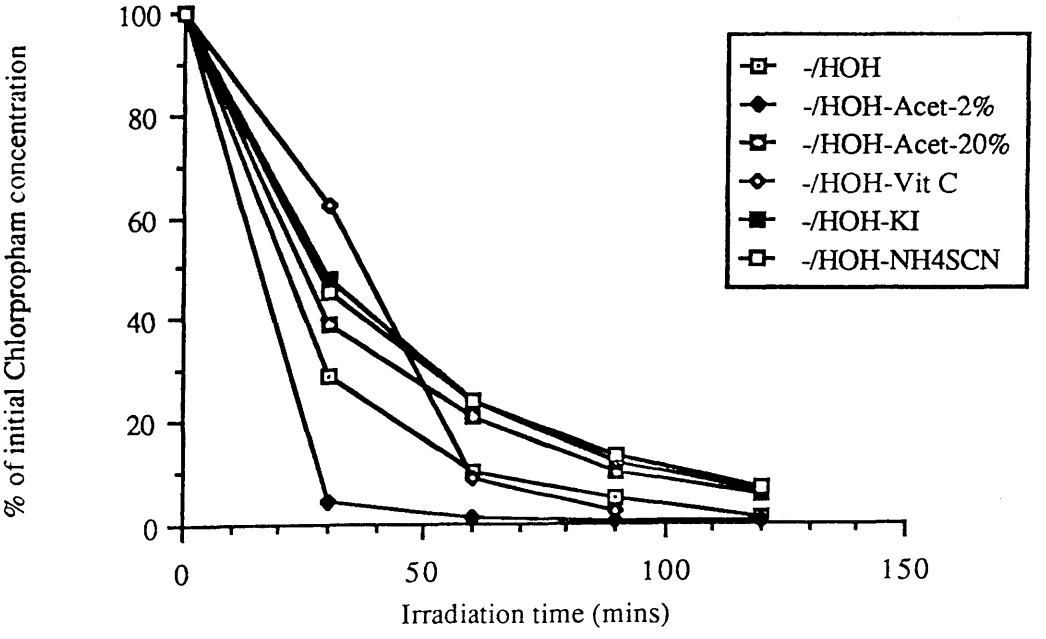


Figure 4.9 Photodegradation pattern of chlorpropham in water and in the presence of other substances of equimolar concentrations.



4.11.3 Identification of Chlorpropham Photoproducts

Chromatographic, mass and GC-MS spectral analysis of the photolyzate in hexane, particularly after concentration and/or chromatographic separation by TLC revealed the formation of propham, with the characteristic molecular ion at M/Z 179, as a major photoproduct, and to a smaller extent eight isomers of hexylpropham with M/Z 263. Two dimers of chlorpropham - propham biphenyl with M/Z 390 and propham-propham biphenyl with M/Z 356, were also obtained. The identity of the propham in the media of hexane, methanol and water was confirmed from its GC-retention time and mass spectrum as compared to that of an authentic sample analysed under the same conditions.

The various isomers of hexylpropham were demonstrated on the GC-MS spectra of the irradiated sample, but not on the respective spectrum of chlorpropham. The absence of isotopic chlorine and the fragmentation pattern of the various isomers complied with the assigned photoproducts. The parent or molecular ion(s) of the proposed derivative(s) did not appear on the GC-MS spectra, while on the mass spectra it did. However this is not without similarities or precedents, since the alkoxy group is a good leaving group especially at high temperature (Saunders and Frisch, 1967). Also the molecular ion of chlorpropham and/or propham, for example either did not appear or appeared in very small abundances on the spectrum of the GC-MS.

The most characteristic fragments for the different isomers were at M/Z 203 and M/Z 133. These fragments may be correlated to the corresponding hexyl phenylisocyanate and methyl phenylisocyanate respectively.

Fox et al. (1973) reported that irradiation of chlorbenzene at 254 nm in organic solvents such as cyclohexane or benzene resulted in a reductive dehalogenation and coupling with the solvent (solvolysis). The authors proposed a high energy caged bi-radical or π-isomer of the type $[Ar^{\cdot}Cl^{\cdot}]^{*}$. The previous authors were not able to answer whether the chlorine atom was localized or was capable of delocalization over the ring moiety. Later on, Arnold and Wong (1977) suggested instead of the π-halogen intermediate, normal free radicals of $Ar^{\cdot}$ and $Cl^{\cdot}$. Also they demonstrated that both of these radicals were capable of hydrogen or solvent abstraction.

In the case of chlorpropham in hexane, a chlorine radical from the homolysis of a C-Cl bond, shows normal selectivity toward tertiary or secondary carbon over a primary carbon. Accordingly one would expect the formation of three kinds of radical resulting from hydrogen abstraction at C_1, C_2 and C_3 of hexane. Since there are three different positions on the ring moiety one would expect to find nine isomers, which was in complete agreement to what has been observed here from the GC-MS spectra of the photolysate in hexane (see figure 4.10). Consequently the formation of free

radicals upon uv-irradiation and the delocalization of the radical over the entire ring system could not be ruled out.

The formation of the biphenyl dimers of chlorpropham-propham and propham-propham, though it has not been reported in any previous study, has similarities with other herbicides. Tanaka et al. (1984) demonstrated the formation of monuron-fenuron and fenuron-fenuron biphenyls upon uv-irradiation of monuron in aqueous solution. The previous authors reported similar dimer formation from anilides and phenylcarbamates in aqueous solution, eg. Tanaka and associates noted the formation of a hydroxylated biphenyl from chlorpropham photolysis. In their explanation for the dimers formation from monuron photolysis. Tanaka and co-workers proposed the generation of phenyl radicals which add readily to an intact molecule forming a fenuron-monuron dimer similar to H11, or to its dechlorinated counterpart forming a fenuron-fenuron dimer, similar to H10. The previous authors, excluded the possibility of a photocoupling among the photoexcited fenuron molecules, as they failed to obtain a fenuron-fenuron dimer from fenuron photolysis.

It is worth mentioning here that in a separate experiment on the photolysis of propham in this laboratory, dimers of propham with molecular ions equal to 356 were isolated by TLC from photolysate in hexane, methanol and water and eventually analysed by mass spectrometry (see table 4.1 and figure 4.11).

Accordingly the possibility of propham or fenuron coupling through the substitution of aromatic hydrogen, although it is rarely documented, can not be ruled out, in contrast to what has been reported by Tanaka et al. (1984).

The photoirradiation of chlorpropham in methanol yielded a variety of products, including propham with M/Z 179 (M1), as a major photoproduct, also two isomers of methoxypropham with M/Z 209 (M2 and M3), three isomers of methoxychlorpropham with M/Z 243, (M4, M5 and M6) and a dimer of chlorpropham-propham with a molecular ion of M/Z 390 (M7). The identity of these photoproducts was determined by their mass spectra after TLC separation, or GC-MS spectra without separation, as compared to the respective mass spectra of synthesised analogues. The right molecular ions and the degradation pattern were in full agreement with the assigned structures (see figure 4.12 and table 4.2).

The photolysis of chlorpropham in water afforded 3-hydroxypropham as a major photoproduct, and propham as a second major product. The meta position of the hydroxy group was deduced from its mass spectrum as compared to that reported by Guzik (1978). Another proof for this came from the absence of a mass fragment at M/Z 107 which corresponds to the iminoquinone from ortho or para derivatives only.

Another four compounds from the concentrated photolysate in water were identified from the GC-MS spectra as compared to their literature analogues as

follows; 3-chlorophenylisocyanate with M/Z 153, benzoxazole-2-one of M/Z 135; and the methylester derivatives of both chlorpropham, M/Z 185 and propham, M/Z 151 (see figure 4.13 and table 4.3)

The formation of benzoxazole-2-one (W1), the poorly represented compound in nature, may have resulted from thermal cyclization of a primary hydroxylated propham ortho to the nitrogen group (Still and Herrett, 1976). Similar photochemical rearrangement and cyclization to the respective lactam from the photolysis of 4-chlorophenoxyacetic acid has been reported by Pinhey and Rigby (1969).

The transesteration or the substitution of a methoxy moiety for the isopropoxy one in N-phenyl-carbamates to the respective W2and W3 may be formed thermally on the GC-column rather than photochemically, since the photolysis was performed in water and no methanol was available.

Similar alkoxy substitution for N,N-dimethyl group in monuron, a phenylurea herbicide, has been reported photochemically in alcohol solution or thermally on the GC-column during its analysis (Gaylord and Stroog, 1953; Lee and Fang, 1971; Mazzochi and Rao, 1972). Tables 5.1-5.3 represent the mass spectral date of chlorpropham photoproducts and their assigned structures in hexane, methanol and water respectively. Figures 4.11-4.13 demonstrate the mass spectra for some of the chlorpropham photoproducts in hexane, methanol and water respectively.

In this context, it is worth noting that the formation of azo and azoxy derivatives from chlorpropham, though they were expected, as such derivatives have been reported as photoproducts from ureides on soil surfaces (Kulshrestha and Mukerjee, 1986), they have not been detected in this study, probably because their formation needs efficient sensitization (Rosen et al., 1970).

As the results from chlorpropham photolysis demonstrated that reductive dehalogenation and solvolysis were common changes in the three studied media, it is interesting to know that such phototransformations are general to all aryl halides and haloatrazines (Plimmer, 1970); several mechanisms were proposed to account for this general trend. Pinhey and Rigby (1969) and Banerjee (1978) rationalized the forementioned changes into three types of mechanism. In the first a proposal of homolysis followed by hydrogen abstraction, while in the second a suggestion of a nucleophilic substitution of the excited aryl cation intermediate ($Ar^{\delta+}-Cl^{\delta-}$) by the solvent, and in the third an electrophilic substitution, by proton for example, with subsequent removal of a halonium ion were made. The third proposal was based on an experimental result which showed that the rate of the reductive dehalogenation of benzidine in water was greater than that in hexane or isopropanol, both of which are better hydrogen donors than water.

Finally as the results of this experiment showed the photolability of chlorpropham into various products under the influence of uv-irradiation it is anticipated

that there may be some questions to be answered concerning the impact and validity of these findings to the natural environment. However, as the conditions of the environment and the laboratory are not comparable, and as the toxicology and persistence of chlorpropham photoproducts are not known, it is difficult to assess. However, as chemicals do not exist in isolation in the environment, and where many substances may act as sensitizers, the transformations observed may give some clues to what might actually occur, especially in arid areas where sunshine predominates for long periods.

4.12 Conclusion

Current knowledge concerning basics of photochemistry and phototransformations of aniline-based herbicides has been summarized. The absorption of the uv-radiation at 254 nm by chlorpropham in polar and non-polar solvents has been investigated. The photochanges of chlorpropham in various media was followed and found to obey first order kinetics, with the highest rate at the lowest concentrations. Rates of chlorpropham phototransformation were affected by the nature and the polarity of the photolyzed media in the following order: water>hexane>methanol. In hexane it was found that acetone cannot act as a sensitizer in contrast to its behaviour in protic solvents, because in hexane, acetone may preferentially abstract hydrogen instead of transfering its triplet excitation energy to chlorpropham. Ascorbic acid, potassium iodide and

ammonium thiocyanate all inhibited chlorpropham phototransformation, probably due to their quenching properties.

The principal pathways of chlorpropham photolysis in aqueous and organic solvents were:

1. Reductive dehalogenation to the more stable propham.
2. Solvolysis, giving rise to hydroxypropham in water, various isomers of methoxypropham and/or methoxy-chlorpropham in methanol and different isomers of hexylpropham in hexane.
3. Coupling or dimerization to the corresponding chlorpropham-propham and/or propham-propham biphenyl dimers.

These findings indicate undoubtedly that chlorpropham is a photolabile herbicide especially under laboratory uv. If some of this behaviour was to happen in the environment, the consequences would be of major significance to public health and environmental pollution, since the toxicology and the persistence of the forementioned photoproducts have not been studied in any detail.

Table 4.1 - Mass spectral data of the most intensive peaks for chlorpropham photoproducts in hexane, accompanied by their assigned names and structures as determined by mass* and/or GC-MS.

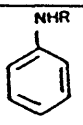
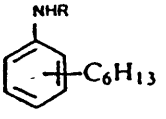
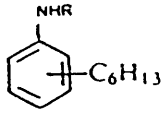
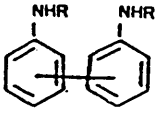
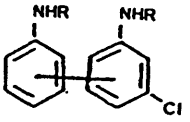
Code number	proposed structure; assigned name	$\underline{M}^{\cdot+}$;M/Z (% intensity)
H1*	 Propham (IPC)	<u>179</u>(30);137(29), 120(25),119(17), 93(84),65(20) 43(100)
H1	Propham	<u>179</u>(2);119(100), 91(26),64(21), 45(60)
H2	 Hexylpropham	<u>263</u>;203(30), 174(4),160(2), 146(4),133(100) 132(13),118(2), 104(2),44(63), 43(44)
H3	Hexylpropham	<u>263</u>;203(22), 147(4),133(68), 132(12),71(37), 44(100)
H4	Hexylpropham	<u>263</u>;203(26), 146(4),133(76), 132(24),44(99), 40(100)
H5	Hexylpropham	<u>263</u>;203(18), 146(6),133(32), 132(16),104(4), 71(36),44(100)

Table 4.1 (continued)

Code number	proposed structure; assigned name	$\underline{M}^{\cdot+}$;M/Z (% intensity)
H6	 Hexylpropham	<u>263</u>;203(33), 146(6),133(53), 132(15),44(100)
H7	Hexylpropham	<u>263</u>;203(34), 160(3),146(100), 133(1),132(16), 128(18),44(52)
H8	Hexylpropham	<u>263</u>;203(33), 160(3),146(3), 133(10),132(100), 90(20),44(38)
H9	Hexylpropham	<u>263</u>;203(28), 160(42),147(46), 146(30),132(56), 128(12),44(100)
H10*	 Propham-propham dimer	<u>356</u>(18);296(15), 236(6),208(12), 179(48),178(37)
H11*	 Chlorpropham-propham dimer	<u>390</u>(2);330(2), 270(8),252(2), 224(6),197(6), 178(19),162(7)

R = $COOCH(CH_3)_2$

Table 4.2 - Mass spectral data of the most intensive peaks for chlorpropham photoproducts in methanol, accompanied by their assigned names and structures as determined by mass and/or GC-MS*.

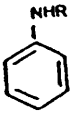
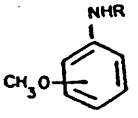
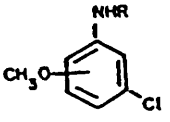
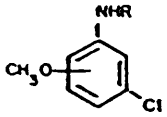
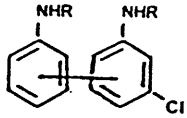
Code number	proposed structure; assigned name	$\underline{M}^{\cdot+}$;M/Z (% intensity)
M1	 Propham (IPC)	<u>179</u>(21);137(21), 120(22),93(82), 77(12),65(30), 43(100)
M2	 Methoxypropham	<u>209</u>(41);167(90), 150(20),123(44), 122(47),108(100), 95(18),80(18), 43(97)
M3	Methoxypropham	<u>209</u>(30);167(58), 150(27),123(56), 122(12),108(4) 95(15),94(35), 80(18),43(100)
M3*	Methoxypropham	<u>209</u>;149(100), 91(15),90(4), 64(4),45(64)
M4	 Methoxychlorprapham	245(8),<u>243</u>(25); 201(44),184(10), 157(31),142(51), 108(57),106(7), 43(100)
M4*	Methoxychlorpropham	<u>243</u>;183(100), 158(13),157(5), 140(21),112(6), 45(79)

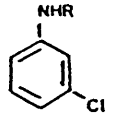
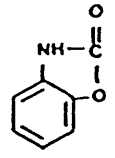
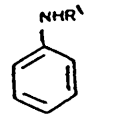
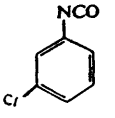
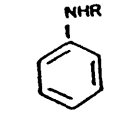
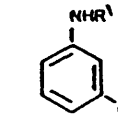
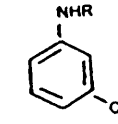
Table 4.2 - (continued)

Code number	proposed structure; assigned name	$\underline{M}^{\cdot+}$;M/Z (% intensity)
M5*	 Methoxychlorpropham	<u>243</u>;183(53), 163(41),157(4), 140(9),132(13) 45(100)
M6*	Methoxychlorpropham	<u>243</u>;183(100), 168(5),157(1), 140(27),112(6), 45(68)
M7	 Chlorpropham-propham dimer	<u>390</u>(8),330(10); 304(8),245(8), 244(8),218(22), 217(9),182(9), 180(11),43(100)

R = $COOCH(CH_3)_2$

Table 4.3 - Mass spectral data of the most intensive peaks for chlorpropham and its photoproducts in water, accompanied by their assigned names and structures as determined by GC-MS.

*For % intensities see Figure 4.13.

Code number	proposed structure; assigned name	$\underline{M}^{\cdot+}$;M/Z (% intensity)*
W	 Chlorpropham (CIPC)	<u>213</u> (50); 171, 154, 127, 99, 75, 63, 43
W1	 Benzoxazole-2-one	<u>135</u> (100); 107, 91, 79, 68, 63, 52, 39
W2	 Methyl N-carbanilate	<u>151</u> (78); 119, 106, 92, 79, 65, 51, 45, 39
W3	 3-Chlorophenyl-isocyanate	<u>153</u> (100); 125, 90, 74, 63, 45
W4	 Propham (IPC)	<u>179</u> (39); 137, 120, 93, 77, 65, 51, 43
W5	 Methyl N-3-chloro-carbanilate	<u>185</u> (100); 153, 140, 126, 99, 90, 75, 63, 43
W8	 Isopropyl N-3-hydroxy-carbanilate,(HO-CIPC)	<u>195</u> (76); 153, 136, 109, 91, 81, 65, 43

R = $COOCH(CH_3)_2$ $R^{\backprime}$ = $COOCH_3$

Figure 4.10 - Gas chromatogram of chloropropham photoproducts in hexane

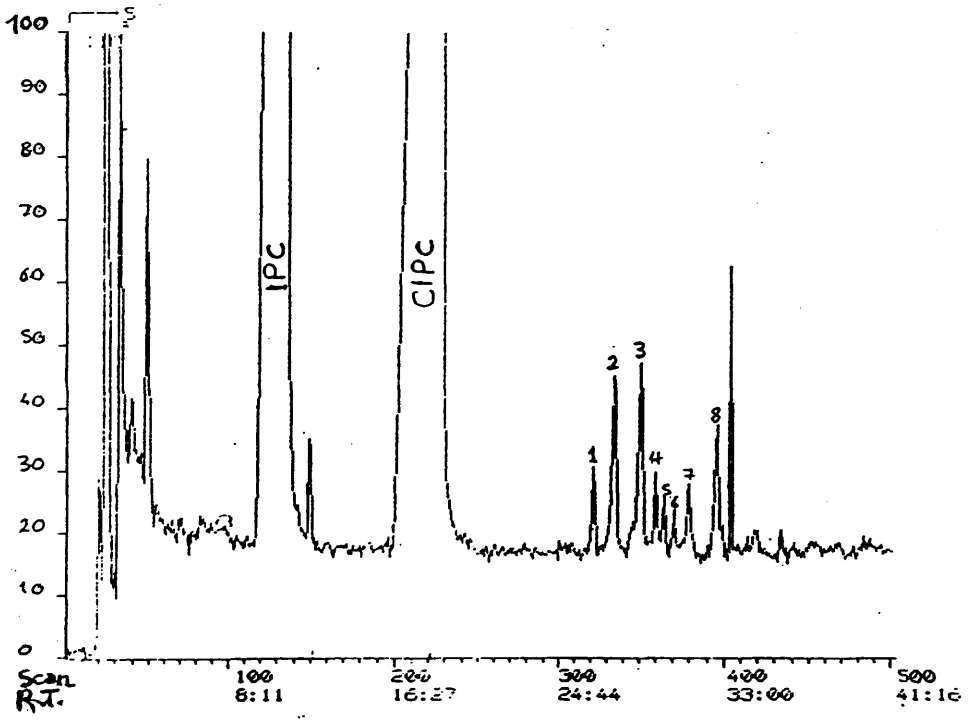


Figure 4.11 - Representative mass spectra of chlorpropham photoproducts in hexane

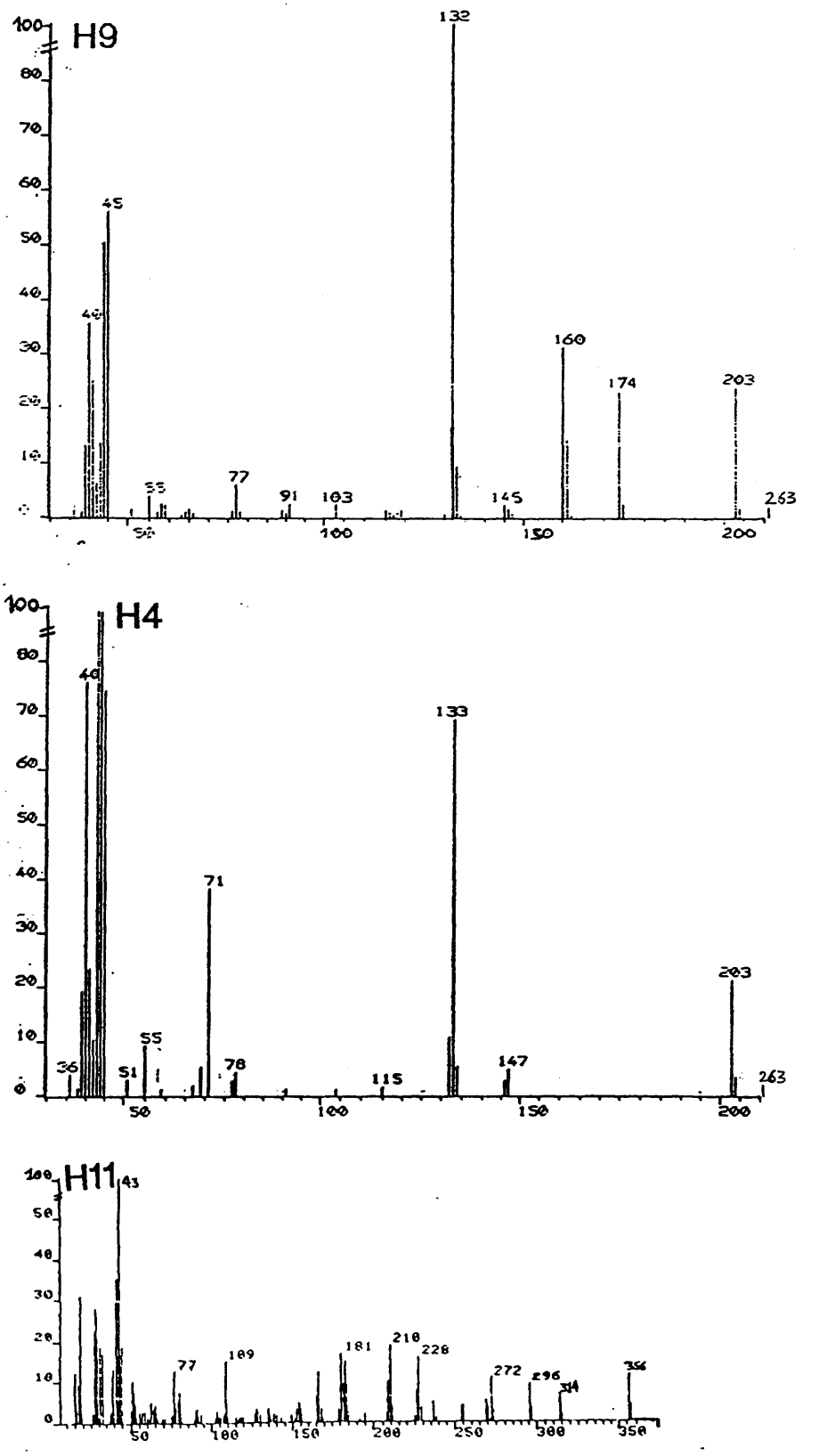


Figure 4.12 - Representative mass spectra of chloroprpham photoproducts in methanol, as determined by mass or GC-MS*

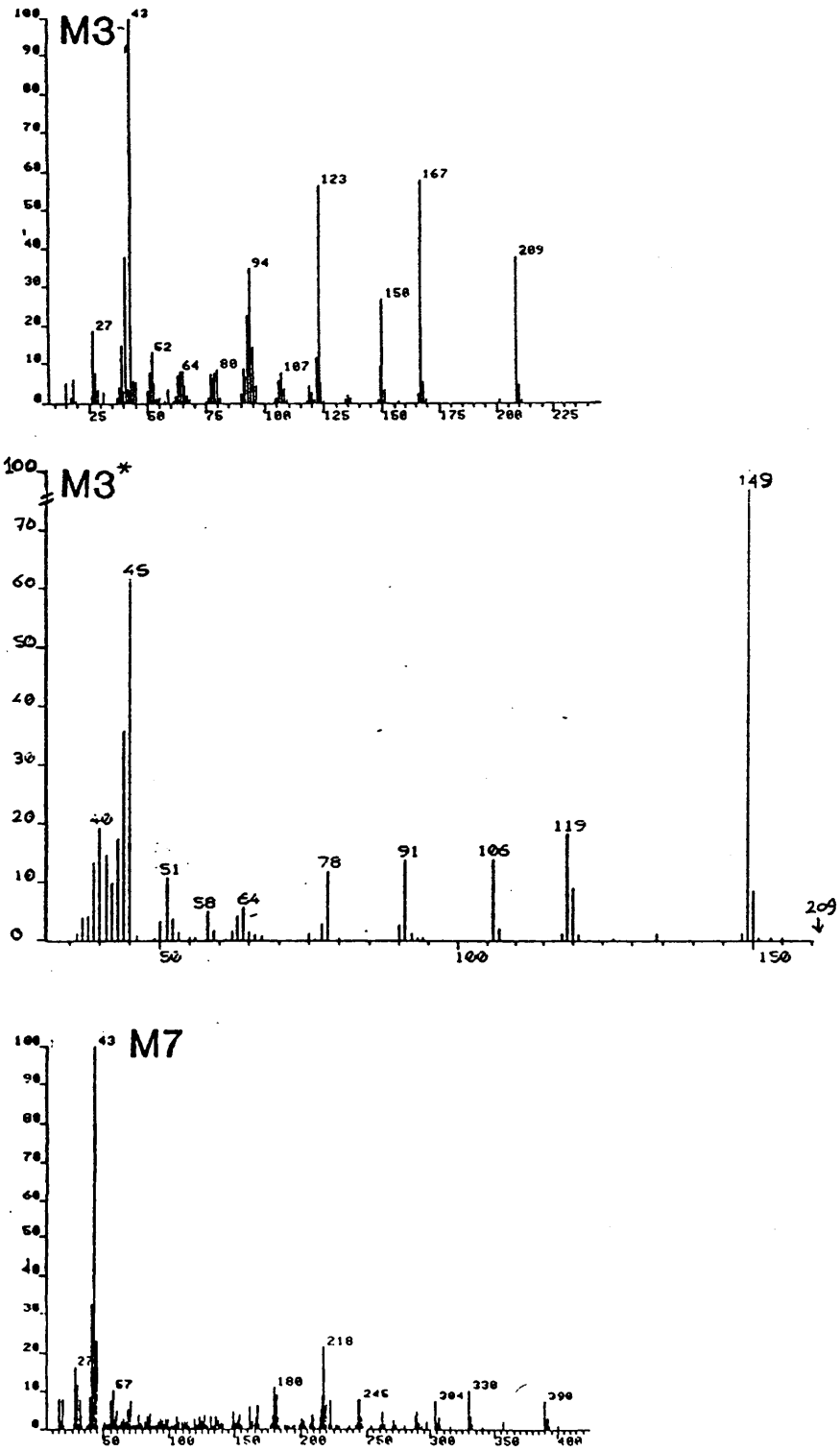


Figure 4.12 (continued)

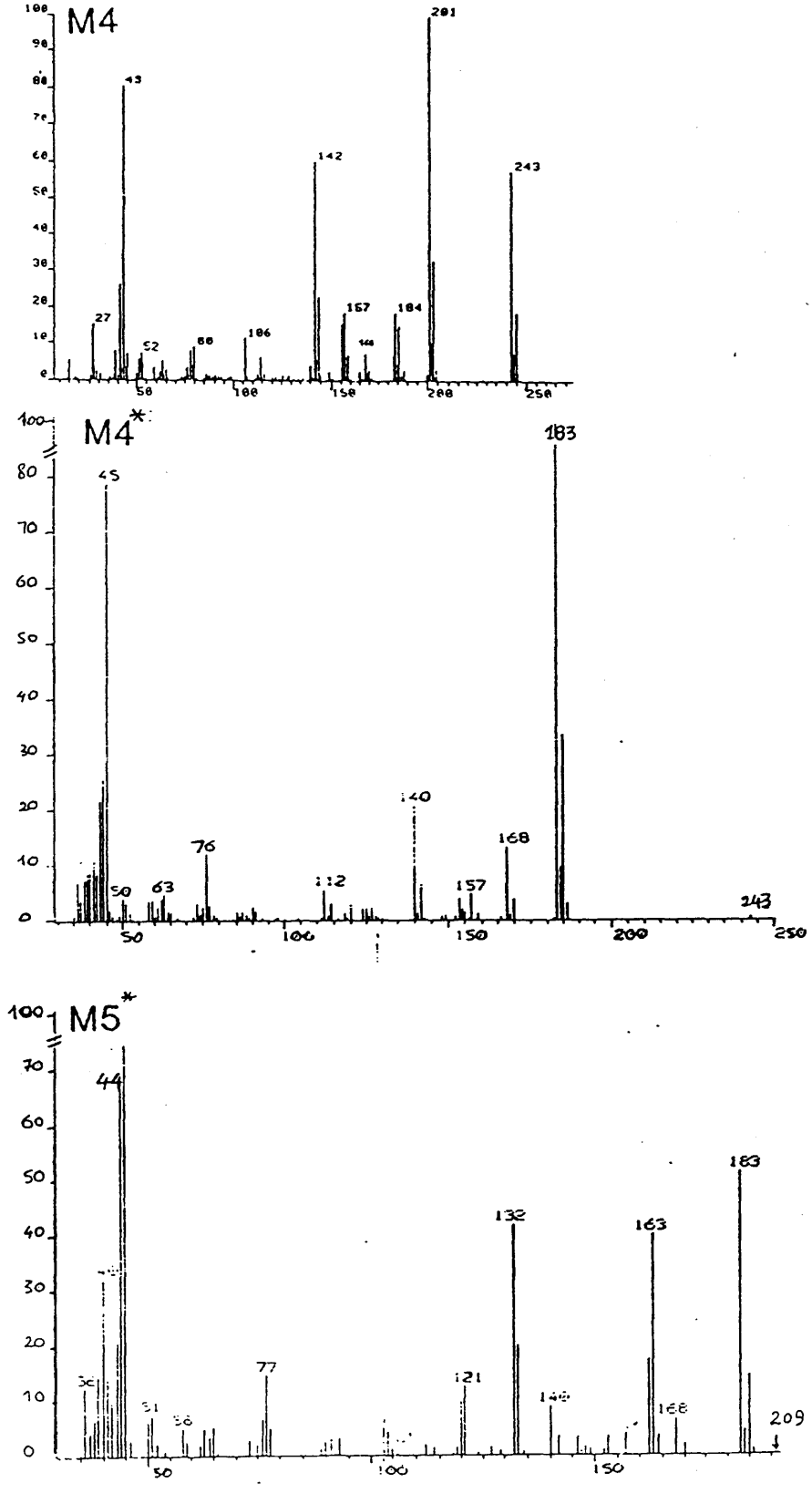
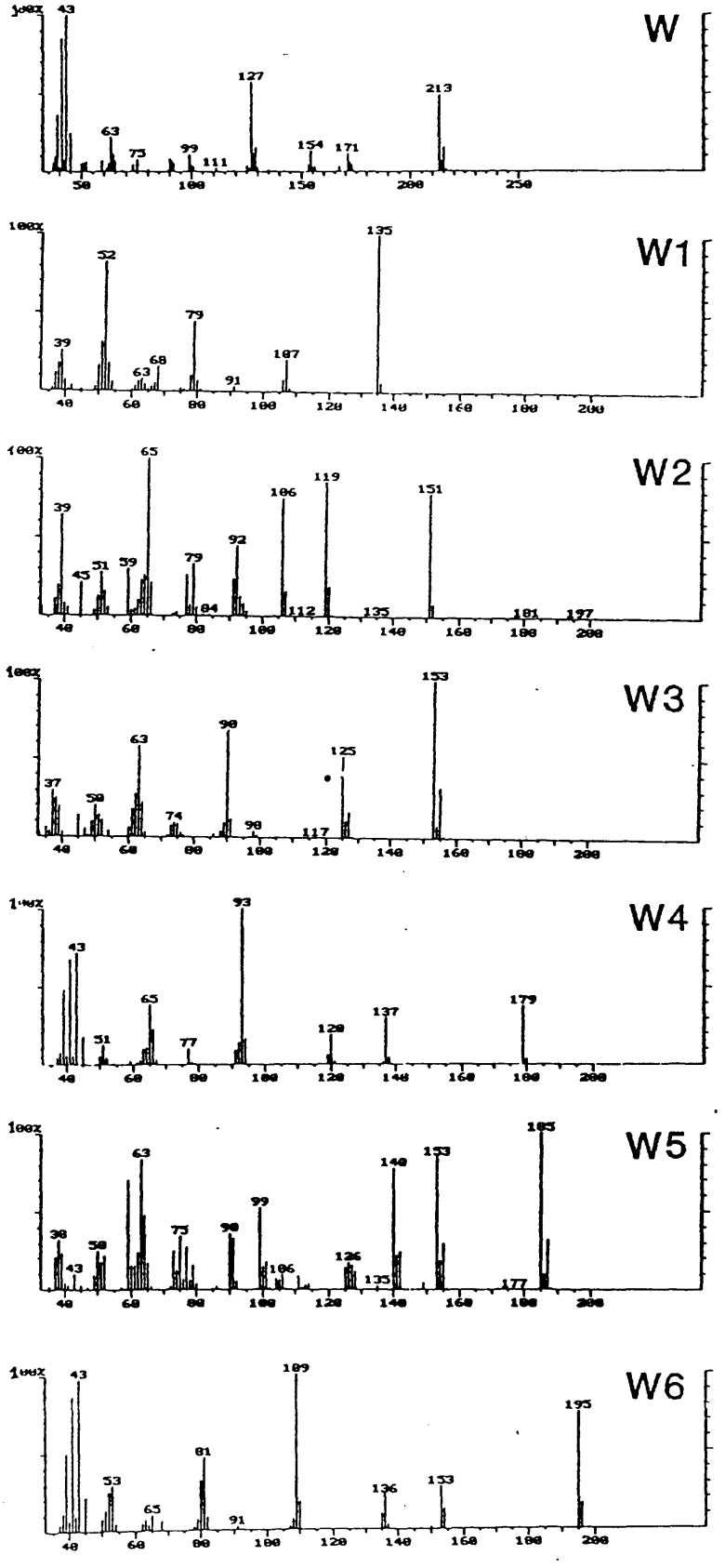


Figure 4.13 Mass Spectra of Chlorpropham and its Photoproducts in Water as Determined by GC-MS.



CHAPTER FIVE

Peroxidase-catalyzed Action on Chlorpropham and Various Related Anilines

5.1 Introduction

The persistence, toxicity and fate of pesticides and their metabolites are matters of environmental and public health concern, because of their widespread use. One group of metabolites, the substituted aniline intermediates, formed in soils, animals and plants by biotic and abiotic action on phenylamide herbicides, are very unstable and may undergo various transformations, producing eventually more or less toxic compounds than their parent analogues.

As various factors are directly or indirectly involved in the transformation processes, it is often not easy to distinguish which factor is responsible for a particular change, especially since all environmental factors are so closely independent and may act simultaneously. Also, the incomplete knowledge on the environmental transformations and the small concentrations present due to sorption, copolymerization, volatilization and mineralization competing processes, all make detection and identification of the transformation products *in situ* quite tedious and complicated. As an alternative, *in vitro* and laboratory model experiments with the advantage of being replicatable and having low level interference, are often

used to investigate the potential transformations of xenobiotics, taking into consideration that their results may or may not represent the actual conditions found in the field.

The degradation of phenylamide herbicides and the transformation of their aniline moieties in soil, microbial and enzymatic cultures has been the subject of intensive studies, and it has been suggested by various workers (Bartha et al., 1968; Bartha and Bordeleau, 1969; Briggs and Ogilvie, 1971; Bordeleau and Bartha, 1972c; Kaufman et al., 1972; Cripps and Roberts, 1978; Simmons and associates, 1986) that peroxidase in soils and microbial cultures are responsible at least in part for the transformations of substituted anilines into various products of oligomers such as substituted azobenzenes, anilinoazobenzenes, biphenylamines, benzoquinoneanils, phenoxazines and azophenines.

Peroxidase-catalysed reactions of aromatic amines have been previously studied (Saunders, 1964; Bordeleau and Bartha, 1972c; Bordeleau et al., 1972), but the products have only been partially clarified, and some contradictions from earlier studies have been noticed. Bearing this in mind, and as no previous study on the effect of chlorpropham has been made, it was felt that an in vitro enzymatic study is worth carrying out. The study aims at an investigation in more detail of the effect of horseradish peroxidase on chlorpropham and various anilines, which have been reported or suspected to be potential metabolites of chlorpropham in soils,

animals or plants (Still and Herrett, 1976; Heikes, 1985). The selected anilines are 3-chloroaniline, p-anisidine, 3-chloro-p-anisidine, 2-amino-p-chlorophenol and nitroaniline.

Also it was felt that giving some insight into the known peroxidase action on various anilines would provide better understanding of the environmental fate of the substituted anilines, and would be very helpful in explaining the consequences obtained.

5.2 Peroxidases

This subject has been fully reviewed and clarified by Saunders et al. (1964) and Paul (1963). Accordingly, peroxidases, of which horseradish peroxidase (HRP) is a typical example, are naturally occurring enzymes of low molecular weights. Their properties are very similar to the other known hemo-proteins such as methemoglobine, catalase and cytochrome-C. Peroxidase contains ferri-protoporphyrin or a heme as prosthetic group attached to its protein moiety via an imidazole group of a histidine residue, and requires for its catalytic action a primary substrate such as hydrogen peroxide (H_2O_2) or an alkyl peroxide (ROOH).

Peroxidases are widely distributed in the biological systems of plants, animals and microorganisms. Frequently they occur extracellular in soils and microbial cultures. It is worth mentioning in this context that peroxidase residues in soils depend on the nature of the microflora and the type of plants growing

in a particular area. Another important aspect is that peroxidases from different sources may have quite different properties. The richest known sources for peroxidases are fig sap and the roots of horseradish. Other sources include saliva, liver leucocytes, milk and intestine juice (Saunders et al. 1964).

Ferriporphyrins play a central role in the action of diverse biological catalysis. Their main metabolic function is to destroy H_2O_2 and/or ROOH which are normally released from biooxidation processes or from ultraviolet irradiation, thereby preventing peroxide build-up in the biocells to the level that is harmful. The destruction of the hydrogen peroxide into water and oxygen is well known in the presence of catalase. However in the case of peroxidase, oxygen is not normally observed. Peroxidases also mediate the oxidative coupling and polymerization of phenols and aromatic amines, especially in the humification process. Moreover, it may be involved in the formation of plant humic matter and lignin probably via free radical oxidation of phenols or coniferyl alcohol respectively (Berry and Boyd, 1984).

Finally, peroxidases may have a valuable selective synthetic role particularly under very mild conditions. In this aspect, the oxidative coupling of aniline(s) to the respective azodyes, the hydroxylation of certain compounds such as salicylic acid , cresols and phenylalanine in the presence of dihydroxy fumarate and oxygen, the redox reactions that take place in stored

wine to improve its taste and the oxidation of the iodide and the iodination of the tyrosine in the thyroid gland are just examples of the peroxidase synthetic role.

5.2.1 Peroxidase Inhibition

Peroxidase inactivation may be brought about by various reagents such as CN^-, F^-, S^{2-}, N_3^-, Mn^{2+} hydroxylamine and high concentrations of H_2O_2. The inhibitory action of these reagents may be attributed to their complexing behaviour with the iron atom in the enzyme prosthetic group. Some reagents like $Na_2S_2O_4$ may reduce the ferriporphyrin to the ferro analogue. Strong acids and bases split the enzyme into its protein and prosthetic components, thereby affecting the enzyme activity. Finally, long storage, heat treatment, mechanical breakdown and uv-irradiation may also decrease peroxidase activity. However Saunders et al. (1964) and references therein, reported that alcohols and glycols up to a concentration of 1% have no detectable effect on peroxidases, especially those of plant origin.

5.2.2 Detection and Assay

Various hydrogen donors may be used for the detection of peroxidase (Saunders et al., 1964; Guilbault et al., 1968). Mesidine, for example, in the presence of H_2O_2 proved to be the most selective and most suitable for this purpose, since it gives a purple product of 2,6-dimethyl benzoquino-4-(2,4,6-trimethyl)-anil. Purgallol or p-anisidine and homovanillic acid also, may be used in peroxidase detection. The amount of

CO_2 from peroxidase-catalysed oxidation of pyrogallol into purpurogallin may be utilized as a measure of the enzyme activity. Related to this, is the so-called purpurogallin number, PN, or the activity unit which is defined (Sigma Chemical Company) as the amount of peroxidase enzyme which forms one mg of purpurogallin from pyrogallol-H_2O_2 in 20 seconds at pH 6 at 20°C. However Bartha and Bordeleau (1969) defined peroxidase activity as the amount of the enzyme which decomposes 1 μM of H_2O_2/min in the presence of 0.5% p-anisidine in 0.05 M phosphate buffer (pH 6) at room temperature.

5.2.3 Peroxidase Mode of Action

Though peroxidases differ in their capability of utilizing a wide range of electron and/or hydrogen donors, they are remarkably specific in their action at least with regard to their primary substrates such as hydrogen peroxide, alkylperoxide or percarboxylic acid RCO-OOH (Lieb and Still, 1969; Bordeleau et al., 1972). Several workers (Saunders et al., 1964; Bordeleau et al., 1972; Taylor and associates, 1984; and Bruice, 1988) studied the mode of action of peroxidase and revealed that the enzyme prosthetic group, upon its reaction with a primary substrate such as H_2O_2, undergoes an irreversible two electron oxid., producing a low spin oxoligated iron(IV) protoporphyrin-IX, π-cation radical which is known as compound I. This intermediate is reactive and may acquire an electron or a hydrogen atom from a secondary substrate (AH_2) such as an aromatic amine, phenol, indoleacetic acid or ascorbic acid,

resulting in the formation of an iron(IV) oxoporphyrin species, termed compound II. This tends upon further reduction by an electron or hydrogen atom to the formation of the free enzyme (E), as demonstrated below (Bordeleau et al., 1972).

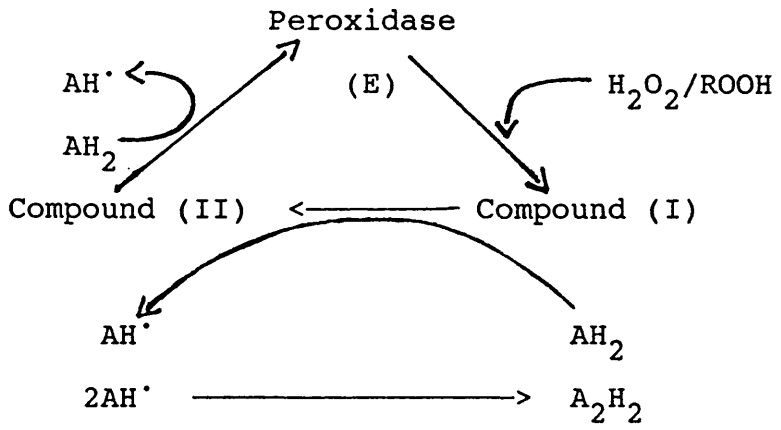


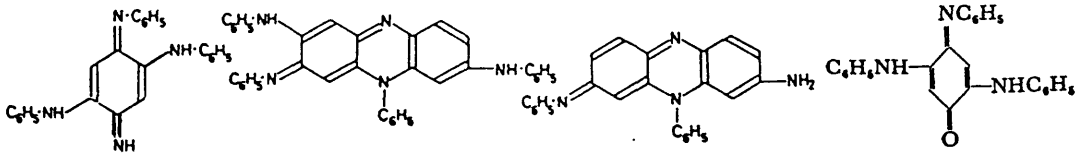
Figure 5.1 - Currently accepted mechanism for the peroxidase-catalysed reaction. AH_2, stands for hydrogen donor, $AH^{\cdot}$, is a free radical species and ROOH, an alkyl peroxide.

The overall enzymatic reaction is influenced by the nature of the reducing agent. Anilines for example, are easier to react with peroxidase than the analogous phenols, because nitrogen is less electronegative than oxygen. Bordeleau and Bartha (1972c); Cripps and Roberts (1978) and many others reported that the susceptibility of arylamines to peroxidase reactions increases as the electron density on the nitrogen atom increases as well, but leads to the formation of high molecular weight polymers rather than dimers of azobenzene compounds. In this field, Brown et al. (1978) demonstrated that HRP-catalyzed oxidations showed an inverse dependence on the hydrogen ion concentration $[H^+]$, ie. the enzyme

activity increases with the pH, but this was to a certain limit, because at high enough pH, the active peroxidase monomers aggregate and change into a pH independent and less reactive oxo-bridge (Fe-O-Fe) dimer. Earlier, Bordeleau and Bartha (1972b) reported that peroxidase-oxidation of arylamines displayed a pH dependence with optimum activity at pH 4.8. The above authors also revealed no peroxidase oxidation in the absence of H_2O_2.

5.2.4 Peroxidase Oxidation of Aromatic Amines

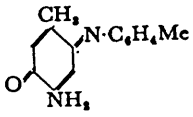
Various studies have been conducted to investigate the action of the peroxidase system on several aromatic amines and phenols (Saunders et al., 1964; Saunders, 1957). Saunders and associates reported that horseradish peroxidase and H_2O_2 oxidize aniline to a coloured complex mixture of related compounds such as aniline black, nitrobenzene, 2,5-dianilino-p-benzoquinoneimide-anil (I), induline (II) and pseudo-mauvine (III). However, with Fenton's reagent, (Fe^{++}/H_2O_2), aniline gave benzoquinone monoanil (IV) as a major product.



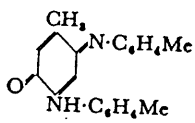
I II III IV

P-toluidine with the peroxidase system yielded various products including dimers, trimers and tetramers identified by Holland and Saunders (1966) as 4,4^{-}-dimethyldiphenylamine; 4,4^{-}-dimethylazobenzene;

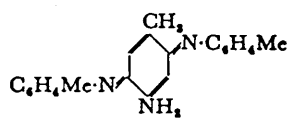
4-amino-2,5-toluquinone-2-p-tolylimine (V);
4-p-toluidino-2,5-toluquinone-2-p-tolylimine (VI),
4-amino-2,5-toluquinone bis-p-tolylimine (VII) and
4-p-toluidino-2,5-toluquinone-bis-p-tolylimine (VIII).



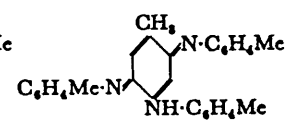
V



VI



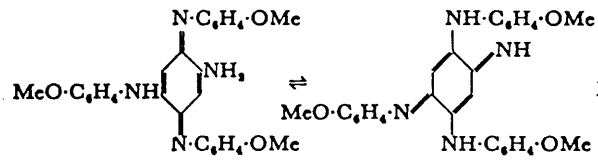
VII



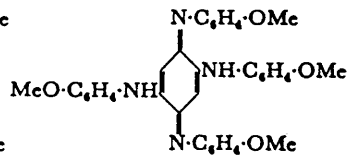
VIII

Holland and co-workers also reported that the relative yield of the formed products depends upon the initial concentration of the arylamine.

The oxidation of p-anisidine and p-chloroaniline by peroxidase/H_2O_2, though they were expected to behave as p-toluidine, they were not found to do so. Daniels and Saunders (1951) reported that the enzyme catalyzed oxidation of p-anisidine yielded three products separated and identified as 4,4^{-}-dimethoxyazobenzene; 2-amino-5-p-anisidino benzoquinone-di-p-methoxyphenylimine (IX) and tetra-p-methoxyazophenine (X).



IX



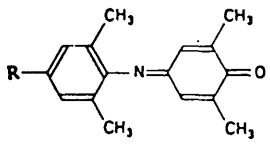
X

The work of Bordeleau and Bartha (1972c) on the three isomers of the anisidine with HRP/H_2O_2 revealed that none of them gave the respective azo derivative as in the case of nitroanilines. Earlier, Briggs and Ogilvie (1971) reported that 3-chloro-p-anisidine in the

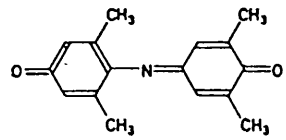
soil or in the presence of the peroxidase system at pH 7 yielded 3-chlorobenzoquinone-4-(3-chloromethoxy)anil and its reduced form to a smaller extent. However at pH 4.5 the enzymatic oxidation gave unidentified trimer and polymeric material in addition to the respective azo derivative.

It is of interest to know that the reduced forms of benzoquinone-anil represented the major metabolite of phenacitine and/or phenetidine, the antipyretic drugs, in human red blood cells (Hawkins, 1988).

In contrast to what has been mentioned, Saunders and Wadak (1967) reported that peroxidase-catalysed oxidations of 2,5-dimethylaniline or its p-substituted analogue gave the corresponding benzoquinone-anil (XI) as a single product in high yield most probably via the respective methylol and formyl intermediates, mesitole and 2,6-dimethylbenzoquinone and the tetra-methyldiphenoquinone (XII) (Saunders and Stark, 1967).



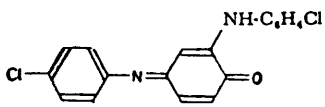
XI



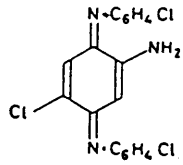
XII

In this field, the oxidation of monohaloaniline such as p-chloro- or p-bromoanaline by peroxidase systems produced three derivatives separated and identified by Daniels and Saunders (1953) as 4,4^{-}-dichloroazobenzene 2-amino-5-p-chloroanilinobenzoquinone and p-tetrachloroazophenine similar to the outcome from p-anisidine (see compounds IX and X).

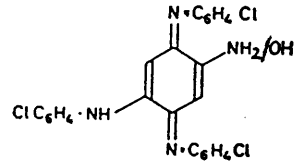
When the above work was repeated by Bordeleau and co-workers (1972) to re-examine the formation of azobenzene from monohaloanilines in soils, microbial or enzymatic cultures, they reported the formation of 4-chloro-4^{-}-(4-chloroanilino)azobenzene in addition to the respective azo compound. Holland and Saunders (1968) and Simmons et al. (1986) revealed the formation of eight oligomers from the action of peroxidase on p-chloroaniline, six of which have not been previously reported. Simmons and associates separated the various products by HPLC and identified them by mass spectroscopy as: N-(4-chlorophenyl)-p-phenylenediamine or p-chlorosemidine; N-(4-chlorophenyl)-p-phenylenediimine; N-(4-chlorophenyl)-benzoquinonemonoimine; 2-(4-chloroanilino)-N-(4-chlorophenyl)benzoquinonemonoimine (XIII); 2-amino-5-chlorobenzoquinone-di-4-chloroanil (XIV) and 2-(4-chloroanilino)5-aminobenzoquinone-di-4-chloroanil (XV) and its hydroxy analogue.



XIII



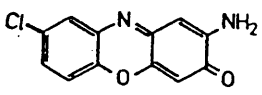
XIV



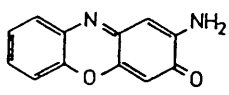
XV

Simmons and co-workers reported that p-chlorosemidine and phenylenediamine compounds did not appear on the TLC probably due to their instability towards oxidation. The authors also suggested that the benzoquinonemonoimine was formed by a non-enzymatic hydrolysis of the terminal imine moiety.

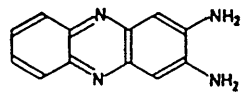
In relation to what has been mentioned, Briggs and Walker (1973) demonstrated that microbial incubation of 4-chloroaniline yielded coloured compounds identified by mass spectrometry as 7-chloro-2-amino-3H-phenoxazine-3-one (XVI). This compound was thought to be formed by condensation of a primary hydroxylated 4-chloroaniline on route to chlorocatechol and ammonia. Nagasawa and co-workers (1955) and Engelhardet et al. (1977) reported the isolation and identification of a similar phenoxazinone (XVII) and its reduced form in the urine of rabbits and in microbial culture containing ortho-aminophenol. Similarly Nagasawa and Gutmann (1959) and Saunders et al. (1964) described the formation of 2,3-diaminophenazine (XVIII) from a peroxidase system containing ortho-phenylenediamine.



XVI



XVII



XVIII

Recently Hamilton (1989) showed that the mechanism of phenoxazinone formation involves oxidation of the aminophenol to the respective iminoquinone followed by tautomerization and cyclization.

Finally it is worth mentioning that careful investigation of the action of HRP/H_2O_2 on arylamines failed to demonstrate the production of nitrosobenzene, although Saunders et al. (1964) and Corbett et al. (1978) mentioned the formation of small percentages of

nitrosobenzene and chloronitrobenzene from aniline and 4-chloroaniline respectively. More recently Corbett and Corbett (1983) demonstrated the enzymatic oxidation of arylamines to the corresponding hydroxylamines, nitroso and nitro derivatives by H_2O_2 and that the microsomal fraction of germinating pea seedlings was comparable to the action of chloroperoxidase.

5.2.5 Mechanism of Peroxidase Oxidation

Aromatic amines and phenols are electron rich substances and may act as reducing agents. Accordingly they are very susceptible to peroxidase-catalyzed oxidation. Several proposals were suggested to account for their oxidative transformation. Saunders and associates (1964); Pordeleau et al. (1972); Parris (1980) and Berry and Boyd (1984) mentioned that arylamine free radical formation of the type (Ar—NH)$^{\bullet}$, via subsequent losses of an electron and proton would be satisfactory to account for most of the symmetrical and asymetrical oxidative coupling. The ($N^{\bullet}$+$N^{\bullet}$) symmetrical condensation eventually leads to the formation of azo type compounds via the corresponding hydrazo labile intermediates as follows:

$$2ArN^{\bullet}H \longrightarrow ArNH\text{-}NHAr \underset{[H]}{\overset{[O]}{\rightleftharpoons}} ArN{=}NAr \quad \text{--------} \quad (5.1)$$

However the asymmetrical coupling of the ($N^{\bullet}$+$C^{\bullet}$) type results in the formation of quinonediimine derivatives, which upon further hydrolysis, addition and/or redox reactions gives various products such as

benzoquinoneanils and/or azophenines. Once formed, these compounds may undergo various nucleophilic and/or coupling reactions at any centre on the aryl moiety, depending on the electron density and hence the effect of the electrophilic site and the approaching nucleophile as well (Brown, 1988).

In this context, it has been reported by many workers (Saunders, 1957; Lieb and Still, 1969; Bordeleau and Bartha, 1972; Berry and Boyd, 1984) that arylamine susceptibility to peroxidase transformation is dependent on the electronic distribution of the molecule, which is primarily determined by the nature, number and position of the substituents on the ring moiety, e.g. electron withdrawing groups (EWG) such as nitro, cyano, 2,5-dichloro, 2,6-dichloro and 2,4,6-trichloro, all inhibit the localization of the free radical on the nitrogen atom, thereby preventing it from enzymatic transformation. However, electron releasing groups (ERG) such as $-OCH_3$, $-CH_3$ increase the electron density on the nitrogen atom, i.e. its basic strength and hence increase the susceptibility of arylamines to peroxidase transformation particularly into high molecular weight polymers rather than azo derivatives.

Another possible route which would account for the formation of the nitroso, nitro, azo type compounds and other iminoquinone, suggests the involvement of the hydroxy radicals $[OH]^{\cdot}$ which could join to the nitrogen radicals forming hydroxylamine intermediates, or to the ring moiety forming aminophenols or iminoquinone.

Bordeleau et al. (1972); Kaufman and associates (1973) and Engelhardet et al. (1977) studied peroxidase oxidation of chloroanilines and were able to observe and identify interchangeable products of hydroxylamine, nitroso and nitro compounds formed as follows:

$$ArNH_2 \rightleftharpoons ArNHOH \rightleftharpoons ArNO \rightleftharpoons ArNO_2 \quad \text{------} \quad (5.2)$$

A third proposal which would account for the formation of hydroxylamine, nitroso, phenoxazinone and benzoquinoneanil and the ability of arylamine to conjugate itself to biological macro molecules such as proteins, DNA and/or RNA, includes the formation of an arylnitrinium ion of the type $(Ar{-}NH)^+$ through subsequent losses of two electrons and a proton. This route is likely to occur mainly in the presence of chloroperoxidase which dominates in the marine environment (Saunders and Wadak, 1970; Parris, 1980).

5.3 Experimental

5.3.1 Materials

Isopropyl N-(3-chlorophenyl)carbamate or chlorpropham, technical grade, horseradish peroxidase (HRP) type II, with RZ (Reinheitszahl) value of 2 and a specific activity of 200 purpurogallin units per mg of the solid, and cysteine, all were purchased from Sigma Chemical Co. and used as they were received. 3-chloro-aniline, B.P. 95-96° (11 mm Hg), 3-chloro-p-anisidine, p-anisidine and 5-chloro-2-hydroxyaniline were obtained from Aldrich Chemical Company, 4-nitroaniline, hydrogen

peroxide, 30% (w/v), sodium acetate (anhydrous), sodium hydroxide, L-ascorbic acid and ammonium thiocyanate were obtained from BDH Ltd. Sulfanilamide and potassium iodide were obtained from May and Baker Ltd. Potassium dihydrogen phosphate and sodium dithionite ($Na_2S_2O_4$) were obtained from Hopkin and Williams Ltd.

All chemicals and other solvents were Analytical Grade or Analar and used as such except 3-chloro-p-anisidine which was purified by vacuum distillation. Pre-coated glass plates, 20x20 cm, for preparative layer chromatography (PLC) and TLC plates were silica gel 60 F_{254} and GF_{254} respectively, with ~ 13% calcium sulphate and fluorescent indicator. Layer thickness for the prepared TLC was 1 mm.

5.3.2 Preparation of Buffer Solutions

Two buffer systems, A - phosphate buffer of pH 6.8, and B - acetate buffer of pH 4.8, were prepared in distilled water according to procedures described for preparation of buffers for pH and use in enzyme studies (Colowick and Kaplan, 1955; Perrin and Dempsy, 1974) as follows:

A: Phosphate Buffer

This buffer was prepared by mixing 25 cm^3 of 0.2 M solution of KH_2PO_4 and 11.7 cm^3 of 0.2 M solution of NaOH in a total volume of 100 cm^3 of distilled water.

B: Acetate Buffer

This buffer was prepared by mixing 20 cm^3 of 0.2 M solution of acetic acid and 30 cm^3 of 0.2 M

solution of CH_3COONa in a total volume of 100 cm^3 of distilled water.

The pH values for both systems were checked by pH meter (Pye Unicam Model 292) and adjusted for the correct values before use.

5.3.3 Peroxidase Catalyzed Reactions

Solutions of horseradish peroxidase (HRP, Type II) and hydrogen peroxide (H_2O_2) were prepared in distilled water to give concentrations of 2.5 mg cm^{-3} (500 unit cm^{-3}) and H_2O_2, (20% w/v).

Chlorpropham and other substituted anilines tested were dissolved and/or suspended in the phosphate and acetate buffers separately at buffer concentrations of 10^{-2} M or mM/100 cm^3. All experiments were carried out in glass flasks open to the atmosphere at room temperature.

5.3.3.1 General Procedure

To a stirred solution and/or suspension of the prepared arylamine derivative in the buffer solution, 0.1 cm^3 of HRP was added followed by a dropwise addition of 1 cm^3 of H_2O_2. Further addition of the same quantities of the enzyme and peroxide were repeated intermittently after 30 and 60 minutes of the initial addition, as too much H_2O_2 may inhibit the enzyme activity. The reaction mixture was set aside and left overnight stirring at room temperature to ensure complete reaction.

A. In the case of chlorpropham, nitroaniline and sulfanilamide, no visible change in either pH media were noticed after the addition of the enzyme and/or the hydrogen peroxide, even after 3 weeks incubation, as compared to a blank of each amine with the enzyme or hydrogen peroxide alone.

B. In the case of 3-chloroaniline, no immediate visible change was observed following the addition of the enzyme alone. However, a faint yellow coloration developed after some time following the addition of hydrogen peroxide. As time passed following the third addition of the enzyme and peroxide, the intensity of the developed colour increased as compared to the blanks in the two buffer systems. No visible changes were ever observed in the blanks.

C. When 3-chloro-p-anisidine and 2-amino-p-chlorophenol were incubated with the peroxidase system, immediate dark red to deep violet colorations were observed as soon as HRP and H_2O_2 came into contact with each other and with the titled anilines in any of the two buffer systems. After a few minutes of the colour development a dark red to brown precipitate was also observed.

5.3.3.2 Inhibitory Studies

Equimolar amounts (10^{-2}M) of para-anisidine, 3-chloro-p-anisidine and 2-amino-p-chlorophenol were placed separately in the two sets of buffer and were treated with equimolar quantities of organic and inorganic reducing agents in the following order:

1) H_2O_2

2) HRP

3) HRP/$Na_2S_2O_4$

4) HRP/Ascorbic acid

5) HRP/Cysteine

6) HRP/KI

7) HRP/NH_4SCN

In the forementioned seven cases, no observable changes were noticed. However, dropwise addition of the hydrogen peroxide to the five sets, 2-7, produced visible changes. The amounts of H_2O_2 needed to initiate the coloration in the various sets were different, depending on the nature of the reducing agent present, as compared to the amount of H_2O_2 needed to initiate the reaction in set number 2, where only the amine and HRP were present. Also it was noticed that the amount of H_2O_2 needed to initiate peroxidase catalysed oxidation increased with the amounts of the reducing agent present in the following order:

Ascorbic acid >> $Na_2S_2O_4$ > Cysteine

However, in the presence of KI or NH_4SCN, the amount of H_2O_2 needed to initiate the enzyme catalysed reactions was not different from that needed to initiate the reactions of the respective blanks.

5.3.3.3 Isolation of the Products

In all cases studied, observable changes were noticed. Products from each reaction medium were partitioned twice into ethyl acetate. The combined organic extracts were then dried over Na_2SO_4, filtered and concentrated under vacuum to a few cm^3 prior to the separation trials.

Chromatographic separations were made on self-coated thin layer chromatographic plates (TLC) 3 mm thickness or on ready or pre-coated ones (PLC) with fluorescent and calcium sulphate as binder. Various developing systems to move the various components from the origin were tested, of which toluene-acetone (7:3 v/v) was chosen and considered the most satisfactory for all subsequent separations. Following plate development, products were examined by the naked eye and localized. Eventually, the various localized bands were scraped off and the components were removed and eluted from the silica gel with methanol, prior to their analysis by mass and/or GC-MS spectrometry. The previous treatment resulted in separation of various coloured bands.

5.3.3.4 Identification of the Products

The products from TLC and/or PLC plates were analysed by positive ion electron-impact mass spectrometry, using direct probe and single focusing technique. Identification from mass spectra were made on the basis of molecular ions, isotopic abundances and fragmentation pattern as compared to the mass spectra of reference standards and/or literature analogues.

In some cases the presence of metastable ions, using the formula in Sternhell and Kalman, (1986),

$$\text{metastable ion} = \frac{(\text{mass of the daughter})^2}{\text{mass of the parent ion}} \quad \text{---- (5.3)}$$

was utilized as a confirmatory mean for certain fragmentations. Due to the small amounts being handled,

only mass spectra provided the necessary information for identification, and hence no further confirmatory studies were made except in a few cases where high resolution mass spectrometry, ultra-violet absorption spectroscopy and colour tests were utilized.

5.4 Results and Discussion

5.4.1 Peroxidase Action on Chlorpropham, para-Nitroaniline and Sulfanilamide

Incubation of the above tested compounds separately in the peroxidase system, afforded no observable changes, as compared to a blank of each, even after three weeks of incubation. This was in agreement with the findings of Briggs and Ogilvie (1971) who stated that soils and slurries containing ureides or ethyl-carbamate, up to more than two months, yielded no observable changes as compared to those incubated with acylanilides. This indicated that ethylcarbamate and ureides degraded too slowly producing not enough aniline to react with peroxidase.

The inertness of the sulfanilamide and nitroaniline toward peroxidase oxidation, may be attributed to the presence of the sulfonamide or the nitro groups on the phenyl ring, which, according to their electron withdrawing nature, tend to stabilize their respective anilines and prevent them from forming anilino radicals, the precursors for their oxidative reaction.

The forementioned results were in agreement with the previous findings on nitroanilines (Bordeleau and Bartha, 1972c) and hence may be regarded as confirmatory.

5.4.2 Peroxidase Action on 3-Chloroaniline (3-CA)

The addition of hydrogen peroxide and peroxidase to a buffer aqueous solution of 3-CA yielded within a few hours a faint yellow to red coloration. When extracted with ethyl acetate and analysed by GC-MS using capillary column of a semipolar stationary phase, this digest revealed the existence of nine metabolites in addition to the starting material. Attempts to isolate the enzymatic products by TLC and identify them by mass spectrometry using direct inlet technique, confirmed some of the GC-MS identified products, and also demonstrated the formation of eight additional metabolites. In table 5.1 is shown the various metabolites of 3-chloroaniline and their assigned identities.

Molecular ions and isotopic abundances of all the various metabolites were in accordance to the assigned structures. Not only was this the case but their mass degradation patterns were in agreement with those of some reference standards and/or literature analogues (Budzikiewicz et al., 1967).

Figure 5.2 demonstrates the mass spectra of 3-CA and its metabolites.

Despite the incomplete reaction of 3-CA, as was revealed from the GC-MS analysis of the crude reaction mixture, peroxidase catalysed oxidation of 3-CA resulted

in the formation of seventeen metabolites ranging in size from one to four units of the chloroaniline (table 5.1).

The formation of 3-chlorophenylisocyanate, C1, may have resulted from thermal degradation of the respective formanilide and/or acetanilide. As the isocyanate group is very reactive, it was not expected to stay free in the reaction media. Accordingly, the formation of the corresponding phenylurea, C10, probably came from the reaction of the isocyanate with the available 3-chloroaniline in the reaction media, or on the GC column.

N-hydroxylation with subsequent oxidation into the nitro derivative through a nitroso labile intermediate could be regarded as the most probable route for the formation of metabolites C3 and C11.

Oxidative coupling with the formation of chloro-substituted azo benzene, C5, phenazine, C12 and 4-amino di-phenylamine in C11 and C16 might be attributed to a symmetrical and unsymmetrical coupling of chloro-substituted anilino radicals as discussed in section 5.2.5.

The formation of the azoxy metabolites, cis and trans in C6 and C7 gave indirect evidence for the formation of hydroxylamines and/or nitroso labile intermediates, the precursors for the azoxy derivative formation.

Metabolite C13, with the molecular ion of 308, was thought to have the formula of p-N,N-diformylamine-3,3^{-}-dichlorodiphenylamine. However, this was not the

case, as the metabolite showed a pronounced mass spectral peak at M/Z 154. Accordingly an alternate with no previous evidence was considered and the product identified as N,N^{-}-diformylbenzidine. This metabolite was thought to have resulted from a rearrangement of 3,3^{-}-dichlorohydrazobenzene, the precursor for azo formation, and then followed by formylation.

Compound C14 with the parent ion at M/Z 375 was thought to have the formula 2-amino-5-chlorobenzoquinone di-m-chloranil or its tautomer, or the formula 2-(m-chloroanilino)-5-chlorobenzoquinon-p-3-chlorophenyl diimine. However, the uv absorption spectrum of C14 revealed two broad absorption bands at 319 and 405 nm, very similar to the uv spectrum of the anilinoazo derivative from 4-chloroaniline. Accordingly, metabolite C14, was identified as 4-(3-chloroanilino) 3,3^{-}-dichloro-azobenzene.

The similarities in the mass spectra of C15 and C14 with only 16 mass units difference in their parent ions, led to the suggestion that C15 is an anilinoazoxy derivative. In this context it was interesting to note that the formation of azo, azoxy and anilinoazo derivatives were not without precedents. However the formation of the anilinoazoxy was considered new, and thought to be formed from the coupling of 3-chloroanilino radical and the respective azoxy metabolite.

Finally, unsymmetrical coupling of the anilino radicals initiated by peroxidase, may lead to the formation of p-aminodiphenylamine. Further oxidation of this into benzoquinonediimine with subsequent

nucleophilic substitution and/or hydrolysis may be considered as the most probable routes for the formation of C8, C9, C16 and C17.

On comparison of these results with those previously reported on the metabolism of the aniline particularly 4-chloroaniline and/or 3,4-dichloroaniline in soil-plant systems or from sensitized photolysis (Kaufman, 1967; Bordeleau and Bartha, 1972c; Bordeleau et al., 1972; Briggs and Walker, 1973; Engelhardet et al., 1977; Pillai and associates, 1982; and Feishbein, 1984), or in the peroxidase system (Saunders, 1957; Simmons et al., 1986). It was evident that the results found here were alike, and most probably followed the same routes of formation, although there were no more than seven metabolites in any one study on haloaniline (see section 5.2.4).

5.4.3 Peroxidase Action on 2-Amino-4-chlorophenol

Treatment of this substituted arylamine with hydrogen peroxide and peroxidase in a buffer aqueous media produced dark red to deep violet coloration, followed by a slurry of reddish brown turbidity. Extraction of the oxidized products by ethyl acetate, followed by separation and purification of the concentrated extract by TLC, and eventually identification by mass spectrometry, using direct insertion probe, revealed the formation of four major metabolites. In table 5.2 is shown the relative flow (R_f) or mobility relative to front values and the molecular ions of the aminochlorophenol and its

metabolites.

The first product of aminochlorophenol, H1, when analysed by mass spectrometry, showed an intense molecular ion peak at M/Z 246, and isotopic abundances with the ratio of ~ 3:1, indicating the presence of one chlorine and an even number of nitrogen atoms. Other imported peaks at M/Z 219, 191 and 155 might be attributed to stepwise losses of HCN, CO and HCl respectively. The mass spectrum of this compound showed great similarities to that of 2-amino-7-chloro-3H-phenoxazinone, a microbial metabolite of 4-chloroaniline. Hence, metabolite H1 was identified as 2-amino-8-chlorophenoxazinone.

The molecular ion at M/Z 280 and the isotopic abundances at M/Z 282, 284 with the ratio of ~ 9:6:1 of the second metabolite, indicated a dimer of an even number of nitrogen and two chlorine atoms. Mass fragmentation at M/Z 252, 217 and 190 may be attributed to subsequent losses of CO, Cl and HCN respectively.

From the gathered data, metabolite H2 was defined as either 4,8-dichloro-4-hydroxyphenazine or 1,8-dichloro-4-hydroxyphenoxazinimine. However, on the basis of mass fragmentation shown, particularly at M/Z 126, 164 and 140, the first choice was ruled out, as the demonstrated peaks may be rationalized from the second choice to represent $C_6H_2OH^+$, M - $C_6H_2OH^+$ and $C_6H_2OHN^+$ respectively in accordance with the general trend of phenazine mass fragmentation.

The third metabolite with a parent ion at M/Z 286 and isotopic abundances, indicated an even number of nitrogen and one chlorine atom which could rise by condensation of two molecules of aminochlorophenol with elimination of one chlorine atom, so that a partial compound with the formula of $C_{12}H_{11}N_2O_2Cl$ and a mass of 250 units may be possible, leaving 46 mass units to be accounted for. Possibilities for this were CH_3CO(43), C_3H_7(43) and COOH(45).

High resolution mass spectroscopy suggested the formula $C_{15}H_{11}N_2O_2Cl$. Hence, the proposals of CH_3CO and COOH were excluded but the question from where the isopropyl group might come still arises. A rational possibility in this respect might result from the utilized acetone in the chromatographic separation which may react with an amino group (Schiff's base reaction) and give the right molecular formula. Accordingly metabolite H3 was assigned as 2-isopropylimine-8-chlorophenoxazinone. The mass fragment at M/Z 271 with isotopic abundance of one chlorine was the most pronounced base peak, and might be correlated with a fission of a methyl group to give a stable methylbenzoxazole fragment. Other mass fragmentations of this metabolite were in accordance with the assigned structure. In this field, it is worth mentioning that a similar Schiff base reaction with metabolite of 4-chloroaniline in peroxidase system was reported and considered as an artifact (Saunders, 1957).

The fourth isolated metabolite showed an odd

molecular ion at M/Z 353 and isotopic abundances indicating one chlorine atom. A trimer of formula $C_{18}H_{12}ClN_3O_3$ and molecular weight of 353 was proposed. Accordingly no mass difference was there to account for. Fragments at M/Z 141 and 213 suggested a rupture of hydroxychloroanilinium ion and hence metabolite H4 was identified as 2-amino-8-(m-chloro-2-hydroxyanilino) -3H-phenoxazincone. In figure 5.3 is demonstrated the mass spectra of 2-amino-4-chlorophenol and its various isolated metabolites.

On comparing these findings with those previously reported (Briggs and Walker, 1973; Engelhardet et al., 1977) in soil and microbial systems, it was evident that phenoxazinone formation represented a common reaction particularly for ortho-hydroxylated anilines. Similarly o-hydroxylated acylanilides and o-hydroxylated phenyl-carbamates underwent thermal cyclization to the corresponding benzoxazole or benzoxazolone (Still and Herrett, 1976; Fletcher and Kaufmann, 1979).

5.4.4 Peroxidase Action on p-Anisidine

The enzymatic oxidation of the above compound was of interest for two reasons, the first, because p-anisidine may represent a potential metabolite of chlorpropham or its related propham analogue *in vivo*, or as a photoproduct of chlorpropham and/or propham particularly in the presence of methanol. The second reason came as a result of the contradiction relating to its transformation into the respective azobenzene derivative (Saunders, 1957; Bordeleau and Bartha,

1972c).

With the peroxidase system, p-anisidine produced an instantaneous deep violet colour within a few minutes. A reddish brown turbidity, and a red-brown solid gradually separated. Partitioning of the crude reaction mixture in ethyl acetate followed by chromatographic separation prior to an eventual mass analysis of the oxidation products afforded eight metabolites, six of which have not previously been described. Table 5.3 represents the assigned structure for the p-anisidine metabolites coupled with their molecular ions as well as R_f values.

The metabolites of p-anisidine were mostly trimers and tetramers in addition to the respective formanilide and 4,4^{-}-dimethoxyazobenzene. The molecular ions and mass fragmentation patterns of the various metabolites were in full agreement with their assigned structures. Fragmentation peaks corresponding to losses of CH_3, CO, HCHO, $CH_3O\text{-}C_6H_4$ and $CH_3O\text{-}C_6H_4NH$ as radicals and/or ions from the parent molecular ions were common among the mass spectra of the various products. Figure 5.4 represents the mass spectra of p-anisidine and its isolated metabolites.

On comparison of these consequences with those previously reported (Saunders, 1957), it was evident the formation of a greater number of metabolites; also, the formation of the respective azobenzene derivatives (Saunders, 1957) were reaffirmed in contrast to the findings of Bordeleau and Bartha (1972c).

5.4.5 Peroxidase Action on 3-Chloro-4-methoxyaniline

The interest in the enzymatic action on this compound arose as it represented a potential intermediate metabolite of chlorpropham in potatoes (Heikes, 1985), and metoxuron, a ureide herbicide (Briggs and Ogilvie, 1971). Another reason for the interest came as this compound afforded in soil, slurries and/or peroxidase systems a limited number of metabolites (Briggs and Ogilvie, 1971), which were thought to be related to the presence of the chlorine and the methoxy groups.

These moieties may counteract each other, as one group is an electron withdrawing, while the other is an electron releasing, group. Also their attachment to the same ring may leave little room for aniline coupling, but this was not found to be the case. With the peroxidase system in a buffer aqueous media, 3-chloro-4-methoxyaniline developed a pink to deep violet colour and within a few minutes there was a dark brown turbidity and eventually a solid material formed. Extracting the reaction mixture with ethyl acetate, followed by concentration under vacuum, and separation of the crude product by TLC led to the isolation of many components. Eventual mass analysis of the separated components revealed eleven metabolites. Table 5.4 demonstrates the molecular ion of these metabolites as it was shown from their respective mass spectra, coupled with their R_f values and proposed identities. The mass spectra of the analysed compounds showed the right molecular ions and isotopic abundances. Also, the mass fragmentation

pattern of various metabolites showed characteristic peaks concomitant with the radical fissions of CH_3, CO, HCHO, Cl and HCN, and all were in accordance with the assigned structures. Representative mass spectra of 3-chloro-4- methoxyaniline and its metabolites are shown in figure 5.5. On comparing the metabolites of 3-chloro-4- methoxyaniline with those previously reported (Briggs and Ogilvie, 1971), it was evident that the methoxy and chlorine groups of the molecule underwent nucleophilic substitution and gave rise to a great number of metabolites (Table 5.4), in contrast to only three metabolites reported by Briggs and Ogilvie. Among the various metabolites identified, were 3-chloro-4-methoxy-formanilide, 3,3^{-}-dichlor-4,4^{-}-dimethoxyazobenzene and its respective anilinoazo in addition to a substituted phenazine type of compound similar to pseudo-mauveine. Additional trimers and tetramers also were clarified.

Of interest in this context were the formation of substituted diphenylamines in A4, A5, D5 and D8, since secondary amines are likely to form nitrosamine types of compounds which are known to be carcinogenic and/or mutagenic agents. The formation of diphenylamine is not without precedent. Hawkins (1988) demostrated the formation of 4-amino-4^{-}-ethoxydiphenylamine from phenitidine and/or phenacitine (analgesic and antipyretic agents) in the red blood cells of humans, and hence caused its substitution by a more safe paracetamol.

5.4.6 Peroxidase Inhibition Studies

Experimental evidences in section 5.3.3.2 revealed that a variety of naturally occuring organic and inorganic reducing agents were capable of retarding the catalytic activity of peroxidase to a varying degree, depending on their nature and their concentration as well. The masking power of the reducing agents were in the order, ascorbic acid >> $Na_2S_2O_4$ > cysteine. However, potassium iodide and ammonium thiocyanate, as compared to the ascorbic acid or the dithionite, showed negligible or no effect on the oxidation of substituted anilines in a peroxidase system. The demonstrated inhibition action of the reducing agents may be attributed to their competition with the enzyme preferentially for hydrogen peroxide, the precursor for peroxidase initiation activity. This assumption was made as the enzyme resumed its activity after certain additions of the peroxide, indicating that the tested reducing agents were preferentially oxidized by peroxide and/or the peroxidase system (Saunders et al., 1964). However, variations in the amounts of the peroxide needed to initiate the enzymetic oxidation of arylamines in the presence of equimolar amounts of the reducing agents indicated that they might inhibit the enzyme directly by coordination and/or reduction of the iron atom in the heme prosthetic group into the ferro analogue.

The negligible effect of I^- or SCN^-, despite their free radical scavenging abilities, may be correlated with their high oxidation potential values, as

compared to that of the anilines in the system. Earlier work on this aspect revealed that iodide but not thiocyanate was oxidizable by the peroxidase system at least in the absence of inhibitory competing substance(s) (Saunders and Stark, 1967; Morrison and Schonbaum, 1976).

5.5 Conclusions

With the continued use of chlorpropham as a herbicide and/or as a sprout suppressant on a staple food item such as potatoes, it was felt important from the toxicological viewpoint, not only to determine the terminal residues of chlorpropham, but also to determine the fate of chlorpropham and its potential aniline intermediates. Since the peroxidase system was reported to be responsible at least in part for the transformation and/or humification of anilines, and providing metabolites in accordance to that observed in soils and microbial cultures, it was thought relevant to study the effect of horseradish peroxidase *in vitro* on chlorpropham and its related anilines. The overall aim was to elucidate the various transformations of these compounds, and to deduce what metabolites are likely to be formed from chlorpropham.

The work in this chapter provided an overall view on peroxidase and its catalytic action on various anilines. Also, it described the oxidative effect of peroxidase system on chlorpropham, sulfanilamide, p-nitroaniline, 3-chloroaniline, 2-amino-4-chlorphenol,

p-anisidine and 3-chloro-p-anisidine. The chapter also described the inhibitory effect of various organic and inorganic reducing agents on peroxidase, not only this, but the mass spectra and the probable structures for an overall forty metabolites were exclusively presented. As an outcome of this study, the following conclusions were made.

1. No reaction was observed when either HRP or H_2O_2 was excluded from the reaction system, thus indicating that both of the reagents were very essential to initiate the catalytic role of peroxidase reaction(s).
2. Reducing agents such as ascorbic acid, sodium dithionite or cysteine, inhibited the action of peroxidase but to varying degrees, probably because they were favourably oxidized by peroxidase or they competed with the enzyme preferentially for H_2O_2. However, KI and NH_4SCN did not show any inhibitory effect, or their effects were so negligible as to be not recognized probably due to their high oxidation potentials as compared to that of anilines present in the reaction media.
3. Chlorpropham did not show any response to the peroxidase system even after three weeks of incubation, most probably because it did not hydrolyze, or hydrolyzed too slowly providing not enough aniline to react with peroxidase.
4. The presence of sulfonamide or nitro groups on the phenyl moiety of the aniline rendered it unreactive

toward peroxidase system. This might be attributed to the electron withdrawing nature of these groups, which tend to stabilize their parent molecules through resonance and by generating a positive charge on the reacting centre, thereby influencing the nucleophilicity of the electron donors to the heme iron of the enzyme.

5. 3-chloroaniline, 2-amino-p-chlorophenol, p-anisidine and 3-chloro-p-anisidine were not stable in the peroxidase system as they showed instantaneous changes when mixed together. However their susceptibility to the enzymatic attack was correlated positively with the electron density on the reacting amino centre of the tested anilines in the order of p-anisidine > 2-amino-4-chlorophenol ≥ 3-chloro-p-anisidine >> 3-chloroaniline.
6. Susceptibilities of the various anilines for the enzymatic attack in the acetate buffer, pH 4.7 were greater than that in the phosphate buffer, pH 6.8, as demonstrated from the intensities of the separated colour bands on the TLC plates. This was in accordance with the peroxidase activity which has a negative correlation with pH. Peroxidase enzymes tend to dimerise at high pH's. Also, at extreme pH's, they may split into individual components.
7. 3-chloroaniline in the peroxidase system demonstrated various changes and afforded seventeen metabolites. They were identified from their mass spectral data as follows: chloro substituted phenylisocyanate,

formanilide, acetanilide, nitrobenzene, anilino-nitrobenzene, disubstituted urea, in addition to various oligomers of chloro-substituted azo, azoxy, anilinoazo, anilinoazoxybenzenes, diformyl-benzidine, phenazine, benzoquinonimine and finally tetramers of amino and hydroxy anilinobenzoquinone dianils.

8. 2-amino-4-hydroxyphenol with the peroxidase system gave four metabolites including chloro substituted dihydroxyphenazine, phenoxazinone, chloroanilino-phenoxazinone and leuco Schiff base of chloro-phenoxazinone.
9. p-anisidine with the electron donating methoxy group in the peroxidase system produced eight metabolites characterized from their mass spectra as methoxy substituted-, formanilide, azobenzene, p-anisidinosemidine, anisidinomethoxydi-phenylamine, anisidinomethoxysemidine, di-anisidino-p-anisidine, di-anisidinomethyoxysemidine and leuco Schiff base of amino-p-anisidinobenzoquinone di-p-methoxyanil.
10. Incubation of 3-chloro-p-anisidine produced eleven products mostly new, separated by TLC and identified from their mass spectra. The various metabolites included the respective formanilide, substituted azo, anilinoazobenzene and phenazine similar to pseudo-mauvine (from aniline), in addition to oligomers of substituted diphenylamines, benzoquinonimine, trimers, tetramers and two Schiff bases of other trimers and tetramers (table 5.4).

11. As the initial interest in this study was primarily concerned with the isolation and identification of the various products resulting from chlorpropham and its related anilines in the peroxidase system, and the aim of shedding some light on the likely metabolites of chlorpropham, the information gathered from the mass spectral data of the various products together with that obtained from some reference standards and literature analogues, was enough to suggest probable structures for more than forty metabolites some of which may not have been exclusively identified.

    It is anticipated in this context that some questions will arise as to the metabolite percentage yields and/or further confirmations of their identities. However, the amounts dealt with were very limited and were not enough to allow further confirmation, except in a few cases. Yield as a function of the initial concentration of the starting materials, pH of the reaction media and nature and position of the substituents were not determined for the same mentioned reason.

12. The great versatility of the reactions that took place and the possibility of constituents separation, implied that such systems may be utilized for synthetic purposes, particularly for some selective reactions under mild conditions, e.g. the phenoxazinone derivative, the basic unit of actienomycin , which is known as the most powerful

antitumor agent ever known. This chemical could be synthesized by the action of peroxidase on the respective hydroxy- aniline. Similarly substituted azo, azoxy and anilinoazobenzene type of compounds may be easily synthesized by this route, thereby avoiding and/or abandoning some of the tedious and dangerous ways of chemical synthesis.

13. Finally, a question is anticipated as to whether such a variety of reactions and transformations occur in nature, and to what extent they may add to the pollution problem. The versatility of changes observed led to the formation of more stable and more persistent compounds and oligomers than their parents, and since some of the metabolites have mutagenic and/or carcinogenic properties or at least undiscovered toxicity and as polymerization and oligomer formation represented the dominant route for peroxidase-substituted aniline transformation, which by one way or another may act to retard or compete with mineralization and/or incorporation or copolymerization, the two major pathways for dissipation and fate of anilines. Therefore, based on all of these considerations it may be advisable to stay alert that such various metabolites may pollute the environment and contaminate human food wherever aniline-based herbicides may be used.

Table 5.1 Molecular ions, $M^{\cdot+}$, and/or GC-MS retention time, t_r, of 3-chloroaniline and its metabolites in the peroxidase system coupled with their proposed structures and names.

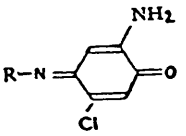
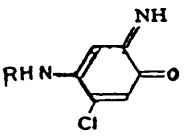
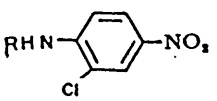
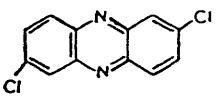
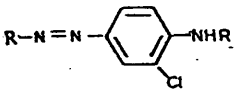
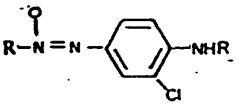
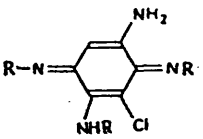
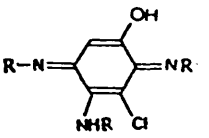
Code No.	$M^{\cdot+}$	t_r	Proposed structure	Chemical name
C	127	5.03	$R-NH_2$	3-chloroaniline
C1	153	4.15	R-NCO	3-chlorophenylisocyanate
C2	155	8.16	R-NHCHO	3-chloroformanilide
C3	157	5.52	$R-NO_2$	3-chloronitrobenzene
C4	169	8.4	$R-NHCOCH_3$	3-chloroacetanilide
C5	250	11.2	R-N=N(R)	3-3¯-dichloroazobenzene
C6	266	13.2	R-N(O)=N(R)	cis-3,3¯-dichloroazoxybenzene
C7	266	13.2	R-N(O)=N(R)	trans-3,3¯-dichloroazoxybenzene
C8	266	12.67		2-amino-5-chlorobenzoquinone-4-m-chloroanil
C9	266	12.89		p-(3-chloroanilino-o-benzoquinone-5-chloro-2-imine
C10	280	-	R-NHCONH-R	N,N¯-bis(3-chlorophenyl)urea
C11	282	-		4-(3-chloroanilino)-3-chloro-nitrobenzene

Table 5.1 Continued

Code No.	$M^{\cdot+}$	t_r	Proposed structure	Chemical name
C12	248	-		2,7-dichlorophenazine
C13	308	-	-(R-NHCHO)$_2$	2,2^{-}-dichloro,N,N^{-}-bisformyl-benzidine
C14	375	-		4-(3-chloroanilino)-3,3^{-}-dichloroazobenzene
C15	301	-		4-(3-chloroanilino)-3-3^{-}-dichloroazoxybenzene
C16	503	-		2-amino-5-(3-chloroanilino)-6-chloro-p-benzoquinone-di-m-chloroanil
C17	505	-		2-hydroxy-5-(3 chloroanilino)-6-chloro-p-benzoquinone-di-m-chloroanil

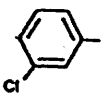
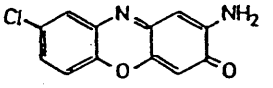
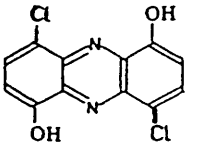
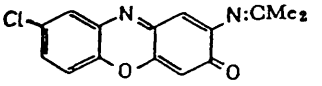
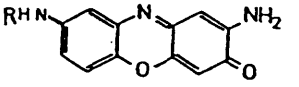
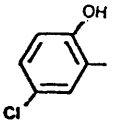
R = 

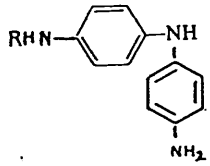
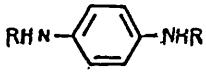
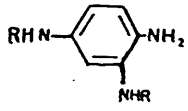
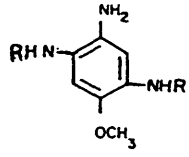
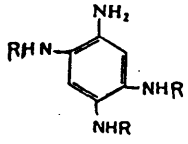
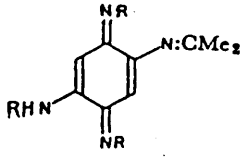
Table 5.2 Molecular ions, $M^{\cdot+}$ and R_f^* values for 2-amino-4-chlorophenol and its metabolites from peroxidase system coupled with their proposed identities.

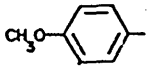
Code No.	$M^{\cdot+}$	R_f	Proposed structure	Chemical name
H	143	-	$R\text{-}NH_2$	2-amino-4-chlorophenol
H1	246	0.22		2-amino-8-chloro-3H-phenoxazinone
H2	280	0.34		4,8-dichloro-1,6-dihydroxy-phenazine
H3	286	0.48		2-isopropylimine-8-chloro-3H-phenoxazinone
H4	353	0.05		2-amino-8-m-chloro-2-hydroxyanilino-3H-phenoxazinone

R = 

R_f values were determined on glass plates self-coated with silica gel G and developed once in hexane-toluene-acetone mixture (7:3:1 v/v).

Table 5.3 Molecular ions, $M^{\cdot+}$, and R_f values for p-anisidine and its various metabolites from peroxidase system, coupled with their proposed structures and names.

Code No.	$M^{\cdot+}$	R_f	Proposed structure	Chemical name
A	123	-	$R\text{-}NH_2$	p-anisidine
A1	151	-	R-NHCHO	4-methoxyformanilide
A2	242	0.58	R-N=NR	4-4^{-}-dimethoxyazobenzene
A3	305	0.39		4-(p-anisidino)semidine
A4	320	0.28		bis(p-anisidino)phenylene
A5	333	0.05		2-anisidino-4-methoxy-semidine
A6	365	0.07		2,5-dianisidino-p-anisidine
A7	454	0.29		2,5-dianisidino-p-methoxysemidine
A8	494	0.22		2-isoprophylimine-5-p-anisidinobenzoquinone di-p-anisidinoanil

R = 

R_f values determined on glass plates of silica gel G and developed in hexane-toluene-acetone (7:3:1 v/v)

Table 5.4 Molecular ions, $M^{\cdot+}$ and R_f values for 3-chloro-4-methoxyaniline and its metabolites from peroxidase system, coupled with their proposed structures and names.

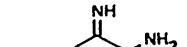
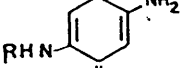
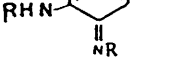
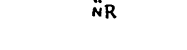
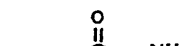
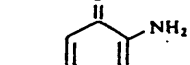
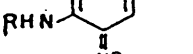
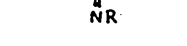
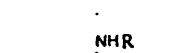
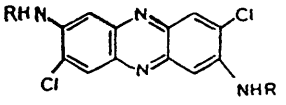
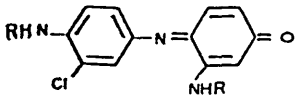
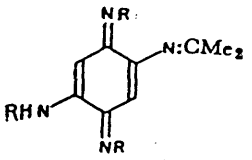
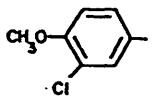
Code No.	$M^{\cdot+}$	R_f	Proposed structure	Chemical name
D	157	-		3-chloro-4-methoxyaniline
D1	185	0.05		3-chloro-4-methoxyformanilide
D2	310	0.4		3-3¯-dichloro-4,4¯-dimethoxy-azobenzene
D3	416	0.06		2-amino-5-(3-chloro-p-anisidino)-3¯-chlorobenzoquinoneimide-anisid
D4	417	0.03		2-amino-5-(3-chloro-p-anisidino)-3¯-chlorobenzoquinone-monoanisid
D5	422	0.25		N,N¯-di(3-chloro-4-methoxy)-3-chlorophenylenediamine
D6	433	-		2,5-bis(3-chloro-p-anisidino)-p-anisidine
D7	435	-		3,3¯-dichloro-4-methoxy-4¯-(3-chloro-p-anisidino)-azobenzene
D8	477	0.22		2-isopropylimine-5-chloro-N,N¯-di(3-chloro-4-methoxy)-phenylenediamine

Table 5.4 Continued

Code No.	$M^{\cdot+}$	R_f	Proposed structure	Chemical name
D9	558	-		2,7-dichloro-3,8-bis-(3-chloro-p-anisidino)-phenazine
D10	561	0.36		3-(3-chloro-p-anisidino)-3¯-chloro-4¯-methoxy-benzoquinone monoanil
D11	596	-		2-isopropylimine-5-(3-chloro-p-anisidino)-benzoquinone-3,3¯-dichloro-bis-p-anisid

R = 

R_f values were determined on glass plates self-coated with silica gel G and developed in hexane-toluene-acetone (7:3:1 v/v).

Figure 5.2 Mass spectra of 3-chloroaniline and its metabolites from peroxidase system.

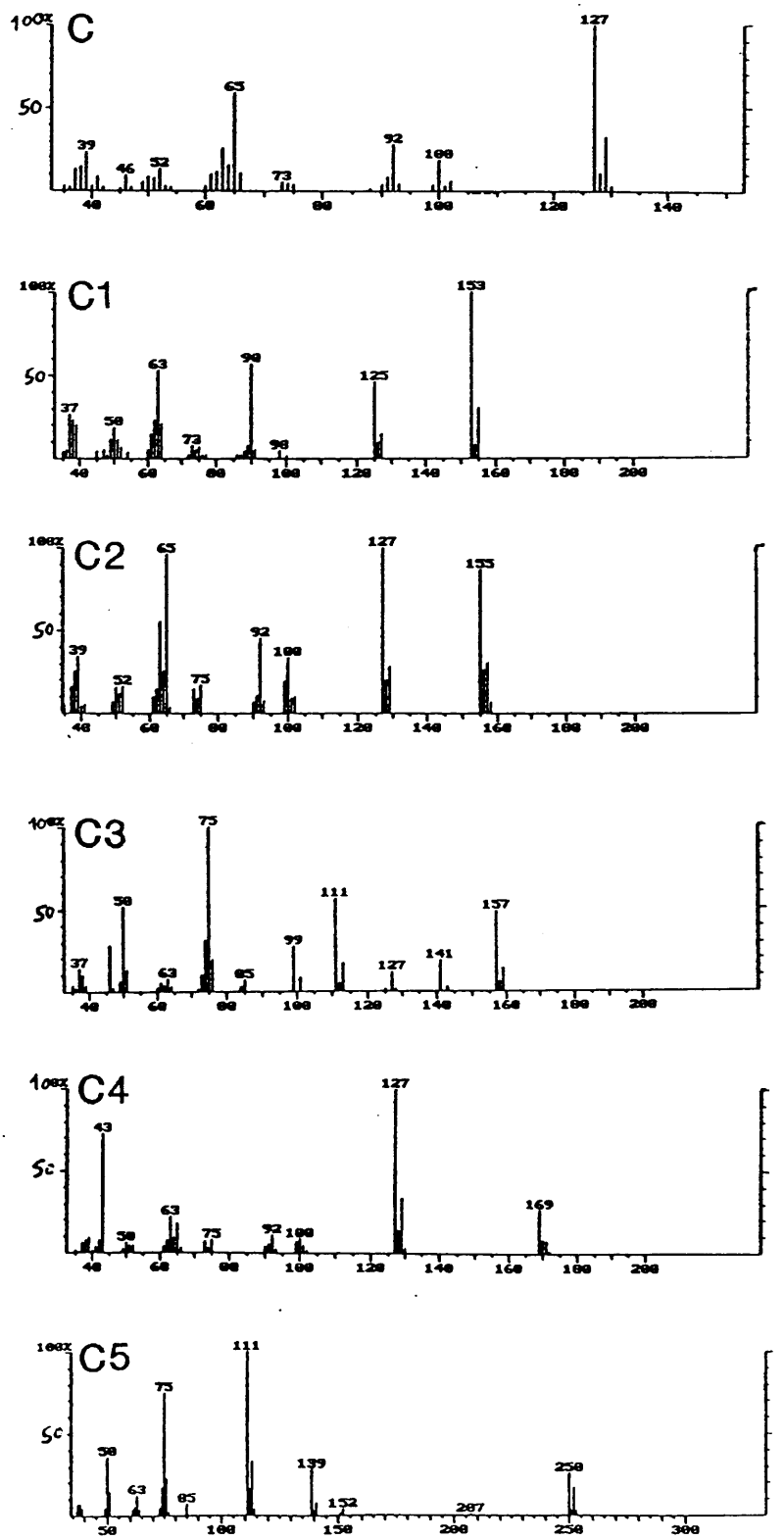
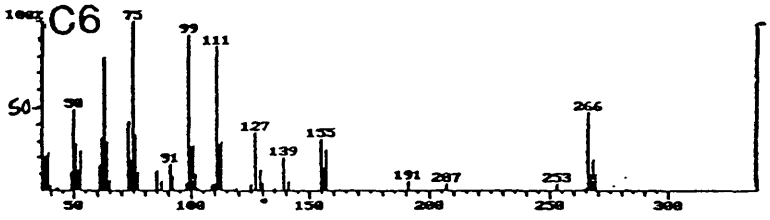
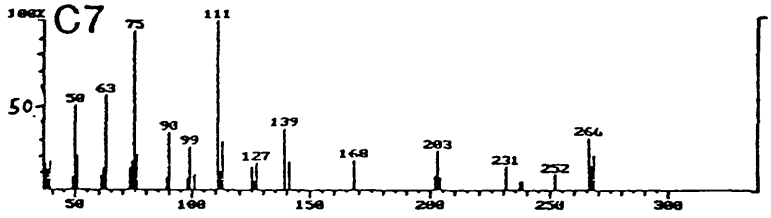
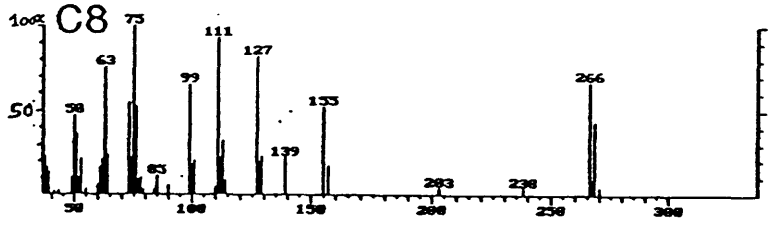
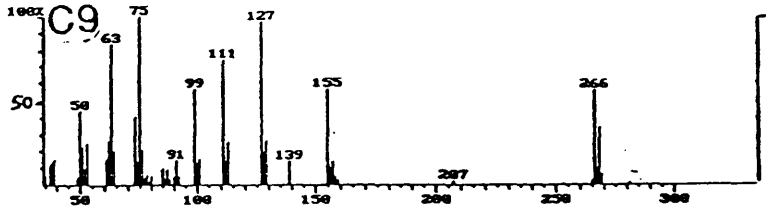


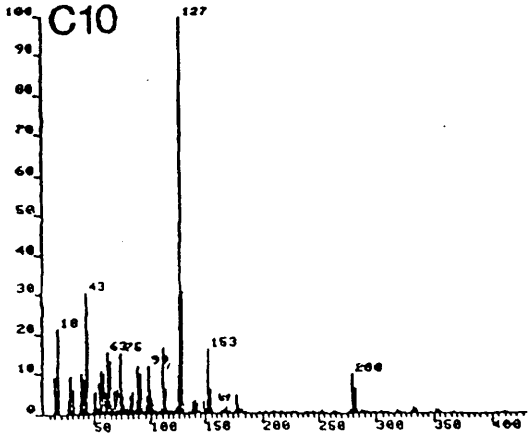
Figure 5.2 Continued











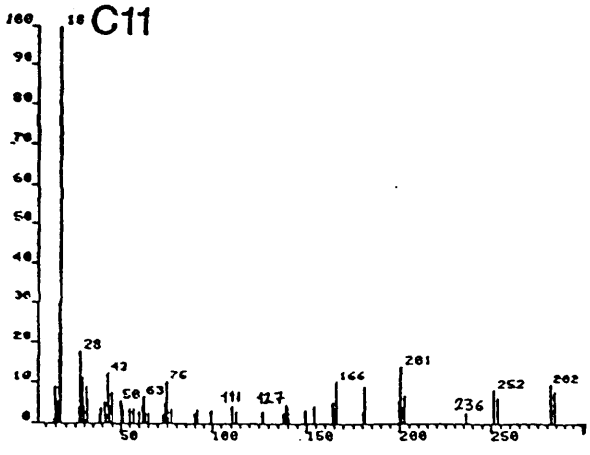


Figure 5.2 Continued

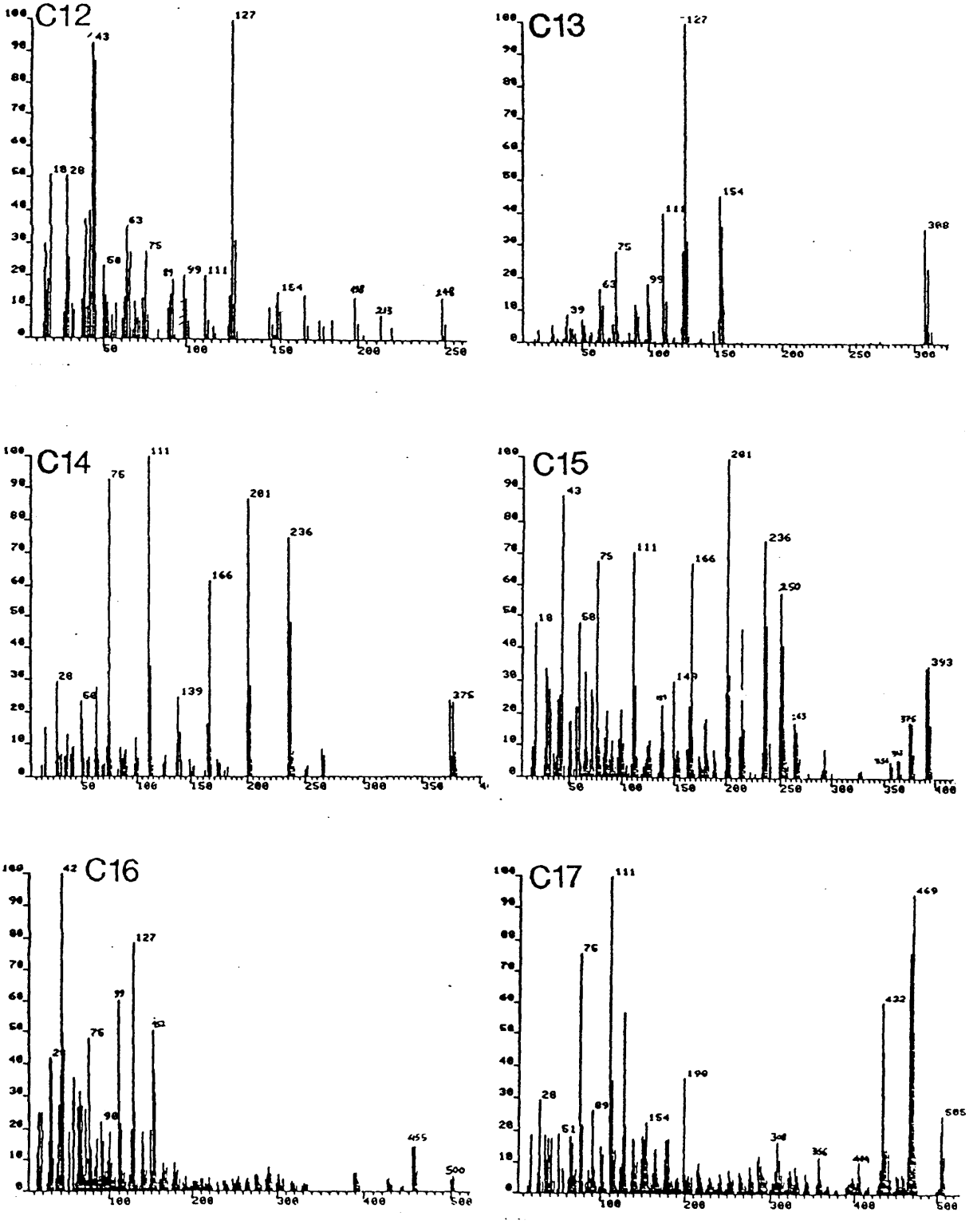


Figure 5.3 Mass spectra of 2-amino-p-hydrosyphenol and its metabolites from peroxidase system.

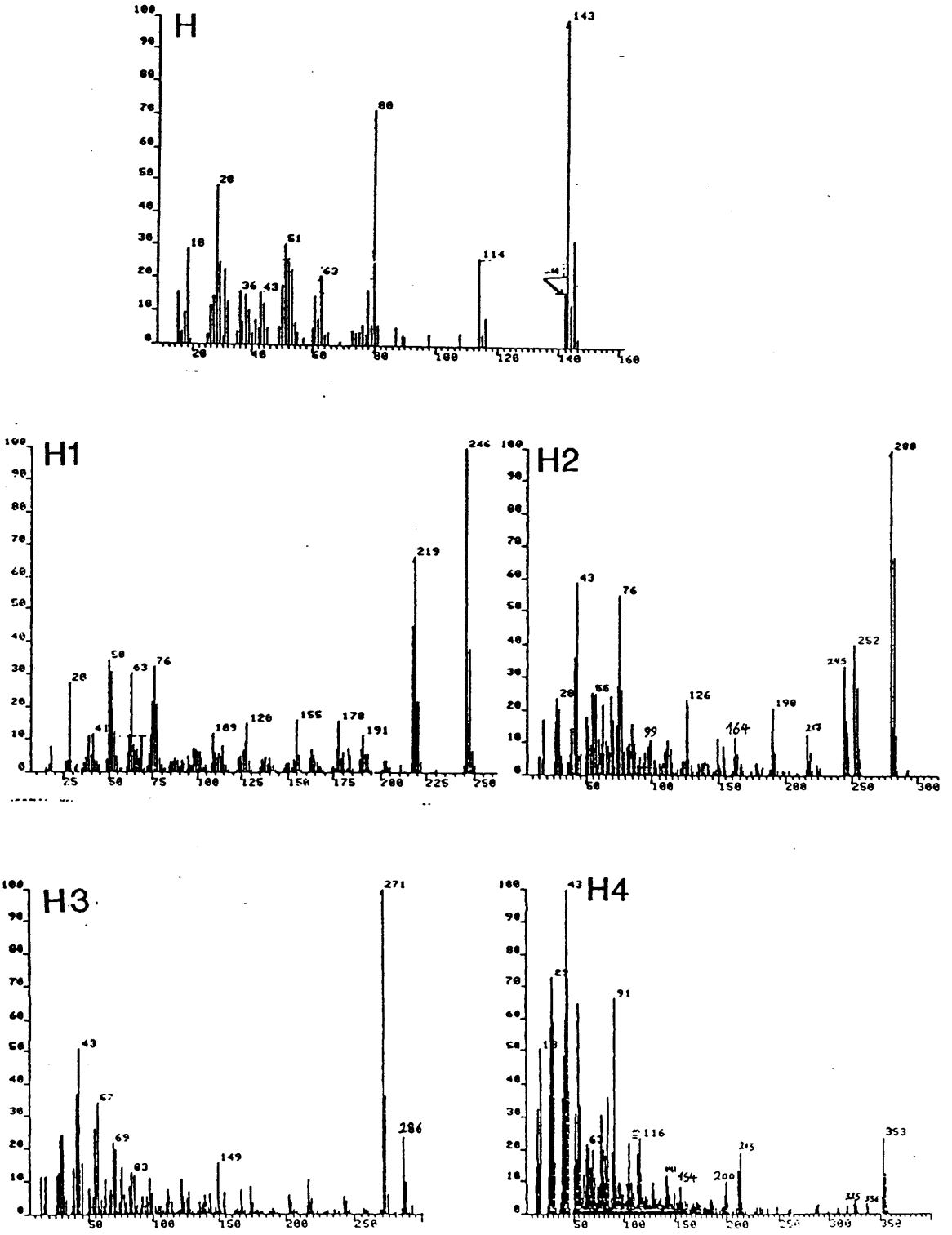


Figure 5.4 Mass spectra of p-anisidine and its metabolites from peroxidase system.

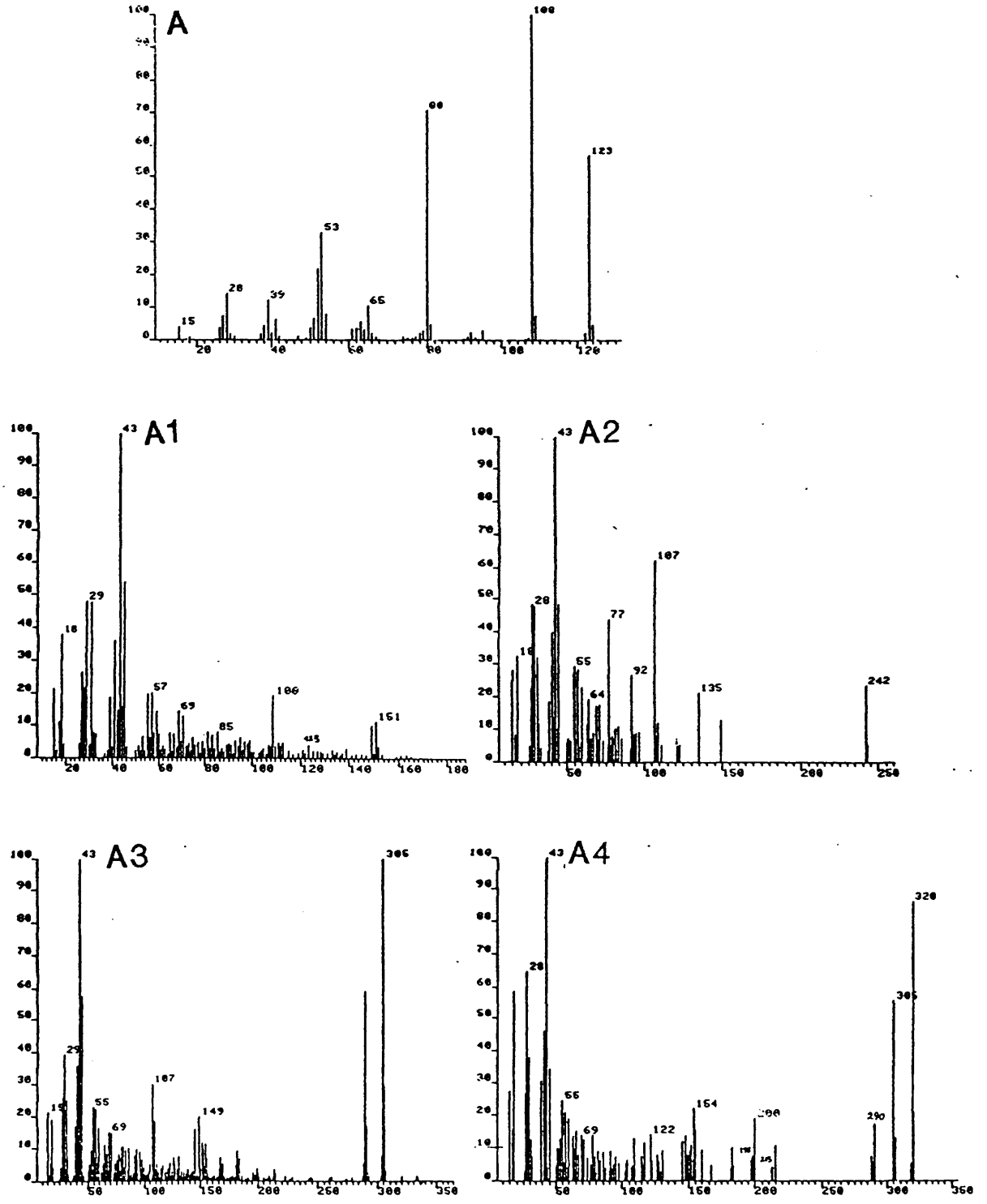


Figure 5.4 Continued

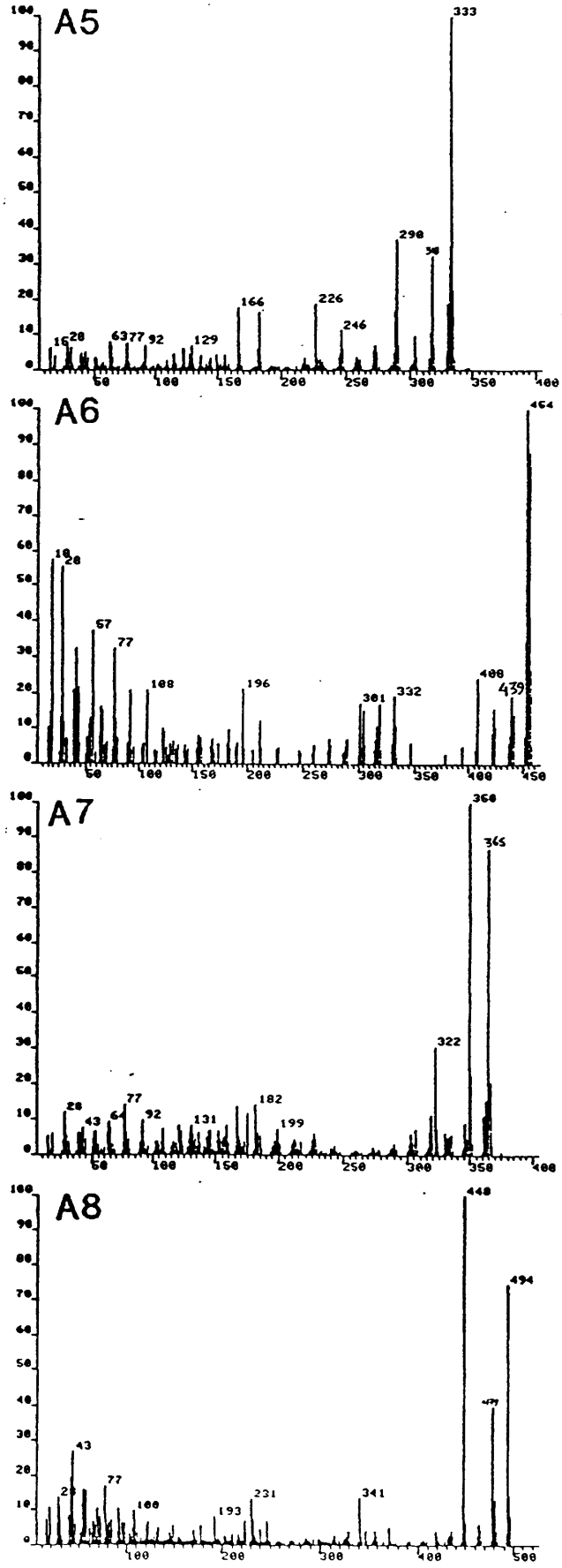


Figure 5.5 Mass spectra of 3-chloro-p-anisidine and its metabolites from peroxidase system.

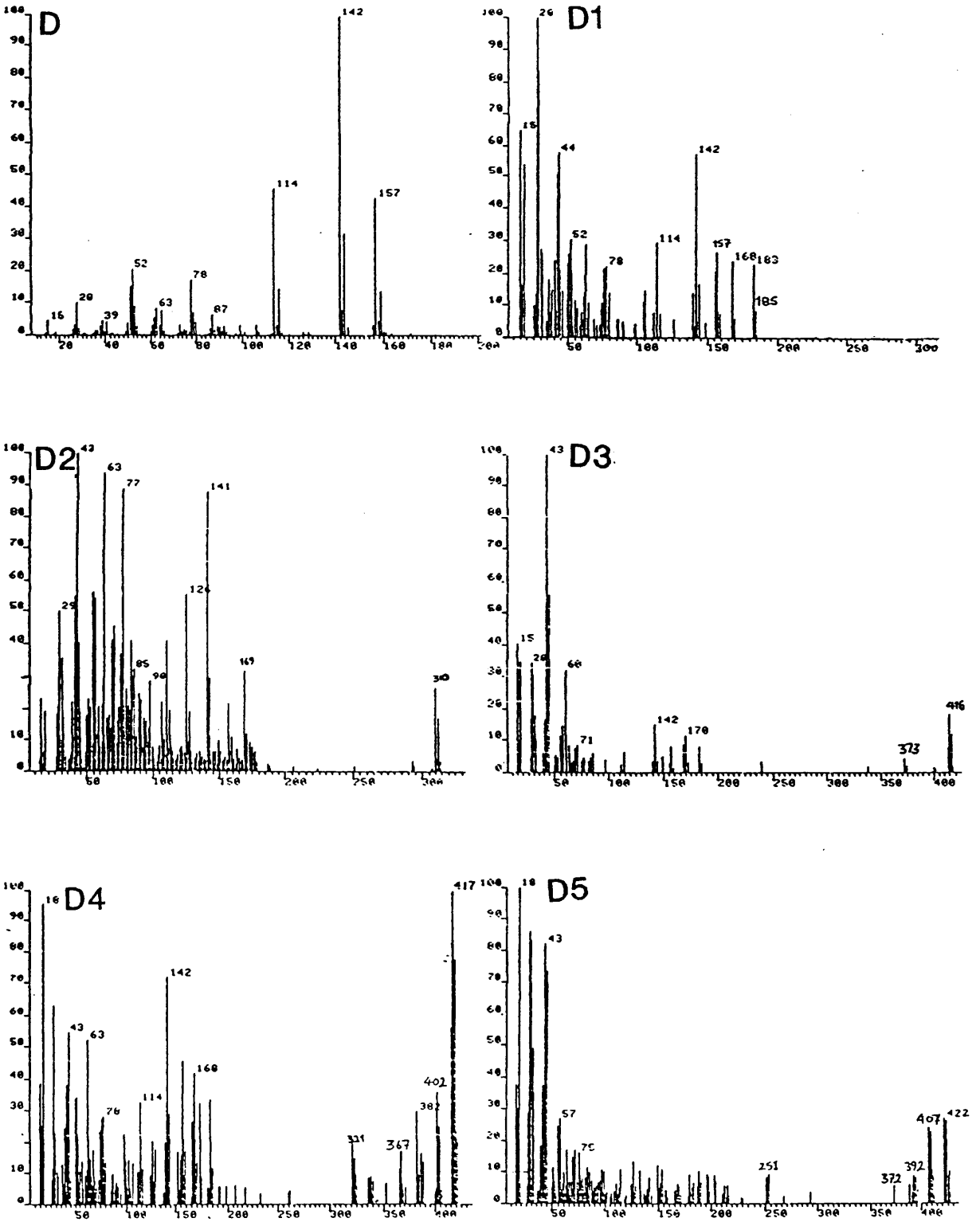
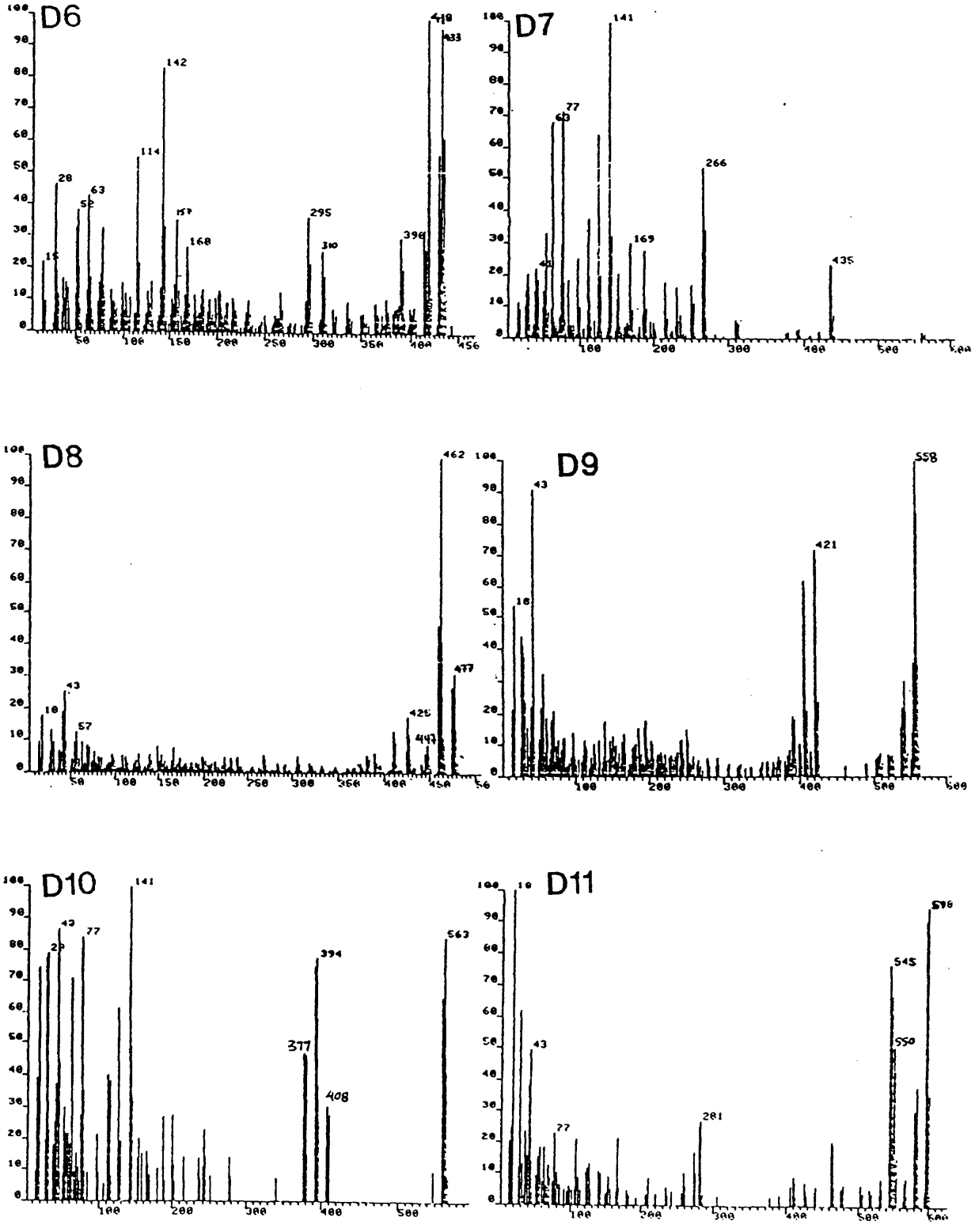


Figure 5.5 Continued



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